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ABSTRACT

Several forthcoming wireless telecommunication systems will use electromagnetic frequencies at millimeter waves (MMWs), and technologies developed around the 60-GHz band will soon know a widespread distribution. Free nerve endings within the skin have been suggested to be the targets of MMW therapy which has been used in the former Soviet Union. So far, no studies have assessed the impact of MMW exposure on neuronal metabolism. Here, we investigated the effects of a 24-h MMW exposure at 60.4 GHz, with an incident power density (IPD) of 5 mW/cm^2, on the dopaminergic turnover of NGF-treated PC12 cells. After MMW exposure, both intracellular and extracellular contents of dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were studied using high performance liquid chromatography. Impact of exposure on the dopamine transporter (DAT) expression was also assessed by immunocytochemistry. We analyzed the dopamine turnover by assessing the ratio of DOPAC to DA, and measuring DOPAC accumulation in the medium. Neither dopamine turnover nor DAT protein expression level were impacted by MMW exposure. However, extracellular accumulation of DOPAC was found to be slightly increased, but not significantly. This result was related to the thermal effect, and overall, no evidence of non-thermal effects of MMW exposure were observed on dopamine metabolism.

KEYWORDS: *in vitro*, 60 GHz, neuron-like model, dopamine turnover

INTRODUCTION

Millimeter waves (MMWs) range from 30 to 300 GHz and constitute the extremely high frequency band of radiofrequency, which will be soon widely used for wireless telecommunications. These frequencies, especially around 60 GHz, will be exploited for the fifth generation mobile networks (5G) and for various applications in the context of local/personal/body area networks—WLANs/WPANs/WBANs [1–3]. MMWs are also promising for emerging biomedical applications [4]. In the context of the MMW therapy used in the former Soviet Union, a biological response to MMWs has been highlighted, focusing on the hypoalgesic effect [5] in both animals and humans with controlled blind conditions [6–8]. In the therapeutic range (42–62 GHz) [9, 10], the peripheral nervous system may have a role in the transduction of the MMW-induced hypoalgesia, because an initiation in free nerve endings located in the epidermis and the upper dermis is suspected [11]. However, no molecular mechanism of non-thermal effects of MMWs on living tissues has yet been found, whereas thermal effects have been clearly observed and investigated. Indeed, researchers have reported heat-mediated effects of MMW exposure of *Xenopus* spinal cord neurons [12] and *Xenopus* oocytes [13], and suggested that MMW-induced thermal effects can impact the microtubule assembly rate or disturb the activity of voltage-gated channels and ionic pumps.

So far, no study has investigated the impact of MMWs on the production of monoamine neurotransmitters, and no clear conclusion can be drawn from the few studies assessing the effects of

radiofrequencies, because of contradictory results. Singh and Kapoor [14] analyzed the plasma catecholamines content in radar military workforce personnel, and no significant differences were registered between the exposed and the control groups. However, in a rat model, several studies found that semichronic radiofrequency (RF) exposure may cause disturbances in monoamine neurotransmitters metabolism [15–17]. Depending on the study, dopamine (DA) levels are either increased [17] or reduced [15, 16], but differences in the choice of explored brain area and exposure durations may explain these contradictory results [16].

