NEUROPROTECTIVE EFFECT OF WEAK STATIC MAGNETIC FIELDS IN PRIMARY NEURONAL CULTURES

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Abstract—Low intensity static magnetic fields (SMFs) interact with various biological tissues including the CNS, thereby affecting key biological processes such as gene expression, cell proliferation and differentiation, as well as apoptosis. Previous studies describing the effect of SMFs on apoptotic cell death in several non-neuronal cell lines, emphasize the importance of such a potential modulation in the case of neurodegenerative disorders, where apoptosis constitutes a major route via which neurons degenerate and die. In this study, we examine the effect of SMFs on neuronal survival in primary cortical and hippocampal neurons that constitute a suitable experimental system for modeling the neurodegenerative state in vitro. We show that weak SMF exposure interferes with the apoptotic program in rat primary cortical and hippocampal neurons, thereby providing protection against etoposide-induced apoptosis in a dose- and time-dependent manner. Primary cortical neurons exposed to SMF (50 G) for 7 days exhibited a 57.1 ± 6.3% decrease in the percentage of cells undergoing apoptosis induced by etoposide (12 μM), accompanied by a marked decrease in the expression of the pro-apoptotic markers: cleaved poly ADP ribose polymerase-1, cleaved caspase-3, active caspase-9 and the phospho-histone H2A variant (Ser139) by 41.0 ± 5.0%, 81.2 ± 5.0%, 72.9 ± 6.4%, 42.75 ± 2.9%, respectively, and by a 57.2 ± 1.0% decrease in the extent of mitochondrial membrane potential collapse. Using the L-type voltage-gated Ca2+ channel inhibitor nifedipine, which is selective to Ca2+ influx through CaV1.2, we found that the anti-apoptotic effect of SMFs was mediated by Ca2+ influx through these channels. Our findings demonstrating altered Ca2+-influx in response to thapsigargin stimulation in SMF-exposed cortical neurons, along with enhanced inhibition of KCl-induced Ca2+-influx through CaV1.2 channels and enhanced expression of CaV1.2 and CaV1.3 channels, allude to the involvement of voltage- and store-operated Ca2+ channels in various aspects of the protective effect exerted by SMFs. These findings show the potential susceptibility of the CNS to weak SMF exposure and have implications for the design of novel strategies for the treatment and/or prevention of neurodegenerative diseases. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: apoptosis, calcium influx, etoposide, neuroprotection, primary neuronal culture, static magnetic fields.

INTRODUCTION

Weak static magnetic fields (SMFs; 0.1–400 mT)1 have raised a growing interest in recent years due to a wide variety of biological effects reported in both preclinical experiments (Ohkubo and Xu, 1997; Okano et al., 1999; Miyakoshi, 2005; Saunders, 2005), and clinical studies in human subjects (Valibona et al., 1997; Man et al., 1999; Carter et al., 2002; Weintraub et al., 2003). In the CNS, SMFs were reported to affect numerous functions such as inhibition of sensory nociceptive action potentials (McLean et al., 1995), alteration in gene expression (Hirai and Yoneda, 2004; Tenuzzo et al., 2009), in cell orientation and morphology (Pacini et al., 2003; Teodori et al., 2006) and glioblastoma (Teodori et al., 2011), lymphocytes and thymocytes (Filip et al., 1998; Tenuzzo et al., 2006) and glioblastoma (Teodori et al., 2002). However, such modulation of apoptosis has not been demonstrated thus far in primary neuronal cultures such as

1 1 mT = 10 Gauss.
cortical and hippocampal neurons, whose progressive degeneration and death in neurodegenerative diseases such as Alzheimer’s disease (AD) have been suggested to occur via apoptosis (Hartmann et al., 2000; Tatton, 2000; Mattson, 2006), and therefore serve as a suitable model for the study of neurodegenerative diseases. Theoretical models aimed at explaining the interaction of SMFs with biological systems often hypothesize a primary physical interaction of the field with a particular molecular target, preceding subsequent events that ultimately affect the movement of charged particles, such as Ca$^{2+}$, in the cell (Rosen, 1993b). Indeed, studies examining the effect of magnetic fields (MFs) on numerous, mostly non-neuronal cell lines, consistently point to the central involvement of a disrupted ionic equilibrium driven by altered Ca$^{2+}$ fluxes (Bawin and Adey, 1976; Bian et al., 1997; Gobba et al., 2003; Liboff et al., 2003; Grassi et al., 2004; Tenuzzo et al., 2006; Piacentini et al., 2008; Wang et al., 2010). For instance, Fanelli et al. (1999) showed that the anti-apoptotic effect elicited by 0.6-mT SMFs in U937 cells was mediated by modulated Ca$^{2+}$ influx from the extracellular medium. A static field strength of 6 mT was reported to inhibit apoptosis and to affect Ca$^{2+}$ influx from the extracellular medium in human glioblastoma cells (Fanelli et al., 1999; Teodori et al., 2002), and pheochromocytoma-derived (PC12) cells exposed to 230–280-mT SMFs were shown to exhibit a decreased Ca$^{2+}$ efflux from the cells to the extracellular medium (Wang et al., 2010). It has been suggested that MF-induced altered Ca$^{2+}$ oscillations are mediated by a selective modulation of Ca$^{2+}$ influx through L-type voltage-gated Ca$^{2+}$ (VGCCs) (Grassi et al., 2004), although SMFs’ effects on Ca$^{2+}$ fluxes operating through other Ca$^{2+}$ channels such as store-operated channels (SOCs), have also been reported (Cerella et al., 2011).

