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Histopathological examinations of rat brains after long-term exposure to GSM-900 mobile phone radiation

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Abstract

In order to mimic the real life situation, with often life-long exposure to the electromagnetic fields emitted by mobile phones, we have investigated in a rat model the effects of repeated exposures under a long period to Global System for Mobile Communication-900 MHz (GSM-900) radiation. Out of a total of 56 rats, 32 were exposed once weekly in a two hours period, for totally 55 weeks, at different specific absorption rates (SAR) (of in average 0.6 and 60 mW/kg at the initiation of the experimental period). The animals were exposed in a transverse electromagnetic transmission line chamber (TEM-cell) to radiation emitted by a GSM-900 test phone. Sixteen animals were sham exposed and eight animals were cage controls, which never left the animal house. After behavioural tests, 5-7 weeks after the last exposure, the brains were evaluated for histopathological alterations such as albumin extravasation, dark neurons, lipofuscin aggregation and signs of cytoskeletal and neuritic neuronal changes of the type seen in human ageing. In this study, no significant alteration of any these histopathological paramteters was found, when comparing the GSM exposed animals to the sham exposed controls.

Key words

Albumin, brain ageing, dark neurons, GFAP, lipofuscin, mobile phone

Abbreviations

BBB	Blood-brain barrier
CW	Continuous wave
EMF	Electromagentic field
GFAP	Glial fibrillary acid protein staining
GSM	Global System for Mobile Communication
RF	Radio frequency
SAR	Specific Absorption Rate
TEM-cell	Transverse electromagnetic transmission line chamber

Introduction

During the last century, an unprecedent, exposure to electromagnetic radiation, emitted from mobile phones, and also many other devices, has increased. The extensive use of mobile phones has been called the world's largest biological experiment [24]. Now more than one third of the world's population, participates in this experiment.

Different aspects of biological effects have been studied; epidemiological investigations of tumour development, animal models focusing on histopathological and functional alterations, and more recently, gene expression analyses (for a reference, see [26] [18]). However, no final consensus has been reached so far and the question, whether or not these effects might actually end up being harmful, still remains to be answered.

Since 1988 our group has studied the effects of exposure to various magnetic and electromagnetic fields upon the blood-brain barrier (BBB) and upon tumour growth in the mammalian brain. While our studies on the effects of continued and pulsed modulated microwaves at 915 MHz upon brain tumour growth have not disclosed any growth-promoting effects in our rodent models [23], the same radio-frequency (RF) electromagnetic fields have been revealed to cause significantly increased leakage of albumin through the BBB of exposed rats as compared to non-exposed animals. We started our RF experiments with the frequency modulation of 16 Hz and its harmonies 4, 8, 16 and also 50 Hz, which is the standard line frequency of the European power system, with a carrier wave of 915 MHz. Also continuos wave (CW) exposure was studied. At an early stage 217 Hz modulation was added as this is the frequency of the GSM system [22][19]. These studies comprised sham or 915 MHz exposure for in most cases 2 hours but in a minority of the experiments lasting between 2 and 960 min (both continuous and pulsed modulated waves). These results based on 246 rats 1994 and more than 1,000 rats 1997 (the majority EMF exposed and about 1/3 sham-exposed) concluded that there was a significant difference between the albumin extravasation

from brain capillaries into the brain tissue between the differently exposed groups and the controls. So, we have concluded that extravasation of endogenous albumin through the BBB is a good measure of the effects of RFs from mobile phones [19][20][21][22][24].

One remarkable observation, which we have made over the years, is that exposure with very low whole-body average power densities, of less than one per cent of those emitted by mobile phones (that is at energy levels below10 mW/kg), gave rise to a more pronounced albumin leakage than higher power densities, all at non-thermal levels. This phenomenon is called the window effect and has been observed earlier in connection to RF exposure [1]. It might explain why, in many studies of pharmacological effects and RF exposure, response is only seen at a certain dose range, and not at higher or lower dosages.

An intact BBB protects the brain from damage, whereas a disrupted BBB allows influx of normally excluded hydrophilic molecules into the brain tissue. This might lead to cerebral oedema, increased intracranial pressure and in the worst case, irreversible brain damage. The normal selective permeability of the BBB can be altered in several pathological conditions such as epileptic seizures [15][16] or extreme hypertension [28] and also transient openings of the BBB might lead to permanent tissue damage [28]. Considering the ensuing leakage of substances from the blood circulation into the brain tissue, harmful substances might disrupt the cellular balance in the brain tissue.

The initial albumin leakage into the brain tissue has been seen within hours in about 40 per cent of exposed animals in our previous studies. These are acute effects of the BBB opening. The time between last exposure and sacrifice is of great importance for the detection of albumin foci, because extravasated albumin rapidly vanishes below concentrations possible to demonstrate immunohistologically. Even if acutely extravasated albumin can be resorbed the initial albumin leakage might possibly start a second BBB opening, leading to a viscious circle. Notably, Hassel et al. [9] have demonstrated that

injection of albumin into the brain parenchyma of rats gives rise to neuronal damage. When 25 microlitres of rat albumin is infused into rat neostriatum, 10 and 30, but not 3 mg/ml albumin causes neuronal cell death and severe axonal damage. It also causes leakage of endogenous albumin in and around the area of neuronal damage In our studies, we have observed albumin leakage out into the brain parenchyma 14 days [7] and 50 days [25] after 2 hours of EMF exposure to a real GSM programmable mobile phone in the 900 MHz band (at specific absorption rates of 200, 20 and 2 mW/kg for the 50 days post-exposure study and the same energy levels plus 0.2 mW/kg for the 14 days post-exposure study).

Another remarkable observation in our studies is the fact that a significant neuronal damage is seen in rat brains 50 days after a 2 hour exposure to GSM at specific absorption rates of 200 and 20 mW/kg [25]. Also 28 days after 2 hours of real GSM exposure (at power density levels of 2 and 0.2 mW/kg) the same type of neuronal damage is seen (article submitted for publication). Our findings may support the hypothesis that albumin leakage into the brain is the cause for the neuronal damage observed after 28 and 50 days. The neuronal damage might be mediated through organelle damage with release of not only hydrolytic lysosomal enzymes but also, for example, sequestred harmful material, such as heavy metals, stored away in lysosomes.

