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## Electromagnetic fields affect transcript levels of apoptosis-related genes in embryonic stem cell-derived neural progenitor cells

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### ABSTRACT

Mouse embryonic stem (ES) cells were used as an experimental model to study the effects of electromagnetic fields (EMF). ES-derived nestin-positive neural progenitor cells were exposed to extremely low frequency EMF simulating power line magnetic fields at 50 Hz (ELF-EMF) and to radiofrequency EMF simulating the Global System for Mobile Communication (GSM) signals at 1.71 GHz (RF-EMF). Following EMF exposure, cells were analyzed for transcript levels of cell cycle regulatory, apoptosis-related, and neural-specific genes and proteins; changes in proliferation; apoptosis; and cytogenetic effects. Quantitative RT-PCR analysis revealed that ELF-EMF exposure to ES-derived neural cells significantly affected transcript levels of the apoptosis-related *bcl-2*, *bax*, and cell cycle regulatory “growth arrest DNA damage inducible” *GADD45* genes, whereas mRNA levels of neural-specific genes were not affected. RF-EMF exposure of neural progenitor cells resulted in down-regulation of neural-specific *Nurr1* and in up-regulation of *bax* and *GADD45* mRNA levels. Short-term RF-EMF exposure for 6 h, but not for 48 h, resulted in a low and transient increase of DNA double-strand breaks. No effects of ELF- and RF-EMF on mitochondrial function, nuclear apoptosis, cell proliferation, and chromosomal alterations were observed. We may conclude that EMF exposure of ES-derived neural progenitor cells transiently affects the transcript level of genes related to apoptosis and cell cycle control. However, these responses are not associated with detectable changes of cell physiology, suggesting compensatory mechanisms at the translational and posttranslational level.

Key words: mouse embryonic stem cells • neuronal differentiation • *GADD45* • *bcl-2* • *bax*

**P**luripotent embryonic stem (ES) cells differentiate into specialized cells of all three primary germ layers. During differentiation, ES cells recapitulate processes of early embryonic development and therefore provide a unique model to analyze in vitro the influence of genetic or environmental factors on cellular differentiation (1–3). ES-derived cells

have been used for the analysis of embryotoxic properties of chemical agents (4, 5) but may also be applied to analyze the effects of physical factors, such as electromagnetic fields (EMF) (6–8).

EMF in the human environment are generated by mobile communication systems, in Europe particularly by the Global System for Mobile Communication [GSM; GSM1800 uplink: 1.71–1.85 GHz; radiofrequency (RF)-EMF; for review, see ref 9], as well as by power lines and consumer devices generating extremely low frequency (ELF) magnetic fields (in Europe: 50 Hz and harmonics, ELF-EMF). The high level of anthropogenic EMF in the environment (“electro smog”) raised questions about their effects on human health. Epidemiological studies performed to elucidate potential hazardous effects of EMF from electrical wiring and equipment (10–12) and from RF-EMF (13–15) revealed controversies about the involvement of EMF in the origin of cancer and/or neurological disorders.

In vitro studies showed molecular and cellular reactions in response to ELF-EMF exposure, including increase of apoptosis (16), the induction of DNA breaks (17), increased intracellular calcium (18) and gene expression levels (19), acceleration of nerve regeneration (20), and cardiac differentiation (21). Other studies presented evidence for ELF-EMF effects on protein phosphorylation (22, 23) and on the activity of stress proteins (24) and kinases (25, 26). In vivo studies demonstrated an increased level of apoptotic cells in mouse brain and liver (27). Carcinogenic effects of RF-EMF exposure have been described in animal models in vivo (28–31), whereas other studies did not reveal correlations between RF-EMF exposure and tumor initiation and promotion (32–34). However, GSM radiation was shown to activate the hsp27/p38MAPK stress response pathway in human endothelial cells (35). Our previous studies using undifferentiated ES cells showed that RF-EMF and ELF-EMF exposure at defined field strength and intermittency schemes resulted in up-regulation of transcript levels of various regulatory genes only in cells deficient for the tumor suppressor p53 (6, 8).

The identification of potential hazardous effects of EMF on neural progenitor and neuronal cells is of special importance. Given the high level of spontaneous activity and the number of interacting neurons, the nervous system is considered to be sensitive to electric fields and the induction of currents. It has been suggested that induced current densities  $>10 \text{ mA/m}^2$  may affect central nervous system functions (36). Recent data showed significant morphological changes in cultured human neuronal cells after short exposure to a 0.2 T static magnetic field (37). Using PC12 cells, it was found that low ELF-EMF (50 Hz) exposure significantly inhibited or stimulated neuronal differentiation dependent on culture conditions, whereas cell division was not altered (38). In vivo, rats exposed to microwaves from GSM mobile phones revealed nerve cell damage due to an increased albumin intake across the blood-brain barrier (39), and most important, 60 Hz magnetic fields caused a significant increase in single- and double-strand DNA breaks (40).

By using the ES cell differentiation model, here, we show effects of RF- and ELF-EMF on neural progenitor cells by quantitative RT-PCR analysis and the COMET (single cell gel electrophoresis) assay. We demonstrate that EMF induces changes of transcript level of apoptosis-related genes and a low, but significant, increase of DNA breaks in neural progenitor cells, suggesting cellular responses to EMF in wild-type neural stem/progenitor cells. However, we could not detect cytogenetic effects by metaphase analysis of chromosomal aberrations (CA) and sister chromatid exchanges (SCE). BrdU labeling did not reveal differences in the cell proliferation rate of EMF- and sham-exposed cells. By a mitochondrion-selective dye, we could

not detect changes in the mitochondrial function, and quantification of apoptosis by FACS analysis of hypodiploid DNA content (“subG1 population”) showed no differences in the percentage of the subG1 fractions of sham and ELF- and RF-EMF-exposed variants, respectively. These findings suggest that EMF are able to induce changes at the transcript level in ES-derived neural progenitor cells, but these responses may not affect cell physiological functions.

## **MATERIALS AND METHODS**

### **EMF exposure**

EMF exposure of neural precursor cells was performed under defined conditions with respect to field strengths, polarization, modulation, and temperature (41). The exposure setups ensured studies under “blind” conditions and the control of temperature differences for sham- and EMF-exposed cultures within a 0.2°C temperature range. Incident and induced field distribution were characterized by numerical simulation and by dosimetric measurements.

### **RF-EMF exposure**

The RF-EMF setup is defined to operate at 1.71 GHz, which is within the up-link band of the GSM1800 system, also named GSM DCS (Digital Communication System; for details, see ref 7). The setup consists of 2 R14 waveguides (1 for EMF exposure, 1 for sham exposure) enclosed in a conventional humidified incubator (5% CO<sub>2</sub>, 37°C). Petri dishes (60 mm diameter) are positioned in the H-field maximum of a standing wave and exposed in E-polarization. To guarantee atmospheric and temperature exchange, sensor-monitored fans are mounted that enforced airflow through the waveguide chamber. The waveguide chambers contain field and temperature sensors. The signal unit consists of an RF generator, arbitrary function generator, frame generator, and 5 W narrow-band amplifier. The RF output is directed via the computer-controlled RF switch to one of the waveguides (blinded). The entire setup is computer controlled, enabling the setting of the modulation scheme as well as the exposure strength for the different media and culture applications. The exposure strength and temperature as well as all commands are logged to an encrypted file, which is evaluated after the experiments in order to ensure studies under blind conditions. The temperature difference between sham- and EMF-exposed cultures never exceeds 0.2°C. The GSM signals are amplitude modulated by rectangular pulses with a repetition frequency of 217 Hz and a duty cycle of 1:8 (pulse width 0.576 ms), corresponding to the dominant modulation component of GSM. Signals are applied to the cells at time-averaged SAR values of 1.5 W/kg (corresponding to slot SAR=12 W/kg) for 6 and 48 h, respectively, with intermittency cycles of 5 min on/30 min off.

