

Effects of radiofrequency electromagnetic field exposure on neuronal differentiation and mitochondrial function in SH-SY5Y cells

Nicole von Niederhäusern^a, Angélique Ducray^a, Jana Zielinski^a, Manuel Murbach^b,
Meike Mevissen^{a,*}

^a Veterinary Pharmacology and Toxicology, Vetsuisse Faculty, University of Bern, Laenggassstrasse 124, 3012 Bern, Switzerland

^b ITIS Foundation, Zeughausstrasse 43, 8004 Zurich, Switzerland

ARTICLE INFO

Keywords:

Radiofrequency electromagnetic fields
SH-SY5Y cells
Neuronal differentiation
Mitochondria

ABSTRACT

Exposure to radiofrequency electromagnetic fields (RF-EMF) has dramatically increased in the last decades with expanding use of mobile phones worldwide. The aim of this study was to evaluate effects of RF-EMF on neuronal differentiation and underlying signaling pathways involved in neuronal differentiation, neurodegeneration, and mitochondrial function.

Differentiation of SH-SY5Y cells was performed using *all-trans* retinoic acid or staurosporine to obtain cholinergic and dopaminergic neurons.

Exposure of SH-SY5Y cells at 935 MHz, 4 W/kg for 24 h did not alter the neuronal phenotypes quantitatively. Markers of the signaling pathways investigated, namely the mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (Erk) 1 and 2 (p-Erk1/2) and protein kinase B (Akt), glycogen synthase kinase 3 β (GSK3 β) and Wnt/ β -catenin were not significantly affected by RF-EMF compared to sham. RF-EMF-impaired mitochondrial respiration in cells under glucose deprivation, but glutathione levels and mitochondrial fission and fusion markers were not altered. These findings indicate that RF-EMF might lead to an impairment of mitochondrial function that is only manifest at maximal respiration and additional stressors such as glucose deprivation.

Further research is needed to investigate the effects of RF-EMF on mitochondrial function in detail because mitochondrial impairment is closely related to the pathogenesis of neurodegenerative diseases.

1. Introduction

Exposure to radiofrequency electromagnetic fields (RF-EMF) has dramatically increased in the last decades with expanding use of mobile phones telecommunication worldwide. The International Agency of Research on Cancer (IARC) has classified RF-EMF as a possible carcinogen. A large number of *in vitro* and *in vivo* studies provided evidence that RF-EMF leads to an increase in reactive oxygen species (ROS) production in cells after RF-EMF exposure (Friedman et al., 2007; Xu et al., 2010; Consales et al., 2012; Wang and Zhang, 2017). ROS

generation was found in SH-SY5Y cells after short-term RF-EMF exposure at a specific absorption rate (SAR) of 5 W/kg at 872 MHz for 1 h (Luukkonen et al., 2009). Ertilav et al. reported an elevation in ROS production in hippocampal and dorsal root ganglion neurons of female Wistar rats after long-term RF-EMF exposure to 900 and 1800 MHz for 1 year, 1 h per day at 0.1 W/kg (Ertilav et al., 2018). The fact that various studies reported an increased production of ROS after RF-EMF exposure, mitochondrial impairment might be a target of RF-EMF (Gupta et al., 2018).

Besides cancer, ROS was shown to be involved in the development

Abbreviations: Akt, protein kinase B; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; CHT1, choline transporter 1; ir, immunoreactive; DIV, day *in vitro*; Drp1, dynamin-related protein 1; ELF, extreme low frequency; Erk1 and 2 (p-Erk1/2), extracellular signal-regulated kinases; FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; GSH, glutathione; GSK3 β , glycogen synthase kinase 3 β ; GSM, Global System for Mobile Communications; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; Mfn2, Mitofusin 2; OCR, oxygen consumption rate; OMA1, metalloendopeptidase; OPA1, optic atrophy 1; PD, Parkinson's disease; p-p44/42 Erk1/2, phosphorylated extracellular signal-regulated kinases; p-Akt, phosphorylated Akt; RA, all-trans retinoic acid; RF-EMF, radiofrequency electromagnetic fields; ROS, reactive oxygen species; SAR, specific absorption rate; ST, staurosporine; TH, tyrosine hydroxylase

* Corresponding author at: Division Veterinary Pharmacology & Toxicology, Department of Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Bern, Laenggassstrasse 124, 3012 Bern, Switzerland.

E-mail addresses: nicole.vonniederhaeusern@vetsuisse.unibe.ch (N. von Niederhäusern), angelique.ducray@vetsuisse.unibe.ch (A. Ducray), murbach@itis.swiss (M. Murbach), meike.mevissen@vetsuisse.unibe.ch (M. Mevissen).

<https://doi.org/10.1016/j.tiv.2019.104609>

Received 14 May 2019; Received in revised form 23 July 2019; Accepted 23 July 2019

Available online 24 July 2019

0887-2333/© 2019 Elsevier Ltd. All rights reserved.

of neurodegenerative diseases such as Parkinson's disease (PD) (Dias et al., 2013; Cacabelos, 2017). In PD patients, a progressive loss of dopaminergic neurons in the substantia nigra pars compacta was demonstrated (Cacabelos, 2017; Martinez et al., 2018). Most of the cases are idiopathic but interactions with α -synuclein, oxidative stress and mitochondrial dysfunction have been suspected (Xie et al., 2010; Perfeito et al., 2014; Roberts and Brown, 2015; Roberts et al., 2016).

Nevertheless, reported effects of RF-EMF on neurodegeneration of neurons or neuron-like cells are conflicting, which is most likely due to the use of different cell types and varying exposure conditions including variations in frequency, amplitude, waveform and polarization of the electromagnetic field (Irmak et al., 2002; Xu et al., 2010; Consales et al., 2012). In particular, there is some evidence that RF-EMF alters neuronal differentiation (Eghlidospour et al., 2017). However, only a few studies on the effects of RF-EMF and neurodegeneration are currently available. A Danish retrospective cohort study reported no correlation between neurodegenerative diseases (Alzheimer's disease, PD, amyotrophic lateral sclerosis, epilepsy) and RF-EMF exposure. The data were based on 420,095 mobile phone subscribers in the years 1982 to 1995 (Schuz et al., 2009). *In vitro* data indicated some evidence for development and progression of neurodegeneration. ROS generation and an increase in a monomer of α -synuclein were found in SH-SY5Y cells after exposure to 1800 MHz for 2 days (total of 6 times for 10 min (min)) at a maximal SAR of 0.21 W/kg (Stefi et al., 2019). Long-time RF-EMF exposure (835 MHz, 4.0 W/kg, 5 h a day for 12 weeks) of mice resulted in a significant increase in autophagy, accumulation of autophagosomes and myelin damage in cortical neurons. Furthermore, RF-EMF-exposed mice showed hyperactive behavior compared to sham-exposed animals (Kim et al., 2017).

Cells undergo major changes during neuronal differentiation including morphological changes accompanied by an activation of various cellular signaling pathways, namely mitogen-activated protein kinase (MAPK) signaling (Biedler et al., 1973; Buttiglione et al., 2007; Lee et al., 2016), Wnt/ β -catenin (Muroyama et al., 2004) and protein kinase B (Akt) and glycogen synthase kinase 3 beta (GSK3 β) (Miloso et al., 2004; Qiao et al., 2012). A metabolic switch from glycolysis to oxidative phosphorylation was demonstrated that was accompanied by an increased mitochondrial mass, adenosine triphosphate (ATP) production and ROS generation (Agostini et al., 2016; Zheng et al., 2016; Almeida and Vieira, 2017). In particular, a stable balance between the dynamic mitochondrial processes, namely fusion and fission is necessary for mitochondrial function and cell survival. Mitochondrial fission is mainly mediated by dynamin-related protein 1 (Drp1), and mitochondrial fusion is mediated through dynamin like GTPases (guanosine triphosphatases), namely mitofusins and optic atrophy 1 (OPA1). These proteins are particularly important for the mitochondria to maintain genetic and biochemical homogeneity and allow for dissipation of ROS (Cid-Castro et al., 2018) and, thereby, prevent damage to oxidative stress. Increased mitochondrial activity was demonstrated in the cortex and hippocampus of mice after whole-body exposure for one month at 918 MHz and 0.25–1.05 W/kg with 1 h of exposure to RF-EMF twice a day (Dragicevic et al., 2011).

To our knowledge, no data on effects of RF-EMF exposure on fusion and mitochondrial respirometry-based function in living neurons are available. The aim of this study was to investigate effects of RF-EMF on neuronal differentiation and the resulting phenotype. Moreover, signaling pathways involved in neuronal differentiation and neurodegeneration, mitochondrial integrity and function during neuronal differentiation were studied in neuron-like SH-SY5Y cells.

2. Material and methods

2.1. Cell culture

The human neuroblastoma cell line SH-SY5Y was obtained from ATCC® (CRL-2266™, Manassas, Virginia, USA). Undifferentiated SH-

SY5Y cells were cultured in 75 cm² tissue culture flasks with filter screw caps (TPP (Techno Plastic Products) AG, Trasadingen, CH) in Dulbecco's Modified Eagle Medium (DMEM) with high glucose [25 mM], GlutaMAX™ and 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ l/ml streptomycin (all from Life Technologies, California, USA) at 37 °C in 5% CO₂. The cells were passaged every 4 to 6 days by scraping them mechanically. SH-SY5Y cells of passages 6 to 23 at a confluency of 70–90% were used for the experiments. The cells were seeded into 35 mm Petri dishes on day *in vitro* 0 (DIV0) before RF-EMF or sham exposure.

Differentiation of the cells was performed using a two-phase protocol. First, the cells were seeded and kept in an undifferentiated state for 24 h. The first-phase of the differentiation procedure was started on DIV2 to DIV4 with a 5% reduction of FBS in the medium. In the second phase from DIV5 to DIV7, FBS was further reduced to 1%. In both phases, the medium was supplemented with *all-trans* retinoic acid (RA) (Sigma-Aldrich, Missouri, USA) or staurosporine (ST) (Sigma-Aldrich, Missouri, USA) at final concentrations of 10 μ M RA and 5 nM ST. Controls consisted of undifferentiated cells that were kept in 10% FBS medium.

2.2. RF-EMF exposure

The fully characterized sXc900 exposure system (IT'IS Foundation, Zurich, CH) (Schuderer et al., 2004) was used for intermittent exposure of the cells with a Global System for Mobile Communications (GSM) modulation envelope (talk mode, 935 MHz carrier frequency, 217 Hz pulse-modulated rate, ON/OFF cycles 120/120 s) after extensive evaluations regarding electromagnetic and thermal dosimetry (IT'IS Foundation, Zurich, CH). SH-SY5Y cells were exposed at an average SAR of 4 W/kg $\pm$ 0.16 SD for 24 h, with a non-uniformity of the SAR distribution of 20% SD (Schuderer et al., 2004), under the culture conditions described above (37 °C $\pm$ 0.15 °C, > 80% humidity, 5% CO₂). All experiments were performed as double-blinded trials with two waveguide chambers for RF-EMF and sham exposure, respectively. Each chamber held 8 cell culture dishes (35 mm) (Thermo Fisher Scientific, Massachusetts, USA) containing 4 ml medium per dish. SH-SY5Y cells were RF-EMF- or sham-exposed before differentiation (DIV1) or on the last differentiation day (DIV7).