In the present study, we examine the pro-survival effect exerted by weak SMFs in primary neuronal cultures that model the neurodegenerative state in vitro. Effects of SMF exposure in primary neuronal cultures are described in a few studies that do not directly address the pro-survival aspect of weak SMFs’ action, but rather focus on cellular signaling cascades or alterations in cellular morphology affected by strong SMFs (Pacini et al., 1999; Prina-Mello et al., 2005, 2006; Teodori et al., 2006), which utilize different mechanisms of action than those activated by weak SMFs (Rosen, 1993b).

We show that SMF exposure reduces apoptosis in primary cortical and hippocampal neurons subjected to either etoposide or amyloid beta (Aβ) toxicity. The reduction in apoptosis in primary cortical neurons is associated with a marked down regulation of caspase-3, caspase-9 and other pro-apoptotic proteins and with the stabilization of the mitochondrial membrane potential (MMP), and is mediated by calcium influx through the voltage-gated calcium channels (VGCCs) whose expression was enhanced by SMF exposure in the absence of neurotoxins.

**EXPERIMENTAL PROCEDURES**

**Animal procedures**

All procedures with animals were authorized by the Technion Animal Care and Use Committee, whose ethical standards are based on those detailed in the National Institutes of Health (Bethesda, MD, USA) Guide for the Care and Use of Laboratory Animals (NIH Publications 80-23 revised 1996), and whose general procedures for animal welfare comply with Israeli law on animal experimentation.

**Materials**

Unless otherwise specified, all reagents and chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA), all primary antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA) and all secondary antibodies were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

**Primary cortical and hippocampal neurons.** Dissociated neurons were prepared from the brain cortices or hippocampi of postnatal day 1 Sprague–Dawley (SD) rats (Harlan laboratories, Jerusalem, Israel), plated on poly-D-lysine coated 24-well plates (2.5 × 10⁶ cells/well) and maintained in neurobasal medium (NBM) supplemented with B27 (Gibco, Grand Island, NY, USA).

**Primary glial cells.** Dissociated glial cells were prepared from the brain cortices of postnatal day 1 SD rats, plated on un-coated 24-well plates and maintained in minimal essential medium supplemented with 40 mM L-glutamine. Two days post-plating, cultures were washed with phosphate-buffered saline (PBS) with gentle shaking for 5 min then returned to the medium. Medium was changed after 72 h.

**SMF exposure system.** SMFs were generated using an array of 24 Ne$_2$Fe$_{14}$B magnetic disks of 11.2-mm diameter and known intensities, placed below the 24-well culture plates containing the cells. Different intensities of SMFs were achieved by placing the magnetic array at varying distances beneath the culture plates, and the intensity of the field in each of the 24 wells was measured at five constant points using a Hirst GM08 Gaussmeter equipped with a transverse Hall probe (Fig. 1b). In all experiments, data were collected from six (for apoptosis detection) or 24 (for qPCR) wells per experimental condition, in which the intensity of the measured field was comparable. The culture plates were kept in an incubator with 5% CO$_2$ atmosphere at 37 °C. Unless otherwise specified, cells were exposed to SMFs over the 7-day culture period.

**Time-course experiments.** In order to determine the effect of different times of culture in the presence of SMFs, cultures were incubated in the presence of SMFs for one (DIV6–DIV7), four (DIV3–DIV7), five (DIV2–DIV7), six (DIV1–DIV7) or seven (DIV0–DIV7) days, treated with 12 μM etoposide on DIV6, and apoptotic cells were quantified on DIV7 using Hoechst-33342 as described in Section ‘Induction, detection and quantification of apoptosis’ (see Fig. 2c). In order to
determine the duration of effect following 7 days of SMF exposure, neurons were treated with 12 μM etoposide on DIV6 and the magnetic array was removed on DIV7 for either 1.5, 2.5, 3.5, 6 or 24 h. Hoescht-33342 was added at the end of the off period (i.e., after 25.5, 26.5, 27.5, 30 or 48 h, respectively, of etoposide exposure, see protocol in Fig. 2d). Appropriate controls were used for each time point.

Induction, detection and quantification of apoptosis
Apoptosis was induced by 3–25 μM etoposide, or 5–10 μM Aβ1-42 (American Peptide, Sunnyvale, CA, USA, dissolved in double-distilled water and aggregated at 37 °C for 7 days), added to cultures on DIV6. Apoptosis quantification with Hoescht-33342 dye (1 μg/ml, 10 min at 37 °C, Biotium, Inc., Hayward, CA, USA) was carried out on DIV7 using a fluorescence microscope (Carl Zeiss, Munich, Germany) equipped with a digital camera. Typical features indicative of cells undergoing apoptosis (nuclear chromatin condensation and appearance of fragmented nuclei) were assessed on DIV7, using fluorescent Hoescht-33342 dye staining (1 μg/ml, 10 min at 37 °C, Biotium, Inc, Hayward, CA, USA), or terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique, according to the manufacturer’s instructions (Roche Diagnostics GmbH, Mannheim, Germany). In all experiments, the ratio between apoptotic and normal cells was determined by counting at least three randomly selected, non-overlapping fields in each of six wells per experimental condition. An average number of 147 ± 2.8 cells per field in a single well was counted. Apoptosis percentage was analyzed and quantified using Lucia software (Laboratory Imaging S.R.O, Praha, Czech Republic). All cell counting was carried out with the experimenter blinded to the experimental conditions, and each experiment was repeated three times.