In the real human situation the exposure to the mobile phone RF fields is often protracted, and in an increasing number of cases, life-long. In order to mimic this situation, we have now studied male and female Fischer 344 rats, exposed to GSM or sham exposed 2 hours weekly, once a week for totally 13 months. We have used the same exposure system as in the majority of our previous studies, with the awake, non-anaesthetised animals placed in transverse electromagnetic transmission line chambers (TEM-cells). After this protracted period, all animals were studied for behavioural and cognitive functions [17]. In summary, effects upon behaviour such as anxiety, locomotor activity and general exploratory behaviour,

were evaluated in an open-field test, in which no difference was seen between the GSM exposed animals as compared to the sham group. With an episodic-like memory test temporal and spatial effects upon memory functions were evaluated. We could conclude that the GSM exposed animals had impaired episodic-like memory functions, with reduced memory for objects and their temporal order of presentation, when comparing their performance to that of the sham exposed animals. The spatial memory however, evaluated as the detection of the place, in which an object was presented, was not affected by GSM exposure. Finally, after these behavioural and cognitive examinations, the animals were sacrificed, about 5 to 6 weeks after the last exposure.

In the present study, we search for the answer to three main questions.

i. Firstly, we now investigate, whether similar alterations as found before exist also after 55 weeks of EMF repeated GSM exposure. Therefore, we have undertaken histopathological examinations of the rat brains, evaluating the albumin extravasation, as a measure of the integrity of the BBB, and the occurrence of dark neurons, in order to see whether any signs of neuronal damage are present.

ii. Secondly, we now actually have quite a different situation as compared to our previous experiments, as in the present study, brains from animals at an age of 18 to 20 months are studied. In these old animals, we expect ageing parameters to be more prominent than in the generally much younger animals of our previous studies. This leads us to the question, whether or not the long-term EMF GSM exposure might influence the ageing process. In order to investigate this, we searched for glial reaction or scarring, the accumulation of "wear and tear" products in the brain, and signs of cytoskeletal and neuritic changes of the type seen in human ageing. The accumulation of the wear and tear product lipofuscin has been evaluated, as an indicator of possible accelerated ageing. In order to look

for signs of cytoskeletal and neuritic changes of the type seen in human ageing, the silver method by Gallyas has been applied. In this context it could be mentioned, that other researchers in the field have found increased amount of lipofuscin in rat brains after microwave exposure (2450 MHz microwaves, 25mW/cm^2, corresponding to about 7 W/kg (which is a thermal effect not corresponding to our studies). This was accompanied by reduced learning and memory by restraint of hippocampus long-term potentiation [31].

iii. Thirdly, in this study we also investigate to what extent histopathological findings might explain the reduced cognitive capacities observed in the episodic-like memory test [17]. Therefore, correlation analyses between each of the histopathological parameters mentioned above and the performance in the episodic-like memory test have been undertaken.

Material and methods

GSM exposure

The animals are exposed to RF EMFs in the same TEM-cells (Figure 1), as previously described and used by Salford et al. [20][21][22][24][25], Persson et al. [19] and Belyaev et al. [5]. These TEM-cells have been designed by dimensional scaling from previously constructed cells at the National Bureau of Standards [6], which are known to generate uniform TEM-fields for standard measurements. The experimental setup has been described in detail in the first part of the present long term study that reported on cognitive effect after repeated exposure [17]. In short, a GSM mobile test phone with a programmable power output, at the frequency of 900 MHz, was connected to a power splitter, which divides the power into equal parts fed into the four TEM-cells used. No voice modulation was

applied. Each of the four TEM-cells is connected to a 50 Ω dummy load, into which the output is terminated. By using these TEM-cells, the pulse modulated exposure fields can be accurately generated without the distortion that is typically introduced when conventional antennas are used to establish impulse test fields. Thus, a relatively homogeneous exposure of the animals is allowed [14].

The TEM-cells were placed in a temperature-controlled room under constant lighting conditions. The temperature of the TEM-cells was kept constant by placing them on a ventilation table.

The rats were placed in plastic trays (14 × 14 × 7 cm) to avoid contact with the central plate and outer conductor. The bottom of the tray was covered with absorbing paper to collect urine and faeces. Each TEM-cell contained two plastic trays, one above and one below the centre septum. Thus two rats can be kept in each TEM-cell simultaneously. All the animals, even the largest male rats, could move and turn around within the TEM-cells.

For the actual experimental situation with one rat in each compartment of the TEM-cell, the conversion factor K for SAR per unit of input power could be fitted to the data as:

$$K = (1.39 \pm 0.17) - (0.85 \pm 0.22) \cdot w \qquad (1)$$

with w the sum of weights in kilograms of the 2 rats in the cell and the variance given as SEM.

Whole-body SAR and brain SAR vary with orientation. In our present set up, the average of SAR for the brain grey matter was 1.06 times the average whole-body SAR, with a standard deviation of 56 % around the average value for the different orientations, as estimated with a FDTD-computation with the freely available FDTD program of Brooks Airforce Base/FDTD99 [13].

Animals

All animal procedures were performed according to the practices of the Swedish Board of Animal Research and were approved by the Animal Ethics Committee, Lund-Malmö.

Fifty-six inbred male and female Fischer 344 rats (the rats were supplied by Scanbur AB, Stockholm, Sweden) were four to six months of age at the initiation of the EMF exposure. Male and female rats had a weight of approximately 350 g and 200 g respectively, as estimated in calibrations of rat weight as a function of age [29]. The rats were housed in rat hutches, two in each cage, under standard conditions of 22 °C room temperature, artificial daylight illumination and rodent chow and tap water ad libitum. Towards the end of the exposure period the male rats had grown in size and therefore were placed in rabbit hutches, two in each cage. The female rats were smaller and could still be kept in the rat hutches.