### **ELF-EMF exposure**

An ELF-EMF exposure setup based on two magnetically shielded four-coil systems (42) is used. Two fans per chamber ensure a rapid CO<sub>2</sub> exchange between chambers and incubator. The airflow temperature is monitored with accurate Pt 100 probes fixed inside the exposure chamber, and the differences between field and sham chambers can be kept below 0.2°C. The custom-designed current source is based on four audio amplifiers and allows arbitrary field variations in the frequency range from direct current (DC) to 1.5 kHz, with field strengths and magnetic flux densities up to 1.83 kA/m and 2.3 mT, respectively. The signal is generated by a computer-

controlled arbitrary function generator. The currents in the bifilar coils can be randomly switched parallel for field exposure or nonparallel for sham control by the computer. This exposure setup is designed to apply blind protocols and additionally to avoid temperature artifacts between exposed and control coils. The coil current and consequently the magnetic field is quasi-continuously (30 s intervals) recorded and regulated using resistors providing very low-temperature sensitivity. The induced electric fields resulting from the ELF-EMF exposure can be expressed as

$$E \text{ [V/m]} = 664 \text{ [V/(Tm}^2\text{)]} * r \text{ [m]} * B \text{ [T]}$$

whereby  $r$  is the radial distance from the dish center, and  $B$  is the average B-field. The estimated acceleration load due to B-field-induced coil vibrations is  $<1 \text{ m/s}^2$  (0.1 g) for the exposed petri dishes. This acceleration is a factor of 20 above the minimal background level for sham petri dishes.

ES-derived neural cells were exposed for 6 and 48 h, respectively, with intermittency cycles of 5 min on/30 min off. A magnetic flux density of 2 mT was applied for the power line signal consisting of a dominant 50 Hz sinusoidal and harmonics up to 1250 Hz. The amplitude distribution of the harmonics was derived by the maximum accepted distortions for power systems by the IEC (43).

## ES cell culture and differentiation

ES R1 cells (44) were grown on feeder layer of mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium (DMEM, Invitrogen GmbH, Karlsruhe, Germany) supplemented with 15% heat-inactivated fetal calf serum (FCS, Invitrogen, selected batches), 2 mmol/l L-glutamine, nonessential amino acids (NEAA, stock solution diluted 1:100), and penicillin-streptomycin (stock solution diluted 1:100; all from Invitrogen), 100  $\mu\text{mol/l}$   $\beta$ -mercaptoethanol (Serva, Heidelberg, Germany), and 10 ng/ml recombinant human leukemia inhibitory factor (LIF; for preparation, see ref 45), as previously described (1).

For differentiation of neural cells (Fig. 1), ES cells were cultivated in "hanging drops" ( $n=200$  cells/drop) as aggregates ("embryoid bodies," EB) for 2 days. EB were transferred into bacteriological petri dishes (Greiner, Frickenhausen, Germany) and cultivated for 2 days in Iscove's modification of DMEM (IMDM, Invitrogen) containing 15% FCS and supplements as previously described (1), with the exception that  $\beta$ -mercaptoethanol is replaced by 450  $\mu\text{mol/l}$   $\alpha$ -monothioglycerol (Sigma, Steinheim, Germany). EB ( $n=20-30$ ) were plated onto tissue culture dishes ( $\varnothing 6 \text{ cm}$ ) at day 4 and cultivated in IMDM + 10% FCS. The selective differentiation into neural progenitor and differentiated neuronal cells was carried out according to ref 46. Briefly, 1 day after attachment of EB, the medium was exchanged by DMEM/F12 medium supplemented with 5  $\mu\text{g/ml}$  insulin, 0.3  $\mu\text{mol/l}$  sodium selenite (all from Sigma), 50  $\mu\text{g/ml}$  transferrin, and 5  $\mu\text{g/ml}$  fibronectin (all from Invitrogen), referred to as ITSFn medium, and cultured for 7 days (4+7 d). Medium was replenished every 2 days. RF-EMF or ELF-EMF exposure was performed at day 4+4 for 6 and 48 h, respectively, at a stage when nestin-positive neural precursor cells develop (Fig. 1B). At day 4+8, EB were dissociated by 1 min incubation with prewarmed ( $37^\circ\text{C}$ ) 0.2% trypsin (Invitrogen) and 0.02% EDTA (Sigma) (1:1, v/v) solution, collected by centrifugation, and replated onto poly-L-ornithine/laminin-coated tissue culture dishes into DMEM/F12 containing 20 nmol/l progesterone, 100  $\mu\text{mol/l}$  putrescine, 1  $\mu\text{g/ml}$  laminin (all from

Sigma), 25 µg/ml insulin, 50 µg/ml transferrin, and 0.3 µmol/l sodium selenite, referred as “nestin cell-expansion medium,” for 6 days until day 4+13. The medium was exchanged every 2 days. Basic fibroblast growth factor (bFGF; 10 ng/ml) and 20 ng/ml epidermal growth factor (EGF) (both from Strathmann Biotech, Hanover, Germany) were added daily. At day 4+14, neuronal differentiation was induced by “Neurobasal” medium plus 2% B27 supplement (Invitrogen), 10% FCS and by the addition of survival promoting factors such as interleukin-1β (IL-1β, 200 pg/ml; supplemented daily; PeproTech, London, UK) and db-cAMP (700 µmol/l, added every 4 days; Sigma). Glial cell line-derived neurotrophic factor (GDNF, 2 ng/ml; R&D Systems, Wiesbaden, Germany) and transforming growth factor (TGF)-β3 (2 ng/ml; PeproTech) were applied at days 4+18 and 4+21. Neurturin (NTN, 10 ng/ml; PeproTech) was added at day 4+21. The application of survival promoting factors during terminal stages was combined with medium changes at 3-day intervals. The total time of neuronal differentiation was 4+23 days.

