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Effects of 60-GHz Millimeter Waves on Neurite Outgrowth in PC12 Cells Using High Content Screening

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Highlights:

- 60 GHz MMW do not impact neuronal tubulin expression during PC12 differentiation.
- Exposure at 10 mW/cm² induces a slight increase in neurite outgrowth due to the thermal effect.
- The thermal effect impacts all parameters of the neurite outgrowth.

Abstract

Technologies for wireless telecommunication systems using millimeter waves (MMW) will be widely deployed in the near future. Forthcoming applications in this band, especially around 60 GHz, are mainly developed for high data-rate local and body-centric telecommunications. At those frequencies, electromagnetic radiations have a very shallow penetration into biological tissues, making skin keratinocytes, and free nerve endings of the upper dermis the main targets of MMW. Only a few studies assessed the impact of MMW on neuronal cells, and none of them investigated a possible effect on neuronal differentiation. We used a neuron-like cell line (PC12), which undergoes neuronal differentiation when treated with the neuronal growth factor (NGF). PC12 cells were exposed at 60.4 GHz for 24 h, at an incident power density averaged over the cell monolayer of 10 mW/cm². Using a

large scale cell-by-cell analysis based on high-content screening microscopy approach, we assessed potential effects of MMW on PC12 neurite outgrowth and cytoskeleton protein expression. No differences were found in protein expression of the neuronal marker β 3-Tubulin nor in internal expression control β -Tubulin. On the other hand, our data showed a slight increase, although insignificant, in neurite outgrowth, induced by MMW exposure. However, experimental controls demonstrated that this increase was related to heating.

Abbreviations: MMW (millimeter waves), HC (heat control).

Key words: Electromagnetic field, Neuron-like cell line, Neuronal differentiation, Neurite Outgrowth.

Introduction

Since the beginning of the 21st century, more sophisticated wireless telecommunication systems have emerged, exploiting higher frequency bands. Ranging from 30 to 300 GHz, millimeter waves (MMW) constitute the extremely high frequency band, which will be used for forthcoming wireless technologies. High data rates (up to 7 Gbit/s), secured networks and a miniaturization of electronic devices are the main advantages brought by those frequencies, especially around 60 GHz, explaining why they have been recently brought back to the fore. This band will be used for a variety of commercial applications, such as for next-generation mobile phone networks 5G or local/personal/body area networks - WLAN/WPAN/WBAN [1–3], as well as for emerging biomedical applications [4]. This new kind of electromagnetic fields (EMF) sources will increase the global anthropogenic exposure constituted by radiofrequencies (RF). Therefore, assessing the potential effects of MMW on living organisms is crucial before any deployment of technologies exploiting this band. The involvement of the nervous system in biological responses to RF-EMF is an extensively studied field [5,6] and lately, the implication of the peripheral nervous system in response to MMW has been highlighted, especially concerning modulation of the pain sensation [7]. MMW therapy has been extensively used since 1960s by the former Soviet Union, and

aroused interest in Western countries [8]. Hypoalgesia is the most reported and the only reproducible effect of MMW exposure on animal models or on human volunteers using well-controlled blind experimental conditions [9–11]. The involvement of the opioid system, triggered by the peripheral nervous system was previously shown [12]. As MMW penetration in the skin is very limited (i.e. between a few tenth to 1 mm in the therapeutic range (42 - 62 GHz)[13,14]), the hypothesis of the activation of free nerve endings from the epidermis and upper dermis by MMW has been proposed [12]. The physiology of the nervous system involves a large panel of cellular processes such as proliferation, migration, differentiation, synaptogenesis and programmed cell death. In this study, we used the PC12 cell line, which is derived from rat pheochromocytoma. PC12 cells have the particularity to differentiate into a sympathetic neuron-like phenotype when treated with the neurotrophic factor nerve growth factor (NGF) [15] and have therefore been extensively used to assess the effects of chemical compounds on neuronal physiology [16]. Many studies assessing the impact of EMF on a wide range of neuronal physiology mechanisms used this model, mostly in the RF range [17–19]. The NGF-induced PC12 differentiation induces a cellular phenomenon called neurite outgrowth. During this phenomenon, cells present a sprouting of cellular extensions called neurites. The number, length and branching complexity of neurites are correlated with the differentiation state of NGF-treated PC12 cells. Neurite outgrowth assay is one of the most common *in vitro* approach in neurotoxicology due to its sensitivity to environmental disruption [20]. This assay has been used several times in bioelectromagnetics research. A study showed that extremely low frequency (ELF) EMF exposure (4.5-15.8 μ T, 50 Hz AC) of PC12 cells can either reduce or increase neurite outgrowth, depending on the culture conditions [21]. For RF, it was reported by Del Vecchio *et al.* [22] that exposure to GSM signal at 900 MHz, at 1W/kg, reduced the number of neurites generated in murine SN56 cholinergic cell line and rat primary cortical neurons. It was also found that 1800 MHz exposure of mouse embryonic neural stem cells, with a specific absorption rate (SAR) of 4 W/kg, could decrease neurite outgrowth [23]. Lately, another team evidenced that radio electric asymmetric conveyor (REAC) technology, involving a 2.4 GHz exposure (SAR = 0.128 W/kg), induced a shift of the exposed PC12 cells toward the neurogenic phenotype, characterized by an increased protein expression of the neuronal marker β III-tubulin [24].