The twenty-eight male and twenty-eight female rats were divided into four groups with an equal number of male and female rats in each group. Each animal was given a number and the division into groups was randomised with reference to these numbers. Sixteen animals were sham exposed. Sixteen animals were exposed to lower power level of GSM, with an average SAR-value for males and females of 0.6 mW/kg. Sixteen animals were exposed to higher power level of GSM, with an average SAR-value of 60 mW/kg. Eight animals were cage controls, which, contrary to all the other animals of the study, never left the animal house. These SAR-values are valid at the initiation of the experimental period.

At the end of the experimental periods, the animals had increased in weight with males weighing 545±24 g and the females 304±23 g. The groups of sham, GSM exposed and cage control animals did not significantly differ in weight. Due to the increase in weight the average SAR-values were reduced to 40 mW/kg and 0.4 mW/kg for males and females together at the higher and lower GSM exposure levels respectively.

For each exposure the rats were assigned different TEM-cells quasi-randomly according to a rolling timetable. The duration of the GSM-900 exposure as well as the sham exposure was two hours at one occasion weekly for totally fifty-five weeks. Behavioural tests were performed during a period from three weeks to seven weeks after the last EMF or sham exposure. Two animals, one male cage control and one male exposed to lower effect GSM, died before the initiation of these tests of unknown reasons.

Six to seven weeks after the last EMF exposure (during this time interval the behavioural and memory tests were performed), the fifty-four animals, at an age of eighteen to twenty months, were anaesthetized with chloralhydrate and sacrificed by perfusion-fixation with 4% formaldehyde. The anesthesia is necessary to avoid stress and blood pressure rise during perfusion–fixation procedure.

Histopathology and methods

The brains were fixed in situ through saline perfusion through the aorta ascendens for 3 minutes followed by 4% formaldehyde for 10 minutes and immersion in 4% formaldehyde for 24 hours and then removed from the skulls by a non-traumatic technique (resection of bone structures at the skull base, followed by a midline incision from the foramen magnum to the nose) and immersion fixed in 4% formaldehyde for more than 24 hours. Whole coronal sections of the brains were dehydrated and embedded in paraffin and sectioned at 5 μm with a microtome. Samples of other organs were frozen or fixed for possible future analysis.

All brains were examined histopathologically by our neuropathologist (A.B.). All microscopic analyses were performed blind to the test situation**.**

Albumin was demonstrated with the IgG fraction of rabbit anti-rat albumin (Dakopatts, Helsingborg, Sweden) diluted 1:8,000. This reveals albumin as brownish spotty

or more diffuse discolorations. Biotinylated swine anti-rabbit IgG was used as a secondary antibody. Then avidin, peroxidase conjugated, was coupled to the biotin and visualised with DAB (diaminobenzidine). Regarding albumin extravastaion, the numbers of immunopositive extravasates (foci) were recorded under a microscope. None or occasional minor leakage was rated as normal, whereas one larger or several leakages were regarded as pathological. Immunopositive sites were, however, disregarded when localised in the hypothalamus, basally from the median eminence and laterally including the nucleus lateralis hypothalami, in the immediate vicinity of the third ventricle and just beneath the pial membrane. These structures are well known for their insufficient blood-brain barrier.

In order to study dark neurons, the same 5μm sections were also stained for RNA/DNA with cresyl violet. Regarding dark neurons, these were judged semiquantitatively as no or occasional (score 0), moderate (score 1) or abundant occurrence (score 2) of dark neurons.

Sections were also stained with the silver method by Gallyas, which in humans is a reliable and widely used method to show cytoskeletal and neuritic changes in ageing (and Alzheimer's disease), in a search for premature or accelerated ageing.

The immunohistochemical method for glial fibrillary acid protein (GFAP) was used to identify astrocytes in the search for a glial reaction or scarring elicited by possible EMF damage such as loss of neurons or alterations in the glial part of the BBB in vessel walls. More in detail it might show up as a focal or general increase in number and density of astrocytes, coarser glial fibrils or as a thickening of the vessel wall.

Staining with Sudan Black B was used to reveal the neuronal content of lipofuscin shown as black granules, sometimes aggregated as heaps in the cytoplasm. Since it varies in amount between animals and also regionally in the brain, an over all quantification was deemed difficult and less accurate. Therefore, the lipofuscin analysis was focused on a

specific group of neurons conveniently compared across the material. For this purpose the hippocampal pyramidal neurons were selected since they are easily defined anatomically and may be of particular interest in view of the animal's behavioural symptoms probably based on a disturbed memory. (+) or + denotes what in this blind study was assumed to be a basic or normal amount and larger amounts as +(+), ++, and ++(+).

The Gallyas silver staining, which in humans is a reliable and widely used method to show cytoskeletal and neuritic changes in ageing (and Alzheimer's disease), was used in a search for premature or accelerated ageing.

Statistics

The Kruskal-Wallis one-way analysis of variance by ranks was used for a simultaneous statistical test of the score distributions for the four exposure conditions. If the null hypothesis could be rejected, the non-parametric Mann-Whitney U-test for independent samples was used to compare each of the groups of GSM exposed, sham exposed and cage control animals to each other.

In order to compare the correlation between the histopathological and cognitive parameters at an individual level, Spearman correlation analysis (non-parametric test; two-tailed t, corrected for ties) was used. The performance in the third test trial of the episodic-like memory teat is compared by using the standardised difference between old familiar object exploration time and recent familiar object exploration time and between displaced and non-displaced objects [17].

Results

Histopathological examinations

Animals were regarded as pathological when showing a semi-quantitatively judged score ≥1 in the case of albumin leakage and dark neurons; and a score ≥1.5 in the case for lipofuscin aggregation and glial reactions.