### Detection of mRNA levels by quantitative RT-PCR

Cells were collected at day 4+4 (control) and after EMF exposure at the days indicated in [Fig. 1](#). Samples were suspended in lysis buffer (4 mol/l guanidinium thiocyanate, 25 mmol/l sodium citrate [pH 7], 0.5% sodium N-lauroyl-sarcosinate, 1% β-mercaptoethanol). Isolation of total RNA, reverse transcription, and semiquantitative PCR amplification of cDNA was done as previously described (1). Quantitative (Q) RT-PCR analysis was performed with specific primers and TaqMan probes (designed by Primer Express 2.0; bax primers were used according to ref 47). The TaqMan probes for Q-RT-PCR were 5'-labeled with FAM (6-carboxyfluorescein) and with 3' quencher, TAMRA ([Table 1](#)). To avoid amplification of contaminating DNA, primers were designed with an intron sequence inside the amplicon, and template-free controls were used as negative controls (48). The melting temperature of the TaqMan probe was adjusted to be 10°C higher than melting temperatures of sense and anti-sense primers. Reactions ( $n=10$  per variant, 2 replicates from 5 separate experiments) were carried out in 96-well plates in the iCycler (Bio-Rad, Hercules, CA) using iCycler IQ Optimal Software, Version 3.0.6070 (Bio-Rad). The cDNA (3 µl) was transferred to an optical microtiter well containing 5 µl 10× PCR buffer, 3 µl 25 mmol/l MgCl<sub>2</sub>, 1 µl AmpliTaq DNA polymerase (all from Applied Biosystems, Foster City, CA), 1 µl 10 mmol/l dNTP (MBI Fermentas, St. Leon-Rot, Germany), 2 µl 10 pmol/µl forward primer, 2 µl 10 pmol/µl reverse primer, 1 µl 10 pmol/µl TaqMan probe (all from Metabion, Martinsried, Germany), and 32 µl DEPC-H<sub>2</sub>O (for preparation, see ref 1). The mouse GAPDH gene was used as reference (amplified in separate wells). The reactions were incubated at 95°C for 10 min, then cycled 50 times between 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The relative amount (in %) of bcl-2, bax, p53, GADD45, Nurr1, TH, and nestin mRNA levels was calculated using the comparative Ct method as described in the Relative Quantitation Of Gene Expression: ABI PRISM 7700 Sequence Detection System (49). Gel electrophoresis was performed to confirm the correct size of the amplified product. The statistical analysis was performed by the nonparametric Mann-Whitney test with the GraphPad InStat v.2.04 program (GraphPad Software, San Diego, CA).

### COMET (single cell gel electrophoresis) assay

The COMET assay was used to detect single-strand DNA breaks and alkali labile sites (alkaline assay) and double-strand DNA breaks (neutral assay). ELF-EMF- and RF-EMF- and sham-exposed ES-derived neural progenitor cells were dissociated by 1 min incubation of cells with prewarmed (37°C) 0.2% trypsin:0.02% EDTA (1:1, v/v) (1). The cells were resuspended into

cold DMEM with 15% FCS to inactivate trypsin. The cell density was adjusted to  $\sim 5 \times 10^5$  cells/ml, and the samples were placed on ice.

The COMET assay (50) was used with modifications (17). After a 6 h exposure, the alkaline and neutral COMET assays were performed immediately after ELF- or RF-EMF exposure ( $t=0$  h) and after a recovery time of 18 h ( $t=18$  h). After 48 h exposure, the alkaline and neutral COMET assays were done immediately after ELF- or RF-EMF exposure ( $t=0$  h) and following a recovery time of 24 and 48 h, respectively. Cells were analyzed using the fluorescence microscope ECLIPSE E600 (Nikon, Germany), appropriate excitation and barrier filters at 200-fold magnification, and the imaging software Lucia (Version 4.71 for Windows). A total of 1000 nuclei ( $n=500$  per slide) was analyzed under blind conditions in exposed and sham-exposed cells, respectively, and classified into 5 groups according to tail length and intensity using the classification according to ref 51. DNA damage was defined by the tail factor (for calculation, see ref 17).

Results of the alkaline and neutral COMET assays obtained from 6 independent experiments are presented as mean  $\pm$  SE. Statistical significance was tested by Student's *t* test. The number of COMETs in EMF- and sham-exposed cells classified into corresponding groups according to the DNA damage percent were compared by Chi-square test analysis of contingency tables.

### **Detection of chromosomal aberrations and sister chromatid exchanges**

For measuring CA and SCE, standard protocols (52) were applied with some modifications. Briefly, immediately after EMF or sham exposure, fresh culture medium containing BrdU (10  $\mu$ mol/l, 5-Bromo-2'-deoxyuridine Labeling and Detection Kit I, Roche, Mannheim, Germany) was added to the cells for 24 h. Colchicine (20 nmol/l) was added to the medium 9 h before harvest. After trypsinization, cells were treated with prewarmed 75 mmol/l KCl at 37°C for 10 min and fixed twice in cold methanol:acetic acid (3:1, v/v) for 2 h at  $-20^\circ\text{C}$ . Cells were collected by centrifugation, resuspended in fresh fixative, and dropped onto clean wet, cooled slides. Sister chromatids of the metaphase chromosomes were differentially stained after mitotic division by a modification of the original FPG technique (53). One hundred metaphases in the first mitosis (M1) were evaluated for chromosomal aberrations, and 30 metaphases in the second mitosis (M2) were evaluated for SCE. Gaps were not included in the final evaluation of CA. Aberration frequencies were expressed as aberrations/cell and percentage of cells with aberrations. SCE were expressed as SCE/cell. The statistical comparison between sham-exposed and ELF-EMF- and RF-EMF-exposed cells, respectively, was performed by the nonparametric Mann-Whitney test.

### **Proliferation analysis by BrdU incorporation**

The neural progenitor cells grown on poly-L-ornithine/laminin-coated cover slips were exposed to ELF- or RF-EMF. After BrdU treatment (10  $\mu$ mol/l, 24 h), the cover slips were washed in phosphate-buffered saline (PBS), incubated in 2 N HCl at room temperature for 30 min, and fixed in methanol:acetone (7:1, v/v) at  $-20^\circ\text{C}$  for 10 min. After washing three times in PBS, 1% BSA in PBS was applied as a blocking agent for 30 min, followed by incubation with primary rat anti-BrdU (Abcam Ltd., Cambridge, UK) and primary mouse anti-nestin antibodies (clone rat-401, Developmental Studies Hybridoma Bank, Iowa City, IA, diluted 1:3 in PBS) for 90 min, then secondary rabbit anti-rat (Cy3-labeled, 1:800 in 1% BSA in PBS, Jackson ImmunoResearch

Laboratories, West Grove, PA), and Alexa 488-labeled goat anti-mouse secondary antibodies (diluted 1:100 with 1% BSA in PBS, Molecular Probes, Eugene, OR) for 60 min. Nuclei were stained by Hoechst 33342, and the cover slips were mounted in Vectashield medium (Vector Laboratories, Burlingame, CA) on SuperFrost glass slides (Menzel, Braunschweig, Germany). BrdU-positive and nestin-positive (BrdU+/nestin+) cells in the EB outgrowths ( $n=10$  fields of  $n=2$  preparations) were estimated as percentage of Hoechst 33342-labeled cells using the fluorescence microscope ECLIPSE E600 (Nikon) and the confocal laser scanning microscope LSM 510 META (Zeiss, Munich, Germany).

In the differentially stained metaphase preparations, a minimum of 200 metaphases was scored per variant, the percentages of metaphases in M1, M2, and M3 were determined and the M1:M2:M3 ratio was used to analyze cell cycle progression. Chi-square test was applied for statistical comparisons.

### **Analysis of mitochondrial function by Mitotracker CM-H2X ROS**

Mitotracker CM-H2X ROS is a cell-permeable mitochondrion-selective stain, which is concentrated by active mitochondria and retained after cell fixation (54). It is a reduced form of the Mitotracker ROS probe, which is nonfluorescent, but becomes fluorescent when oxidized by reactive oxygen species (ROS). Parts of the oxidized products bind to thiol groups and proteins within the cell (55).