Here, we wanted to explore the potential effects of MMWs on DA metabolism in a neuron-like model, the PC12 cell line. This model has been used several times for assessing the effects of electromagnetic fields, especially in the RF range [18–20]. PC12 cells are derived from a rat adrenal tumor [21] and are widely used as an *in vitro* neuronal cell model [22]. They are able to differentiate and to extend neurites when treated with nerve growth factor (NGF) [23]. Moreover, PC12 cells possess a catecholamine metabolism (synthesis, release, uptake and catabolism) similar to the one observed *in vivo*: stored into vesicles, DA can be released from PC12 cells by exocytosis. The major pathway of DA inactivation is initiated by specific 'reuptake mechanisms' using presynaptic dopamine transporters (DAT) [24], with a subsequent partial intracellular catabolism mediated by the monoamine oxidase (MAO), leading to the formation of 3,4-dihydroxyphenylacetic acid (DOPAC) [25, 26]. DA turnover involves a full set of cellular processes such as vesicular trafficking, monoamines synthesis, liberation, uptake, and enzymatic activities. Therefore, DA turnover is considered to be a good reflection of the global neuronal activity and metabolism. It is reported in the literature that DOPAC contents can be used as a criterion for the determination of DA turnover, as reviewed by Arbuthnott *et al.* [27]. Thus, the aim of this study was to determine the effects of acute exposure to MMWs on DA metabolism, by assessing this turnover in an exposed NGF-treated PC12 cell line. We focused on the determination of the intracellular ratio of DOPAC to DA, while assessing the accumulation of aminergic compounds in the culture medium. As DAT protein membrane density has been reported to regulate DA turnover in the human brain [28], DAT protein expression was also studied here.

MATERIALS AND METHODS

Antibodies and pharmacological compounds

Nerve growth factor beta (NGF-β) and pargyline were obtained from Sigma-Aldrich (St Louis, MO, USA). Among the primary antibodies, the mouse monoclonal anti-β-Actin (AC15, A5141) was obtained from Sigma-Aldrich, and rabbit polyclonal anti-dopamine transporter (ab111468) from Abcam (Cambridge, UK). 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 purchased from Thermo Fisher (Waltham, MA, USA).

Cell culture and exposure protocol

Neuroscreen-1 (NS-1), a PC12 cell line subclone, was obtained from Thermo Fisher. Cells were maintained in Roswell Park Memorial Institute 1640 Medium (RPMI medium 1640, 21875, Thermo Fisher) containing 10% horse serum (Biowest, Nuaillé, France), 5% fetal bovine serum (Biowest), 1 mM sodium pyruvate (Thermo Fisher) and antibiotics (Thermo Fisher) at a temperature of 37°C in 5% CO_2. For exposure experiments, cells were seeded in collagen I-coated Falcon 6- or 96-well plates (Corning, Corning, NY, USA) at 5×10^4 and 6×10^3 cells per well respectively, and maintained in 15% serum medium for 24 h. One day before exposure, the medium was replaced with RPMI 1640 medium containing 1% horse serum and 0.5% fetal bovine serum. It was performed concomitantly with the treatment with NGF (200 ng/ml) in an exposure medium designed to keep the pH buffering in the non-gassed incubator of the exposure system. It consisted of 1.5% serum powder reconstituted RPMI 1640 medium, without $NaHCO_3$, with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and with antibiotics. During exposures, culture plates were sealed with AeraSeal sealing films (Excel Scientific, Victorville, CA, USA) to avoid extended medium evaporation.

Exposure system, dosimetry and experimental set-up

The exposure system used in this study was designed to expose cell cultures to MMWs at 60.4 GHz. It consists of two main units: (i) a signal generation subunit and (ii) an exposure chamber, previously described in detail [29]. The exposure chamber was a compact temperature-controlled anechoic chamber located inside a MEMMERT UNE400 incubator (Memmert GmbH, Schwabach, DE) and lined inside by absorbing materials. The cells located in one well of a 6-well tissue-culture plate or in four wells of a 96-well tissue-culture plate were exposed from the bottom for 24 h, under near-field conditions. For the exposure of 6-well culture plates, an averaged incident power density (IPD) of 5 mW/cm² was used, corresponding to the maximal exposure limit recommended by the International Commission on Non-ionizing Radiation Protection (ICNIRP) for workers exposed on at least 20 cm² of their bodies. The exposure scenario and the associated dosimetry have previously been described in detail [30]. Use of 96-well plates changes the exposure levels. A numerical dosimetry study showed that the IPD for the four wells containing cells is 10.1 ± 0.1 mW/cm² [31]. Sham exposures were performed under identical conditions with the generator switched off. Since MMW exposure induces a slight temperature increase in the medium (~1°C and ~1–2°C for 6-well and 96-well tissue-culture plates respectively), a heat control (HC) was done in parallel for each experiment. It consists of the exact same culture plate in another MEMMERT UNE400 incubator, with the temperature set in order to be the same in the medium and in the exposed wells. During the experiments, the temperature was monitored using a 4-channel Reflex fiber optic thermometer (NEOPTIX, Quebec, Canada). The experimental protocol is described in Fig. 1.