Regarding albumin positive foci, generally one animal in each exposure group of high GSM, low GSM, sham exposure and cage controls, respectively, showed albumin extravasation. These findings are occasional and rare, and thus judged as normal variants (Figure 2).

Dark neurons with score >1.0 were observed in about 40 % of the animals, irrespective of exposure.

Glial reactions and scarring, as detected by the immunohistochemical method for glial fibrillary acid protein (GFAP) was compared to a control group of animals of the same age (see Figure 3 and 4). As for GFAP, the lipofuscin aggregation (see Figure 5), as viewed with the Sudan black B staining as an indicator of ageing was compared to a reference group of rats in the same age. The percentage of animals with a score >1.5 is shown in Table 1.

Regarding the signs of cytoskeletal and neuritic neuronal changes of the type seen in human ageing, as visualized by the silver method by Gallyas (see Figure 6), no differences between the animals in terms of ageing changes were observed. A few animals showed occasional neurons with a more conspicuous cytoskeleton compared to the majority of the neurons in the same section. However, these were never convincingly pathological and showed no tangle formation. Also, there were no plaques and no grains or granulovacuolar bodies in the hippocampal neurons.

When statistically testing the degree of histopathological alterations, as graded with the semi-quantitative scoring methods described above, the Kruskall-Wallis test revealed that there were no significant differences between the exposure groups of high GSM, low GSM, sham exposed or cage control animals. This was true for all the parameters studied, that is albumin leakage, the occurrence of dark neurons, glial reactions, lipofuscin aggregation and cytoskeletal and neuritic changes (as visualised by the silver method by Gallyas).

Individual correlation between memory and behavioural functions and histopathological alterations

We have previously seen in the same animals that the performance in the test trial of the episodic-like memory test (as seen by the standardised difference between old familiar object exploration time and recent familiar object exploration time) is significantly impaired in the GSM exposed animals as compared to the sham exposed animals [17]. There was no statistically significant difference in the exploration time between the higher and lower GSM exposed animals. When comparing the exposed animals to the sham animals, they spent a significantly shorter time exploring the old familiar objects as compared to the recent familiar objects. Thus, the temporal order memory is significantly impaired in GSM exposed rats, as compared to sham exposed controls. However, an even larger impairement of temporal order memory is observed in the inexperienced cage control animals.

A question of great interest is whether possible signs of neuronal damage and premature ageing can be related to impairement seen in the episodic-like memory test. For this purpose a non-parametric correlation test (Spearman correlation) was performed between the different morphological parameters and the standardised temporal and spatial memory parameters. No correlation was found between the performance in the episodic-like memory test or the spatial memory test (the standardised difference of the third test trial), to the

occurrence of albumin extravasation or dark neurons. Concerning the lipofuscin aggregation, as seen with the Sudan black B staining, the temporal order memory was weakly correlated to this parameter: a higher lipofuscin score leads to a higher impairment, r=-0.23, p (one-sided) =0.05). However, when analysing the correlation for spatial memory, significant effects were seen. Animals with higher amounts of lipofuscin aggregation had impaired spatial memory (Spearman 2-tailed : $r_s = -0.43$, $p = 0.001$).

Discussion

In the present study, rats have been exposed to GSM-900 radiation for a long-term period of 55 weeks. The whole-body SAR-values of 0.6mW/kg and 60mW/kg are well below the ICNIRP limit [10] of 2 W/kg for thermal effects in connection to RF exposure. We have previously reported that cognitive alterations could be observed in the GSM exposed animals as compared to the sham exposed animals after this exposure period [17]. Now histopathological examination shows no significant difference when comparing the brains of the GSM exposed rats to those of the sham exposed rats, regarding the amount of albumin leakage, the occurrence of dark neurons, glial reactions, lipofuscin aggregation, premature or accelerated ageing.

How could the present long term EMF exposure changes be distinguished from those occurring as a natural phenomenon in the ageing rat. We hypothesise that if there actually were an increased level of cellular damage, observable at a histopathological level, it should be more prominent in the EMF exposed rats, and although it could not be separated from damage resulting from natural ageing, the quantity of ageing processes should indicate an effect seen as a result of the EMF exposure.

The effects of RF exposure upon the nervous system involve many different aspects, such as cognitive functions, electrophysiology, morphology, neurotransmitter release

and neuronal response to different pharmacological compounds. However, in most studies, these effects are investigated separately. In our present study, behavioural and cognitive functions of the rats have been well documented. It is remarkable, that cognitive alterations can be observed in the episodic-like memory test performed 5 to 6 weeks after the last GSM exposure session, whereas no histopathological alterations can be seen in the brains taken from the sacrificed animals less than two weeks after these episodic-like memory tests were terminated. It seems most unlikely, that morphological alterations should have been present at the time of the episodic-like memory test, and that these alterations should have disappeared less than two weeks later. Rather, behavioural parameters seem to be much more sensitive to the RF exposure. The histopathological alterations, which we search for here, are those of a damaged brain, and even though functional alterations exist, there is a wide gap to what can be seen through observations in light microscope. Indeed, the hypothesis that behavioural parameters are the most sensitive measures of RF effects has been suggested earlier [12].

There was an interval of 6 to 7 weeks between last EMF exposure and sacrifice of the animals. This, of course, might affect histopathological examinations. On the other hand, only a week passed after the last cognitive memory test until the sacrifice of the animals. Therefore, correlation analyses between the histopathological alterations and the episodic-like memory functions are feasable. Notably, we did not expect any correlation, keeping in mind the generally very occasional histopathological findings and and indeed, as a confirmation of this, few correlations were revealed. However, a negative correlation between the amount of lipofuscin aggregation and spatial memory functions was found. This means, that animals with reduced spatial memory, had an increased amount of lipofuscin aggregation. Similar findings of increased amount of lipofuscin in rat brains after microwave exposure (2450 MHz microwaves, 25mW/cm^2) have been found previously, and also in that study the

increased amount of lipofuscin was accompanied by reduced learning and memory [31] due to restraint of hippocampal long-term potentiation.