The ES-derived neural progenitor cells were seeded onto poly-L-ornithine/laminin-coated cover slips (18×18 mm). After EMF exposure, the medium was changed with freshly prepared Mitotracker probe (500 nmol/l, Molecular Probes) in prewarmed (37°C) ITSFn medium for 15 min. The medium with the probe was removed, and the cells were washed twice in prewarmed medium to remove any unbound pool of oxidized MitoTracker Red held in the mitochondria. The cells on the cover slips were immediately fixed in prewarmed 4% paraformaldehyde in PBS for 15 min at 37°C, washed three times in PBS, and fixed in ice-cold acetone for 5 min at −20°C for permeabilization and better signal retention. The cells were then blocked with 1% bovine serum albumin for 30 min at room temperature, and immunostaining was performed with the primary mouse anti-nestin antibody at 37°C for 90 min, followed by a 60 min incubation with a secondary Alexa 488-labeled goat anti-mouse antibody, nuclei staining with Hoechst 33342 for 10 min at room temperature, and mounting in Vectashield medium.

### **Quantification of apoptosis by FACS subG1 analysis**

For quantification of apoptosis by subG1 analysis (according to ref 56), cells were fixed in 70% ethanol at −20°C and stained with propidium iodide (16.5 µg/ml) in PBS after RNase (0.03 µg/ml, Qiagen, Hilden, Germany) digestion. A total of 30,000 cells were analyzed for each sample on a FACS Aria flowcytometer (Becton-Dickinson, Franklin Lakes, NJ), and the number of apoptotic cells was calculated using the software FACSDiva (Becton-Dickinson). Statistical comparison was performed by the paired two-tailed *t* test.

## RESULTS

### ELF-EMF exposure affects bcl-2, bax, and GADD45 transcript levels

Differentiation of ES cells according to the experimental protocol (Fig. 1) via EBs (Fig. 1A) revealed up to 85% of nestin-positive neural progenitor cells (Fig. 1B) at the stage 4+4 days to 4+6 days. Q-RT-PCR analysis showed that bcl-2 and bax mRNA levels were up-regulated at day 4+11 and GADD45 mRNA levels were down-regulated at terminal stage of 4+23 days (Fig. 2A). However, p53 and nestin mRNA levels showed no differences between the experimental groups. Immunofluorescence analysis of cells after ELF-EMF exposure showed no changes in the number of cells expressing neuronal ( $\beta$ -III-tubulin, TH) or astrocytic (GFAP) proteins and their intracellular distribution (see Fig. 1C, 1D).

### RF-EMF exposure affects Nurr1 and GADD45 transcript levels

Q-RT-PCR analysis of ES-derived neural cells after RF-EMF exposure showed the up-regulation of GADD45 mRNA levels at terminal stage of 4+23 days. bax mRNA level was up-regulated at day 4+17 (Fig. 2B), whereas no significant changes of bcl-2 transcript levels were found. Nurr1 levels were significantly down-regulated at day 4+7, but no modifications of p53, nestin, and TH transcript levels were detected in EMF- and sham-exposed cells (Fig. 2B). By immunofluorescence analysis, we found no differences in the number and intracellular distribution of cells expressing neuron- and glial-specific proteins, such as  $\beta$ III-tubulin, TH, and GFAP, suggesting that RF-EMF may transiently affect transcript levels of neural cells, but not the abundance of neuronal and glial proteins.

### RF-EMF induces double-strand DNA breaks in neural progenitors at low level

To study primary DNA damage, ES-derived neural progenitor cells were exposed to EMF for short (6 h) and prolonged (48 h) duration, followed by the alkaline ( $\approx$  single-strand breaks) and neutral ( $\approx$  double-strand breaks) COMET assay with and without recovery time (Fig. 3).

We observed a low, but statistically significant increase of double-strand DNA breaks in ES-derived neural progenitor cells (by the neutral COMET assay) after 6 h RF-EMF exposure without recovery time ( $P < 0.05$ , Fig. 3A). Statistical evaluation of the COMETs in RF-EMF- and sham-exposed cells (distributed into the groups according to the percentage of DNA damage, see Fig. 3C) showed a significant increase of heavily damaged cells (group E with  $>95\%$  DNA damage,  $P < 0.05$ , Chi-square test, Fig. 3C) immediately after exposure ( $t = 0$  h) and in groups B, C, and D, 18 h after exposure. Examples of COMETs are shown in Fig. 3D. However, no increase in DNA break induction was observed after 48 h RF-EMF exposure by the alkaline and the neutral COMET assay, respectively (not shown), suggesting the activation of only transient and short-term responses by RF-EMF. We also did not detect single- or double-strand DNA breaks in ES-derived neural progenitor cells after 6 h (Fig. 3B) and 48 h (not shown) ELF-EMF exposure.

## **RF- and ELF-EMF exposure does not induce cytogenetic effects and does not affect proliferation and apoptosis**

ES-derived neural progenitor cells after 48 h RF- and ELF-EMF exposure were analyzed for the induction of CA and SCE. Analysis of CA in M1 metaphases and of SCE in M2 metaphases did not reveal chromosomal alterations in the EMF-exposed cells ([Table 2](#)).

To investigate the proliferation rate of nestin-positive neural progenitor cells, EMF- and sham-exposed cells were analyzed by the BrdU incorporation assay. The number of BrdU+/nestin+ cells ([Table 3](#)) and the M1:M2:M3 ratio (used to analyze cell cycle progression, [Table 2](#)) did not reveal significant differences between EMF- and sham-exposed variants. These findings suggest that RF- and ELF-EMF exposure to ES-derived cells exerts no effects on the proliferation of nestin-positive neural cells.

To determine potential effects on apoptosis, we analyzed by FACS the apoptotic subG1 fraction of ES-derived cells. RF- and ELF-EMF did not induce significant differences in the apoptotic subG1 fraction ([Table 4](#)). However, the interpretation of the FACS data is difficult because of the high level of apoptotic cells in the control population (without EMF exposition). According to the neuronal differentiation protocol, neural cells are selected by cultivation in ITSFn medium between days 4+1 and 4+8. In this growth-selective medium, the proliferation of neural cells is supported, whereas cells of other phenotypes are suppressed, leading to a high level of apoptosis and necrosis in the cell population.

Also, the Mitotracker CM-H2X ROS assay used to investigate the mitochondrial membrane potential did not reveal detectable changes of mitochondrial function in ELF-EMF- and RF-EMF-exposed cells compared with sham-exposed cells (data not shown).

## **DISCUSSION**

Here, we show that exposure of neural progenitor cells derived from ES cells to EMF results in specific alterations of transcript levels of various regulatory genes ([Table 5](#)). We analyzed genes that regulate cell cycle activity (p53, GADD45) and anti-apoptotic (bcl-2) and pro-apoptotic (bax) responses based on previous experiments with p53-deficient ES cells. In these studies, RF-EMF exposure to p53-deficient ES cells resulted in long-term up-regulation of hsp70 transcript levels paralleled by a transient increase of c-jun, c-myc, and p21 levels (8), and ELF-EMF exposure resulted in the up-regulation of egr-1 (early growth response-1), p21, and c-jun transcript levels (6). However, in the present study, EMF-induced effects on transcript levels of cell cycle regulatory and apoptosis-related genes were detected in neural progenitor cells differentiated from wild-type ES cells.