2.3. Phenotypical characterization and quantification

Quantification of dopaminergic and cholinergic phenotypes was performed using high-content analyses with the IN Cell Analyzer 2000 (General Electric Healthcare, Illinois, USA). Cells were scraped mechanically, the cell suspension was centrifuged (201 $\times$ g, 5 min, at room temperature (RT)), and the pellet was resuspended in 1 ml medium prior to seeding into a 96-well microplate (μ Clear® CELL-STAR®, Greiner Bio-One, Kremsmünster, Austria) at a cell density of 5.3×10^4 cells per well. On DIV8, the cells were sequentially fixed in 2% and 4% paraformaldehyde (Sigma-Aldrich, Missouri, USA) for 10 min each. The cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) (Life Technologies, California, USA) before the cells were blocked for 2 h with Dulbecco's phosphate buffered saline (DPBS) containing 0.4% Triton X-100 (Sigma-Aldrich, Missouri, USA) and 10% horse serum (Life Technologies, California, USA). Dopaminergic and cholinergic phenotypes were identified with antibodies against tyrosine hydroxylase (TH) and choline transporter 1 (CHT1), respectively, using Western blotting or immunofluorescence. Incubation with the primary antibody was performed overnight at 4 °C (Table 1). The following day, cells were washed four times with DPBS before they were incubated with the respective secondary antibody (Table 2) and Hoechst 33342 nucleic acid stain reagent (1.25 μ g/ml) (Life Technologies, California, USA) for 2 h at RT in the dark. SH-SY5Y cells were washed again four times with DPBS prior the analyses. Images were taken from three wells

Table 1
Primary antibodies.

Primary antibody	Host	Dilution WB	IF	Company
p44/42 MAPK	Mouse	1:1000		Cell Signaling
phospho-p44/42 MAPK	Mouse	1:500		Cell Signaling
Akt	Rabbit	1:1000		Cell Signaling
phospho-Akt	Rabbit	1:1000		Cell Signaling
GSK3β	Rabbit	1:1000		Cell Signaling
β-actin	Mouse	1: 20,000		Sigma-Aldrich
OPA1	Mouse	1:3000		BD Biosciences
Mfn2	Mouse	1:10,000		Abcam
β-catenin	Rabbit		1:400	Cell Signaling
TH	Rabbit		1:1000	Cell Signaling
CHT1	Mouse		1:250	Santa Cruz

Akt: protein kinase B, CHT1: choline transporter 1, GSK3β: glycogen synthase kinase 3β, IF: immuno-fluorescence, MAPK: mitogen activated protein kinase, Mfn2: mitofusin 2, OPA1: optic atrophy 1, TH: tyrosine hydroxylase, WB: Western blot.

of RF-EMF and sham-exposed cells and 30 randomly assigned areas in each well. The software, InCell Developer Toolbox 1.9.2 (General Electric Healthcare, Illinois, USA) was used to calculate the total number of cells as well as the TH- and CHT1-immunoreactive (ir) cells.

2.4. Western blot analyses of differentiation and mitochondrial markers

Markers for neuronal differentiation (Erk1/2, Akt, GSK3β, β-catenin) and mitochondrial fusion and fission (OPA1, Mfn2) were analysed by Western blotting. SH-SY5Y cells were exposed to RF-EMF on DIV1 or DIV7. The seeding density was adapted to obtain a cell density of 90% on DIV8. Hydrogen peroxide (Dr. Grogg Chemie AG, Deisswil, CH) at a final concentration of 250 μM was used as positive control with incubation times of hydrogen peroxide of 3 and 6 h. Total cellular protein was extracted on DIV8 using cold protein lysis buffer (Thermo Fisher Scientific, Massachusetts, USA) containing phosphatase (Sigma-Aldrich, Missouri, USA) and protease (Thermo Fisher Scientific, Massachusetts, USA) inhibitor cocktails. SH-SY5Y cells were scraped, the cell suspension was incubated on ice for 30 min and subsequently sonicated for 10 s at an amplitude of 40%. Subsequently, the cells were centrifuged at $18,506 \times g$ for 15 min at 4 °C. The protein concentration was determined with the Pierce protein assay reagent (Bio-Rad Laboratories, California, USA). Protein extracts (10 μg) of each sample were resolved with 8 to 12% sodium dodecylsulfate polyacrylamide (SDS) gel electrophoresis and transferred to a polyvinylidene difluoride membrane (PVDF) (Bio-Rad Laboratories, California, USA). Blocking was performed for two hours in phosphate-buffered saline (PBS) containing 0.2% TWEEN 20 (Sigma-Aldrich, Missouri, USA) and 5% skimmed milk powder (Migros, Zurich, CH) before incubating the samples with the respective primary antibody (Table 1) overnight at 4 °C. The membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Table 2) for two hours at RT after a washing step with PBS 0.2% Tween 20. Chemiluminescent substrate (WesternBright Sirius, Vitec, Sursee, CH) was used to detect the respective signal using a luminescent imaging system (LAS-3000 Imaging system by Fuji, Tokyo, J). Quantification of the

Table 2
Secondary antibodies.

Secondary antibody	Dilution WB	IF	Use with primary antibody	Company
dk anti-rabbit IgG HRP	1:5000		Akt; phospho-Akt; GSK3β;	Novex
dk anti-rabbit IgG AF 488 nm		1:200	β-catenin; TH	Invitrogen
dk anti-mouse IgG HRP	1:5000		p44/42 MAPK; phospho-p44/42 MAPK; OPA1; Mfn2	Novex
	1:50,000		β-actin	
dk anti-mouse IgG AF 647 nm		1:200	CHT1	Invitrogen

AF: Alexa Fluor, dk: donkey, HRP: horseradish peroxidase, IF: immunofluorescence, WB: Western blot.

respective protein was performed with ImageJ (National Institutes of Health, Washington, USA) by measuring the signal ratio of the specific marker to the corresponding β-actin signal.

2.5. Immunofluorescence analyses

On DIV2, SH-SY5Y cells were seeded at a cell density of 6.1×10^5 cells per well in 24 well tissue culture plates (TPP (Techno Plastic Products), Trasadingen, CH) on glass coverslips (VWR International, Pennsylvania, USA) primed with a poly-L-ornithine-laminin coating (Sigma-Aldrich, Missouri, USA). On DIV8, the cells were fixed and stained with the respective primary and secondary antibodies, respectively (Tables 1 and 2) as described above. Secondary antibodies were incubated together with Hoechst 33342 for nuclear staining (1.25 μg/ml) (Life Technologies, California, USA) and Acti-stain 555 phalloidin (0.23 μM) (Lubioscience, Zurich, CH). After mounting the cover slips on slides with Glycergel Mounting Medium (Agilent Technologies, California, USA), immunofluorescence images were captured with a Zeiss Axio Imager Z1 coupled with an Apotome 1 (Carl Zeiss Vision Swiss AG, Feldbach, CH).

2.6. β-catenin enzyme-linked immunosorbent assay (ELISA)

SH-SY5Y cells were exposed to RF-EMF on DIV1 and protein extraction was performed on DIV8 as described for the Western blot analyses. The ELISA was performed according to the manufacturer's instructions (Enzo Life Sciences, New York, USA). The β-catenin amount was normalized to the protein concentration in the sample obtained with Pierce protein assay reagent (Bio-Rad Laboratories, California, USA).

2.7. Microplate-based respirometry

The mitochondrial oxygen consumption rate (OCR) was analysed with the Seahorse Bioscience XF-24 analyzer (Agilent Technologies, California, USA) using a standard mitochondrial stress test. Cells were RF-EMF-exposed or sham-exposed for 24 h, followed by differentiation with RA or ST (see first-phase conditions) for 24 h. Cells were washed twice with DMEM medium (Sigma-Aldrich, Missouri, USA) containing of 25 mM D(+)-glucose, GlutaMAX™, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μl/ml streptomycin (all from Life Technologies, California, USA) before they were incubated in the same medium for 1 h at 37 °C without CO₂. Basal OCR was measured before a sequential exposure to modulators of mitochondrial activity was carried out. First, the ATP synthase inhibitor oligomycin [1 μM] was added to determine ATP-linked respiration. Second, the uncoupler, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), was sequentially added twice [each 0.3 μM]. FCCP shuttles protons across the inner membrane and, consequently, leads to maximal activity of the electron transport chain. Finally, mitochondrial electron transfer was completely inhibited by addition of rotenone [1.2 μM], an inhibitor of complex I, and antimycin A [0.6 μM], an inhibitor of complex III. The data were adjusted to the cell count in the respective well using the CyQuant assay (Molecular Probes, Oregon, USA). Experiments performed under limited glucose conditions were performed in DMEM medium deprived of

D(+)-glucose to observe mitochondrial function under additional cellular stress.

2.8. Oxidative stress - glutathione Assay

Decreases in cellular glutathione (GSH) levels were measured in order to detect an induction of oxidative stress. The assay is based on the conversion of a luciferin derivative into luciferin in the presence of GSH. Luminescence is proportional to the amount of glutathione present in the cells.

Following a 2 h exposure to RF-EMF at 1, 2 and 4 W/kg or sham on DIV1 under normal condition (medium with glucose) and under glucose deprivation, cells were harvested and washed once with $1 \times$ PBS. Exposed and sham-exposed cells (50 μ l) were added to a 96-well plate (density: 10,000 cells/well), 50 μ l of GSH-Glo™ Glutathione Assay (Promega, Switzerland) were added, followed by an incubation of 30 min at RT. Finally, 100 μ l of reconstituted luciferase detection reagent (Promega, Switzerland) were added and incubated for 15 min at RT before luminescence was measured on a microplate reader (Synergy HT, Bio-Tek, Switzerland). Menadione [75 μ M] was used as positive control.

2.9. Statistical analyses

For each evaluation, at least three independent experiments were performed. Data were analysed using a one-way ANOVA followed by Tukey's multiple comparison test to compare the means from RF-EMF-exposed samples and the respective sham-exposed controls (GraphPad Software Inc., California, USA). Data are presented as mean $\pm$ standard error of the mean (SEM). *P*-values of ≤ 0.05 were considered statistically significant.

3. Results

In the present study, neuronal differentiation and mitochondrial function were investigated in SH-SY5Y cells after RF-EMF or sham exposure as both are important in the development of neuronal differentiation and neurodegeneration. Moreover, related signaling-pathways were assessed.

3.1. Quantification of neuronal phenotypes

Undifferentiated neuron-like SH-SY5Y cells, exposed to RF-EMF or sham-exposed on DIV1, were differentiated with RA or ST and the neuronal phenotypes, namely numbers of TH-ir dopaminergic neurons and CHT1-ir cholinergic cells, were analysed at the end of the differentiation on DIV8. The number of dopaminergic neurons increased significantly ($p \leq .0001$) in ST-differentiated cells when compared to undifferentiated SH-SY5Y cells and RA-differentiated cells (Fig. 1A). RF-EMF exposure resulted in $2.0 \pm 0.7\%$ TH-ir cells in undifferentiated cells and $2.5 \pm 1.0\%$ in sham-exposed cells with the difference not being statistically significant. Likewise, RF-EMF exposure led to $1.7 \pm 0.6\%$ TH-ir cells in RA-differentiated cells and $1.6 \pm 0.5\%$ TH-ir cells in sham-exposed cells. In ST-differentiated cells, $24.8 \pm 2.8\%$ TH-ir cells were found after RF-EMF-exposure and $23.9 \pm 3.3\%$ TH-ir cells were obtained after sham-exposure (Fig. 1A). Nevertheless, no statistically significant differences were found in TH-ir cell counts when RF-EMF-exposed and sham-exposed cells were compared in any of the experimental conditions (undifferentiated cells, RA- or ST-differentiation) used. CHT1-ir cell counts of about 65% were found in undifferentiated cells and in cells differentiated with RA or ST regardless of the exposure (RF-EMF or sham) as shown in Fig. 1B. In RA-differentiated SH-SY5Y cells, similar CHT1-ir cells counts, namely $63.4 \pm 2.5\%$ and $60.7 \pm 3.1\%$ were obtained in cells exposed to RF-EMF and sham-exposed cells, respectively. Likewise, differentiation with ST resulted in $65.3 \pm 4.3\%$ and $63.5 \pm 5.2\%$ CHT1-ir cells after

RF-EMF and sham exposure, respectively. No statistically significant differences were obtained when RF-EMF-exposed cells were compared to sham-exposed cells regardless of the differentiation supplement.