Remarkably few studies of long-term GSM exposure have been performed. Two-year PERFORM-A bioassays were undertaken in a collaboration between the GSM association, the Mobile Manufacturers forum and the EU Commission [27][30]. 1,170 mice [30] and 1,170 rats [27] were studied in two separate arms, comparing the effects of GSM and DCS (Digital Personal Communications System) exposure 2 hours daily, 5 days weekly for totally 104 weeks (at average whole-body SAR-values of 0.4, 1.3 and 4.0 W/kg), to those seen in sham controls and cage control animals. The main focus in these studies was to evaluate possible carcinogenic effects. Neither the rat nor the mouse study showed any increase in the organ-related tumour incidence when comparing the GSM and DCS exposed rats to the sham exposed controls. In male mice, increasing levels of EMF exposure resulted in a decreased incidence of liver adenomas. In rats, a higher incidence of dilated ducts in the Zymbal's glands (an auditory sebaceous gland that opens into each external ear canal), bone marrow atrophy and focal C-cell hyperplasia in the thyroid gland were seen in the high-level GSM exposed animals. However, these findings were considered to be incidental.

Another study of tumour incidence in F344 rats in connection to RF EMF long-term exposure of the type emitted by mobile phones, reached the same conclusion, that no significant differences in the incidence of any kinds of tumours could be seen when comparing the exposed rats to sham exposed controls [11]. In this study, comprising totally 480 animals, 80 male and 80 female rats were exposed to either FDMA (Frequency Division Multiple Access) or CDMA (Code Division Multiple Access) RF radiation 4 hours daily, 5 days weekly for totally 2 years (at an average SAR-value of 1.3 W/kg). It was pointed out that the rat weight is a major indicator of overall well being, and in this context it was also concluded that no difference of the weight was seen between the EMF exposed rats and the

sham exposed controls. A similar lack of difference was found for overall survival, the incidence of neoplasia, both as far as brain/spinal cord tumours, and non-CNS tumours, were concerned.

A further long-term examination of tumour development in connection to EMF exposure, simulating the radiation emitted from mobile phones (frequency-modulated signal at 836.55 Hz, at brain SAR-values of 1-1.2 W/kg), showed no increase in the incidence of brain tumours after 731-734 days [3]. In this study, the neurocarcinogen ENU (ethylnitrosurea) had been injected into pregnant rats, leading to an exposure of the offspring (totally 540 rats) *in utero*. Following this neurocarcinogen exposure, the incidence of spontaneous tumourigenicity of CNS tumours was tested in the offspring. Absence of tumourigenic effects was also noted after CNS exposure to intermittent digital-phone fields for 24 months (SAR levels simulating localized peak brain exposures of a mobile phone user) [2]. However, evidence of tumour-inhibiting effects as a result of the EMF exposure was observed, with reduced incidence of ENU-induced CNS tumours after digital mobile phone exposure in preterm animals, that is animals where primary neural tumours were determined to be the cause of death.

These conclusions referred to above are consistent with our previous study [23], in which no increase in the growth rate of implanted brain tumour cells could be found in animals exposed to RF EMFs.

Regarding long-term exposure and blood-brain barrier permeability, an even smaller number of studies have been undertaken. At 900 MHz, mice have been exposed 1 hour daily, 5 days weekly, for totally104 weeks (at SAR-values of 0.25, 1.0, 2.0 and 4.0 W/kg) [8]. Albumin extravasation after the termination of this exposure was studied and considered to be minimal and of no significant increase in any of the different exposure groups as compared to the sham exposed animals.

As concluded above, no albumin extravasation and no increase in the amount of dark neurons can be seen after 55 weeks of exposure to the GSM radiation in our present study as compared to sham exposed animals. However, we do not know whether there might have been an observable albumin leakage during the earlier stages of the whole-year exposure period, comparable to the albumin leakage we have observed in previous studies [7][19][20][21][22][25]. In our previous studies, albumin leakage and neuronal damage seem to follow dynamical courses over time after the termination of the EMF exposure. Albumin leakage can be seen 14 days and 50 days after terminated 2-hour-EMF exposure, but not after 28 days. On the other hand, neuronal damage, seen as dark neurons, can be observed 28 days and 50 days after terminated 2-hour-EMF exposure, but not after 14 days. In our present study, the animals were allowed to live 42 days (males) and 49 days (females) after the last EMF exposure. After these time intervals, neither albumin leakage nor increase in the numbers of dark neurons were observed. Whether this is due to restorative mechanisms, or damage might still remain, although not observable with our techniques, is an important question. Keeping in mind the reduced memory functions it however seems more likely that there has been irreversible damage, and although compensatory mechanisms have covered up for visible alterations, not all neuronal functions have been maintained. Thus, we hypothesise, that during the 55 weeks of repeated exposure, the BBB permeability alterations shown in our earlier studies after 14 and 50 days have occurred. The albumin leakage at an initial stage of the experimental period could have been absorbed after some time. Furthermore, dark neurons have most likely occurred after 28 and 50 days of exposure, and possibly also later on. However, at a certain but unknown time point during this protracted, more than one year long exposure period some adaptation process has been activated. Possibly, damaged neurons might have been removed by phagocytosis and could have been replaced.

In summary, we may conclude that the long-term GSM EMF exposure has at least not resulted in any significant histopathological alterations. The permeability and occurrence of dark neurons seen in our previous BBB studies as acute effects of short-term exposure, are not seen in this long term study**.** Furthermore, the occurrence of ageing parameters is not accelerated in this protracted, almost life-long, EMF exposure even if admittedly the parameters used are difficult to quantitate and compare. However, the fact that the cognitive functions are impaired, still remains. Which mechanisms might lay behind this is an interesting and important question. We have previously shown, that suitable combinations of static and time varying magnetic fields directly interact with the Ca^{2+}-channel protein in the cell membrane, and thus, that the Ca^{2+}-efflux over plasma membranes in plasma vesicles from spinach is affected by exposure to extremely low frequency (ELF) electromagnetic fields [4]. Further work in this field, exposing plasma membrane vesicles for amplitude modulated RF-radiation, is presently performed at our laboratory. Possibly, this might explain how the biological effects of EMF exposure, seen by us in many studies during 20 years, are mediated.