Specifically, we found significant alterations in the transcript level of the two opposing members of the bcl-2 family, bcl-2 and bax, when neural progenitor cells were exposed to ELF-EMF. Apoptosis-related pathways are common during embryonic development, especially in the nervous system (57), and apoptosis is induced in response to environmental stress factors (58). In differentiating mouse ES cells, apoptotic cell death is regulated by members of the bcl-2 family (59). The bcl-2 family includes several members, which are abundant at the contact site of the mitochondrial permeability transition pore between the mitochondrial inner and outer membranes. It is considered that bcl-2 protein prevents the early stage of apoptosis, including the

disruption of the inner mitochondrial transmembrane potential and the release of apoptogenic proteases from mitochondria (60). Studies applying the central axotomy model in mice showed the degeneration of nearly 70% of the affected nigral neurons posttransection due to activation of c-jun, whereas bcl-2 overexpression led to a reduced c-jun phosphorylation in neurons and protection against cell death (61). Bcl-2 overexpression resulted in the elimination of deprivation-induced cell death of brainstem auditory neurons (62), and in situ hybridization revealed a rapid and transient increase of bcl-2 mRNA levels in neurons following deafferentation (63).

In the experiments presented here, the up-regulation of bcl-2 mRNA levels induced by ELF-EMF in ES-derived neural progenitor cells was opposed by the up-regulation of bax transcript levels at the same stage of differentiation. Because the increase of the (anti-apoptotic) bcl-2 mRNA level was only slightly higher in comparison to (pro-apoptotic) bax levels, we would not expect significant effects on apoptosis in mouse neural progenitor cells. Indeed, both the microscopic analysis of selectively stained active mitochondria and the FACS analysis of nuclear apoptosis did not reveal significant differences between EMF- and sham-exposed neural progenitors. Moreover, our cellular model comprised many cell types (i.e., ES cells differentiating either into neuronal or into glial-like cells), which may differentially react to EMF. The parallel up-regulation of the transcript level of pro- and anti-apoptotic genes after ELF-EMF exposure might be a possible consequence. The down-regulation of GADD45 transcript level may be considered as an indication of subtle stimulatory effects on cell proliferation. Other data provided evidence of increased cell proliferation following exposure to 50 Hz EMF in human neuroblastoma and rat pituitary cells (64). However, neither the BrdU incorporation assay of nestin-positive cells nor the ratio of M1:M2:M3 metaphases indicated any effects by EMF on proliferation of neural progenitors derived from ES cells.

We observed no changes of neuron (Nurr1, TH)- and glial (GFAP)-specific genes at the transcript and protein level. Therefore, we speculate that these ELF-EMF-dependent effects are not correlated with processes of neuronal differentiation. The heterogeneity of the ES cell-derived cell population, however, could mask subtle effects on differentiation, which might be phenotypically observable (at the protein level) in other neural cell models. For example, exposure by 5 and 20 Hz EMF resulted in differentiation induction of midbrain stem cells into neurons (65). However, similar to our data, no alterations of transcript levels of central nervous system-specific genes were detected in newborn rats after maternal continuous exposure to 60 Hz magnetic fields in vivo (66).

RF-EMF exposure, in contrast, resulted in down-regulation of transcript levels of the neural-specific gene Nurr1 at early stage of differentiation, which might transiently affect neuronal differentiation. Nurr1 is a transcription factor involved in the determination and development of dopaminergic neurons in vivo (67, 68) and in vitro (69, 70). Because this effect was observed only following RF-EMF exposure, we may also speculate that specific cellular responses are dependent on the EMF frequency.

RF-EMF induced an up-regulation of bax at intermediate and of GADD45 levels at the terminal stage of differentiation, however, without alterations of p53 transcript levels. Members of the GADD protein family are considered to play an important role in maintaining genomic stability and regulating cell cycle activity (71). GADD45 was found to regulate G2/M arrest, DNA repair, and cell death in keratinocytes following ultraviolet exposure (72). The phenotype of GADD45-

deficient mice is similar to those of p53-deficient mice, characterized by genomic instability and sensitivity to radiation-induced carcinogenesis (73). The GADD45-mediated G2/M arrest is considered to be p53-dependent, because no G2/M arrest was observed in p53 null fibroblasts (74). However, DNA damage-induced transcription of the GADD45 gene may be mediated by both p53-dependent (75) and p53-independent mechanisms (76).

Furthermore, we found a low, but transient increase of double-strand DNA breaks immediately after short-term 6 h exposure to GSM signals (but not to ELF-EMF). Some authors reported an increase in both single- and double-strand DNA breaks in rat brain cells (40), whereas others could not find EMF effects on DNA damage after acute exposure to high-frequency EMF (pulsed-wave 2450 MHz) (77). Despite the (low) induction of DNA double-strand breaks and the up-regulation of the DNA damage-inducible GADD45 gene by GSM signals, we did not observe increased frequencies of CA and SCE. These findings suggest that RF-EMF-induced DNA breaks may be repaired, and no conversion into chromosomal damage and homologous recombination events may occur.

In summary, we conclude that EMF signals are able to trigger responses at the transcript level of cell cycle regulatory and apoptosis-related genes in neural progenitor cells derived from pluripotent ES cells in vitro. However, due to the lack of effects on other cellular processes, including proliferation, chromosomal stability, and apoptosis, we postulate that EMF responses at the mRNA level may be gradually compensated at the translational and posttranslational level and do not lead to detectable changes of cell physiology.

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**Table 1****Primer and TaqMan probe sequences used for quantitative RT-PCR<sup>a</sup>**

| <b>Gene</b> | <b>Forward primer</b>    | <b>TaqMan probe<sup>b</sup></b>      | <b>Reverse primer</b>   |
|-------------|--------------------------|--------------------------------------|-------------------------|
| bcl-2       | TGGGATGCCTTTGTGGAAC      | FAM-TATGGCCCCAGCATGCGACCTC-TAMRA     | GAGACAGCCAGGAGAAATCAAAC |
| bax         | GGAGCAGCTTGGGAGCG        | FAM-CGGGCCCCACCAGCTCTGAACA-TAMRA     | AAAAGGCCCTGTCTTCATGA    |
| GADD45      | GCTGGCTGCTGACGAAGAC      | FAM-ACGACCGGGATGTGGCTCCGCA-TAMRA     | CGGATGAGGGTGAAATGGAT    |
| p53         | CCTTACCATCATCACACTGGAAGA | FAM-CAGTGGGAACCTTCTGGGACGG-TAMRA     | CACAAACACGAACCTCAAAGCT  |
| Nurr1       | GCGCTTAGCATACAGGTCCAA    | FAM-CCAGTGGAGGGTAAACTCATCTTT-TAMRA   | GTGCAAGACCACCCCATG      |
| TH          | GGAACGGACTGGCTTCCA       | FAM-CTGCGACCCGTGGCCGGT-TAMRA         | AGAAAATCACGGGCAGACAGTA  |
| nestin      | CAGCCCACTGAAAAGTTCCA     | FAM-TGGAGCAGGAGAAGCAGGGTCTACAG-TAMRA | TCCAGGATCTGAGCGATCTG    |
| GAPDH       | CCTGCACCACCAACTGCTTA     | FAM-CCTGGCCAAGGTCATCCATGACAA-TAMRA   | TCATGAGCCCTTCCACCATG    |

<sup>a</sup>Sequences in 5' to 3' orientation. <sup>b</sup>FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.