Measurement of intracellular and extracellular contents of catecholamines

The 6-well plates containing PC12 cells were scraped immediately after the exposure experiments, and cell monolayers were dissociated and quantified using a TC20 automated cell counter (Biorad, Hercules, CA, USA). After centrifugation (150*g*, 5 min), supernatants and cell pellets were used for determination of extracellular (medium) and intracellular contents of catecholamines, respectively. Cell pellets were lysed in an Elvehjem Potter homogenizer in 150 µl

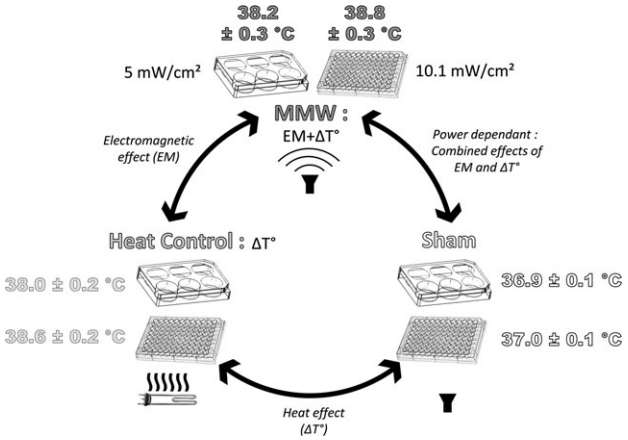

Fig. 1. Experimental protocol. In order to assess the impact of MMWs on cell metabolism, while identifying any outcome unrelated to the thermal effect, PC12 cells in 6- or 96-well tissue-culture plates were exposed to three different sets of experimental conditions: an exposure to MMWs at 60 GHz, a heat increase in the medium induced by the thermostat setting of the incubator (heat control: HC) or a sham-exposure (MMW generator turned off).

of an antioxidant medium (0.05% EDTA, 0.05% $Na_2S_2O_5$, 0.1% cysteine) containing 0.2 M perchloric acid, and then sonicated three times for 15 s. After centrifugation (10 000*g*, 3°C, 10 min), the supernatants were filtered on a 0.22 µm filter (Merck Millipore, Darmstadt, Germany) and subjected to HPLC. Concerning extracellular catecholamines contents, culture media were twice diluted with the antioxidant medium, centrifuged (10 000*g*, 3°C, 10 min), filtered on a 0.22 µm filter (Merck Millipore), then the supernatants were subjected to HPLC.

Kinetics

In a separate experiment, PC12 cells were incubated as described above from 0 to 24 h. At different times (0, 10 min, 2 h, 8 h and 24 h), culture media were collected and subjected to HPLC. The same experiments were conducted in the presence of 10 µM pargyline, a monoamine oxidase inhibitor (MAOi) [32]. It is important to notice that most pharmaceutical studies using MAOi to inhibit DA catabolism use short durations (for 30 min to 1 h) of treatment [33], because pargyline treatment is effective during a short time span. Therefore, the effect of MAOi on catecholamines contents was only assessed after 2 h of treatment. Amines were detected by HPLC–electrochemical detection (ECD) [34, 35]. The effluent [0.1 M phosphate buffer containing 6% methanol and sodium 1-heptanesulfonate solution (Sigma-Aldrich) at a final concentration of 6 mM, adjusted to pH 3.6] was monitored at a flow rate of 1 ml/min in a Merck Cl8 reverse-phase column (250 × 4 mm inner diameter, 5 µM average particle size; Merck, Darmstadt, Germany). Sample analysis consisted of the injection of 20 µl of sample into the column. The output signals from a bioanalytical system amperometric detector (model 2465 Waters; working electrode potential maintained at +0.8 V versus Ag/AgCl ISAAC reference electrode) were recorded, and amines were determined based on the peak to peak distances and peak heights, as compared with those of standard solutions of DA and DOPAC (Sigma-Aldrich). A linear relationship was observed between the peak lengths and the amounts of aminergic compounds from 50 fmol to 100 pmol. The limit of detection for amines in our HPLC-ECD procedure was in the range of 0.5 pmol [35, 36]. Results were expressed as ng/ml ± SEM of culture medium (extracellular contents) or ng/10^6 cells ± SEM (intracellular contents), and statistical analysis was performed using the Wilcoxon signed-rank test.