MMP measurement
The extent of MMP loss was measured using the potentiometric cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1, Life Technologies, Grand Island, NY, USA). Following 7 days of SMF exposure and treatment with 12 μM etoposide on DIV6, cultures were incubated on DIV7 with JC-1 (3 μM) for 30 min at 37 °C. The dual emission of the dye was observed using the fluorescence microscope at excitation 488 nm, emission 529 nm and 590 nm. The 529/590-nm emission ratio was used as a measure for free cytosolic [Ca^{2+}]. For positive control,
cultures were incubated for 10 min with the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (10 μM). Quantification of the fluorescence intensities in the green and red channels was accomplished by ImageJ software (NIH, Bethesda, Maryland, USA).

Western blot

Cultured cells (SMF-exposed or control) were harvested 5 or 10 h following etoposide (25 μM) treatment on DIV6. Cultures were washed with ice-cold PBS, scraped from wells and homogenized in Radio-Immunoprecipitation Assay (RIPA) buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetra-acetic acid, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM betaglycerophosphate, 1 mM Na3VO4, 1% protease inhibitor cocktail (Sigma–Aldrich), and 1% phosphatase inhibitor cocktail (Sigma–Aldrich). Following centrifugation at 3000g, supernatants were collected and protein concentration was determined by Lowry’s method (Waterborg and Matthews, 1984), with bovine serum albumin (BSA) as standard. Following heating at 95°C for 10 min, 30–80 μg protein was loaded and fractionated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then transferred onto a 0.2- or 0.45-μm nitrocellulose membrane. Membranes were blocked with either 5% non-fat dry milk or 5% BSA in PBS-Tween 20 (PBST) 0.1%, and then incubated with the following primary antibodies: anti-cleaved caspase-3 (1:1000), anti-cleaved caspase-9 (1:1000), anti-phospho-histone H2A.X (Ser139) (pH2A.X, 1:750, Millipore, Billerica, MA, USA), anti-H2A.X (1:1000), anti-cleaved poly ADP ribose polymerase-1 (PARP1; 1:1000), anti α-actin (1:1000), anti Cav1.2 (1:250, Alomone Labs, Jerusalem, Israel), and anti Ca v1.3 (1:200, Alomone Labs, Jerusalem, Israel), diluted in blocking buffer for 16–20 h at 4°C. Membranes were then washed with 0.1% PBST and the bound antibodies were detected by horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit 1:2000 or goat anti-mouse 1:2000) diluted in blocking buffer for 1 h at room temperature. Supersignal West Pico Chemiluminescent substrate kit (Pierce, Thermo scientific, Rochford, IL, USA) was used for detection, according to the manufacturer’s instructions. The density of the bands was quantified using Totallab software (Newcastle upon Tyne, UK).

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Real-time quantitative PCR (qPCR) analysis

Cultures were sham- or SMF-exposed for 72 h (DIV4–DIV6) and treated with/without 25 μM etoposide on DIV6 for 24 h. On DIV7, cells were washed with PBS and scraped from the wells using trypsin/EDTA (0.25% trypsin/0.05% EDTA; Biological Industries), and total RNA was isolated using a commercial kit according to the manufacturer’s instructions (5 Prime, Hilden, Germany). DNA was digested using On-Column RNase-free DNase (5 Prime). Following determination of nucleic acid concentration by Nanodrop (Thermo Fisher scientific Inc., Wilmington, DE, USA), RNA was reverse-transcribed to cDNA using a commercial kit (Applied Biosystems, Life Technologies, Grand Island, NY, USA), according to the manufacturer’s instructions. For qPCR, cDNA was reacted with Taqman mix and primers of interest (Life Technologies, Grand Island, NY, USA). Data were analyzed using the Real-Time PCR 7500 software (Applied Biosystems, Life Technologies) and the fold increase in gene expression was determined according to the ΔΔCT method. In each experiment, all samples were run in triplicate and normalized to the level of the house-keeping gene glucuronidase beta (GusB NM_017015.2). Three independent experiments were performed to determine the change in expression of two subtypes of the L-type VGCCs: alpha 1C subunit Ca,1.2 (CACNA1C, NM_012517.2), and alpha 1D subunit Ca,1.3 (CACNA1D NM_017298.1).

Ca2+ imaging

Following 7 days of SMF exposure, cultured cortical neurons (2.5 × 105 cells/well) were loaded with the fluorescent calcium sensitive dye Indo-1 acetomethyl ester (3 μM, Molecular Probes, Invitrogen, Eugene, OR, USA) in NBM for 20 min at 37 °C, diluted with NBM and incubated for an additional 20 min at 37 °C to allow a complete de-esterification of the dye. Cells were then washed three times with cold modified Tyrode’s solution (25 mM HEPES, 125 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 50 mM MgCl2, BSA 0.1%, w/v, glucose 0.1%, w/v, at pH 7.4), and taken to kinetic measurements in a 37 °C pre-warmed plate reader (Tecan 200 Pro, Männedorf, Switzerland). The 410/480-nm emission ratio at 355-nm excitation served as a measure for intracellular free Ca2+ concentration ([Ca2+]i). All compounds were added to the cultures using automatic injectors. For experiments using thapsigargin (ThG), cells were maintained in the modified Tyrode’s solution without CaCl2. ThG (10 nM) was added after a 70-s baseline recording, followed 100 s later by 2.5 mM CaCl2. In KCl-induced depolarization experiments, cells were maintained in the modified Tyrode’s solution supplemented with CaCl2 throughout the experimental period. Following the 70-s baseline recording, a pulse of KCl (50 mM) was injected followed 180 s later by the addition of vehicle or nifedipine (20 μM), and a second pulse of KCl was injected after 100 s following media change. The numbers of cells measured per well by the plate reader at the time of experiment (DIV7) were 7.77 × 104 ± 2.47 × 103 and 7.79 × 104 ± 3.2 × 103 in the control and SMF-exposed cultures, respectively. Additionally, in order to ensure that comparable cell numbers were examined in all treatments, protein count was performed at the end of the experiment.