**Table 2**

**Frequency of chromosomal aberrations (CA) and sister chromatid exchanges (SCE) and percentage of metaphases in the first (M1), second (M2), and third (M3) mitosis in ELF-EMF vs. sham-exposed cells and RF-EMF vs. sham-exposed cells, respectively<sup>a</sup>**

| Exposure          | Number of CA/cell<br>(±SEM) | Cells with CA<br>(%±SEM) | Number of SCE/cell<br>(±SEM) | Cells in M1<br>(%±SEM) | Cells in M2<br>(%±SEM) | Cells in M3<br>(%±SEM) |
|-------------------|-----------------------------|--------------------------|------------------------------|------------------------|------------------------|------------------------|
| ELF-EMF (exposed) | 0.034 ± 0.004               | 3.35 ± 0.40              | 7.83 ± 0.46                  | 56.06 ± 4.96           | 32.16 ± 3.42           | 11.78 ± 2.37           |
| ELF-EMF (sham)    | 0.047 ± 0.015               | 3.59 ± 1.14              | 7.66 ± 0.54                  | 58.28 ± 3.88           | 30.86 ± 3.17           | 10.86 ± 1.58           |
| RF-EMF (exposed)  | 0.110 ± 0.033               | 5.20 ± 0.47              | 7.73 ± 0.86                  | 55.90 ± 3.19           | 33.92 ± 2.70           | 10.58 ± 2.05           |
| RF-EMF (sham)     | 0.066 ± 0.019               | 4.38 ± 0.53              | 7.37 ± 0.67                  | 56.66 ± 2.79           | 32.24 ± 2.01           | 10.98 ± 2.48           |

<sup>a</sup>ES-derived neural progenitor cells were EMF exposed at stage 4+4 days for 48 h and immediately after exposure were labeled with BrdU (5-bromo-2'-deoxyuridine). Sister chromatids were differentially stained by the FPG technique (see Materials and Methods). The number and percentage of CA were analyzed in M1, and SCEs were counted in M2 metaphases. The percent number of metaphases in M1, M2, and M3 was calculated. The statistical evaluation between sham- vs. EMF-exposed variants was performed by the nonparametric Mann-Whitney test.

**Table 3****Percentage of BrdU+/nestin+ cells following ELF vs. sham exposure and RF vs. sham exposure<sup>a</sup>**

| <b>Exposure (48 h)</b> | <b>BrdU+/nestin+ cells (%±SEM)</b> |
|------------------------|------------------------------------|
| ELF-EMF (exposed)      | 48.91 ± 5.98                       |
| ELF-EMF (sham)         | 48.71 ± 14.46                      |
| RF-EMF (exposed)       | 25.54 ± 4.46                       |
| RF-EMF (sham)          | 26.31 ± 5.10                       |

<sup>a</sup>ES-derived neural progenitor cells were grown on poly-L-ornithine/laminin-coated coverslips, EMF exposed at stage 4+4 days for 48 h. Nestin-positive cells were labeled immediately after exposure with BrdU and detected by anti-BrdU and anti-nestin antibodies. The nuclei were stained by Hoechst 33342. The BrdU+/nestin+ cells were counted, the percent was calculated, and the differences were statistically evaluated (paired two-tailed *t* test).

**Table 4****Percentage of cells with hypodiploid DNA content (subG1 fraction) following EMF vs. sham exposure<sup>a</sup>**

| Differentiation time<br>(days after EB plating) | subG1 after 48 h RF-EMF (%±SD) |             | subG1 after 48 h ELF-EMF (%±SD) |              |
|-------------------------------------------------|--------------------------------|-------------|---------------------------------|--------------|
|                                                 | sham                           | exposed     | sham                            | exposed      |
| 6 days                                          | 16.6 ± 5.44                    | 15.8 ± 4.17 | 17.6 ± 5.30                     | 19.2 ± 8.05  |
| 7 days                                          | 17.8 ± 1.70                    | 17.9 ± 5.02 | 18.6 ± 3.04                     | 15.9 ± 5.37  |
| 8 days                                          | 20.1 ± 10.68                   | 20.4 ± 8.06 | 22.3 ± 15.80                    | 24.2 ± 19.09 |
| 11 days                                         | 5.8 ± 1.13                     | 5.6 ± 1.70  | 5.4 ± 2.48                      | 6.1 ± 0.92   |
| 23 days                                         | 5.45 ± 2.05                    | 4.6 ± 1.70  | 5.0 ± 0.50                      | 4.8 ± 2.05   |

<sup>a</sup>The subG1 population (in %) of EMF- vs. sham-exposed cells was quantified by flowcytometry at defined time points of differentiation. Statistical evaluation was done by the paired two-tailed *t* test.

**Table 5**

**Summary of effects by ELF-EMF and RF-EMF exposure to ES-derived neural cells analyzed by Q-RT-PCR analysis; COMET assay; and analysis of chromosomal aberrations (CA), sister-chromatid exchange (SCE), and apoptosis<sup>a</sup>**

| Intermittent exposure (5 min on/30 min off) |                                                                                                                                                                                                                                                                                                                |                                            |                                                                                                                                                                                                                                                                                                               |
|---------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| ELF-EMF<br>(powerline, 50 Hz, 2.0 mT)       |                                                                                                                                                                                                                                                                                                                | RF-EMF<br>(GSM 217 Hz, 1.71 GHz, 1.5 W/kg) |                                                                                                                                                                                                                                                                                                               |
| 48 h                                        | <ul style="list-style-type: none"> <li>• Up-regulation of bcl-2 at 4+11 days</li> <li>• Up-regulation of bax at 4+11 days</li> <li>• Down-regulation of GADD45 at 4+23 days</li> <li>• No effect on DNA break, CA, SCE induction, cell proliferation, apoptosis, and mitochondrial function (n=5–6)</li> </ul> | 48 h                                       | <ul style="list-style-type: none"> <li>• Up-regulation of bax at 4+17 days</li> <li>• Up-regulation of GADD45 at 4+23 days</li> <li>• Down-regulation of Nurr1 at 4+7 days</li> <li>• No effect on DNA break, CA, SCE induction, cell proliferation, apoptosis, and mitochondrial function (n=5–7)</li> </ul> |
| 6 h                                         | <ul style="list-style-type: none"> <li>• No effect on DNA break induction (n=6)</li> </ul>                                                                                                                                                                                                                     | 6 h                                        | <ul style="list-style-type: none"> <li>• Low, transient induction of double-strand DNA breaks (n=6)</li> </ul>                                                                                                                                                                                                |

<sup>a</sup>n = number of experiments.