Immunocytochemistry

Immunocytochemistry (ICC) was done directly inside the 96-well culture plates as previously described [31]. Cells were incubated overnight with primary antibodies at 1:200 dilution each, at 4°C. After three successive washes, cells were incubated 1 h with secondary antibodies at 1:1000 dilution and 10 µg/ml Hoechst 33342 (Sigma-Aldrich) for nuclei counterstaining. Pictures were taken and fluorescence was analyzed in a blind manner using a Cellomics ArrayScan VTI HCS Reader (Thermo Fisher) in the ImPACcell technologic platform (Rennes1 University-Biosit, Rennes, France). For each well, 10 to 20 high-definition pictures were randomly taken, and cell-by-cell fluorescence values were obtained. Statistical analysis was performed by comparing the mean of cell-by-cell fluorescence intensity means measured in all experiments, for each condition, using an analysis of variance (ANOVA) and the Wilcoxon signed-rank test.

RESULTS

Impact of MMW exposure on dopamine turnover of NGF-treated PC12 cells

Intracellular and extracellular contents of DA and DOPAC were analyzed by HPLC-ECD. First, the metabolic phenotype was assessed, considering the fact that many PC12 subclones exist, and that they can present different kinds of catechol release. The PC12 subclone Neuroscreen-1 is a dopaminergic cell line that contains almost 20 times more DA than norepinephrine (NE), as shown on Fig. 2A. DA and DOPAC intracellular contents were not significantly modified by 24 h of 5 mW/cm² MMW exposure at 60.4 GHz or by heat increase, even if a slight increase in the intracellular DOPAC content was observed in the HC condition (Fig. 2B and C). Then, the DOPAC to DA ratio was determined, as it is commonly used for estimating the DA turnover rate. Neither MMW exposure, nor heat increase significantly altered this ratio (Fig. 2D).

Impact of MMW exposure on extracellular accumulation of dopamine compounds

Culture media were removed 24 h after the exposure of NGF-treated PC12 cells in the 96-well culture plates, in the three different experimental conditions. DOPAC levels were ~10 times higher than DA levels, as shown by the scale difference between Fig. 3A and B. The accumulation of DOPAC in the medium was time-related, as demonstrated by the analysis of media samples of NGF-treated PC12 cells, obtained at different times of incubation (Fig. 3C). Moreover, DOPAC early accumulation was significantly inhibited when cells were treated with the MAOi pargyline

