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Research article:

"*Non-Thermal*" Effects on the Blood-Brain Barrier in Fischer rats by exposure to microwaves.

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Abstract. Effect of 915 MHz electromagnetic fields (EMF) on the blood brain-barrier (BBB) permeability has been studied in Fischer 344 rats of both sexes. Male and female Fischer 344 rats were exposed in a Transverse Electromagnetic Transmission line chamber to microwaves of 915 MHz as continuous wave (CW) and pulse-modulated with different pulse power and at various time intervals. The CW-pulse power varied from 0.001W to 10 W and the exposure time from 2 min. to 960 min. In each experiment we randomly placed 4 rats in excited and 4 control rats in non-excited TEM-cells respectively. The rats were not anaesthetised during the exposure.

The rats were exposed to 915 MHz microwaves, either continuous wave (CW) or pulse modulated at 4,8,16 or 217 Hz with 0.57 ms pulse width, or pulse modulated at 50 Hz with 6.6 ms pulse width as well as from a real GSM-900 telephone.

All animals were sacrificed by perfusion-fixation of the brains under chloralhydrate anaesthesia after the exposure. The brains were then perfused, first with saline for 3-4 minutes, and then with 4% formaldehyde for 5-6 minutes. Whole coronal sections of the brains were dehydrated and embedded in paraffin and sectioned at 5 μm. The degree of albumin leakage was demonstrated immune-histo-chemically and classified in order of increased number of albumin extravasations by a rank number: 0 - 0.5 - 1.0 - 1.5 - 2 - 3. Pathological albumin leakage was judged as albumin extravasations equal to or larger than 1.

The frequency of pathological rats in all control groups was about 17%. Among rats exposed to pulse modulated microwaves the ratio of pathological rats was 170/481(0.35±0.03) and among rats exposed to continuous wave exposure (CW) it was 74/149 (0.50±0.07). These results are both highly significantly different to their corresponding controls ($p<0.0001$).

The rats were exposed to SAR various values: 0.2; 2; (20-40); (100-500); (1000-3000) mW/kg. In the 217 Hz modulated group (GSM simulated) we found the most increased ratio of albumin extravasations OR= 4 at 0.2 mW/kg. But no significant increased ratio at SAR 2000 mW/kg. The response curve of OR versus log(SAR) had the shape of a bathtub, with a minimum at a100 mW/kg. A similar curve was recorded for OR versus Specific Absorbed Energy (SAE Joule / kg) with a minimum at 100 J/kg. Similar response curves were recorded for the various modulation frequencies 4; 8; 16; 50 Hz. We found no pronounced difference between the various modulation frequencies other than the effect of CW exposure seems to be more effective than pulse modulated exposure in opening the BBB at high SAR values 100-2000 mW/kg.

Conclusion: The opening of the BBB is most effective at SAR values in the range of 0.1-0.5 $mW.kg^{-1}$ and less effective in the range of 50-500 $mW.kg^{-1}$. In this low SAR range thermal effects are unlikely. Thus there seems to be a non-thermal mechanism involved triggering the opening of the BBB.

Keywords: Blood-brain barrier, Albumin leakage, **Fischer rats,** electromagnetic field, microwaves, non-thermal effects, Mobile phone GSM-900, GSM.1800

1 Introduction

The mammalian brain is protected from exposure to potentially harmful compounds in the blood by the so-called "*Blood-brain barrier*" (BBB). The BBB is a barrier formed by vascular endothelial cells with very tight junctions between endothelial cells. This results in a highly restricted passage of blood components through the endothelial lining. Selective transport mechanisms mediated by receptors in the cell membrane guarantee the import of essential compounds such as glucose, and export of metabolites through the BBB. Astrocytes are covering the inner of the endothelial cells with their end feet and are implicated in the

maintenance, functional regulation and repair of the blood-brain barrier. Thus BBB also serves as a regulatory system that stabilises and optimises the fluid environment of the brain's intracellular compartment (Oldendorf 1975; Rapoport 1976; Zlokovic 2008).

