Calcium Measurements in Hamster Pinealocytes and Human Retinoblastoma Cells during Exposure to RF Fields

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INTRODUCTION

According to the melatonin hypothesis the nightly rise of the melatonin concentration in blood plasma can be influenced by weak ELF fields. RF fields are also suspected to influence the melatonin homeostasis in the pineal gland. Visible light, which synchronizes the melatonin secretion in a day/night rhythm, affects the melatonin producing pineal cells via the photoreceptors in the eye. It is still unknown which signal transduction pathway may be influenced by EMF, and if the pineal cells themselves or other cells in connection to them can be directly affected by EMF.
The aim of the present work was to investigate possible influences of RF fields on the melatonin production of **pinealocytes** and **cells of the retina**, which are also able to produce melatonin. Differentiated and undifferentiated cells of the human retinoblastoma cell line Y79 were used as a model for retina cells.
Calcium ions are decisively involved in the biochemical pathway of melatonin production in pinealocytes. Therefore, the **intracellular calcium ion concentration, [Ca^{2+}]$_i$**, was measured to detect acute effects of RF fields on the melatonin homeostasis at an early step in the concerning signal transduction cascade. The use of two different cell types should give an indication of the way in which melatonin production may be disturbed by RF fields.
The highest possible SAR values (available from the present equipment) and much lower values of 0.08 W/kg (recommended whole body SAR limit) were applied to the cells.

METHODS

Pinealocytes were isolated by mechanical and enzymatic treatment of whole pineal glands from Djungarian hamsters (*Phodopus sungorus*) and were kept on untreated round cover slips in primary culture for up to 14 days.
Undifferentiated human **retinoblastoma cells** (line Y79) were cultured in suspension culture and plated on poly-D-lysine- and fibronectin-treated round cover slips for differentiation (a modified differentiation procedure according to *Kyritsis et al., Nature 307, 471-473, 1984*). The cells were measured in the differentiated status.
The **[Ca^{2+}]$_i$** was analyzed in a microscope setup by use of the fluorescent calcium indicator **fura-2** and digital **video imaging** technique. During the 30 min. calcium measurement the cells were exposed for 8.5 min. to a **pulse modulated 900 MHz or 1800 MHz RF-field** in one of the two **rectangular waveguides** therefore constructed.
The cells on the cover slips were transferred into the experimental chamber which was mounted centrally on the bottom of the rectangular waveguide. The [Ca^{2+}]$_i$ in up to 72 (pinealocytes, mean = 7.3) or up to 35 (differentiated Y79 cells, mean = 11.9) cells/experiment was measured simultaneously in measuring buffer at 36.5°C ±0.5°C with a time resolution of 10s. Sham exposures were carried out in the same arrangement with the field being turned off. **Positive controls** at the end of the runs were carried out with calcium mobilizers (adenylate cyclase activator **Forskolin**, 10 µM, with retinoblastoma cells, α/ß-adrenergic substance **Norepinephrine**, 1 µM, with pinealocytes).

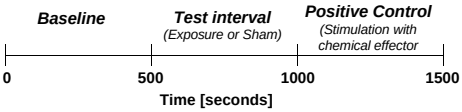
SUMMARIZED RESULTS

Single measurement of **755** **hamster pinealocytes** (448 exposed, 307 sham exposed) at **900 MHz (SAR 13 W/kg and 0.08 W/kg)** and **1800 MHz (SAR 24 W/kg and 0.08 W/kg)** exposure as well as **214** **differentiated human retinoblastoma cells** (139 exposed, 75 sham exposed) at **900 MHz (SAR 13 W/kg)** exposure **revealed no clear changes of the [Ca^{2+}]$_i$ under field influence**.
In some not reproducible „special cases" cells partly produced clear but contradictory effects during exposure, which could not be explained.
For statistical evaluation, we regarded 91% of the pinealocytes and 72% of the retinoblastoma cells, which at the end of each experiment responded to Norepinephrine or Forskolin stimulation, respectively. So, all regarded cells were thoroughly verified for their basic ability to mobilize calcium in response to physiological stimuli.
The statistical evaluation yielded no indication of any field effect.

CONCLUSIONS

We got no hint regarding any statistically relevant effect of the so far tested field types and strengths on the regulatory calcium metabolism in hamster pinealocytes and in human retinoblastoma cells.

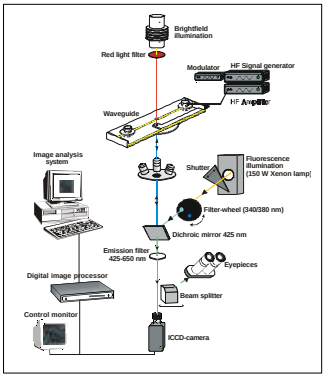
1 Experiments were divided into three parts



Each experiment consisted of three intervals of 500 s duration, a **baseline interval** (sham conditions) at the beginning, a **test interval** in the middle (sham conditions or field exposure), and a **positive control interval** at the end, where calcium mobilizing reagents (see 'Methods') were applied without field exposure.