**Fig. 1**

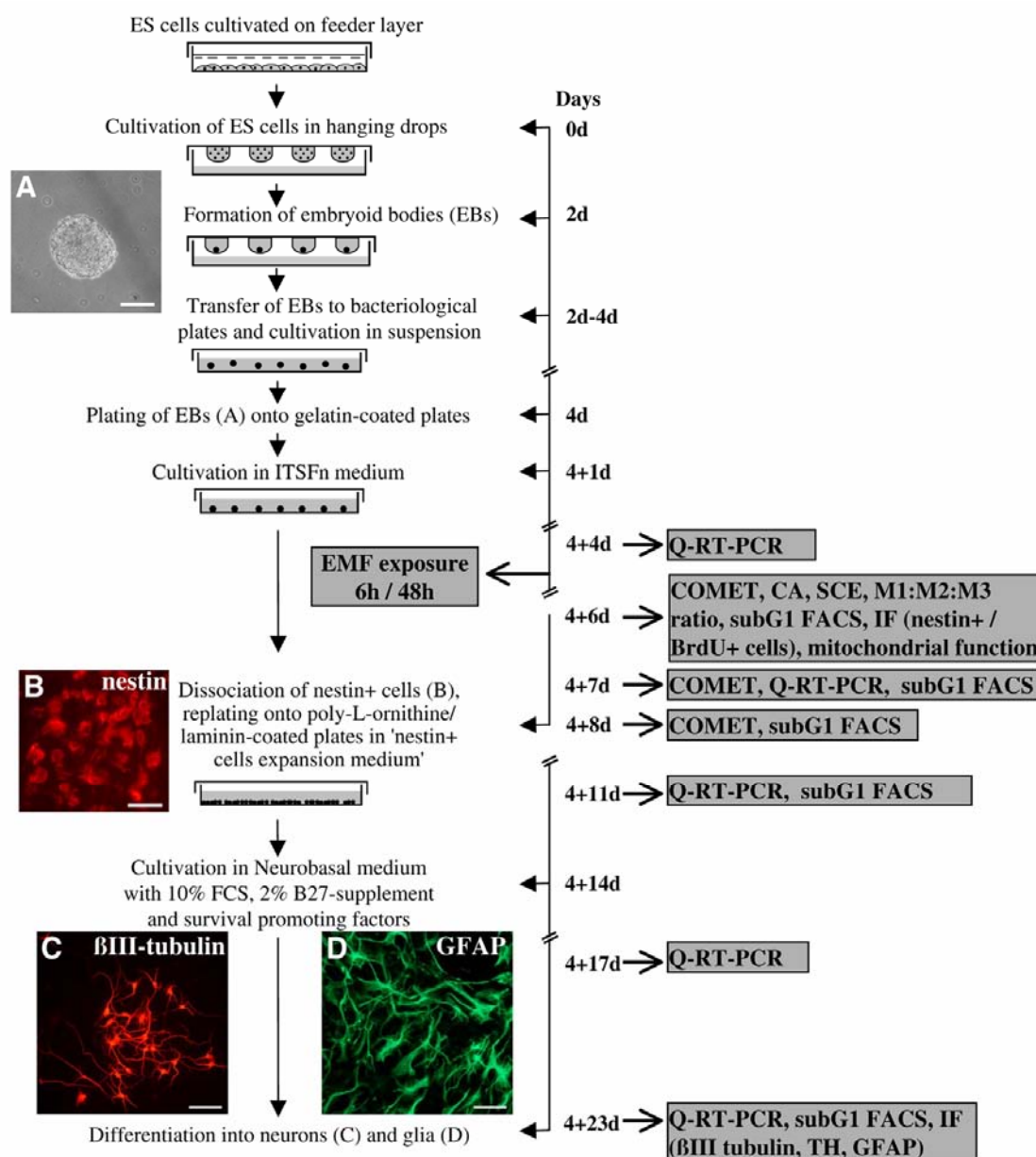


**Figure 1.** Experimental protocol applied to analyze the effects of EMF exposure in ES-derived neural progenitor cells. Undifferentiated ES (R1) cells grown on feeder layer of mouse embryonic fibroblasts were cultivated in “hanging drops” as embryoid bodies (EBs) for 2 days and then transferred to bacteriological petri dishes and cultivated for another 2 days. EBs (A) were plated onto gelatin-coated tissue culture dishes at day 4. One day after attachment of EBs, the medium was exchanged by ITSFn medium containing insulin, transferrin, selenium, and fibronectin and replenished every 2 days until day 4+8. Nestin-positive neural precursor cells (B) developed from EBs. At day 4+14, differentiation induction into neuronal (C) and glial (D) cells was induced by cultivation in “Neurobasal” medium supplemented by neurotrophic (B27 supplement) and survival promoting factors. Cell samples were analyzed for primary DNA damage by the COMET assay and for transcript levels of regulatory genes by quantitative (Q)-RT-PCR at the indicated stages of differentiation and for quantification of apoptosis by subG1 FACS analysis. Cytotoxic effects were estimated by metaphase analysis of chromosomal aberrations (CA) in cells of first mitosis (M1) and sister chromatid exchanges (SCE) in second mitoses (M2). The proliferation rate was determined by the estimation of the percentage of metaphases in M1, M2, and M3 and by immunofluorescence (IF) analysis of nestin+/BrdU+ cells after 24 h incubation with BrdU. GFAP = glial fibrillary acidic protein. Bar = 100 μm (A) and 50 μm (B–D).