3.2. Expression of differentiation markers

The differentiation markers, Erk1/2, Akt and GSK3 β were analysed on DIV8 after RF-EMF exposure or sham exposure on DIV1. The ratio of the active phosphorylated (p-) form, p-p44/42 Erk1/2 to the inactive non-phosphorylated form, p44/42 Erk1/2, was significantly increased in RA-differentiated cells (4-fold; $p \leq .05$ – 0.01) when compared to undifferentiated SH-SY5Y cells (Fig. 2A). RF-EMF exposure resulted in a ratio of 0.28 in undifferentiated cells and 0.36 in sham-exposed cells with the difference not being statistically significant. RA-differentiated cells exposed to RF-EMF led to a ratio of 1.7 and a ratio of 1.4 in sham-exposed cells. Finally, ratios of 0.60 and 0.69 were obtained when ST-differentiated cells were exposed to RF-EMF and sham-exposed, respectively. No statistically significant differences were found for the ratio of p-p44/42 Erk1/2 to p44/42 Erk1/2 in any of the experimental conditions used (undifferentiated cells, RA- or ST-differentiated cells) when RF-EMF-exposed and sham-exposed cells were compared (Fig. 2A).

Furthermore, completely differentiated cells (RA and ST) and undifferentiated control SH-SY5Y cells were exposed to RF-EMF or sham-exposed on DIV7 for 24 h, respectively. The ratio of p-p44/42 Erk1/2 to p44/42 Erk1/2 was significantly higher in RA-differentiated cells when compared to undifferentiated SH-SY5Y cells after sham-exposure (2.7-fold; $p \leq .05$) (Fig. 2B). In contrast, differentiation with ST did not result in a significant difference in the ratio of Erk1/2 when compared to undifferentiated cells. A significantly higher ratio was obtained in RA-differentiated cells compared to ST-differentiated SH-SY5Y cells ($p \leq .01$). Ratios of 0.51 and 0.44 were obtained in RF-EMF-exposed cells and sham-exposed undifferentiated SH-SY5Y cells, respectively. RF-EMF exposure resulted in ratio of 0.89 in RA-differentiated cells and 1.2 in sham-exposed and RA-differentiated cells with the effect not being statistically significant. A ratio of 0.28 for p-p44/42 Erk1/2 to p44/42 Erk1/2 was found in RF-EMF-exposed cells after ST-differentiation and a slightly higher ratio of 0.34 was obtained in sham-exposed cells, but this effect was not statistically significant.

Effects of RF-EMF exposure on Akt, known to be involved in neurite elongation during neuronal differentiation, were investigated with and without neuronal differentiation. Undifferentiated SH-SY5Y cells and cells differentiated with RA or ST were RF-EMF-exposed or sham-exposed on DIV1 for 24 h and the ratio of activated phosphorylated Akt (p-Akt) to the inactive form of this kinase, Akt was analysed. Despite the higher ratio in p-Akt/Akt seen in sham-exposed cells differentiated with RA (1.6-fold) and ST (1.9-fold) when compared to undifferentiated cells, this difference was not statistically significant as shown in Fig. 3. In undifferentiated SH-SY5Y cells, the ratio of p-Akt/Akt was 0.43 after RF-EMF exposure and 0.34 after sham exposure, the effect not being statistically significant. In RA-differentiated cells, the ratio of p-Akt to Akt was lower in RF-EMF-exposed cells (0.21) as compared to sham-exposed cells (0.54). However, this tendency was not statistically significant. Similar to the results obtained after differentiation with RA, ST differentiation did not lead to a significant difference between RF-EMF-exposed cells (ratio p-Akt/Akt = 0.73) and sham-exposed cells (ratio p-Akt/Akt = 0.64).

GSK3 β levels were increased in RA- and ST-differentiated cells when compared to undifferentiated SH-SY5Y cells (1.5-fold), but these differences were not statistically significant (Fig. 4). RF-EMF exposure did not result in an increase in the active non-phosphorylated form of GSK3 β in undifferentiated cells when compared to undifferentiated sham-exposed cells. No significant differences were found in RA- and ST-differentiated RF-EMF-exposed cells compared to sham-exposed cells as shown in Fig. 4.

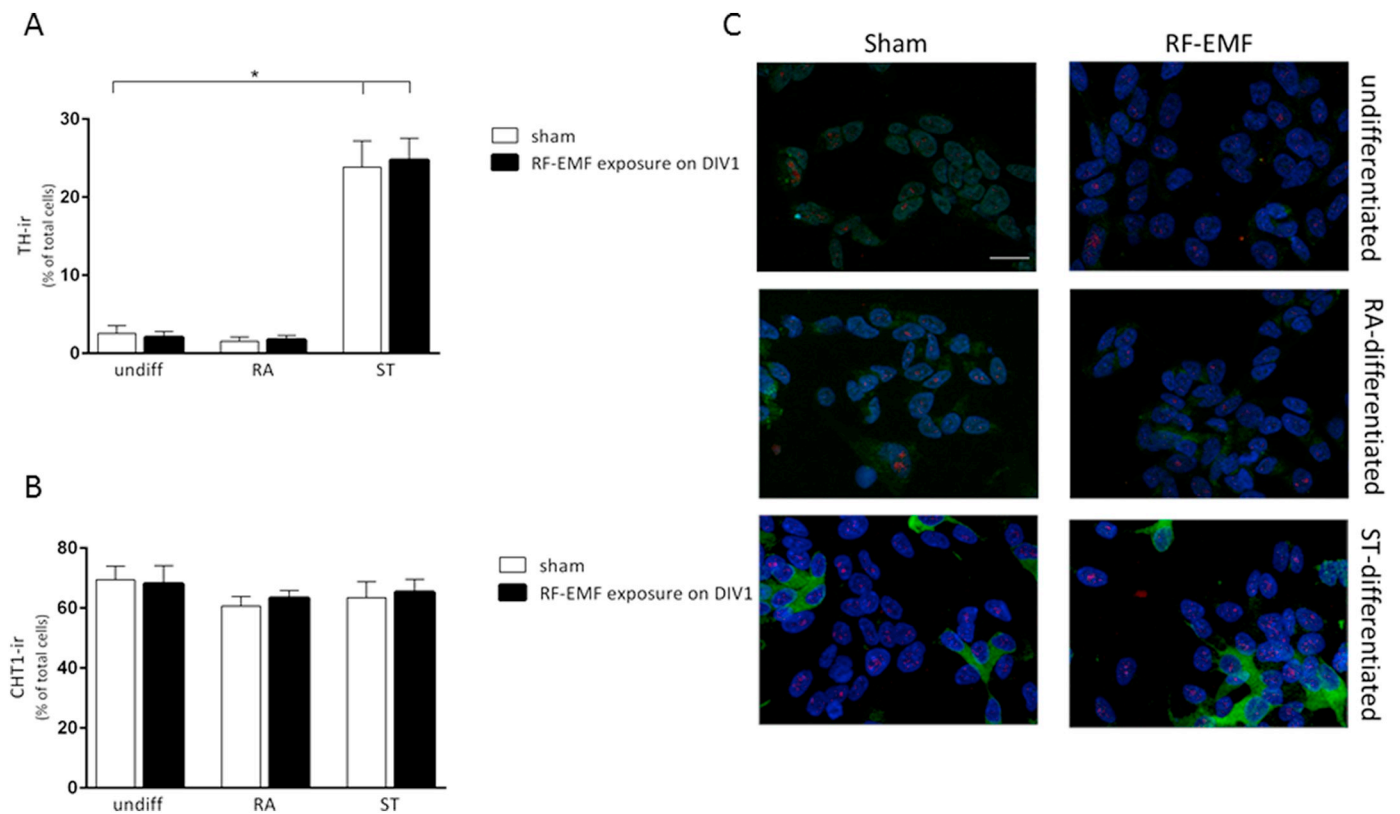


Fig. 1. SH-SY5Y cells were RF-EMF-exposed at 4 W/kg or sham-exposed for 24 h on day *in vitro* 1 (DIV1). Tyrosine hydroxylase immunoreactive (TH-ir) cells (A) and choline transporter 1 immunoreactive (CHT1-ir) cells (B) were analysed and quantified on DIV8 in undifferentiated cells (undiff), *all-trans* retinoic acid (RA)- and staurosporine (ST)-differentiated cells. Data are shown as mean percentage $\pm$ standard error of the mean (SEM) (%) of TH-ir and CHT1-ir cells in relation to the total number of cells; error bars represent the SEM ($n = 11$ to 24). Statistically significant differences are labeled with asterisks ($* = p \leq .0001$). Representative immunofluorescence images are depicted in C; TH-stained cells (green), CHT1 (red) and cell nuclei in blue. Scale bar = 20 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Analyses of β -catenin

Protein expression of β -catenin, a major player in the Wnt pathway that is involved in neurodegeneration, was investigated. β -catenin levels were significantly increased in sham-exposed RA-differentiated cells when compared to undifferentiated SH-SY5Y cells (45%) with the effect being statistically significant ($p \leq .05$) as illustrated in Fig. 5. A significant increase in β -catenin was also found in RF-EMF-exposed undifferentiated cells compared to RF-EMF-exposed differentiated cells ($p \leq .0001$). β -catenin levels in undifferentiated RF-EMF-exposed cells were not significantly different from undifferentiated sham-exposed SH-SY5Y cells as shown in Fig. 5.

In contrast to the differentiation with RA, differentiation with ST did not result in a significant increase in β -catenin concentrations in controls. RF-EMF-exposure increased the β -catenin levels in ST-differentiated cells when compared to sham-exposed ST-differentiated cells. However, this effect was not statistically significant (Fig. 5).

3.4. Microplate-based respirometry

The OCR was analysed in RF-EMF-exposed and sham-exposed undifferentiated SH-SY5Y cells and cells at the beginning of the differentiation in either DMEM medium with and without 25 mM glucose, respectively (Fig. 6A and B).

In experiments with DMEM medium containing 25 mM glucose, no significant differences in basal, leak and maximal respiration were found in cells differentiated with RA and ST compared to undifferentiated cells regardless of the exposure. However, differentiation with RA resulted in higher, but not significantly different basal OCR

levels. This increase in OCR levels was not seen in RF-EMF-exposed SH-SY5Y cells. In contrast, differentiation with ST led to a slight decrease in basal OCR levels. No difference was found in basal OCR levels after RF-EMF exposure when compared to sham exposure in any of the experimental conditions (undifferentiated, RA-, ST-differentiated) (Fig. 6A). At maximal respiration, an increase in the OCR was seen in RA-differentiated cells (sham) when compared to undifferentiated cells (sham), but this difference was not statistically significant. On the other hand, a decrease was found in ST-differentiated SH-SY5Y cells (sham) compared to undifferentiated sham-exposed cells as illustrated in Fig. 6A. RF-EMF exposure of undifferentiated cells showed no variation compared to sham-exposure. On the contrary, the OCR of RA- and ST-differentiated cells was decreased after RF-EMF exposure. In any case, no statistically significant differences were found between RF-EMF-exposed and sham-exposed cells in DMEM medium regardless of the differentiation status (Fig. 6A).