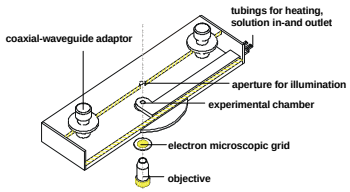
The **time resolution** was 0.1 Hz, i.e. the frequency of digital image double acquisitions (at excitation light 340 nm and 380 nm), needed for the calculation of ratio images that indicate the internal free calcium distribution in the cells according to the fura-2 ratio imaging method. This was fast enough to register all possible calcium changes in the treated cell types.

2 Microscope Setup with HF application



Experimental setup with Zeiss Axiovert 100 TV inverted fluorescence microscope (middle), custom made UV-illumination equipment with step-motor driven shutter and filter wheel (right), Hamamatsu intensifying CCD-camera (below the microscope), and Hamamatsu signal control, digital image acquisition and analysis (left). In the upper part the brightfield illumination pathway, the HF-field generation and the rectangular waveguide with object chamber inside (mounted on the stage of the microscope) are symbolized. A very broad-band fura-2 emission filter (425-650 nm) was used to maximize the emission light intensity for the low-light camera.

3 Rectangular Waveguides were used for HF Exposure

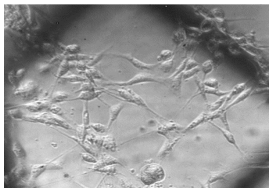


Two different rectangular waveguides were available for exposures to pulsed EMFs of **900 MHz** or **1800 MHz (GSM standard, see box [4]: generic GSM modulation signal)**. Signal generator (Rohde & Schwarz SME 06) and two suitable amplifiers (SSB Electronic) provided maximum input powers of the pulsed signals yielding **SAR values** of **13 W/kg at 900 MHz** or **24 W/kg at 1800 MHz** in the plane of the cells, derived from extensive numerical simulations of the whole test setup.
Top: Schematic drawing of the 3 cm high, 50 cm long and 21 cm (900 MHz) or 12 cm (1800 MHz) wide rectangular waveguide (inside measurements), constructed to fit on the stage of the inverted Zeiss microscope. During experiments, cells were observed with a special UV-usable long-distance objective (Zeiss F-LD 32/0.4 Ph1) through the bottom of the waveguide which was HF-tightened by a tightly inserted electron microscopic grid (440 µm mesh width). Feed point and decoupling point were connected to a coaxial connection to the amplifier and to a terminal load, respectively.
Bottom: Photograph of the 1800 MHz waveguide. For demonstration the removed plexiglass object chamber with glass bottom lies on top of the waveguide.

4 A generic „FGF-standard" GSM modulation signal was used:

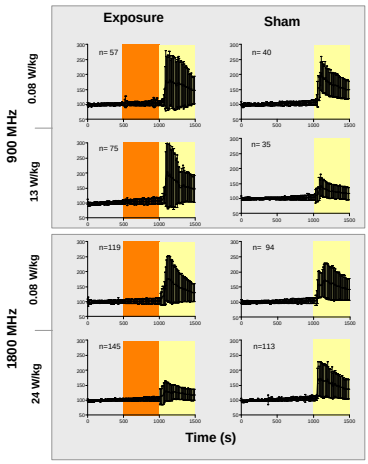
- Generated by a custom made **external bit pattern generator**, connected to the HF signal generator
- The generated **synthetic mobile communication signal** imitates a GSM modulation signal with a „cocktail" of different frequencies at a **duty cycle of 7:8**
- **Frequency „cocktail":**
  - Typical downlink frequencies 1733 Hz and 217 Hz
  - 2 Hz and 8.3 Hz components of a GSM hand set used in DTX-mode

5 Hamster Pinealocytes from Primary Culture

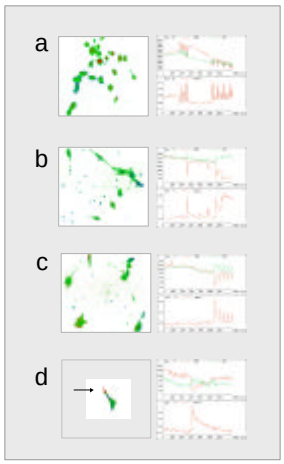


Brightfield image of one field of view with many **pinealocytes** after 6 days in primary culture, attached to a glass cover slip that was laid on the glass bottom of the object chamber. Parts of one 440 µm mesh of the metal grid in the bottom of the waveguide (i.e., directly below the two glass layers) can be seen.