Data and statistical analysis

Data were analyzed by two-tailed Student’s t-test or a one-way analysis of variance (ANOVA) and are presented as the mean value ± SEM. Significant ANOVAs were followed by Dunnett’s multiple comparison post hoc test. Differences between treated cultures and controls were considered significant when P < 0.05.

RESULTS

SMFs protect primary cortical neuronal cultures against etoposide-induced apoptosis in a dose- and time-dependent manner

Incubation of control (zero MF) cultures with 3–25 μM of the topoisomerase-II inhibitor etoposide added on DIV6 for 24 h resulted in a dose-dependent induction of apoptosis assessed on DIV7 by the Hoechst-33342 technique (Fig. 1a), optimally achieved using 12 or 25 μM etoposide, which induced a similar degree of apoptosis (37.3 ± 5.6% and 38.1 ± 2.1% apoptotic cells, respectively). Exposing the neurons to various intensities of SMFs (4–50 G) by placing the cultures at varying distances from magnets (Fig. 1b) yielded a field intensity-dependent decrease (of 25.2 ± 11.1% to 57.1 ± 6.3%) in the numbers of apoptotic cells in cultures treated with 12 μM etoposide (Fig. 1c). This was reflected by the reduced appearance of fragmented nuclei that appear as highly fluorescent white dots as a result of chromatin condensation, observed with Hoechst-33342 staining (Fig. 1d). The decrease in apoptosis level in cultures exposed to SMF intensity of 50 G and 12 μM etoposide was further verified using the TUNEL technique (Gavrieli et al., 1992), yielding 63.2 ± 9.7% less apoptosis compared with control cultures (Fig. 1e, f), which showed a very good agreement between the two methods used for the detection of apoptosis.

We next studied the time-course of the observed effect by exposing the neurons to 50-G SMFs for varying time durations (Fig. 2a), according to the protocol shown graphically in Fig. 2c. A 1-day exposure resulted in a 39.3 ± 4.3% reduction in the apoptotic cell fraction, which intensified on extended exposure, resulting in a 39.3 ± 4.3% reduction in the apoptotic cell fraction after 7 days of continuous exposure. We have recorded no incremental significant effect when extending the duration of exposure beyond 7 days (data not shown).

In order to examine the duration of the anti-apoptotic effect induced by 50-G SMFs, cultures were maintained off -SMFs for distinct time periods following the routine 7-day exposure, as detailed in the protocol depicted in Fig. 2d. Placing the cultures off-SMFs for 1.5, 2.5, 3.5, 6 or 24 h following 7 days of SMF exposure, we found a
gradual decrease in the extent of protection. Although reduced, the anti-apoptotic effect remained significant after 6 h off-SMF. By 24 h off-SMF, only a minor, statistically insignificant, reduction of 15.01 ± 3.03% in apoptosis was seen (Fig. 2b).

**SMFs reduce the expression of apoptotic markers in primary cortical neuronal cultures**

The possible interaction of SMFs with the neuronal apoptotic cascade was studied through the activation of several key proteins in this cascade, such as the cysteine proteases caspases 3 and 9. Caspase-3, which is cleaved and activated as a result of auto-activation of the upstream effector caspase-9, cleaves and inactivates the downstream PARP1, a protein involved in DNA repair. Incubating the cultures with either 12 or 25 μM etoposide resulted in similar levels of both apoptosis induction and SMF protection. However, due to enhanced detection of cleaved pro-apoptotic protein fragments in 25 μM etoposide-treated cultures, we used the latter concentration for apoptosis induction throughout western blot experiments. Extended time-course analysis revealed the activation pattern of these pro-apoptotic proteins, detected by the appearance of their cleaved forms in a shorter time course than that required for the detection of apoptotic cell death by microscopic analysis shown in Fig. 1a. We found that the expression of the pro-apoptotic proteins, which was either very low or undetectable in the absence of etoposide, peaked 5 h following apoptosis induction, decreasing 5 h later (Fig. 3a, b), and was no longer detected 24 h following etoposide treatment (data not shown). The expression of the above proteins after etoposide addition was markedly reduced in cultures exposed to 50-G SMFs. Caspase-9 activation was reduced by 72.9 ± 6.4% 10 h after apoptosis induction in SMF-exposed cells. The level of cleaved caspase-3 was decreased by 20.4 ± 5.4% and 81.2 ± 5.0% in SMF-exposed cells, 5 h and 10 h post apoptosis induction, respectively. A similar pattern was observed in the expression of cleaved PARP1 which was unaltered by SMF 5 h after apoptosis induction but was reduced by 41 ± 5.0% 10 h after apoptosis induction. SMF exposure also decreased the extent of H2A.X phosphorylation by 42.75 ± 2.9%, 10 h following apoptosis induction (Fig. 3b).