A few studies have also explored the potential effects of MMW on neuronal models *in vitro*. Samsonov et al. reported that 94 GHz exposure of *Xenopus* spinal cord neurons with a nominal power density of 31 mW/cm² induced an increased microtubule assembly rate, which was related to a thermal effect [25]. A similar thermal effect of MMW was observed when irradiating *Xenopus* oocytes, a model that shares similar membrane excitable phenotype with neurons. In this study, a 60 GHz exposure with a maximum power density of 600 mW/cm² altered activity levels of voltage-gated channels and ionic pumps, and increased the action potential firing rate of exposed oocytes [26]. Eventually, another team observed that a 60 GHz MMW exposure at 4 mW/cm² of individual neurons in the leech midbody ganglia could dramatically increase the narrowing of the neurons action potential half-width, while transiently reducing their firing rate [27]. Those observations could not be fully reproduced by equivalent bath heating. However, no study has yet used a neurite outgrowth assay to examine the potential impact of MMW on neuronal cells.

Our aim was to investigate potential impact of MMW exposure on the neurite outgrowth of NGF-treated PC12 cells. Using a high-content screening method, we developed an informatics tool in order to create an automated analysis of the different parameters describing neurite outgrowth. We also assessed the potential effect of MMW on neuronal differentiation by measuring the protein expression of β -III-tubulin, a specific marker of neuronal phenotype. To this end, cells were exposed to 60.4 GHz MMW at 10 mW/cm², which is a typical incident power density (IPD) for therapeutic applications [7], and two times above the ICNIRP exposure limit for workers exposed on a wide surface (above 20 cm²) [28].

Materials and Methods

Reagents and Antibodies

Nerve growth factor beta (NGF- β , N2513) was acquired from Sigma-Aldrich Co. (St Louis, MO, USA).

Specific primary antibodies were used for immunocytochemistry detection: rabbit polyclonal anti- β III-tubulin (ab18207) was purchased from Abcam (Cambridge, UK); the mouse monoclonal anti- β -tubulin (AA2, T4026) and anti- β -actin (AC15, A5141) were purchased from Sigma-Aldrich Co. The secondary antibodies labeled with fluorescent dyes Alexa Fluor 488

goat anti-rabbit IgG antibody (A11034) and Alexa Fluor 594 goat anti-mouse IgG antibody (A11005) were acquired from Life Technologies (Carlsbad, CA).

Cell Culture

The PC12 cell line subclone Neuroscreen-1 (NS-1) was acquired from Thermo Fisher Scientific (Waltham, MA). For experiments, cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI medium 1640, 21875, Life Technologies) supplemented with 10% horse serum (Biowest, Nuaille, France), 5% fetal bovine serum (Biowest), 1 mM sodium pyruvate and antibiotics (Life Technologies) and put in an incubator at 37°C in 5% CO₂. Before any exposure experiment, cells were seeded in collagen I (Thermo Fisher)-coated Falcon™ 96-wells plates (Corning, Corning, NY) at the density of 6000 cells per well, and left for 24h in the 15% serum supplemented medium. The 24 h-MMW exposure during the 24 h-NGF treatment at 200 ng/mL was preceded with a 24 h-serum deprivation stage in RPMI 1640 medium containing 1% horse serum and 0.5% fetal bovine serum. Another exposure medium was designed to maintain the pH buffering in the non-gassed incubator of the exposure system. This one consisted in powder reconstituted RPMI 1640 medium deprived in NaHCO₃, containing 1.5% serum, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and antibiotics (Life Technologies). Throughout the exposures, plates were sealed with AeraSeal sealing films (Excel Scientific, Victorville, CA) in order to avoid medium evaporation.