In a future perspective, investigations of EMF effects upon humans, and biology as a whole, are increasingly important. Applications of EMFs constantly increase the level of exposure of the human population as well as most ecosystems. Regarding mobile phones, new communicating systems such as the third generation Universal Mobile Telecommunication System (UMTS) are in use and developments of higher frequency applications are to come. The use of wireless computer networks, mobile internet, and applications using Bluetooth wireless connections is exploding. Thus, to further investigate the mechanisms and the effects of increasing EMF eposure, are more important than ever.

Table 1.

Exposure condition	**Dark neurons (Score>1.0)**	**GFAP (Score>1.5)**	**Lipofuscin (Score>1.5)**
Cage controls	3/7 (43 %)	3/7 (43 %)	5/7 (71 %)
Sham exposed	7/16 (44 %)	9/13 (69 %)	7/16 (44 %)
SAR=0.6 mW/kg	7/15 (47 %)	8/15 (53 %)	7/15 (47 %)
SAR=60 mW/kg	6/16 (38 %)	5/16 (31 %)	8/16 (50 %)

Figure Legends

Figure 1. Rats in TEM-cell.

Figure 2. White matter capillary surrounded by faint brownish area due to leakage of albumin. Uptake of albumin in several astrocytes. Immunostaining for albumin, counterstained with Cresyl violet. Magnification 100.

Figure 3. Animal 5977, high exposure. Cortex with slight increase in stellate astrocytes, a sign of gliosis or scarring, related to EMF exposure or ageing. Glial fibrillary acid protein (GFAP) immunostaining for glial protein. Magnification 150.

Figure 4. Animal 5978, cage control. Cortex with normal amounts of astrocytes. Glial fibrillary acid protein (GFAP) immunostaining for glial protein. Magnification 150.

Figure 5. Animal 5977, high exposure. Cortex with neurons containing small amounts of lipofuscin shown as black dots in the cytoplasm. Sudan Black B for lipofuscin. Magnification 100.

Figure 6. Animal 6028, low exposure. Cortex with axons shown in black. No plaques, no tangles but for questionable condensations in the periphery of some neurons. Gallyas silver method. Magnification 150.

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The authors declare that they have no competing financial interests.

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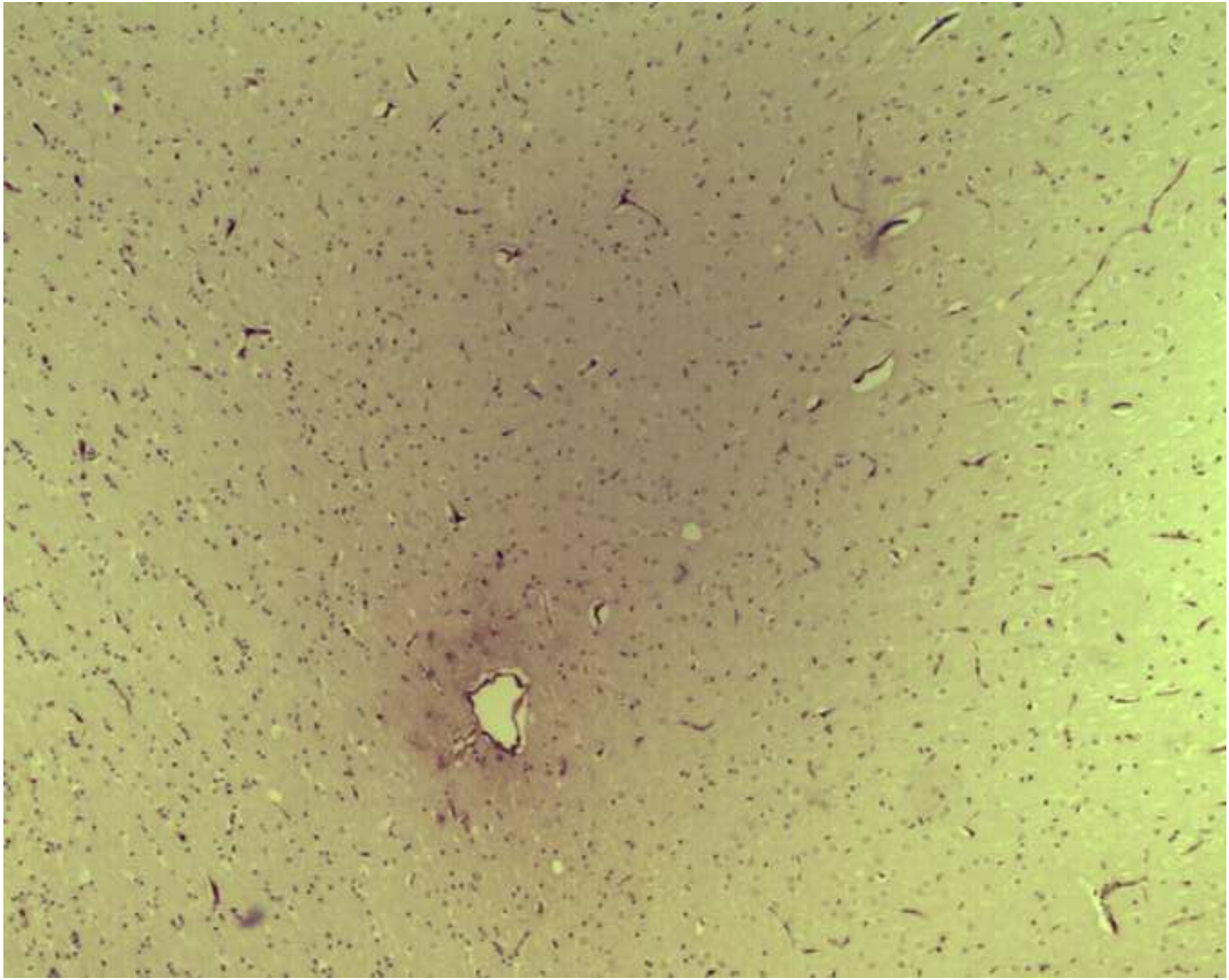