As expected, absolute OCR levels were considerably lower (2- to 8-fold) in undifferentiated and differentiated SH-SY5Y cells in the glucose-deprived medium (Fig. 6B). A trend towards a reduction in basal respiration levels was found in all RF-EMF exposed cells (undifferentiated, RA- and ST-differentiated) with the most prominent effect in ST-differentiated cells. Similarly, leak respiration was decreased in RF-EMF-exposed cells compared to the respective sham-exposed cells, but none of these effects were statistically significant. At maximal respiration, RF-EMF-exposed undifferentiated cells showing significantly decreased OCR levels after FCCP injection, when compared to sham-exposed undifferentiated cells ($p \leq .05$) as shown in Fig. 6B. OCR levels in RA-differentiated cells were not significantly different when RA-differentiated RF-EMF-exposed and RA-differentiated sham-exposed

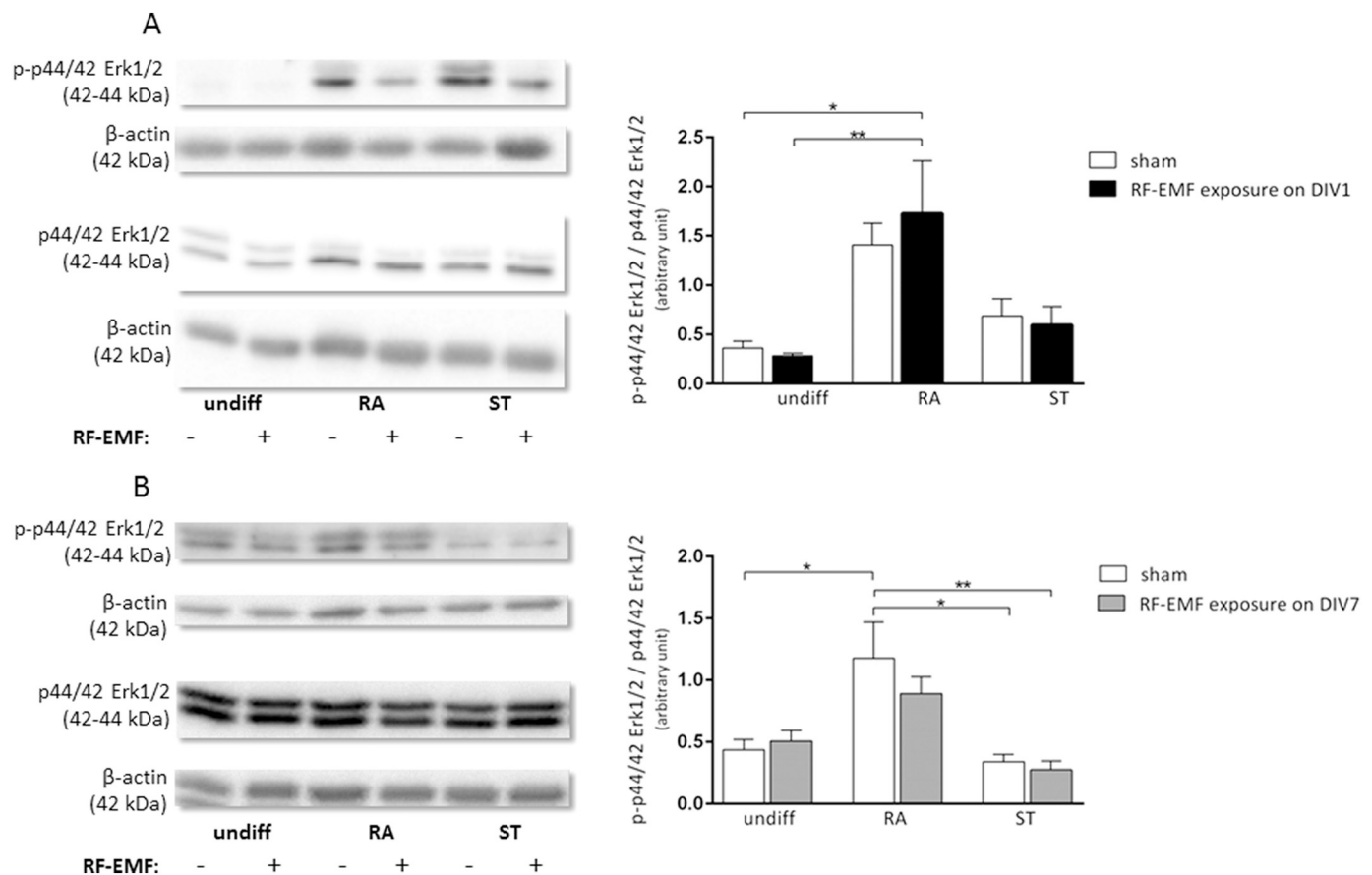


Fig. 2. SH-SY5Y cells were RF-EMF-exposed at 4 W/kg or sham-exposed for 24 h on day *in vitro* 1 (DIV1) (A) or DIV7 (B). The ratio of the active phosphorylated form of the extracellular signal-regulated kinases, p-p44/42 Erk1/2, and the inactive non-phosphorylated form, p44/42 Erk1/2, were analysed in undifferentiated cells (undiff), *all-trans* retinoic acid (RA)- and staurosporine (ST)-differentiated cells. Representative images of Western blots are shown on the left. Error bars represent the standard error of the mean (SEM). Significant differences are labeled with asterisks (* = $p \leq .05$; ** = $p \leq .01$).

cells were compared. RF-EMF exposure, followed by ST differentiation, resulted in a significant decrease in OCR levels ($p \leq .01$) when compared to the respective sham-exposed SH-SY5Y cells (Fig. 6B).

Oxidative stress was investigated by measurements of glutathione (GSH) levels under normal condition and glucose deprivation for 2 h in RF-EMF-exposed cells at 1, 2 and 4 W/kg and sham-exposed cells on DIV1 (Fig. 7). The data show that glucose deprivation in sham-exposed cells for 2 h resulted in significantly lower GSH levels at 1 and 4 W/kg when compared to cells under normal conditions. A trend for decreased

GSH levels was also apparent at 2 W/kg even though this difference was not statistically significant. Similarly, RF-EMF exposure under glucose deprivation resulted in significantly lower GSH values compared to RF-EMF exposure under normal conditions at 1 and 4 W/kg even though a trend was also found at 2 W/kg. No significant reductions of GSH levels were found in RF-EMF-exposed cells compared to sham-exposed cells under normal conditions and glucose deprivation as illustrated in Fig. 7. Mendadione led to significantly different GSH levels when compared to the respective sham-exposed cells at all SAR levels.

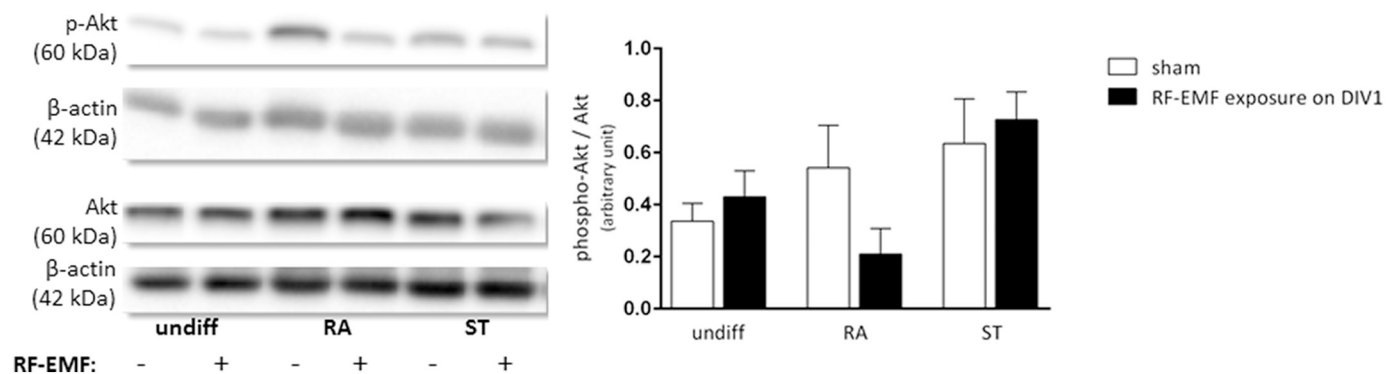


Fig. 3. SH-SY5Y cells were RF-EMF-exposed at 4 W/kg or sham-exposed for 24 h on day *in vitro* 1 (DIV1). The ratio of the active phosphorylated form of the protein kinase B (p-Akt) to the inactive non-phosphorylated form (Akt) was analysed on DIV8 in undifferentiated cells (undiff), *all-trans* retinoic acid (RA)- and staurosporine (ST)-differentiated cells. Representative images of Western blots are given on the left. Error bars represent the standard error of the mean (SEM).

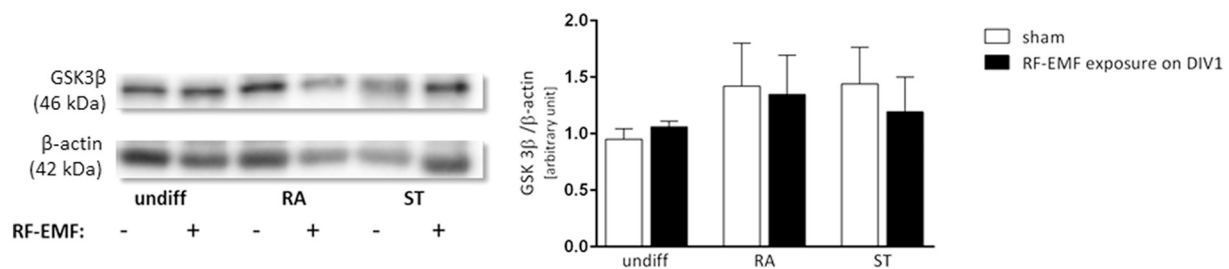


Fig. 4. SH-SY5Y cells were RF-EMF-exposed at 4 W/kg or sham-exposed for 24 h on day *in vitro* 1 (DIV1). Protein levels of the active non-phosphorylated glycogen synthase kinase 3 beta (GSK3β) were analysed on DIV8 in undifferentiated cells (undiff), *all-trans* retinoic acid (RA)- and staurosporine (ST)-differentiated cells. Representative images of the Western blots are given on the left. Error bars represent the standard error of the mean (SEM).

3.5. Expression of proteins involved in mitochondrial fusion

Comparable expression levels of OPA1 in undifferentiated cells were obtained regardless of the membrane-bound long form of OPA1 (l-OPA1) and the cleaved short form of OPA1 (s-OPA1) and the exposure (RF-EMF or sham) as depicted in Fig. 8A. However, a slight, but not significant increase of s-OPA1 compared to l-OPA1 was seen in undifferentiated RF-EMF-exposed cells and in sham-exposed cells, but these differences were not statistically significant (Fig. 8A). Similar data were obtained for SH-SY5Y cells differentiated with RA or ST. No significant differences were obtained between both OPA1 forms regardless of the exposure or the differentiation supplement used.

Furthermore, undifferentiated control SH-SY5Y cells and fully differentiated cells (RA and ST) were RF-EMF- and sham-exposed on DIV7 for 24 h, respectively (Fig. 8B). No significant differences were obtained

when the l- and the s-forms of OPA1 were compared in sham-exposed undifferentiated cells. RF-EMF exposure of undifferentiated SH-SY5Y cells resulted in l-OPA1 and s-OPA1 levels of 83% and 72% s-OPA1, respectively, with the difference not being statistically significant. In RA-differentiated cells, exposure to RF-EMF led to similar l-OPA1 and s-OPA1 levels as those obtained in sham-exposed differentiated cells (Fig. 8B). Similarly, no significant differences were found in ST-differentiated cells regardless of the OPA1 form or the exposure (RF-EMF or sham) examined as shown in Fig. 8B. Positive controls were run using hydrogen peroxide (Fig. 8C). Hydrogen peroxide induced a decrease in l-OPA1 of about 50% with the effect not being time-dependent.