Exposure system, dosimetry and experimental setup

A specific exposure system was designed in order to expose cells to MMW at 60.4 GHz. It includes a signal generation subunit and an exposure chamber. The first subunit had already been described in detail [29]. The anechoic exposure chamber is located in a temperature-controlled anechoic MEMMERT UNE400 incubator (Memmert GmbH, Schwabach, DE) covered inside by absorbing materials. The exposure levels were described in detail for a 6-well tissue culture plate [30]. However, since we used 96-well plates, the exposure conditions were therefore changed. The finite-difference time domain method implemented

in SEMCAD X was used here to perform a numerical dosimetry study. We calculated an equivalent incident power density using the following formula [13]:

$$IPD_{eq} = \frac{\rho \cdot \delta \cdot SAR(z) \cdot e^{2z/\delta}}{2(1 - R)}$$

Where ρ is the mass density, δ is the penetration depth, $SAR(z)$ represents SAR at the depth z , and R is the coefficient of the power reflection. The average IPD_{eq} for the wells containing cells was calculated, and was found to be 10.1 ± 0.1 mW/cm². This level is above the ICNIRP exposure limit when a large surface of the body (over 20 cm²) is exposed [28], but beyond the limit for near-field exposure conditions, corresponding to some emerging body-centric application. When exposure area is small (over 1 cm²), the exposure level is limited to 20 mW/cm².

Four wells of the 96-well tissue culture plates were exposed from the bottom under near-field conditions. Sham exposures were performed under same conditions, but with the generator switched off. Since we noticed a slight increase in the medium temperature due to MMW exposure ($\approx 1-2^\circ\text{C}$), a heat control (HC) was simultaneously performed for each experiment. Sham plates were seeded exactly as exposed plates, put in another MEMMERT UNE400 incubator that was in the same room, and the temperature was set so it would be similar to the one of the medium and the exposed cells wells. Temperature was monitored all along the experiments, by using a 4-channel Reflex fiber optic thermometer (NEOPTIX, Quebec, Canada).

Immunocytochemistry and fluorescence analysis

Immunocytochemistry (ICC) was performed inside the 96-wells culture plates, after exposure experiments. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, then washed twice with phosphate-buffered saline (PBS). Permeabilization was done for 10 min with 0.25% Triton X-100 in PBS. Blocking of antibodies unspecific binding was done by incubating cells during 20 min in 1% bovine serum albumin (BSA, MP Biomedicals, Santa Ana, CA), 0.1 % gelatin from cold water fish skin (Sigma-Aldrich Co.) and 0.1% Triton X-100 (MP Biomedicals) in PBS. After an overnight incubation with primary antibodies at 1:200 dilution each, wells were washed three times using PBS supplemented with 0.1% Tween 20 (MP Biomedicals). Then, cells were incubated 1 h with secondary antibodies at 1:1000 dilution and 10 $\mu\text{g}/\text{mL}$ Hoechst 33342 (Sigma-Aldrich) for nuclei counterstaining. Using a Cellomics

ArrayScan VTI HCS Reader (Thermo Fisher Scientific) in the ImPACcell technologic platform (Rennes 1 University-Biosit, Rennes, France), pictures were taken and fluorescence was analyzed in blind manner. 10 to 20 high-definition pictures were randomly taken for each well, and poor quality pictures were visually screened and deleted in blind condition. Cell-by-cell fluorescence values in each picture were obtained and a data filter was applied in order to remove outliers created by cellular debris and artifactual antibodies aggregates. Values kept for further data treatment were between 0.01 and 30 fold the values median for each staining, in each condition, and in each experiment. Using a Wilcoxon signed-rank test, statistical analysis was performed by comparing the mean of cell-by-cell fluorescence intensity means measured in all experiments, for each condition.

Neurite outgrowth assay

The ImageJ software (NIH, Bethesda, MD) was used to create an original custom macro in order to have an automated tool for assessing the potential impact of MMW exposure on NGF-treated PC12 cells [31]. The pipeline developed on that purpose consists in a series of masks discriminating cellular objects at each step. Starting from the original raw pictures of β -III-tubulin, β -tubulin and β -actin immunofluorescence taken with the Cellomics Arrayscan VTI HCS Reader, cell nuclei are outlined and counted, then neurites are distinguished from the cell bodies. As described on Figure 1, each mask provides a specific numerical information corresponding to a parameter describing the neurite outgrowth. Those parameters are: the number of cells per picture given by the nucleus mask, the neurite length score and the neurites angles given by the neurites mask, the differentiation score and the number of neurites per cell distribution of cell population given by the fusion of the cell bodies mask and the neurites mask. For each parameter, except neurites angles, statistical analysis compared the mean of cell-by-cell data means in all experiment, for each condition, using a Wilcoxon signed-rank test. Neurites angles data from each condition were compared with a chi-squared test.

Results

Impact of MMW exposure on the expression of the neuronal phenotype marker β 3-tubulin

In order to study the potential effect of a 24 h MMW exposure at 60.4 GHz with an average IPD_{eq} of 10 mW/cm² on the NGF-induced neuronal differentiation of PC12 cells, we first assessed the impact of exposure on a neuronal marker expression: the cytoskeleton protein β 3-tubulin. Unlike β -Tubulin which is an ubiquitous protein, β 3-tubulin is only expressed in neuronal cells. No effect of MMW exposure was observed on β 3-tubulin expression in PC12 cells, in terms of marker fluorescence pattern or in terms of mean global expression within cells (Fig. 2A and 2B). NGF induced a slight enhancement of protein expression, regardless of the condition. The low amplitude of the increase showed that the phenotype of the PC12 subclone used in this study was already in an advanced state of differentiation. A control of protein expression level, the housekeeping protein β -Tubulin, was also analyzed (Fig. 2C). The mean of fluorescence intensities results were confirmed by the fluorescence intensity distribution of cell populations: no subpopulation expressing a different level of fluorescence intensity was found in any of the NGF-treated conditions (Fig. 2D).