Averaged Results

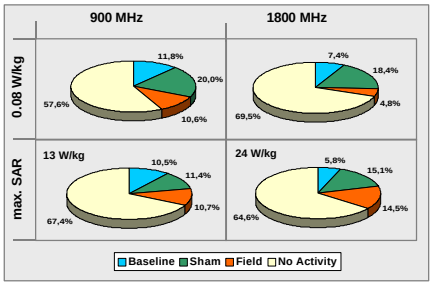


Special Cases



Left: Many single cell registrations, like demonstrated under ‚Special Cases' on the right, were averaged in plots that show the **mean of all calcium changes ±S.D.** (scaled to the mean during the first 500 s =100%) during several experiments under 4 different exposure conditions (each with belonging sham experiments, measured in alternating order). Only cells responding to the final Norepinephrine (NE) stimulation were regarded (91% of all on average). Exposure intervals (**red**) and positive controls by NE application (**yellow**) are indicated. Basically, effects could appear as shift of the mean line or/and as change of S.D. size. The small but distinct effects at the beginning and the end of 900 MHz, 0.08 W/kg exposure was due to *one* experiment with several cells responding like shown in (b) on the right. Varying strengths of responses to NE were randomly distributed.
Right: Four **special cases** demonstrate reaction patterns apart from the majority of all NE-positive single cell registrations (each shows one selected registration from **one unique experiment**). (**a**) 14 of 15 NE-respondent cells (total amount = 28) stopped calcium spiking activity exactly during exposure to 1800 MHz, 0.08 W/kg. (**b**) 3 of 8 cells responded with one or more calcium spikes to 900 MHz, 0.08 W/kg exposure. (**c**) Ca^{2+}-*oscillations* in response to NE-stimulation (3 of 10 cells). (**d**) Large Ca^{2+}-transient only in the very tip of the cell (**arrow**) under sham exposure conditions (but no response to NE). *(Intervals like shown in red and yellow on the left)*

Statistics of Calcium Spiking Activity

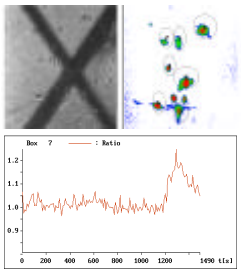


Manual counting of the appearance of single calcium spikes (in the same experimental runs shown above as ‚Averaged Results') yielded a relation of the cells' calcium activity within the different intervals (**baseline, sham, or exposure interval**). ‚No activity' means no activity at all, except for the positive control interval. Baseline intervals of respective Exposure and Sham runs were pooled and averaged in each pie chart. No condition revealed an increased activity under field exposure.

6 Differentiated Y79 Retinoblastoma cells

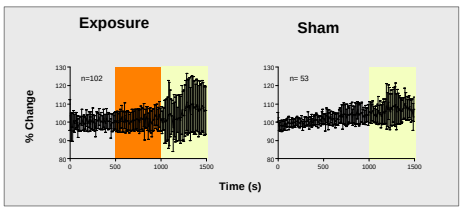
Brightfield image of a field of five **differentiated retinoblastoma** cells besides a number of undifferentiated round retinoblastoma cells, after 15 days in differentiation medium.

Sample Experiment



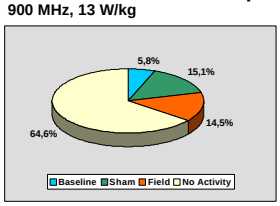
Representative display of a measurement on differentiated retinoblastoma cells under 900 MHz, 13 W/kg exposure.
Top left: Brightfield image of the field of view. Parts of the webs of the metal grid in the bottom of the waveguide can be seen.
Top right: Pseudo-coloured ratio image of the fluorescent cells in the same field of view. For evaluation, each evaluable cell was overlayed with a digital measurement window (1 to 7). Only object pixels (no background) within the windows were evaluated.
Bottom: Analysis graph of window No. 7 in the ratio image above, indicating the $[Ca^{2+}]_i$ changes over the time. No field effects are visible in the middle interval, whereas the cell clearly responds to Forskolin stimulation.

Averaged Results 900 MHz, 13 W/kg



Many single cell registrations, like demonstrated in the example above, were averaged in plots that show the **mean of all calcium changes ±S.D.** (scaled to the mean during the first 500 s =100%) during several experiments under 900 MHz, 13 W/kg exposure (with belonging sham experiments, measured in alternating order). Only cells responding to the final Forskolin stimulation were regarded (72% of all on average). Exposure interval (**red**) and positive controls by Forskolin application (**yellow**) are indicated. Basically, effects could appear as shift of the mean line or/and as change of S.D. size. Response to Forskolin was much weaker and more delicate than response of pinealocytes to NE (cf. scaling). So, slight differences in the response to Forskolin between exposure and sham experiments were not judged to be relevant. No effect of field exposure could be detected.

Statistics of Calcium Spiking Activity
900 MHz, 13 W/kg



Manual counting of the appearance of single calcium spikes (in the same experimental runs shown above as ‚Averaged Results') yielded a relation of the cells' calcium activity within the different intervals (**baseline, sham, or exposure interval**). ‚No activity' means no activity at all, except for the positive control interval. Baseline intervals of respective Exposure and Sham runs were pooled and averaged in the pie chart. A changed activity due to field exposure was not observed.

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