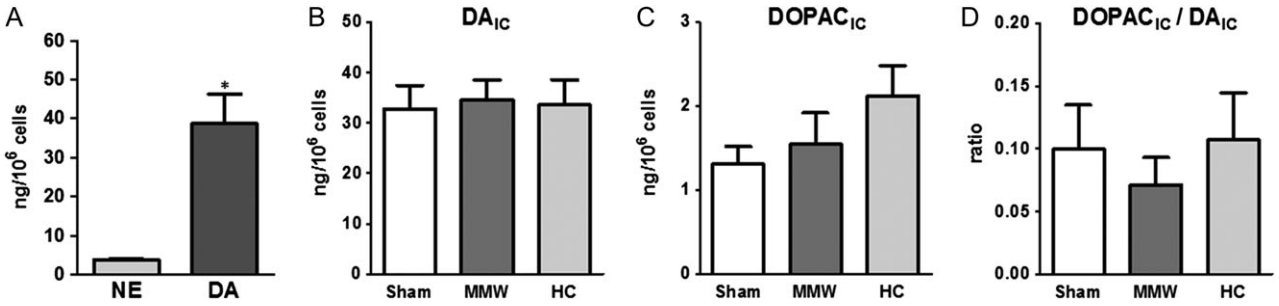

Fig. 2. Impact of MMWs exposure on intracellular DA contents in NGF-treated PC12 cells. (A) The metabolic phenotype of NS-1 cells was assessed by measuring the intracellular DA and NE contents after 24 h of NGF treatment ($N = 4$, mean ± SEM *$P < 0{,}05$). Statistical significance is represented by an asterisk. (B) Intracellular contents of DA and (C) intracellular contents of DOPAC, were determined by HPLC-ECD following a 24 h exposure to MMWs. The results are expressed as ng/10^6 cells ($N = 5$, mean ± SEM). (D) The DOPAC/DA ratio was also determined (Fig. 2D) ($N = 7$, mean of ratios from each experiment ± SEM).

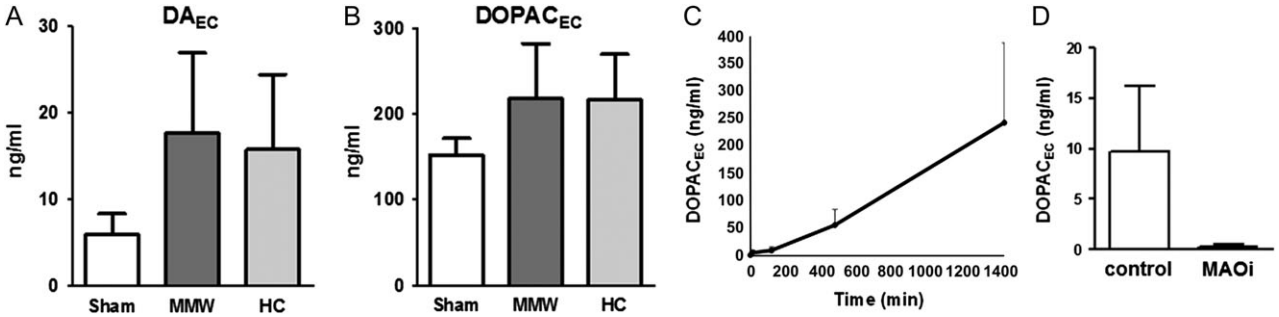

Fig. 3. Impact of MMWs exposure on extracellular DA and DOPAC contents in NGF-treated PC12 cell media. (A) DA and (B) DOPAC contents determined by HPLC-ECD in the medium of differentiated PC12 cell cultures after 24 h exposure to MMWs. The results are expressed in ng/ml of medium ($N = 10$, mean ± SEM). (C) Kinetics of DOPAC accumulation in the medium of NGF-treated PC12 cell cultures: DOPAC contents were measured 0, 10, 120, 480 and 1440 min (24 h) after initial medium change at $t = 0$ min ($N = 4$, mean + SEM). (D) DOPAC accumulation inhibition was assessed by adding 10 µM of the MAO inhibitor pargyline to the media ($t = 0$). DOPAC contents in the media were measured at $t = 180$ min ($N = 4$, mean ± SEM).

(Fig. 3D), emphasizing that the extracellular DOPAC is from cytosolic origin. As pargyline was added only once at $t = 0$ min and is active in a short span of time, its effect was only assessed at $t = 180$ min. Our data show that an exposure to MMWs for 24 h induced a slight increase in both DA (Fig. 3A) and DOPAC (Fig. 3B) contents, although this was not significant. However, this increase was also observed in the HC condition, suggesting that this increase was related to cell heating.