Impact of MMW on parameters characterizing the NGF-induced PC12 neurite outgrowth

Neurite outgrowth is a main feature of neuronal differentiation, and mechanisms underlying it cover a wide range of cell physiological processes, which could be affected by MMW exposure. Thus, we developed an automated macro for the ImageJ software in order to obtain several parameters reflecting the neurite outgrowth from microscopy pictures of NGF-treated PC12 cells, in all experimental conditions. All data shown concerning the potential effect of MMW on PC12 neurite outgrowth were obtained using this tool. It is important to mention that a manual neurite outgrowth assay was previously done with a visual counting of differentiated cells (data not shown). Results obtained showed similar results. The first studied parameter was the impact of MMW exposure on PC12 cells raw differentiation. This feature was expressed as a score based on the number of cells bearing at least one neurite with a size greater than one cell body, in each picture, and for each condition (Fig. 3A). The score is then expressed as the mean percentage of differentiated cells. MMW exposure induced a slight increase in the differentiation score, which was related to the thermal effect. Interestingly, the HC condition presented slightly more differentiation than the MMW condition. However, this difference was not found to be significant (Fig. 3B)

The effect of exposure on neurite length was also assessed by using another scoring approach applied in each picture of each condition. The score is obtained with the mean of total length for all neurites in one picture, divided by the number of cells in the analyzed picture (Fig 4A). As found for the differentiation score, the neurite length score was also slightly enhanced by MMW exposure, but not significantly. This outcome was also related to the thermal effect (Fig. 4B). Once again, the HC condition showed a slightly higher score than the MMW condition. The number of neurites per cell was also assessed as presented in Fig. 5A. The results were expressed as the number of neurites per cell distribution of NGF-treated PC12 cells, with the number going from 1 to over 5 neurites. Cell populations bearing 2 neurites or more were increased by the exposure, although this increase was not found to be significant. As for the previous scorings, this upward trend induced by MMW was also related to the thermal effect (Fig. 5B). It is interesting to notice that the upward trend was again higher in the HC condition than in the MMW condition for populations bearing 4 and 5 or more neurites.

Finally, regarding the fact that in our near-field experimental conditions, the field at the bottom of the exposed plate is polarized, we wanted to assess if MMW could disturb neuritogenesis by influencing the axis of neurite growth. Here, the polarization is as follows: the H-field vector \vec{H} aligns with the horizontal axis of the well (0° angle) and the E-field \vec{E} aligns with the vertical one (90° angle). The angle of a representative sample of neurites taken from all conditions of all experiments was measured using the macro (Fig. 6). The 0° angle corresponds here to the horizontal axis crossing the exposed wells, and the 90° angle corresponds to the vertical one. A slight decrease in the proportion of neurites bearing an angle between 80° and 100° was observed in the MMW condition. However, this result was not found to be significant.

Discussion

In previous investigations, we evaluated the cellular stress response in human keratinocyte cells after acute MMW exposure. We reported that levels of several stress-inducible genes were not significantly enhanced [29,32], suggesting that MMW around 60 GHz do not affect the cellular homeostasis of this model. However, although MMW has a

shallow penetration depth within the skin, the keratinocytes are not the only possible cellular targets. The hypothesis of nerve endings activation by MMW has been proposed [9], but only few studies focused on MMW effect on neuronal models. Here, we tested the impact of a continuous 60.4 GHz signal on morphological neuronal differentiation in a PC12 subclone (NS-1). Those cells are particularly suitable for the quantification of neurite outgrowth, since they present a lower level of aggregation [33]. Neuritogenesis is a critical process for the nervous system development, but it is also involved in neuronal plasticity by maintaining or remodeling the synaptic connections. In the PC12 model, NGF has been widely accepted as a key factor which induces their terminal differentiation toward a neural phenotype [34]. Experimental conditions were optimized in order to obtain a noticeable and quantifiable neurite outgrowth after 24 h of treatment. After MMW co-exposure with NGF, image acquisition was performed in blind manner, by using an automated microscopy screening system (Cellomics ArrayScan). We also developed an ImageJ plug-in to analyze accurately the neuronal morphology of each individual cell, in blind manner. 4000 to 6000 cells were analyzed for each experimental condition.