Mitofusin 2 (Mfn2), a protein involved in mitochondrial fusion, was analysed in undifferentiated and differentiated SH-SY5Y cells after RF-EMF and sham exposure. A decrease in Mfn2 was found in undifferentiated SH-SY5Y cells exposed to RF-EMF-exposure on DIV1

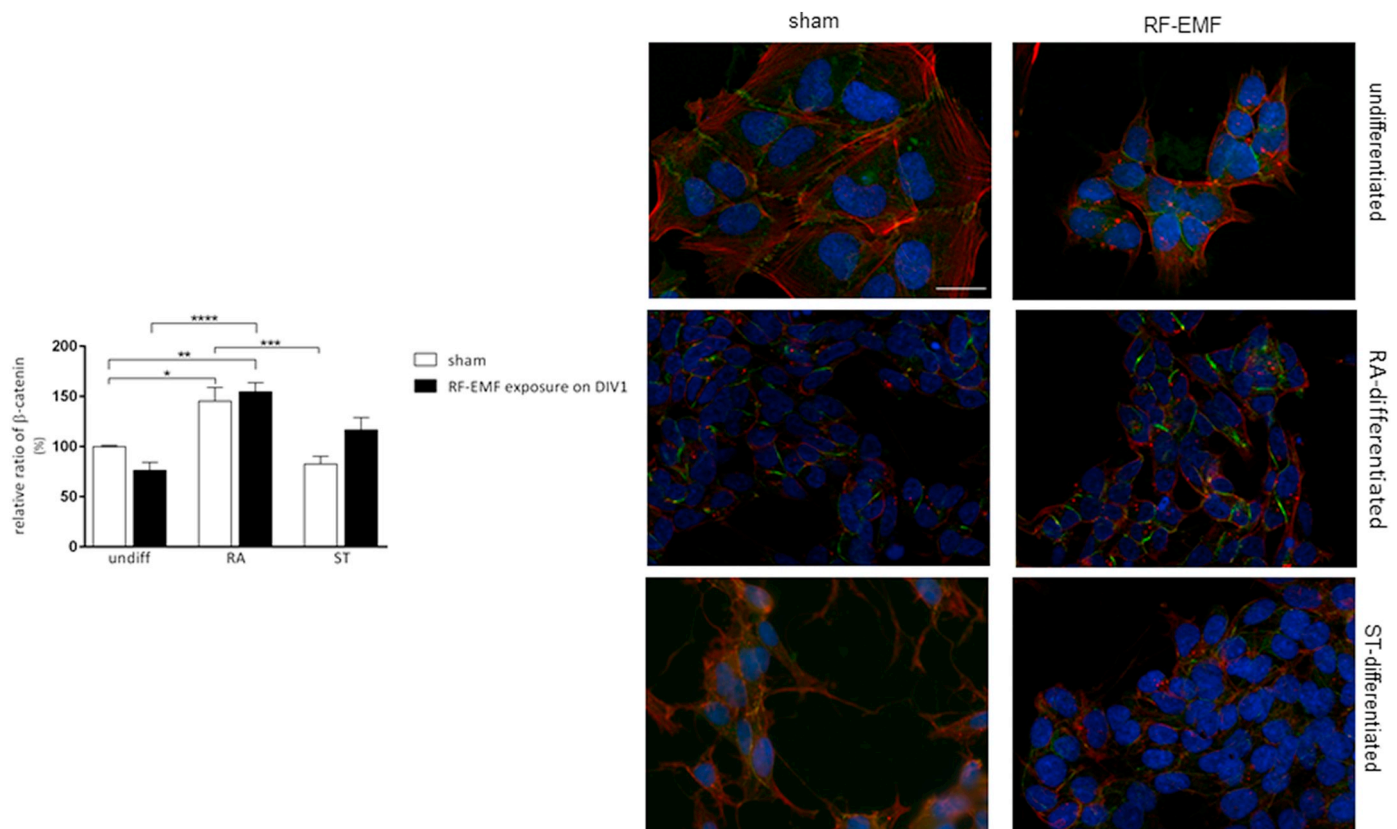
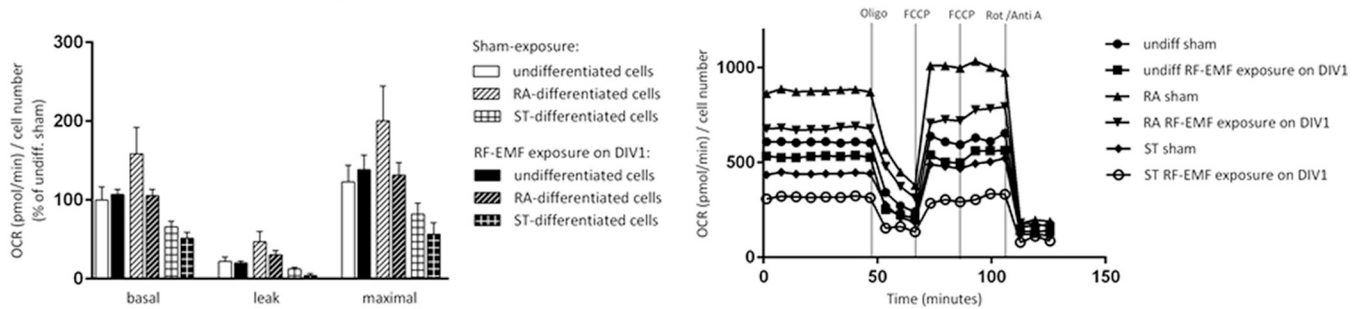


Fig. 5. SH-SY5Y cells were RF-EMF-exposed at 4 W/kg or sham-exposed for 24 h on day *in vitro* 1 (DIV1). Protein levels of the active non-phosphorylated β-catenin were analysed on DIV8 in undifferentiated cells (undiff), *all-trans* retinoic acid (RA)- and staurosporine (ST)-differentiated cells. The β-catenin content was normalized by protein concentration. Data are given as mean of the relative ratio to undifferentiated sham-exposed cells (100%). Error bars represent the standard error of the mean (SEM) ($n = 10$ to 12). Significant differences are labeled with asterisks (* = $p \leq .05$; ** = $p \leq .01$; *** = $p \leq .001$; **** = $p \leq .0001$). Representative immunofluorescence images are depicted on the right; β-catenin-stained cells (green), Hoechst 33342 nucleic acid stain (red) and cell nuclei in blue. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A: DMEM medium (25 mM D(+)-glucose)



B: DMEM medium deprived of D(+)-glucose

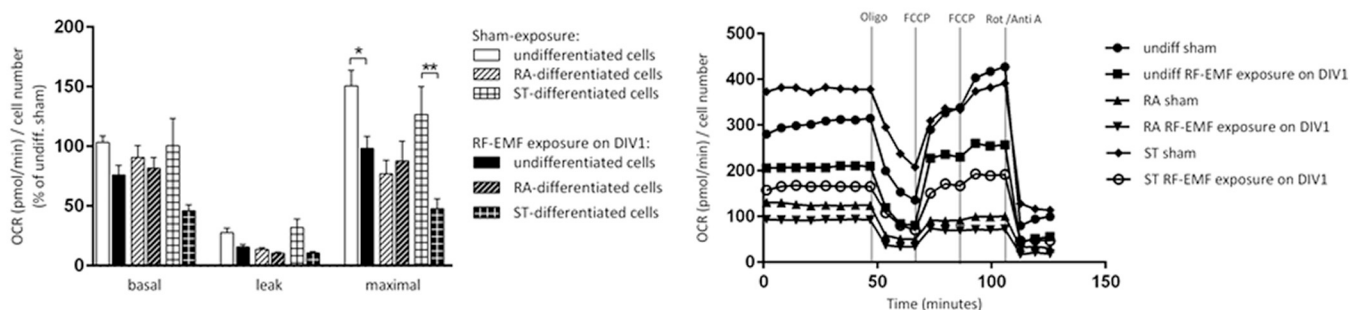


Fig. 6. SH-SY5Y cells were RF-EMF-exposed at 4 W/kg or sham-exposed for 24 h on day *in vitro* 1 (DIV1) and mitochondrial oxygen consumption rates (OCR) were measured on DIV3 in undifferentiated cells (undiff) and differentiated SH-SY5Y cells with *all-trans* retinoic acid (RA)- and staurosporine (ST) for 24 h, respectively. SH-SY5Y cells were sequentially exposed to each mitochondrial modulator (oligomycin = Oligo, FCCP and rotenone/antimycin A = Rot/Anti A). Representative runs of the mitochondrial stress test on the OCR are depicted on the right. Data are shown as mean of the relative ratio to basal respiration of undifferentiated sham-exposed cells (100%) for basal, leak and maximal respiration on the left. Error bars represent the standard error of the mean (SEM). Significant differences are labeled with asterisks (* = $p \leq .05$; ** = $p \leq .01$).

when compared to sham-exposed cells. However, this effect was not statistically significant as shown in Fig. 9. In cells differentiated with RA or ST, RF-EMF exposure did not result in significant alterations of Mfn2 levels when compared to sham-exposed cells. Similarly, Mfn2 levels between undifferentiated or differentiated cells, exposed to RF-EMF or sham-exposed at the end of the differentiation (DIV7), did not differ significantly (Fig. 9).

4. Discussion

The present study was designed to evaluate the effects of RF-EMF exposure, emitted from a mobile phone at GSM 900 MHz and at a SAR value of 4 W/kg on neuronal differentiation, mitochondrial integrity and function and underlying signaling pathways involved in neuronal differentiation and neurodegeneration.

The maximal number of dopaminergic neurons was obtained by ST differentiation, a finding that was also reported in a previously published study (Wiedmer et al., 2019). No difference in the number of TH-ir was found when RF-EMF-exposed cells were compared to sham-exposed cells indicating that RF-EMF exposure does not alter the neuronal phenotype in SH-SY5Y cells under differentiation. In contrast to our findings, a significant increase in TH-ir cells has been reported for the hippocampus of male rats exposed to 900 MHz at 5.95×10^{-4} W/kg (2 h/day, 5 days/week) for 30 days (Megha et al., 2015). The differences between the findings might be explained by variations in the exposure conditions. In addition, *in vivo* exposure represents a higher complexity compared to *in vitro* experiments, which may lead to a different outcome. Extreme low frequency (ELF)-EMF was

demonstrated to promote neuronal differentiation in neuroblastoma cells (SH-SY5Y) after exposure to 1 mT, 50 Hz for up to 196 h (Falone et al., 2007). Besides the difference in the EMF exposure, namely the frequency, the resulting neuronal phenotype was not characterized in that study.

The cells used in the present study, namely SH-SY5Y, have been widely used in PD research because of their human origin, the ease of maintenance and the possibility to differentiate them into various phenotypes, especially dopaminergic neurons (Xicoy et al., 2017). Furthermore, it has been demonstrated that major signaling pathways involved in neurodegeneration are intact in these cells (Krishna et al., 2014). However, using this model to investigate possible effects related to differentiation and neurodegeneration, a characterization of the resulting phenotype is important as reported previously (Wiedmer et al., 2019).