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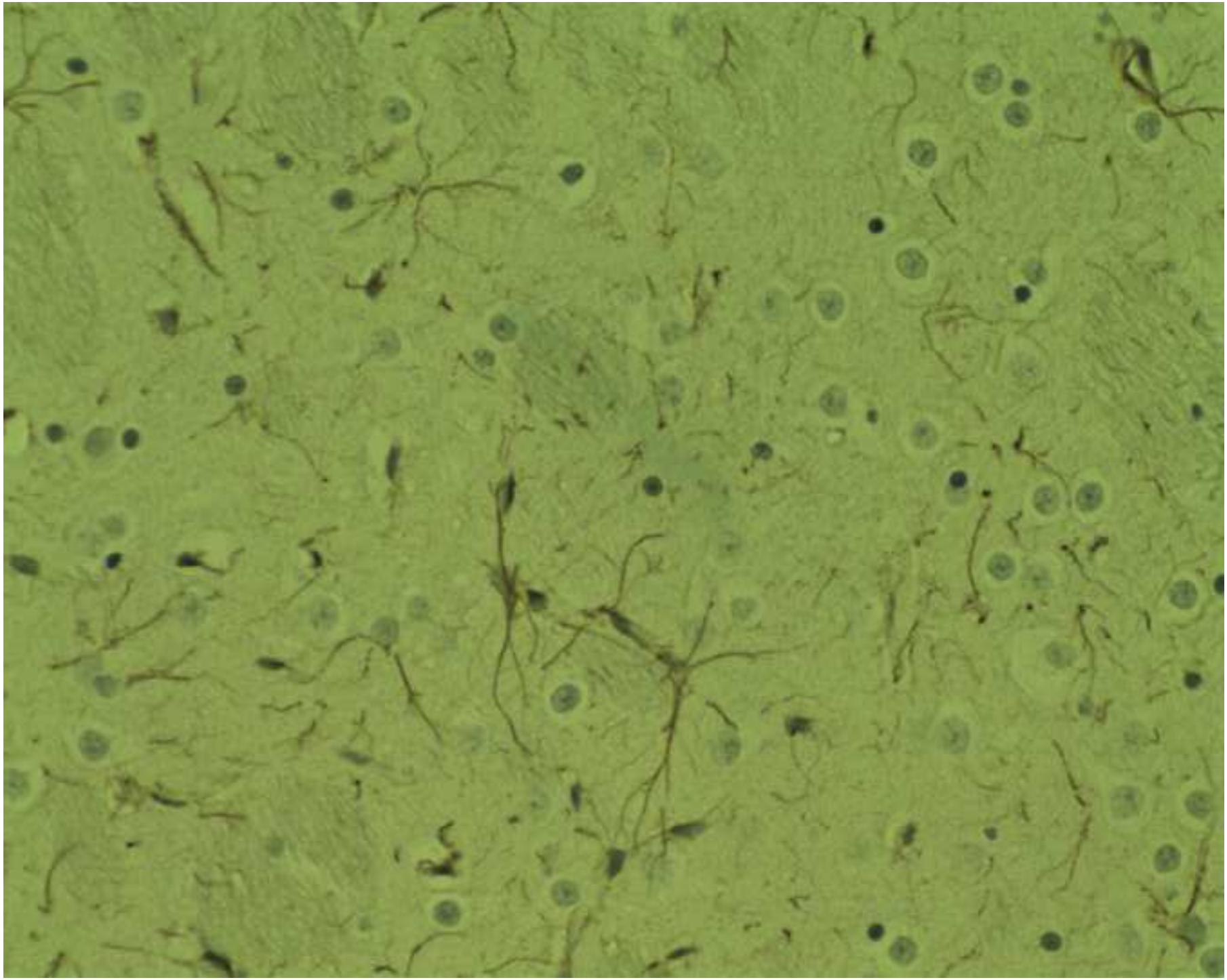