As expected, we found that NGF treatment slightly increased the protein expression of the neuronal marker β 3-tubulin [35]. However, NGF-induced increase of expression was low, probably because the unstimulated PC12 cells already had a high basal level of expression. MMW exposure had no significant effect, neither on β 3-tubulin expression nor on neurite outgrowth, whatever the parameter analyzed (length, number or orientation of the neurites). In the other hand, our data showed that exposure at 60.4 GHz and 10 mW/cm² induced very modest increases of each neurite outgrowth parameter, but that this insignificant effect can be explained by the temperature increase elicited by the exposure. Interestingly, increased parameters levels were higher in the heat controls than in the MMW condition. However, as we did not find any significant differences between our 3 conditions for all neurite outgrowth parameters, we can only speculate that the way cells are heated by MMW may be slightly different than the incubator-mediated heat in the HC condition, while keeping the same exact temperature in media of both conditions. Nevertheless, these heat-mediated tendencies are in line with the fact that temperature has a great influence on cellular processes of neuronal growth [36].

The effects of electromagnetic exposure on neurite outgrowth were already studied in other frequency bands. Most of those studies were showing a significant effect of EMF on

neurite outgrowth. However, depending on the model and the exposure conditions, the effect can be an enhancement, or a decrease of neuronal differentiation [21,23,37]. A recent study proposed that the potential mechanism by which radiofrequencies interfere with neurite outgrowth is the disruption of specific transcription factor expression that control this process [23]. Unlike these studies, our data did not suggest that neurite outgrowth is sensitive to EMF. However, our particular exposure conditions may be an explanation, as previous works were assessing the impact of lower frequencies. Moreover, we used continuous waves, and it is possible that a modulated or pulsed electromagnetic signal is required to observe an impact on neurite outgrowth [38]. Very few work has been done using MMW and neuronal models. In 1996, Kolosava and collaborators showed that low-power MMW (4 mW/cm² at 54 GHz) enhanced regeneration of sciatic nerve after transection [39]. Our work suggests that MMW exposure do not directly activate neuronal regeneration processes in neuronal models. Hence, it is possible that previously observed regeneration was a consequence of more complex and indirect phenomenon. In conclusion, our data are consistent with the well-known notion that the MMW effects are mediated by cell heating, which allows to conclude that, as least in our conditions, acute MMW exposure does not directly interfere with neurite outgrowth and differentiation of NGF-treated PC12 cells.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Captions

Fig. 1. Schematic pipeline of the macro developed for assessing the impact of MMW on PC12 cells neurite outgrowth. A custom macro was created with the ImageJ software, in order to obtain several numerical parameters characterizing the neurite outgrowth. The pipeline consists in a series of morphological discriminations, which can be visualized on masks obtained at each step of the analysis.