Quantitative measurements of the CHT1 expression showed a comparable number of CHT1-ir SH-SY5Y cells in RF-EMF-exposed and sham-exposed cells at any of the experimental conditions used (undifferentiated SH-SY5Y cells, RA- or ST-differentiation) suggesting that RF-EMF exposure does not alter the cholinergic phenotype. Other studies corroborate these findings, demonstrating that whole-body exposure of male rats to 900 MHz RF-EMF at 0.023 W/kg for 1 h per day and 28 consecutive days did not change acetylcholine protein expression levels (Gupta et al., 2018). In contrary, RF-EMF exposure to 2450 MHz resulted in cognitive deficits in the rats that were accompanied by an activation of apoptotic factors in the hippocampus, a decrease in acetylcholine levels and a loss of mitochondrial function and integrity indicating an impaired mitochondrial and cholinergic

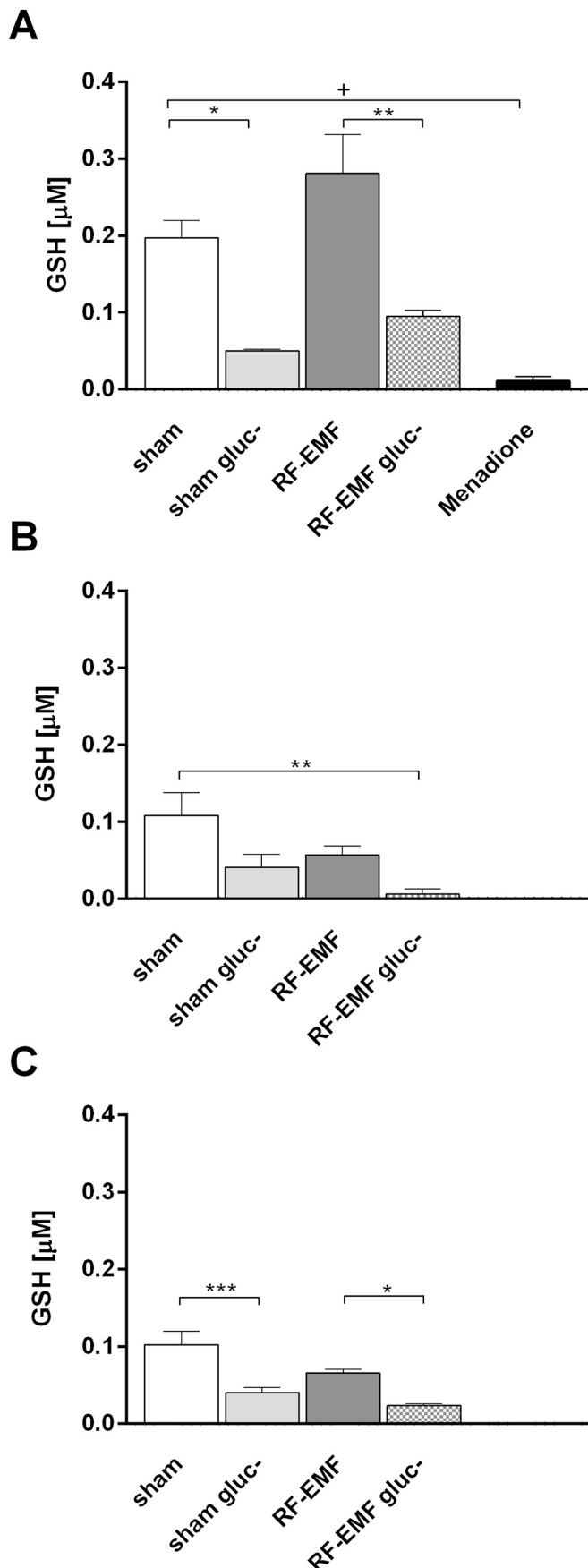


Fig. 7. SH-SY5Y cells were sham- or RF-EMF-exposed at 1 (A), 2 (B) or 4 (C) W/kg for 2 h on day *in vitro* 1 (DIV1) under normal conditions and glucose-deprivation (gluc-). Glutathione levels (GSH) were measured at the end of the RF-EMF- or sham exposure. Menadione [75 μ M] was used as a positive control and comparisons were made to the respective sham control. Data are shown as extrapolated GSH levels [μ M] for each group normalized to the total protein content. Error bars represent the standard error of the mean (SEM) ($n = 4$). Significant differences are labeled with asterisks (* = $p \leq .05$; ** = $p \leq .01$; *** = $p \leq .001$). Differences between the positive control and sham-exposed cells under normal conditions were labeled as + (+ = $p \leq .001$).

function (Gupta et al., 2018).

MAPKs were reported to be involved in neuronal differentiation (Lee et al., 2016). Levels of p-Erk1/2 were not different in RF-EMF-exposed cells compared to sham-exposed in undifferentiated, RA- or ST-differentiated cells. In contrast to our findings, elevated levels of p-Erk1/2 were demonstrated in SH-SY5Y cells after exposure to 900 MHz 1 W/kg for 5 to 15 min with the effect being transient as basal levels of p-Erk1/2 were restored after 30 min to 24 h (Buttiglione et al., 2007). The most prominent increase in Erk1/2 was demonstrated to occur within 10–15 min in Rat1 and HeLa cells after exposure to 0.1 to 0.31 mW/cm² at 875 MHz, but no alterations of the MAPK, c-Jun N-terminal kinase (JNK) and p38, were found (Friedman et al., 2007). In contrast to our findings, increases in protein levels of the mitogen activated protein kinase (MAPK) p38 and stress response genes were demonstrated in Wistar rats exposed to 3G RF-EMF mobile phones for 2 h daily for 60 consecutive days (Kesari et al., 2014). Besides the frequency used in that study, average SAR values (0.26 W/kg) were lower compared to those used in the present study. Corroborating our findings, RF-EMF exposure did not change the phosphorylation of p38 and Erk1/2 MAPK in primary human and rat astrocytes. In contrast, RF EMF (918 MHz, 0.25 W/kg) significantly decreased hydrogen peroxide-induced phosphorylation of these MAPK (Tsoy et al., 2019).

Increased levels of p-Akt, an anti-apoptotic kinase supporting cellular survival, were found in ST- and RA-differentiated cells. RF-EMF exposure did not alter p-Akt in undifferentiated and differentiated SH-SY5Y cells suggesting that RF-EMF exposure does not increase apoptosis under the experimental conditions used in this study. Furthermore, GSK3 β levels remained similar in undifferentiated and differentiated SH-SY5Y cells after RF-EMF exposure when compared to sham exposure. In line with previously published findings, RF-EMF did not affect Akt/GSK3 β signaling. However, there is some evidence that RF-EMF exposure alters apoptosis. Increased levels of Bcl-2-associated X protein (Bax) and decreased levels of B-cell lymphoma 2 (Bcl-2) were demonstrated in the brain of male rats exposed to 900 MHz at 1.13 W/kg for 2 h daily and 60 consecutive days (Motawi et al., 2014). Long-term exposure of rats to 900 MHz and 1800 MHz (1 year for 60 min/5 days per week) resulted in increases in caspases 3 and 9 in hippocampal and dorsal root ganglion neurons (Ertlav et al., 2018). In contrast, caspase 3 levels were not elevated in murine neuronal stem cells after a short-term exposure (120 min) at 900 MHz and 2.3 W/kg (Eghlidospour et al., 2017). Different exposure times and experimental models, namely *in vivo* versus *in vitro* experiments and the use of different apoptosis markers could explain this divergence.

The Akt/GSK3 β pathway was also demonstrated to be activated during neuronal differentiation (Miloso et al., 2004), and an inhibition of this pathway was reported to prevent differentiation (Qiao et al., 2012). In the present study, RF-EMF did not affect the key markers of this signaling pathway, Akt and GSK3 β , leading to the conclusion that RF-EMF does not interfere with this pathway. This result is in line with our findings that RF-EMF exposure did not alter the neuronal phenotype obtained after differentiation.

The Wnt/ β -catenin pathway was shown to be critical for neuronal

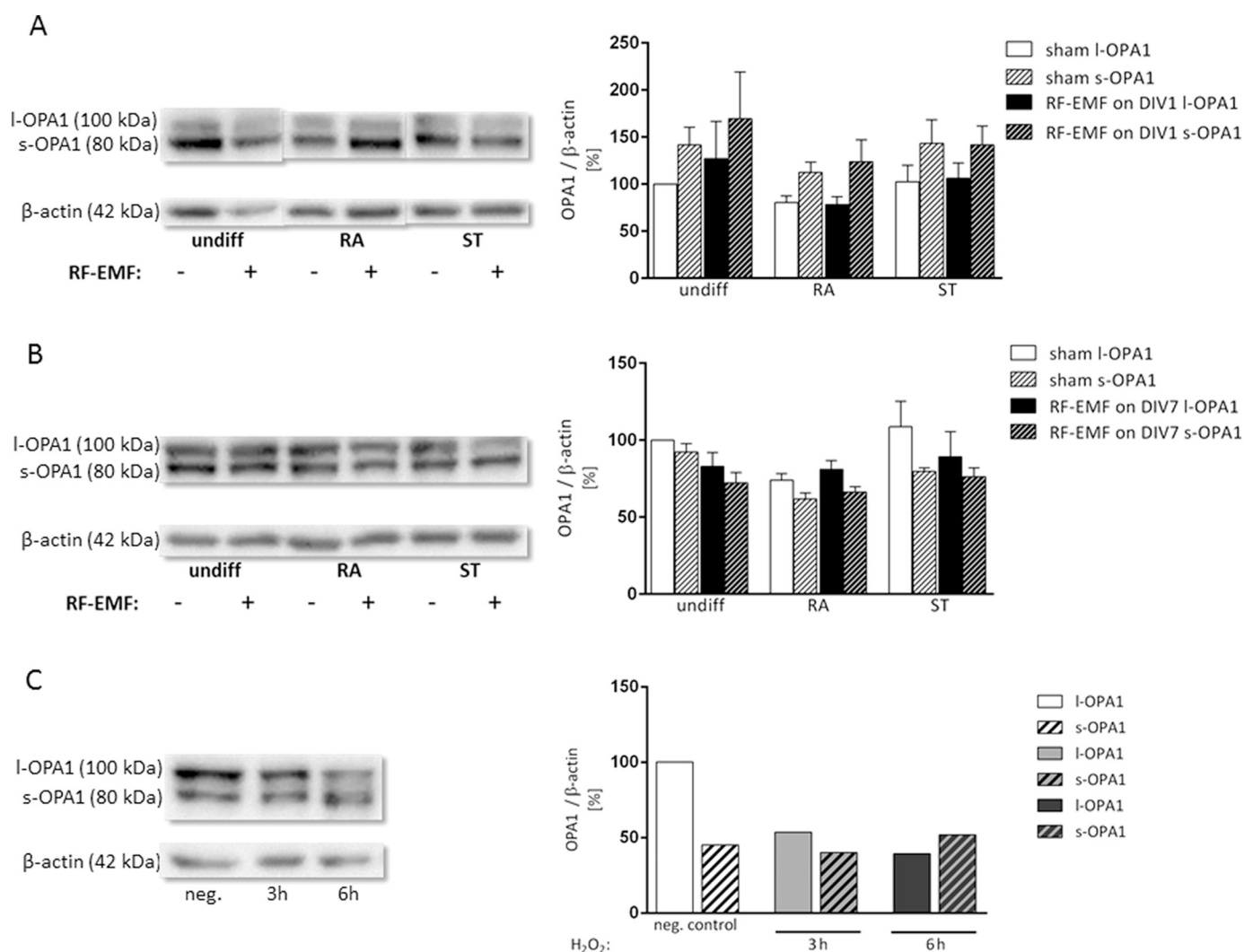


Fig. 8. SH-SY5Y cells were RF-EMF-exposed at 4 W/kg or sham-exposed for 24 h on day *in vitro* 1 (DIV1) (A) and DIV7 (B). Membrane-bound I-OPA1 and cleaved s-OPA1 levels were analysed on DIV8 in undifferentiated cells (undiff), *all-trans* retinoic acid (RA)- and staurosporine (ST)-differentiated SH-SY5Y cells ($n = 3$). I-OPA1 in sham-exposed undifferentiated cells was defined as 100% and used as a reference value. Positive controls were obtained in SH-SY5Y cells treated with 250 μ M hydrogen peroxide for 3 h and 6 h (C). Representative images of the Western blots are given on the left. Error bars represent the standard error of the mean (SEM).