**Fig. 2**

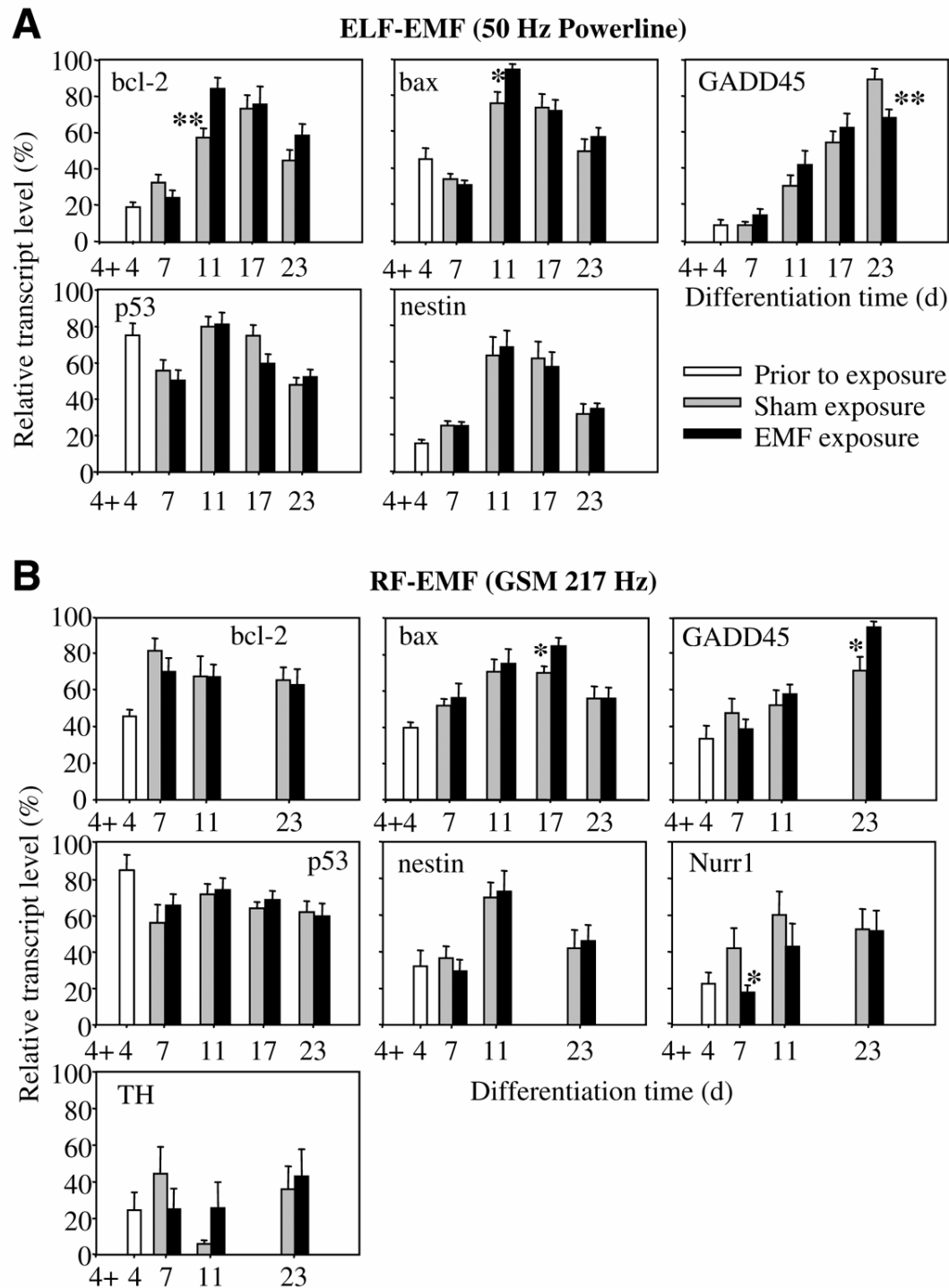


**Figure 2.** Results of Q-RT-PCR analysis using specific primers and TaqMan probes for the estimation of relative mRNA levels of selected genes in ES-derived neural progenitor cells exposed to ELF- or RF-EMF for 48h. Control samples (without exposure) were collected at day 4+4 and EMF- and sham-exposed cells were collected 7, 11, 17 and 23 days after plating. Bcl-2, bax, p53, GADD45, nestin, Nurr1 and TH (tyrosine hydroxylase) transcript levels were evaluated. The mouse GAPDH gene was used as endogenous reference. The relative mRNA levels were calculated using the comparative Ct method (see Material and Methods). Error bars represent standard deviations of  $n = 10$  RT-PCR reactions (5 experiments with 2 replicates per experiment). Statistical significance was tested by the nonparametric Mann-Whitney test (\* $P < 0.05$  and \*\* $P < 0.01$ ).

**Fig. 3**

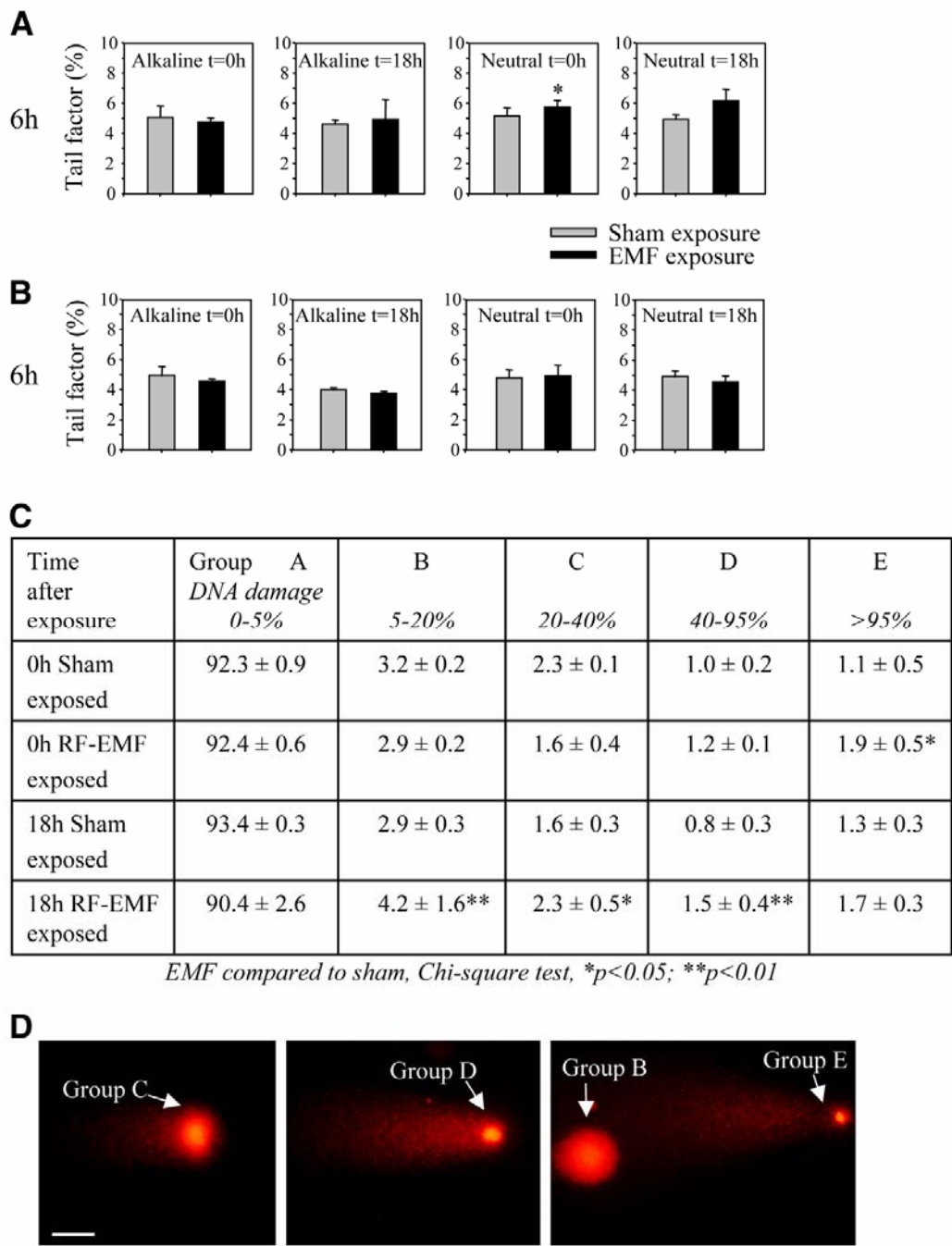


**Figure 3.** COMET assay for detection of single- and double-strand DNA breaks in EMF- and sham-exposed ES-derived neural progenitor cells. Cells were exposed at stage 4+4 days. After 6 h RF-EMF (**A**) and ELF-EMF (**B**) exposure, alkaline and neutral COMET assays, respectively, were performed immediately after exposure ( $t=0$  h) or following a recovery time of 18 h ( $t=18$  h). Ethidium bromide-stained nuclei ( $n=1000$ ) of cells from EMF-exposed and sham-exposed variants were analyzed by fluorescence microscopy and classified into 5 groups according to tail length and intensity, and the tail factor was calculated as % value (see Materials and Methods). All data are presented as mean values  $\pm$  SEM. Statistically significant differences between the tail factors of sham- and EMF-exposed cells were evaluated by Student's  $t$  test for significance level of 5% ( $*P \leq 0.05$ ). **C**) The number of COMETs in RF-EMF- and sham-exposed cells classified into group A, B, C, D, and E according to the DNA damage (%) were compared by Chi-square analysis of contingency tables for a significance level of 5% ( $*P \leq 0.05$ ) and 1% ( $**P \leq 0.01$ ). **D**) Examples of COMETs found in ES-derived neural progenitor cells immediately after 6 h RF-EMF exposure classified into group B (5–20%), C (20–40%), D (4–95%), and E (>95%). Bar = 50  $\mu$ m.