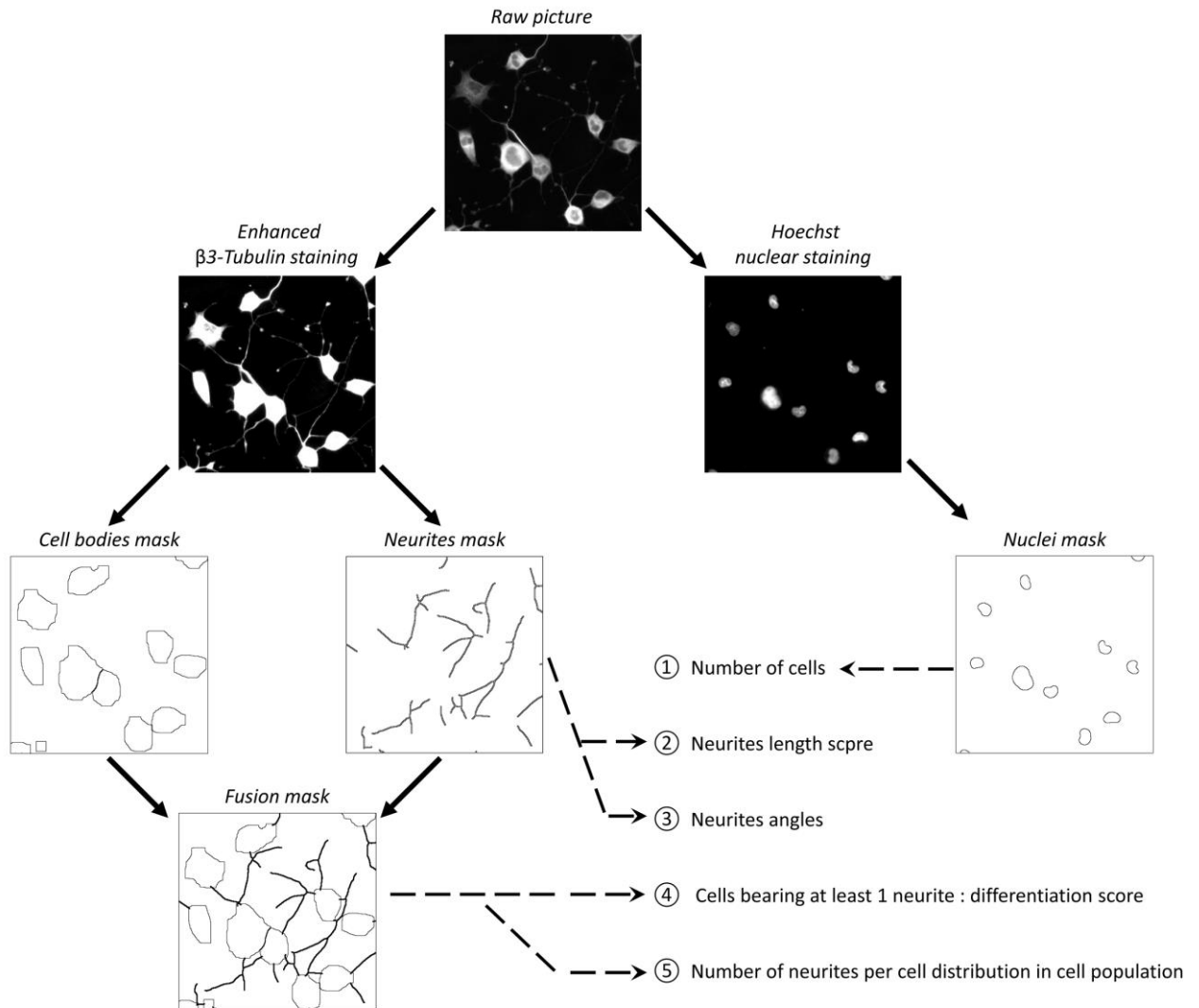


Fig. 2. Impact of MMW exposure on β 3-Tubulin protein expression. A) Representative microscopy pictures of Hoechst (left panel) and β 3-tubulin (right panel) fluorescent staining in NGF-treated cells: sham condition (top panel) vs. MMW condition (bottom panel), scale bar = 10 μ m. B) Mean β 3-tubulin (N=4) and C) mean housekeeping protein β -tubulin (N=8) fluorescence intensities analyzed cell-by-cell in all conditions, in absence or presence of NGF. Data expressed as fold of induction, compared to sham condition, mean of means (error bars = SD). D) Distribution of β 3-tubulin fluorescent staining intensity within NGF-treated cell population, for each condition (N=4, pull of all experiments).

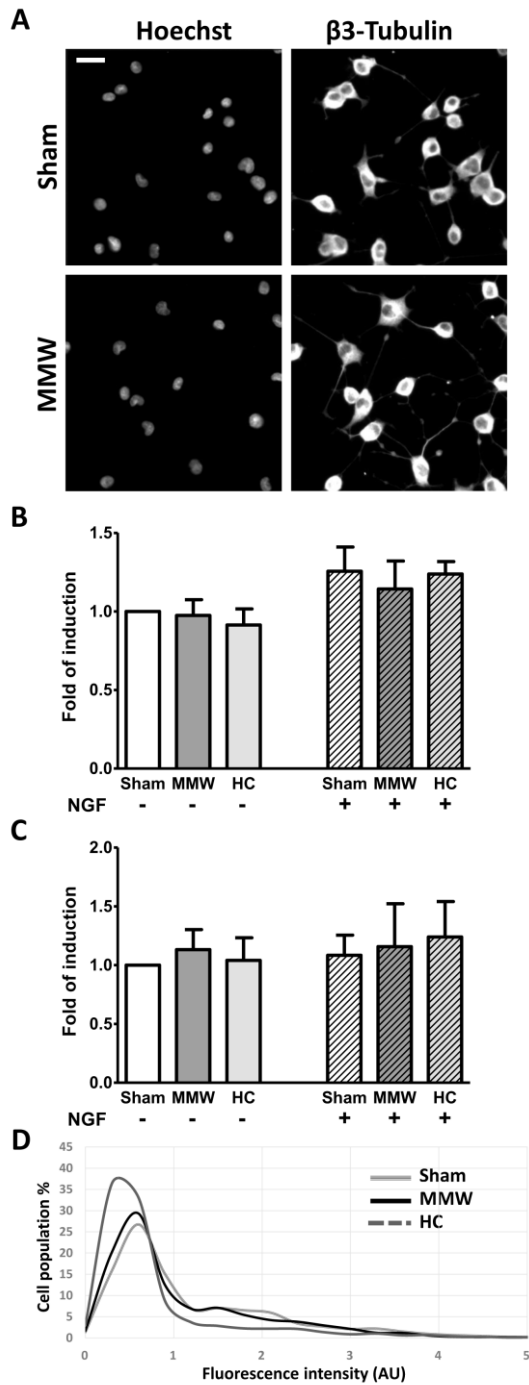


Fig. 3. Impact of MMW exposure on NGF-treated PC12 differentiation score. A) Representative nucleus mask from pictures of NGF-treated cells (left panel) and fusion of raw multichannel pictures with the final fusion masks of the macro (right panel): sham condition (top panel) vs. MMW condition (middle panel) vs. heat control condition (bottom panel), scale bar = 10 μ m. **B)** Mean differentiation score of NGF-treated cells. The score corresponds to the proportion of cells bearing at least one neurite with a length equal or superior to a PC12 cell body. N=7, mean of means (error bars = SEM).