**SMFs reduce the extent of etoposide-induced MMP collapse in primary cortical neuronal cultures**

Neuronal apoptosis induced by etoposide is mediated by mitochondrial dysfunction, hallmarkd by the collapse of the MMP occurring early in the process. We employed the fluorescent lipophylic cationic molecule JC-1 to monitor the changes in MMP induced by SMF exposure (Salvioli et al., 1997). The aggregation of this probe in the mitochondria of healthy non-apoptotic cells results in red fluorescence emission (590 nm), whereas its diffusion from the mitochondria to the cytosol of damaged cells due to diminished MMP results in green fluorescence emission (529 nm) of the dye's monomeric form, indicative of low MMP characterizing apoptotic cells. The ratio between 529- and 590-nm emission of the dye (Fig. 4a) serves as a measure for the MMP. As depicted in Fig. 4b, SMF exposure did not affect the basal level of

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**Fig. 3.** SMF exposure reduces the expression of pro-apoptotic proteins in primary cortical neurons. Neurons were either sham-or 50-G SMF-exposed for 6 days, treated with 25 μM etoposide on DIV6 and harvested at the indicated time points (5 or 10 h) following etoposide treatment. The corresponding whole-cell lysates were extracted and subjected to western blotting analysis of the pro-apoptotic proteins. (a) Representative blots of cleaved PARP1, cleaved caspase-9, cleaved caspase-3 and H2A.X (Ser139). Actin was used as loading control. (b) Corresponding densitometric analyses of western blots obtained from lysates from three independent experiments. Proteins’ expression level was normalized to actin. The expression of H2A.X (Ser139) was normalized to the total expression of H2A.X. Data were quantified using Totallab software, and presented as mean ± SEM. *P < 0.05, **P < 0.01.
MMP compared with control (529/590-nm emission ratio: 0.0230 ± 0.009 and 0.0231 ± 0.009, respectively). Apoptosis induction by etoposide significantly increased the population of cells exhibiting low MMP in both control and SMF-exposed cultures, as reflected by the enhanced mean 529/590-nm fluorescence ratio in both sham- and SMF-exposed cultures, compared with their respective controls. Exposure to SMF decreased the extent of MMP collapse by 57.2 ± 1.0% compared with control cultures (529/590-nm emission ratio: 0.14 ± 0.03 and 0.33 ± 0.04, respectively; Fig 4b).

Differential protective effect of SMFs in primary cortical glial and neuronal cultures

Given the cell-type diversity characterizing neuronal cultures obtained from a primary source, our next goal was to examine whether the observed protective effect of SMFs may be attributed solely to its selective action on specific neuronal cell types, or could emanate from a combined action on neurons and other cell types, such as glial cells, comprising the primary cortical culture. For this purpose, we designed a series of experiments aimed at examining SMFs’ effect on apoptosis of cortical-derived glial cells in the same experimental conditions as those employed with primary cortical neurons. The extent of apoptosis induced in cortical-derived glial cells by 12 μM etoposide was lower than that induced in primary cortical neuronal cultures, which contain 12–15% glial cells (9.5 ± 0.8% vs. 37.3 ± 5.6%, respectively). Exposing glial cultures pretreated with etoposide to 50-G SMFs for 7 days elicited a 56.2 ± 0.2% decrease in the percentage of apoptotic cell fraction. In order to gain a better understanding of the differential effect of SMFs in different cell types and to further substantiate our findings on SMFs’ effect on the neurons, we decreased the percentage of the glial cells in the primary neuronal culture by selectively inhibiting their proliferation using the anti-proliferative compound cytosine arabinoside (Ara-c) (Sagara et al., 1993; Takeshima et al., 1994). Control cultures pre-incubated with Ara-c, as described in the Methodology section, and exposed to 12 μM etoposide exhibited a similar percentage of apoptosis as etoposide-treated cultures naïve to Ara-c (30.2 ± 3.0% vs. 29.6 ± 2.7%, respectively). Upon SMF exposure, primary

Fig. 4. SMF exposure decreases the extent of MMP loss in primary cortical neurons. Neurons were either sham- or 50-G SMF-exposed for 7 days, treated with 12 μM etoposide on DIV6 for 24 h and then loaded with JC-1 (3 μM). The dual emission of the dye was detected by a fluorescence microscope at 529 nm (green; reflects the diffusion of JC-1 monomers from the mitochondria to the cytosol in apoptotic cells) and 590 nm (red; reflects the accumulation of JC-1 aggregates in the mitochondria of healthy cells). Representative fluorescence images at 529 and 590 nm are shown in (a). (b) Relative fluorescence intensities were quantified using ImageJ software, and presented as Green: Red fluorescence ratio, reflecting the cells’ MMP status. ***P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
SMFs enhance ThG-induced capacitative Ca\(^{2+}\) influx in primary cortical neuronal cultures

In light of the link between the anti-apoptotic efficacy of SMFs seen here, and the existing evidence for altered Ca\(^{2+}\) influx in various cell lines following SMF exposure (see Introduction), we analyzed the effect of SMF exposure on free cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) in primary neuronal cultures following various stimuli. By determining the dual emission peak intensities of the Ca\(^{2+}\)-sensitive molecule Indo-1, we first examined the possibility that SMFs alter intracellular Ca\(^{2+}\) re-distribution due to the mobilization of Ca\(^{2+}\) from the endoplasmic reticulum (ER). As reflected by the similar ratio of emission intensities of Indo-1 at 410/480 nm, (Fig. 7a), SMFs did not affect the extent of passive Ca\(^{2+}\) release from the ER resulting from antagonizing the ER Ca\(^{2+}\)-ATPase with its irreversible inhibitor ThG (2.047 ± 0.004 in SMF exposure versus 2.034 ± 0.003 in control) (Thastrup et al., 1990). However, supplementing the medium with CaCl\(_{2}\), SMF-exposed cultures were able to increase their capacitative Ca\(^{2+}\) entry (following ER Ca\(^{2+}\) depletion) by 38.4 ± 0.4% (2.04 ± 0.003 and 2.82 ± 0.007 before and after Ca\(^{2+}\) addition), compared with 21.27 ± 0.29% (2.166 ± 0.003 and 2.619 ± 0.006 before and after Ca\(^{2+}\) addition) in the control (P < 0.0001).