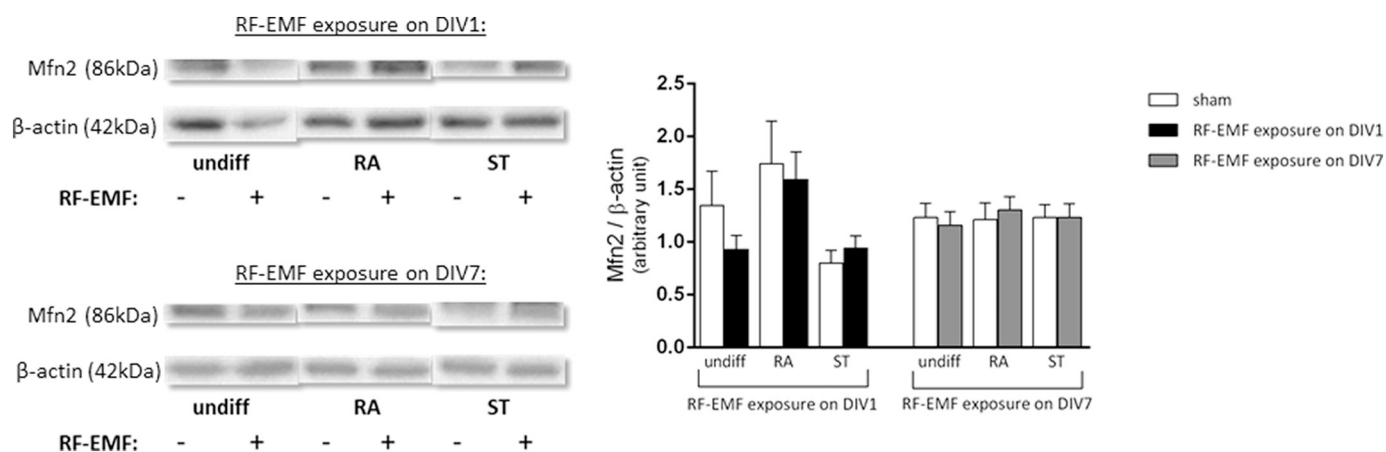


Fig. 9. SH-SY5Y cells were RF-EMF-exposed at 4 W/kg or sham-exposed for 24 h on day *in vitro* 1 (DIV1) and DIV7 ($n = 3$). Mitofusin 2 (Mfn2) levels were analysed on DIV8 in undifferentiated cells (undiff), *all-trans* retinoic acid (RA)- and staurosporine (ST)-differentiated SH-SY5Y cells. Representative images of Western blots are given on the left. Error bars represent the standard error of the mean (SEM).

differentiation (Muroyama et al., 2004). The slight increase in β -catenin that was found after differentiation with RA and a tendency towards elevated β -catenin levels observed in ST-differentiated cells exposed to RF-EMF compared to sham-exposed cells might indicate that RF-EMF exposure alters neuronal differentiation. However, these effects were not statistically significant and therefore, a possible association between RF-EMF exposure and an impairment of neuronal differentiation or a promotion of neurodegeneration remains rather speculative. To our knowledge, no data on effects of RF-EMF exposure on Wnt/ β -catenin signaling in neurons are currently available. However, ELF-EMF exposure of mice to 1 mT, 50 Hz for 3.5 h daily for 12 consecutive days stimulated the Wnt/ β -catenin signaling as increased mRNA levels of Wnt3 and raised the protein expression of nuclear β -catenin were obtained in cells of the subventricular zone of the brain, this reflecting a stimulation of neurogenesis (Mastrodonato et al., 2018).

Mitochondrial integrity and function are closely related to neuronal differentiation and neurodegeneration as mitochondrial dynamics, trafficking, turnover and biogenesis play key roles in both (Agostini et al., 2016; Zheng et al., 2016; Almeida and Vieira, 2017; Golpich et al., 2017; Franco-Iborra et al., 2018; Panchal and Tiwari, 2018). A tendency towards decreased mitochondrial respiration was found after RF-EMF exposure in RA- and ST-differentiated cells cultured in medium containing D(+)-glucose. The decrease in mitochondrial respiration was even more pronounced in cells grown in medium lacking D(+)-glucose as a significant reduction was seen at the maximal respiration rate in undifferentiated and ST-differentiated cells exposed to RF-EMF. In contrast, no alterations of the mitochondrial fission and fusion protein levels, OPA1 and Mfn2, were found after RF-EMF exposure. To our knowledge, this is the first time that effects of RF-EMF on mitochondrial fission and fusion proteins were studied. Our findings indicate that RF-EMF does not impair mitochondrial fission and fusion. A significant reduction of the OCR levels was found at maximal respiration in undifferentiated SH-SY5Y cells and ST-differentiated cells deprived of glucose after RF-EMF exposure. These findings suggest that RF-EMF leads to an impairment of mitochondrial function that is only manifest at maximal respiration and in combination with additional stressors such as glucose deprivation. Impairment of mitochondrial fission and fusion could be anticipated after RF-EMF exposure because an increase in ROS production has often been reported after RF-EMF exposure (Luukkonen et al., 2009; Ertilav et al., 2018). On the other hand, a significant reduction in DNA damage *via* ROS formation was shown in RF-EMF (1950 Hz, 0.3–1.25 W/kg)-exposed SH-SY5Y cells for 20 h treated with menadione, a compound known to induce DNA damage by ROS production. This effect was absent in cells that were not treated with menadione, indicating an adaptive response in the menadione-treated cells (Falone et al., 2018). Similarly, menadione resulted in significant decreased GSH levels in the present study. Glutathione levels did not differ between RF-EMF-exposed cells and sham-exposed cells after 2 h of exposure on DIV1. The reduction in GSH levels under glucose deprivation indicates higher oxidative stress in these cells. However, no clear dose-/SAR response was apparent as the effect was already seen at 1 W/kg. This finding might explain the decrease in mitochondrial respiration in cells grown under limited glucose for 2 h. Direct ROS measurements could not be performed because all the available assays require live cell measurements in 96-well plates. The SH-SY5Y cells were grown and differentiated in 35 mm Petri dishes and could not be transferred to 96-well plates without generating additional stress for the cells. The exposure in the waveguides had to be done in 35 mm Petri dishes to create a homogenous RF-EMF exposure. Therefore, ROS could not be measured at the end of the differentiation. Contrary to our findings, in primary cortical neuronal cultures of rats, ROS production was found after RF-EMF exposure to 1800 MHz, 2 W/kg for 24 h (Xu et al., 2010). Increased ROS generation was also demonstrated in brains of male rats after RF-EMF exposure for 2 h per day and 30 days at 900 and 1800 MHz, 5.95×10^{-4} W/kg and 5.84×10^{-4} W/kg, respectively (Megha et al., 2012). ROS-induced

cleavage of l-OPA1 into s-OPA1 was found in primary mouse embryonic fibroblasts that was due to activation of metalloendopeptidase (OMA1) after exposure to hydrogen peroxide (0.5 mM) for 2 h (Baker et al., 2014). OPA1 impairment was shown to be involved in neurodegeneration as OPA1 haploinsufficient induced pluripotent stem cells (iPSC) obtained from PD patients resulted in a reduction of OPA1 levels after differentiation into dopaminergic neurons, leading to reduced complex I levels and impaired oxidative phosphorylation. Reduced basal and maximal OCR levels were found at late (DIV65), but not early (DIV25) differentiation times in the dopaminergic neurons (Jonikas et al., 2018). It can be speculated that in the present study, RF-EMF exposure did not result in ROS formation in the SH-SY5Y cells or the ROS production might have been transient as has been shown in other *in vitro* studies (Luukkonen et al., 2009; Xu et al., 2010). Hence, the mitochondrial proteins OPA1 and Mfn2 remained unchanged.

Besides mitochondrial fission and fusion, mitochondrial respiration is of particular importance during neuronal differentiation as the metabolic switch from glycolysis to oxidative phosphorylation was demonstrated to be accompanied by an increase in mitochondrial mass, ATP production and ROS generation (Agostini et al., 2016; Zheng et al., 2016; Almeida and Vieira, 2017). Decreased oxidative phosphorylation, namely a reduction in the activity of respiratory chain complexes I, II, IV and V, was reported to be frequency-dependent. Whole-body exposure of male rats to 900 MHz, 0.023 W/kg and 2450 MHz, 0.062 W/kg for 1 h per day and 28 consecutive days reduced the activity of all respiratory chain complexes when exposed to 2450 MHz but not to 900 MHz (Gupta et al., 2018). Even short-term exposure for 24 h to 1800 MHz at 2 W/kg increased the expression of 8-hydroxyguanine, a common biomarker of deoxyribonucleic acid (DNA) oxidative damage, in mitochondria of primary cortical neurons (Xu et al., 2010). In line with the findings by Gupta et al. and Xu et al., the reduced OCR levels found at maximal respiration after RF-EMF exposure in the present study might indicate that RF-EMF exposure leads to an impairment of the mitochondrial function in neuron-like cells (Xu et al., 2010; Gupta et al., 2018).

5. Conclusions

RF-EMF exposure at 935 MHz and an SAR of 4 W/kg for 24 h did not alter the neuronal phenotype after neuronal differentiation in SH-SY5Y cells. The data on markers of the signaling pathways (Akt/GSK3 β ; MAPK; Wnt/ β -catenin) involved in neuronal differentiation and neurodegeneration corroborate the hypothesis that RF-EMF exposure does not interfere with neuronal differentiation. Defective mitochondrial function is often related to neurodegeneration. RF-EMF exposure altered mitochondrial respiration only in glucose-deprived SH-SY5Y cells. GSH levels were substantially lower in cells under glucose deprivation, but RF-EMF did not change GSH production. Proteins responsible for mitochondrial fission and fusion were not altered by RF-EMF exposure, supporting the findings that mitochondrial integrity and function is not impaired.

Further research is needed to investigate the effects of RF-EMF on mitochondrial function in detail because mitochondrial impairment is closely related to the pathogenesis of neurodegenerative diseases.

Acknowledgments

The authors thank Nadja Peduto for helping with the cell culture work and the radiation system maintenance and Andrea Felser for her scientific support for the Seahorse experiments. The authors also thank Prof. Dr. Michael Stoffel for editing the manuscript. Microscopy was performed on equipment supported by the Microscopy Imaging Center (MIC), University of Bern, Switzerland.

This study was funded by the Swiss Research Foundation for Electricity and Mobile Communication, Swiss Federal Institute of Technology (ETH).

Declaration of Competing Interest

None.