Impact of MMW exposure on DAT expression

A low-intensity, well-spread DAT protein expression was observed on microscopy pictures (Fig. 4A) in PC12 cells. Cells treated with NGF during 24 h expressed significantly more DAT than untreated cells (Fig. 4B), whereas the housekeeping protein β-Actin immunostaining was not modified (Fig. 4C). The 24 h of MMW exposure did not significantly modify DAT and β-Actin expression in both undifferentiated and differentiated PC12 cells (Fig. 4B and C). Moreover, exposure did not significantly modify the distribution of fluorescent staining intensity within the NGF-treated PC12 cell population (Fig. 4D). HC showed that the slight exposure-related temperature increase in the media had no consequence on these parameters.

DISCUSSION

In this study, we wanted to explore the direct effects of MMW exposure on the DA metabolism of neuron-like cells. This investigation was motivated by the fact that EMF exposure can modify monoamine contents in a rat model [15–17]. This effect could be involved in reported cognitive process impairment, and it has been hypothesized that catecholamine dysregulation could participate in electromagnetic hypersensitivity [37]. However, no serious study supports this hypothesis so far.

In order to perform our experiments on a model with a well-defined catecholamine metabolism, we first phenotyped the Neuroscreeen-1 PC12 subclone, and confirmed that it was dopaminergic. Indeed, the intracellular NE contents were found to be

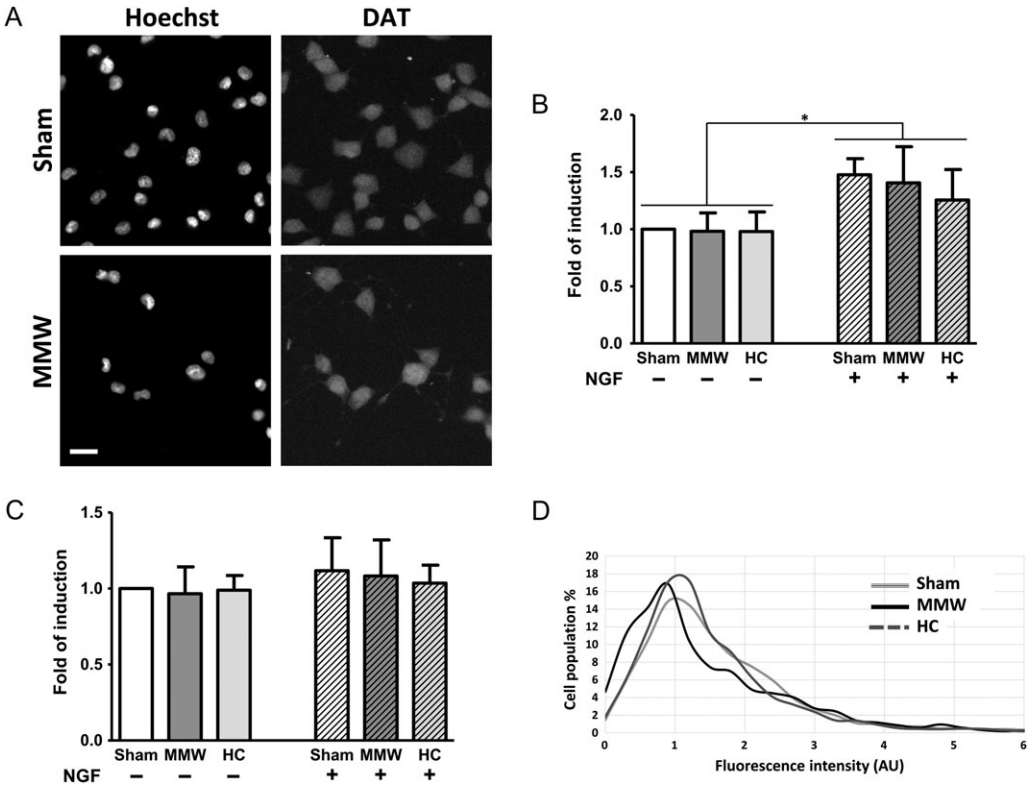

Fig. 4. Impact of MMWs exposure on DAT protein expression in NGF-treated PC12 cells. (A) Microscopy images of DAPI (left panel) and DAT (right panel) fluorescent staining in NGF-treated cells: sham condition (top panel) vs MMWs exposure condition (bottom panel); scale bar = 10 µm. (B) Mean DAT and (C) mean housekeeping protein β-actin fluorescence intensity analyzed cell by cell. Data are expressed as fold of sham condition expression ($N = 4$, mean of means, ± SD $^*P <$ 0,05). Statistical significance found using one-way ANOVA is represented by an asterisk. (D) Distribution of DAT fluorescent staining intensity within NGF-treated cell populations ($N = 4$, pooling of the data from of all experiments).