**SMFs protect primary hippocampal neuronal cultures against etoposide- and Aβ\(^{1-42}\)-induced apoptosis**

We next examined the pro-survival effect of SMFs in primary hippocampal neurons relevant to neurodegenerative disorders such as AD, in which the formation and the extracellular accumulation of amyloid plaques predominantly composed of Aβ\(^{1-42}\) and Aβ\(^{1-40}\) peptides in hippocampal and cortical brain areas, is associated with neuronal degeneration by apoptosis (Masters et al., 1985; Smith et al., 2000). The extent of protection provided by 50-G SMF exposure in hippocampal neurons in the etoside-induced cell death model was similar to that observed in cortial cultures (55.9 ± 5.9% reduction in apoptotic cells Fig. 6a), although a higher concentration of the neurotoxin (50 μM) was required to induce apoptosis in the former.

Additionally, SMF exposure decreased the extent of Aβ\(^{1-42}\)-induced apoptosis by 59.0 ± 4.0% in cultures pre-incubated with 25 μM Aβ\(^{1-42}\) (25.8 ± 3.2% versus 10.6 ± 1.0% in control and SMF-exposed cultures, respectively) (Fig. 6b).

![Fig. 5. SMFs’ differential protective effect in primary giall cells and cortical neurons. Primary cortical glial cells (a) or primary cortical cultures (gla and neurons, b) were sham- or 50-G SMF-exposed for 7 days with or without 0.25 μM Ara-c, added to the cortical neuronal culture 48 h post seeding. The concentration of Ara-c was halved on DIV4, and maintained at 0.12 μM until DIV7. All cultures were incubated with 12 μM etoposide on DIV6 for 24 h. Nuclei were then stained and visualized by fluorescent Hoechst-33342 dye. The apoptotic sub-population of each treatment was quantified and the ratio of apoptotic nuclei/total nuclei is presented. **P < 0.01.](image)

**[Fig. 6. SMF exposure protects primary hippocampal neuronal cultures against etoposide and Aβ\(^{1-42}\)-induced cell death. Cultures were incubated in the presence or absence of 50-G SMFs for 7 days, and received either etoposide (12, 25 or 50 μM) (a) or Aβ\(^{1-42}\) (5, 10 or 25 μM) (b) on DIV6 followed by Hoechst-33342 staining on DIV7. The apoptotic nuclei sub-population was analyzed and presented as percentage of total cell number. **P < 0.01, ***P < 0.001.](image)
SMF exposure enhances both the mRNA and protein expression of L-type VGCCs and the extent of inhibition of KCl-induced Ca\(^{2+}\) influx by nifedipine in primary cortical neuronal cultures

Next, we considered the possibility that SMFs may affect Ca\(^{2+}\) influx through VGCCs in response to depolarization. To test this hypothesis and determine the contribution of L-type VGCCs to the observed Ca\(^{2+}\) signals, we first compared the level of [Ca\(^{2+}\)]\(\text{cyt}\) after KCl-depolarization stimuli before and after treatment with the selective reversible L-type VGCC inhibitor nifedipine (Tsien et al., 1988).

Depolarizing the neurons with 50 mM KCl evoked VGCC-mediated Ca\(^{2+}\) influx in both SMF-exposed and control neurons, with a similar ability to generate intracellular Ca\(^{2+}\) increase in response to membrane depolarization. However, antagonizing KCl-induced Ca\(^{2+}\)-influx with nifedipine caused a more profound inhibition of Ca\(^{2+}\)-influx in SMF-exposed cultures, resulting in a 22.7 ± 0.05% decrease in the ability of SMF-exposed neurons to maintain Ca\(^{2+}\) influx, compared with a 10.4% decrease in control (2.6 ± 0.007 versus 2.0 ± 0.005 in SMF-exposed cells before and after nifedipine, and 2.5 ± 0.005 versus. 2.2 ± 0.008 in control cultures before and after nifedipine, respectively (Fig. 7b). The difference between the decrements (0.329 ± 0.007) is statistically significant at the level of \(P < 0.0001\). Moreover, we found that SMF exposure increases the mRNA expression of the L-type VGCCs Ca\(_{1.2}\) and Ca\(_{1.3}\) after 72 h of exposure (2.30 ± 0.12-fold and 1.65 ± 0.07-fold, respectively, Fig. 7c). Additionally, the protein expression of Ca\(_{1.2}\) and Ca\(_{1.3}\) was increased following 7 days of SMF exposure (2.30 ± 0.12-fold and 2.58 ± 0.15-fold, respectively, Fig. 7d).
The anti-apoptotic effect of SMFs in primary cortical neurons is blocked by nifedipine

Exposure of primary cortical neurons to SMFs induced a reduction of 54.64 ± 1.64% in etoposide-induced apoptosis. Blocking Ca_2+ channels by pre-treating the neurons with 20 μM nifedipine resulted in diminished protection by SMFs, yielding no difference in apoptosis level between SMF-exposed and control cultures (Fig. 7e, 25.38 ± 1.68% and 22.10 ± 1.50%, respectively, apoptotic cells as% of total cells in culture).

**DISCUSSION**

A growing body of evidence accumulated over the past few decades suggesting a wide range of biological effects exerted by weak SMFs, raises the intriguing possibility of utilizing the anti-apoptotic capacity of SMFs, demonstrated thus far mainly in non-neuronal cell types, in treating neurodegenerative disorders.