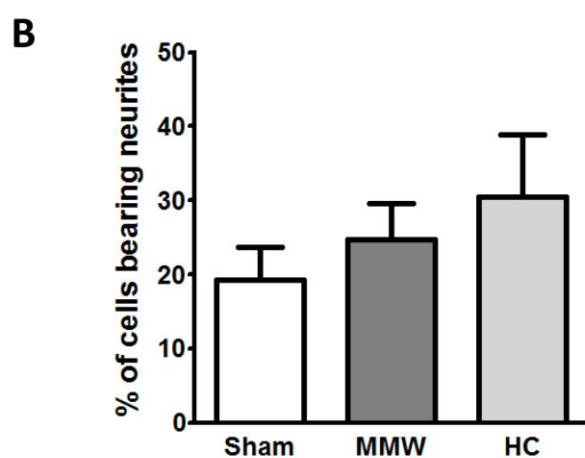
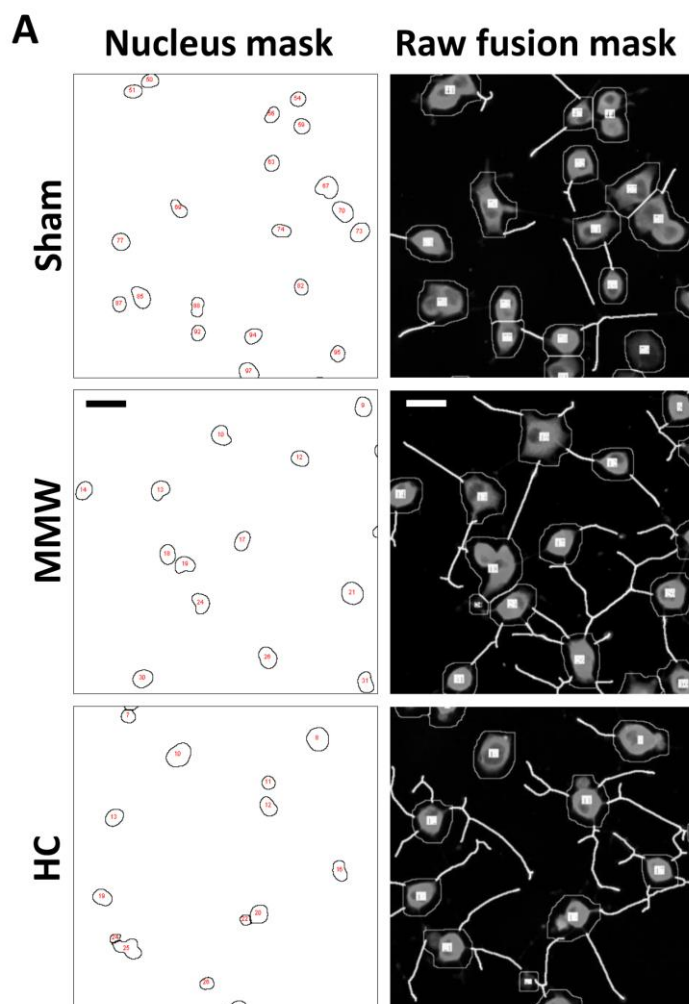


Fig. 4. Impact of MMW exposure on NGF-treated PC12 neurite length score. A)

Representative neurites mask from pictures of NGF-treated cells: sham condition (top panel) vs. MMW condition (middle panel) vs. heat control condition (bottom panel), scale bar = 10 μm . B) Mean neurite length score of NGF-treated cells. The score corresponds to the mean of total length of all neurites per pictures divided by the number of cells in the picture. N=7, mean of means (error bars = SEM).