much lower than the intracellular DA contents, showing that DA is the major released transmitter. DA metabolism is a complex phenomenon involving synthesis, secretion, uptake and catabolism. Simply measuring the whole cellular DA content is not sufficient for investigating this metabolism, because small cytoplasmic changes can be masked by the high DA granular storage, both *in vitro* and *in vivo*. Therefore, we also assessed DA release and DA catabolites. DA catabolites appear both in the extracellular compartment, from the activity of catechol-O-methyl transferase (COMT) [38], and in the cytosol (following DA uptake via DAT), from the action of the intracellular enzyme MAO [39], with formation of the respective catabolites 3-methoxytyramine (3MT) and DOPAC. In the present study, the 3MT contents were under the limit of detection (data not shown), which is in accordance with several studies showing that 3MT contents are low in mammalian neurons [40, 41]. Therefore, DOPAC is the major DA catabolite, and any changes in the DOPAC/DA ratio for the cytosolic contents are related to the DA turnover. We also assessed the extracellular DA and DOPAC contents in the culture medium. DOPAC quantities were found to be much higher than the DA quantities, due to the extracellular DOPAC accumulation over time. Such accumulation can be seen as another index of DA turnover, and we confirmed this mechanism by showing that the extracellular DOPAC stock was lowered by the MAOi treatment, inhibiting the activity of intracellular MAO. Moreover, this accumulation has been reported in the literature, both *in vivo* [42] and *in vitro* [43]. Finally, intracellular DOPAC content is also controlled by DAT protein, which is the primary component of DA recycling in the synaptic cleft, making DAT a major psychostimulant target in the brain [44]. DAT is expressed in PC12 cells [45], and is intensively recycled to the plasma membrane [46]. When PC12 cells are treated with NGF, the immunohistochemical labeling of DAT has been reported to be increased on the growth cones of growing neurites, for example [45]. Our results showed that 24 h of NGF treatment induced a slight but significant increase in DAT expression, which is in accordance with these previous studies. Therefore, and to summarize, our *in vitro* model was appropriate for studying the various parameters involved in DA turnover (which was close to the one observed in neuronal metabolism *in vivo*) and for assessing the impact of MMW exposure on them.

We found that cytosolic DA and DOPAC contents were not significantly modified by an exposure to MMWs for 24 h. Concerning intracellular DOPAC contents, a slight but insignificant increase was observed in the HC group, but the DOPAC/DA ratio was not

modified. We observed a slightly increased accumulation of extracellular DOPAC in NGF-treated PC12 cells, after 24 h of MMW exposure. However, this increase was not found to be significant, and as the HC condition showed the same result, we concluded that it was caused by the thermal effect. Moreover, exposure had no effect on DAT expression in NGF-treated PC12 cells. Altogether our data are consistent with the fact that acute MMW exposure at 60 GHz, with IPDs within the range allowed by ICNIRP, has no impact on the DA turnover. Our study did not reproduce the effects observed *in vivo* at the cellular level. This may be explained by the fact that we exposed cells to a continuous MMW signal and not to modulated radiofrequency. It is also possible that the potential effect does not involve neuronal cells alone, and may require longer exposure durations (chronic exposure) and an undergoing systemic response. Nevertheless, our findings are rather reassuring, as they dismiss the idea of a direct mechanism of action of MMWs on neuronal metabolism. However, they do not rule out the hypothesis of a possible indirect effect induced by chronic MMW exposure.

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