In the present study, we show that weak static field strength antagonizes toxin-induced apoptosis in primary cortical and hippocampal neuronal cultures and provides a time-dependent protection against toxic insult in primary cortical neurons. Moreover, the protection, found to be optimally induced after 7 days of continuous exposure, was preserved up to 6 h in the absence of SMFs, reducing after 24 h off the field. The fact that the anti-apoptotic effect was sustained for several hours after removing the SMF alludes to the induction of long-term alterations, such as in protein expression. It should be noted, however, that the different exposure times of cultures to SMFs necessary to characterize the time dependency of the protective effect, entails dissimilarity in the stage of development and differentiation between the cultures, which were initially exposed to SMFs on different DIVs.

During neuronal apoptosis, which is characterized by a conserved morphological phenotype, the dying neuron exhibits shrinkage, plasma membrane (PM) blebbing, nuclear chromatin condensation and DNA fragmentation, resulting in the formation of apoptotic bodies (Kerr et al., 1972). Apoptosis induced at the mitochondrial level (intrinsic pathway) is hallmarked by a collapse of the MMP, the maintenance of which is critical for cell survival (Chong and Maiese, 2004), and by the downstream recruitment and activation of several caspases, notably, caspase-9 and caspase-3 (Hengartner, 2000; Degterev et al., 2003), major executioners of apoptosis (Charriaut-Martiange, 2004). As the vast majority of neurons have limited regenerative capacity throughout life, the prevention of, and protection against, DNA damage is of paramount importance. Indeed, a substantial fraction of the neurons’ proteome is targeted at apoptosis prevention. This defense array includes proteins that remove damaged proteins, others aimed at decreasing oxidative stress, as well as proteins involved in DNA repair such as PARP1, a caspase-3 substrate DNA binding-enzyme that detects and binds to single-strand DNA breaks in response to DNA damage (Decker and Muller, 2002), and H2A.X, a DNA-double strand breaks marker which is phosphorylated at Ser139 position in response to DNA damage (Orth et al., 1996; Fernandez-Capetillo et al., 2004). Here, we show for the first time that 50-G SMF exposure reduces the expression of active caspase-9, cleaved caspase-3, cleaved PARP1 and pH2A.X in primary cortical neurons in which apoptosis was stimulated with etoposide. The stabilization of the MMP found in the SMF-exposed neurons further supports the interference of SMFs with key events in the apoptotic cascade. This is consistent with other studies reporting a reduction of toxin-induced apoptosis by SMF exposure in non-neuronal cells (Fanelli et al., 1999; Teodori et al., 2002). In the absence of etoposide, however, SMF did not provide protection against the spontaneous, low-level apoptosis which occurs in neuronal cultures, nor did it alter the biochemical expression profile of the above mentioned pro-apoptotic proteins, whose activation was minor, and below the quantification threshold (data not shown). Similarly, Hirai et al. (2005) found that repetitive SMF exposure (15 min daily, 100 mT, for up to 9 days) prevented the reduction in MAP-2 expression in primary hippocampal neurons in response to the glutamate receptor antagonist MK-801, but produced no effect on the neurons’ survival. It should be noted, however, that several studies do provide evidence for modulation of SMF’s effect on spontaneous apoptosis (Dini and Abbro, 2005). The modulation of apoptosis by SMFs as well as by extremely low frequency MFs (ELMFs) is a well-known phenomenon demonstrated in a variety of cell lines. However, the reported findings are ambiguous. Whereas some studies demonstrate the inhibition of apoptosis in response to MF-exposure (Fanelli et al., 1999; Teodori et al., 2002; Pirozzoli et al., 2003; Oda and Koike, 2004; Tenuzzo et al., 2006), others attest to enhanced apoptosis rate (Zmlysony et al., 2000; Tofani et al., 2001; Tenuzzo et al., 2006; Ahmadianpour et al., 2013). These conflicting findings may be attributed to the marked variation in experimental conditions across different studies, in terms of physical and biological parameters such as type of field (static, time varying or combination of the two), the duration and pattern of exposure (continuous or periodic) and the specific neurotoxin used to induce apoptosis. Additionally, the variation in the type and origin of the exposed cells (primary source versus stabilized or transformed cell lines, and excitable or non-excitatory membranes), may explain, at least partially, the conflicting findings regarding the pro-survival action of MFs. Most research in the field is carried out in the context of possible carcinogenic or mutagenic effects of MFs, and is therefore based on transformed or stabilized cell lines such as human neuroblastoma, PC12, astrocytoma and glioblastoma (Tofani et al., 2001; Pirozzoli et al., 2003; Grassi et al., 2004). Such rapidly proliferating cell lines are known to possess altered electrical membrane properties including differently depolarized cell membranes, compared with normal cells obtained from a primary source (Marino et al., 1994; Cuzick et al., 1998; Tofani et al., 2003). Moreover, the concentration of free radicals, well-known apoptosis mediators whose excessive generation contributes to the oxidative stress involved in the patho-etiology of neurodegenerative diseases, is higher in transformed versus non-transformed cell lines (Sztadowski and Nathan,
Given the mechanical forces exerted by SMFs on charged particles (Rosen 1993a) such as free radicals, such cell lines may be affected differently by SMFs, compared with primary cells.