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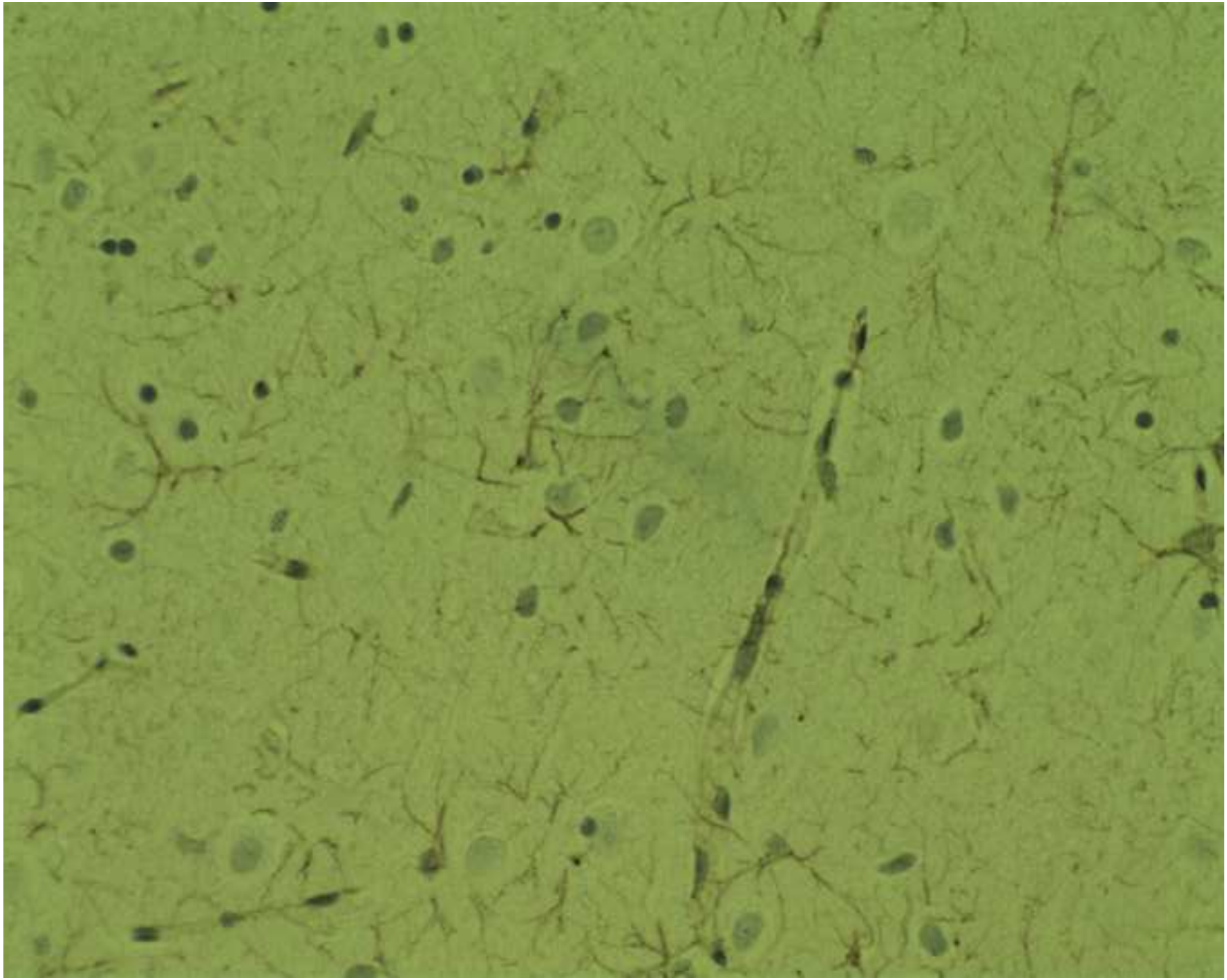

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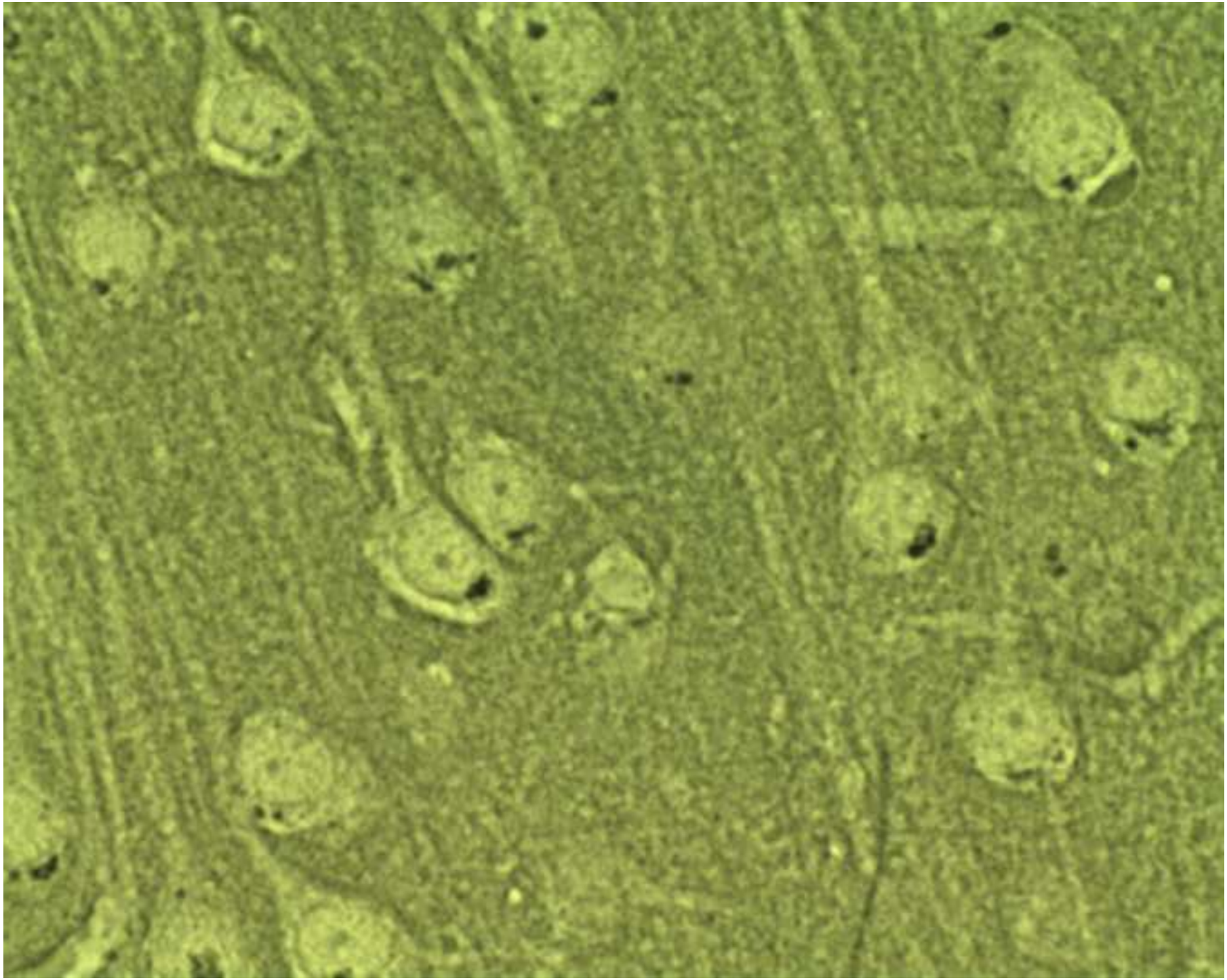

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