References

- Agostini, M., Romeo, F., Inoue, S., Niklison-Chirou, M.V., Elia, A.J., Dinsdale, D., Morone, N., Knight, R.A., Mak, T.W., Melino, G., 2016. Metabolic reprogramming during neuronal differentiation. *Cell Death Differ.* 23, 1502–1514.
- Almeida, A.S., Vieira, H.L.A., 2017. Role of cell metabolism and mitochondrial function during adult neurogenesis. *Neurochem. Res.* 42, 1787–1794.
- Baker, M.J., Lampe, P.A., Stojanovski, D., Korwitz, A., Anand, R., Tatsuta, T., Langer, T., 2014. Stress-induced OMA1 activation and autocatalytic turnover regulate OPA1-dependent mitochondrial dynamics. *EMBO J.* 33, 578–593.
- Biedler, J., Helson, L., Spengler, B., 1973. Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res.* 33, 2643–2652.
- Buttiglione, M., Roca, L., Montemurro, E., Vitiello, F., Capozzi, V., Cibelli, G., 2007. Radiofrequency radiation (900 MHz) induces Egr-1 gene expression and affects cell-cycle control in human neuroblastoma cells. *J. Cell. Physiol.* 213, 759–767.
- Cacabelos, R., 2017. Parkinson's disease: from pathogenesis to pharmacogenomics. *Int. J. Mol. Sci.* 18.
- Cid-Castro, C., Hernandez-Espinosa, D.R., Moran, J., 2018. ROS as regulators of mitochondrial dynamics in neurons. *Cell. Mol. Neurobiol.* 38, 995–1007.
- Consales, C., Merla, C., Marino, C., Benassi, B., 2012. Electromagnetic fields, oxidative stress, and neurodegeneration. *Int. J. Cell Biol.* 2012, 683897.
- Dias, V., Junn, E., Mouradian, M.M., 2013. The role of oxidative stress in Parkinson's disease. *J. Park. Dis.* 3, 461–491.
- Dragicevic, N., Bradshaw, P.C., Mamcarz, M., Lin, X., Wang, L., Cao, C., Arendash, G.W., 2011. Long-term electromagnetic field treatment enhances brain mitochondrial function of both Alzheimer's transgenic mice and normal mice: a mechanism for electromagnetic field-induced cognitive benefit? *Neuroscience* 185, 135–149.
- Eghlidospour, M., Ghanbari, A., Mortazavi, S.M.J., Azari, H., 2017. Effects of radio-frequency exposure emitted from a GSM mobile phone on proliferation, differentiation, and apoptosis of neural stem cells. *Anat. Cell Biol.* 50, 115–123.
- Ertlav, K., Uslusoy, F., Ataizi, S., Naziroglu, M., 2018. Long term exposure to cell phone frequencies (900 and 1800 MHz) induces apoptosis, mitochondrial oxidative stress and TRPV1 channel activation in the hippocampus and dorsal root ganglion of rats. *Metab. Brain Dis.* 33, 753–763.
- Falzone, S., Grossi, M.R., Cinque, B., D'Angelo, B., Tettamanti, E., Cimini, A., Di Ilio, C., Amicarelli, F., 2007. Fifty hertz extremely low-frequency electromagnetic field causes changes in redox and differentiative status in neuroblastoma cells. *Int. J. Biochem. Cell Biol.* 39, 2093–2106.
- Falzone, S., Sannino, A., Romeo, S., Zeni, O., Santini, S.J., Rispoli, R., Amicarelli, F., Scarfi, M.R., 2018. Protective effect of 1950 MHz electromagnetic field in human neuroblastoma cells challenged with menadione. *Sci. Rep.* 8, 13234.
- Franco-Iborra, S., Vila, M., Perier, C., 2018. Mitochondrial quality control in neurodegenerative diseases: focus on Parkinson's disease and Huntington's disease. *Front. Neurosci.* 12, 342.
- Friedman, J., Kraus, S., Hauptman, Y., Schiff, Y., Seger, R., 2007. Mechanism of short-term ERK activation by electromagnetic fields at mobile phone frequencies. *Biochem. J.* 405, 559–568.
- Golpich, M., Amini, E., Mohamed, Z., Azman Ali, R., Mohamed Ibrahim, N., Ahmadiani, A., 2017. Mitochondrial dysfunction and biogenesis in neurodegenerative diseases: pathogenesis and treatment. *CNS Neurosci. Ther.* 23, 5–22.
- Gupta, S.K., Mesharam, M.K., Krishnamurthy, S., 2018. Electromagnetic radiation 2450 MHz exposure causes cognition deficit with mitochondrial dysfunction and activation of intrinsic pathway of apoptosis in rats. *J. Biosci.* 43, 263–276.
- Irmak, M.K., Fadillioglu, E., Gulec, M., Erdogan, H., Yagmurca, M., Akyol, O., 2002. Effects of electromagnetic radiation from a cellular telephone on the oxidant and antioxidant levels in rabbits. *Cell Biochem. Funct.* 20, 279–283.
- Jonikas, M., Madill, M., Mathy, A., Zekol, T., Zois, C.E., Wigfield, S., Kurzawa-Akanbi, M., Browne, C., Sims, D., Chinnery, P.F., Cowley, S.A., Tofaris, G.K., 2018. Stem cell modeling of mitochondrial parkinsonism reveals key functions of OPA1. *Ann. Neurol.* 83, 915–925.
- Kesari, K.K., Meena, R., Nirala, J., Kumar, J., Verma, H.N., 2014. Effect of 3G cell phone exposure with computer controlled 2-D stepper motor on non-thermal activation of the hsp27/p38MAPK stress pathway in rat brain. *Cell Biochem. Biophys.* 68, 347–358.
- Kim, J.H., Yu, D.H., Huh, Y.H., Lee, E.H., Kim, H.G., Kim, H.R., 2017. Long-term exposure to 835 MHz RF-EMF induces hyperactivity, autophagy and demyelination in the cortical neurons of mice. *Sci. Rep.* 7, 41129.
- Krishna, A., Biryukov, M., Trefois, C., Antony, P.M., Hussong, R., Lin, J., Heinäniemi, M., Glusman, G., Köglberger, S., Boyd, O., van den Berg, B.H., Linke, D., Huang, D., Wang, K., Hood, L., Tholey, A., Schneider, R., Galas, D.J., Balling, R., May, P., 2014. Systems genomics evaluation of the SH-SY5Y neuroblastoma cell line as a model for Parkinson's disease. *BMC Genomics* 15, 1–21.
- Lee, J.E., Lim, M.S., Park, J.H., Park, C.H., Koh, H.C., 2016. PTEN promotes dopaminergic neuronal differentiation through regulation of ERK-dependent inhibition of S6K signaling in human neural stem cells. *Stem Cells Transl. Med.* 5, 1319–1329.
- Luukkainen, J., Hakulinen, P., Maki-Paakkanen, J., Juutilainen, J., Naarala, J., 2009. Enhancement of chemically induced reactive oxygen species production and DNA damage in human SH-SY5Y neuroblastoma cells by 872 MHz radiofrequency radiation. *Mutat. Res.* 662, 54–58.
- Martinez, J.H., Alaimo, A., Gorjod, R.M., Porte Alcon, S., Fuentes, F., Coluccio Leskow, F., Kotler, M.L., 2018. Drp-1 dependent mitochondrial fragmentation and protective autophagy in dopaminergic SH-SY5Y cells overexpressing alpha-synuclein. *Mol. Cell. Neurosci.* 88, 107–117.
- Mastrodonato, A., Barbati, S.A., Leone, L., Colussi, C., Gironi, K., Rinaudo, M., Piacentini, R., Denny, C.A., Grassi, C., 2018. Olfactory memory is enhanced in mice exposed to extremely low-frequency electromagnetic fields via Wnt/beta-catenin dependent modulation of subventricular zone neurogenesis. *Sci. Rep.* 8, 262.
- Megha, K., Deshmukh, P.S., Banerjee, B.D., Tripathi, A.K., Abegaonkar, M.P., 2012. Microwave radiation induced oxidative stress, cognitive impairment and inflammation in brain of Fischer rats. *Indian J. Exp. Biol.* 50, 889–896.
- Megha, K., Deshmukh, P.S., Ravi, A.K., Tripathi, A.K., Abegaonkar, M.P., Banerjee, B.D., 2015. Effect of low-intensity microwave radiation on monoamine neurotransmitters and their key regulating enzymes in rat brain. *Cell Biochem. Biophys.* 73, 93–100.
- Miloso, M., Villa, D., Crimi, M., Galbiati, S., Donzelli, E., Nicolini, G., Tedricci, G., 2004. Retinoic acid-induced Neurogenesis of human neuroblastoma SH-SY5Y cells is ERK independent and PKC dependent. *J. Neurosci. Res.* 75, 241–252.
- Motawi, T.K., Darwish, H.A., Moustafa, Y.M., Labib, M.M., 2014. Biochemical modifications and neuronal damage in brain of young and adult rats after long-term exposure to mobile phone radiations. *Cell Biochem. Biophys.* 70, 845–855.
- Muroyama, Y., Kondoh, H., Takada, S., 2004. Wnt proteins promote neuronal differentiation in neural stem cell culture. *Biochem. Biophys. Res. Commun.* 313, 915–921.
- Panchal, K., Tiwari, A.K., 2018. Mitochondrial dynamics, a key executioner in neurodegenerative diseases. *Mitochondrion* (47), 151–173.
- Perfeito, R., Lazaro, D.F., Outeiro, T.F., Rego, A.C., 2014. Linking alpha-synuclein phosphorylation to reactive oxygen species formation and mitochondrial dysfunction in SH-SY5Y cells. *Mol. Cell. Neurosci.* 62, 51–59.
- Qiao, J., Paul, P., Lee, S., Qiao, L., Josifi, E., Tiao, J.R., Chung, D.H., 2012. PI3K/AKT and ERK regulate retinoic acid-induced neuroblastoma cellular differentiation. *Biochem. Biophys. Res. Commun.* 424, 421–426.
- Roberts, H.L., Brown, D.R., 2015. Seeking a mechanism for the toxicity of oligomeric alpha-synuclein. *Biomolecules* 5, 282–305.
- Roberts, R.F., Tang, M.Y., Fon, E.A., Durcan, T.M., 2016. Defending the mitochondria: the pathways of mitophagy and mitochondrial-derived vesicles. *Int. J. Biochem. Cell Biol.* 79, 427–436.
- Schuderer, J., Spat, D., Samaras, T., Oesch, W., Kuster, N., 2004. In vitro exposure systems for RF exposures at 900 MHz. In: *IEEE Transactions on Microwave Theory and Techniques*. 52, pp. 2067–2075.
- Schuz, J., Waldemar, G., Olsen, J.H., Johansen, C., 2009. Risks for central nervous system diseases among mobile phone subscribers: a Danish retrospective cohort study. *PLoS One* 4, e4389.
- Stefi, A.L., Margaritis, L.H., Skouroliaou, A.S., Vassilacopoulou, D., 2019. Mobile phone electromagnetic radiation affects Amyloid Precursor Protein and alpha-synuclein metabolism in SH-SY5Y cells. *Pathophysiology*. <https://doi.org/10.1016/j.pathophys.2019.02.004>.
- Tsoy, A., Saliev, T., Abzhanova, E., Turgambayeva, A., Kairlyrkyzy, A., Akishev, M., Saparbayev, S., Umbayev, B., Askarova, S., 2019. The effects of mobile phone radiofrequency electromagnetic fields on beta-amyloid-induced oxidative stress in human and rat primary astrocytes. *Neuroscience* 408, 46–57.
- Wang, H., Zhang, X., 2017. Magnetic fields and reactive oxygen species. *Int. J. Mol. Sci.* 18.
- Wiedmer, L., Ducray, A.D., Frenz, M., Stoffel, M.H., Widmer, H.R., Mevissen, M., 2019. Silica nanoparticle-exposure during neuronal differentiation modulates dopaminergic and cholinergic phenotypes in SH-SY5Y cells. *J. Nanobiotechnol.* 17, 46.
- Xicoy, H., Wieringa, B., Martens, G.J., 2017. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Mol. Neurodegener.* 12, 10.
- Xie, W., Wan, O.W., Chung, K.K., 2010. New insights into the role of mitochondrial dysfunction and protein aggregation in Parkinson's disease. *Biochim. Biophys. Acta* 1802, 935–941.
- Xu, S., Zhou, Z., Zhang, L., Yu, Z., Zhang, W., Wang, Y., Wang, X., Li, M., Chen, Y., Chen, C., He, M., Zhang, G., Zhong, M., 2010. Exposure to 1800 MHz radiofrequency radiation induces oxidative damage to mitochondrial DNA in primary cultured neurons. *Brain Res.* 1311, 189–196.
- Zheng, X., Boyer, L., Jin, M., Mertens, J., Kim, Y., Ma, L., Ma, L., Hamm, M., Gage, F.H., Hunter, T., 2016. Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation. *Elife* 5.