Our results also indicate a protective effect exerted by SMFs on glial cells, whose apoptosis is implicated in the pathogenesis of neurodegenerative disorders (Kitamura et al., 1999; Shimohama, 2000). The lower extent of apoptosis induced by etoposide in primary glial cells compared with the primary cortical cultures consisting of neurons and 15% glial cells, is in line with other studies reporting that less apoptosis is induced in astrocytes by topoisomerase inhibitors such as camptothecin (Morris and Geller, 1996). The lower sensitivity of astrocytes to neurotoxins may be attributed to a greater rate of gene transcription in astrocytes than in neurons (Morris and Geller, 1996; Flangas and Bowman, 1970) as well as to the difference in the apoptotic machinery suggested by the reduced sensitivity of astrocytes to various apoptotic inducers [Dwyer et al., 1995; Koh et al., 1995]. Despite the lower apoptotic induction rate, we show here that SMFs protected glial cells against etoposide-induced apoptosis to a similar extent to that seen in the primary cortical culture. Experiments done in the presence of Ara-c, that reduced the fraction of glial cells in the primary cortical culture to 5.0 ± 1.1%, showed a similar reduction in apoptosis in response to SMFs in the absence of Ara-c, suggesting only a secondary contribution of glial cell protection to the overall observed effect. In our model system, therefore, SMFs act primarily as neuroprotective agents. Nevertheless, the possibility that other actively proliferating cells, such as neuroblasts, present in the primary culture may also be affected by SMFs should not be excluded at this stage, and is currently being investigated in our laboratory.

Our findings showing enhanced gene expression of the L-type VGCCs α subunits are in line with altered gene expression commonly described in response to MF exposure (Hiraoka et al., 1992; Potenza et al., 2004; Hirai and Yoneda, 2005; Tenuzzo et al., 2009; Wooseok et al., 2012). Taken together with the enhanced VGCC protein expression, and the twofold inhibition of KCl-induced Ca$^{2+}$ influx by nifedipine along with the complete abolishment of the ability of SMF exposure to reduce the pro-apoptotic effect of etoposide in cortical neurons, our findings strongly indicate the involvement of L-type VGCCs in both the cellular response to SMFs and the defense provided by the field against toxin-induced cell death. Influx of Ca$^{2+}$ through L-type VGCCs in cortical neurons has been shown to activate downstream signaling that affects gene expression involved in cell proliferation, neuronal differentiation and apoptosis (Berridge, 1998; Marshall et al., 2003; Grassi et al., 2004; Toescu et al., 2004). In particular, Ca$^{2+}$ influx through Ca1.2 and Ca1.3 was shown to activate signaling cascades from the PM to the nucleus (Hardingham et al., 1998; Berridge et al., 2000; Toescu et al., 2004) associated with promotion of cell survival (West et al., 2001). It was also shown that repetitive SMF exposure (100 mT) in primary cultures of hippocampal neurons increases the expression of N-methyl-D-aspartate (NMDA) receptor subtypes (Hirai and Yoneda, 2005a), and enhances the effect of NMDA to increase cytoplasmic free Ca$^{2+}$ levels. Rosen (1993b, 2003) has previously suggested that SMFs may directly affect PM proteins, such as calcium channels, thereby increasing their ion permeability. In addition to a voltage-gated Ca$^{2+}$ entry through transmembrane Ca$^{2+}$ channels in response to depolarization, or to Ca$^{2+}$ entry via receptor-operated channels (e.g., NMDA), Ca$^{2+}$ may also enter excitatory cells via SOCs (Penner et al., 1993; Putney, 2001; Uehara et al., 2002). These channels are activated as a result of intracellular Ca$^{2+}$ mobilization, such as ER Ca$^{2+}$ depletion by ThG, that induces a subsequent Ca$^{2+}$ entry termed capacitative Ca$^{2+}$ influx (Putney, 2003), aimed at replenishing ER Ca$^{2+}$ stores (Emptage et al., 2001; Parekh and Putney, 2005). This form of Ca$^{2+}$ entry is considered ubiquitous in non-excitable cells, and has also been recognized in some neuronal cell types (Usachev and Thayer, 1999; Emptage et al., 2001). The significance of enhanced capacitative Ca$^{2+}$ entry in cortical neurons in response to SMF exposure described in this study is emphasized due to the involvement of impaired capacitative Ca$^{2+}$ entry in the patho-physiology of neurodegenerative diseases. For instance, it has been shown that gene mutations associated with AD resulted in diminished capacitative calcium entry and reduced agonist-induced Ca$^{2+}$ release from stores (Yoo et al., 2000). The role of increased cytosolic Ca$^{2+}$, however, is ambiguous because it depends on both the duration of elevated Ca$^{2+}$ concentrations and the response of the specific cell type to this elevation. Thus, increased cytosolic Ca$^{2+}$, may either trigger apoptosis if the [Ca$^{2+}$], reaches toxic levels (McConkey et al., 1989; Choi, 1992), or serve to protect cells against apoptosis (Franklin and Johnson, 1992). Additional experiments aimed at clarifying the exact pathway via which Ca$^{2+}$ influx through these channels is involved in SMF’s effects in primary cortical neurons are required, and are currently being carried out in our laboratory.

CONCLUSION

We show here that SMFs provide neuroprotective activity related to their anti-apoptotic capacity, which is mediated by Ca$^{2+}$ influx through L-type VGCCs. Our findings show that SMFs provide a dose-dependent protection to neurons against neurotoxin-induced apoptosis, accompanied by the stabilization of the MMP and the decrease in the expression of the pro-apoptotic cleaved caspase-9 and -3, PARP1 and pH2A.X. Furthermore, we show that cortical neurons exposed to SMFs exhibit altered Ca$^{2+}$ influx in response to different stimuli. These findings emphasize the susceptibility of the CNS to weak SMF exposure and may provide a basis for new strategies in treating neurodegenerative disorders.

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