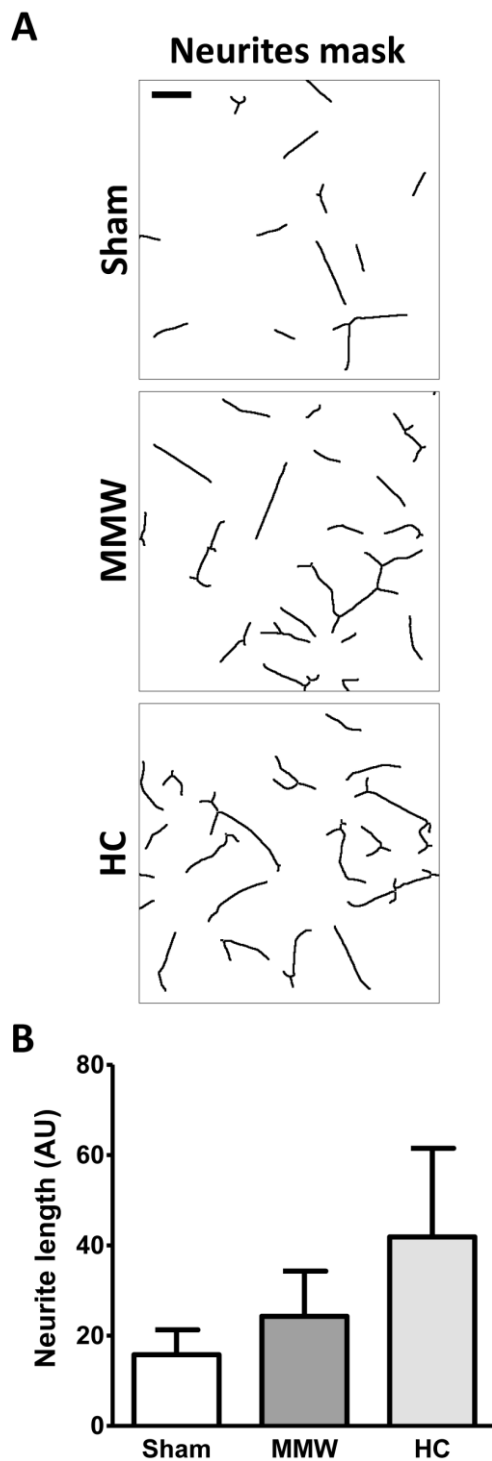


Fig. 5. Impact of MMW exposure on number of neurites per cell distribution of NGF-treated PC12 population. A) Representative fusion mask of neurites and cell bodies mask from pictures of NGF-treated cells: sham condition (left) vs. MMW condition (middle panel) vs. heat control condition (right panel), scale bar = 10 μ m. Numbers inside cell bodies tag cells bearing x neurites where x is the written number. B) Number of neurites per cell distribution of NGF-treated cells. The histogram represents the proportion of cells bearing x neurites ($0 < x \leq 5$). Data expressed as mean percentage of the population. The fold of sham induction is noted on top of MMW and HC histograms. $N=7$, mean of means (error bars = SEM).

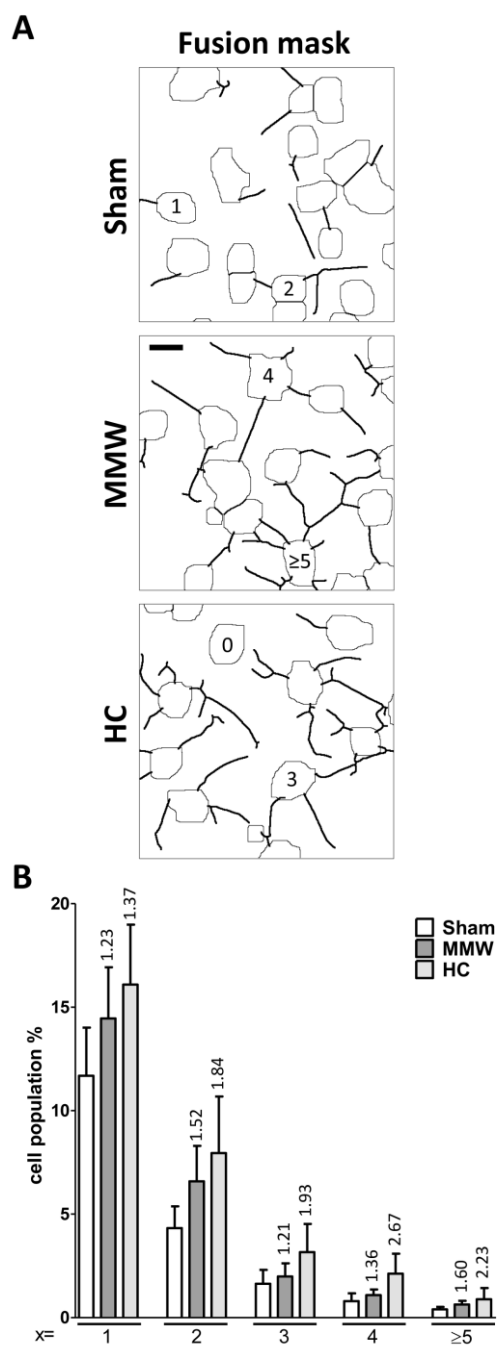


Fig. 6. Impact of MMW exposure on neurites angle of NGF-treated PC12 population. The angle of 1000 neurites taken randomly from all conditions of all experiments (N=7) were measured. The vertical axis of the 96-wells culture plate did correspond to the 90° angle (orientation of the electric field).