The intact BBB protects the brain from chemical damage, whereas a dys-functioning BBB allows influx of potentially toxic compounds such as albumin into the brain tissue. This might in the worst case lead to irreversible brain damage. The permeability of the blood-brain barrier (BBB) can be altered in several pathological conditions such as epileptic seizures or extreme hypertension (Mihaly & Bozoky 1984; Sokrab T-E, et al. 1988; Sokrab T-E, et al. 1989,1990).

The blood brain barrier system has been used to investigate effects on CNS of various types of physiological activity and stress and has also been found to be sensitive to electromagnetic energy. In **Table 1** a review is presented of investigations reporting that microwave exposure of rats increases BBB permeability to various compounds.

In **Table 2** a review is presented of those studies that did not reveal any increased BBB permeability from microwave or RF exposure. In a Japanese study rats were exposed to 1.439 GHz microwaves from cellular phone at an average SAR of 0.25 W/kg for 2-4 weeks. But no evidence of albumin leakage was found by using the same method that we have used in the present study (Tsurita, et al. 1999a; Tsurita, et al. 2000; Tsurita, et al. 1999b).

Since 1988 we have studied the permeability of BBB to endogenous albumin and fibrinogen during exposure to electromagnetic fields. We have exposed rats to various magnetic fields as well as 915 MHz microwaves, continuous waves (CW) and pulse-modulated waves at the various repetition rates (4-200 pulses per s) (Eberhardt, et al. 2008; Nittby, et al. 2011; Nittby, et al. 2008a; Nittby, et al. 2008c; Persson, et al. 2005; Persson, et al. 1999a,b; Persson, et al. 1997; Persson, et al. 1992; Salford, et al. 1992; Salford, et al. 2003a; Salford, et al. 1993; Salford, et al. 1994; Salford, et al. 2003b; Salford, et al. 2007; Salford, et al. 2008; Salford, et al. 2006; Salford, et al. 2000).

Table 1

Increased Blood-Brain Barrier permeability at microwave exposure of rats and mice at various frequencies.

Reference	Microwave Frequency MHz	Modu-lation Pulses per s "pps"	SAR W/kg	Tracer studied	Remark
(Neilly & Lin 1986)	3150		3 W/cm^2	Ethanol & Evans Blue	Ethanol inhibits MW-induced BBB permeation of Evans Blue
(Albert, E. N. 1979)	2800	0/CW	10 mW/cm^2	HRP[1)]	Reversed after 2 h delay
(Sutton, et al. 1973)	2450	0/CW	high	HRP[1)]	42°C in < 5min
(Sutton & Carrol 1979)	2450	0/CW	high	HRP[1)]	Temp. rise
(Albert, E. N. 1977)	2450	0/CW	10 mW/cm^2	HRP[1)]	Permeability increase
(Albert, E. E. & Kerns 1981)	2450	0/CW	2.5	HRP[1)]	Permeability increase
(Lin & Lin 1982)	2450	25-500	0.08-240	Evans Blue and Flourescine	Permeability increase at 240 W/kg not at lower SAR
(Goldman, et al. 1984)	2450	500	240	Rubidium-86	Temp. increase
(Quock, et al. 1986)	2450	0/CW	24	Methylatropine	10 min exposure enhance BBB permeability
(Quock, et al. 1987)	2450	0/CW	24	Domperidone	10 min exposure enhance BBB permeability
(Neubauer, et al. 1990)	2450	100	2	rhodamine-ferritin	A pino-cytotic-like mechanism is presumed responsible for the MW induced increase in BBB permeability
(Nagaraja, et al. 2008)	2450	CW interstitial		MR T2 imaging	BBB disruption >43°C
(Frey, et al. 1975)	1200	1000 &CW	0.2-2.4	Fluorescine	Permeability increase
(Oscar & Hawkins 1977)	1300	50-1000	0.3-2 mW/cm^2	mannitol, inulin, and dextran	Inversed U-shape dose/response
(Merritt, et al. 1978)	1200	1000	2-75 mW/cm^2	Fluorescine and ^{14}C-mannitol	Permeability increase only in hyper-thermal rats
(Salford, et al. 1992)	915	8-215		Evans Blue, albumin, fibrinogen	Permeability increase for albumin but not for fibrinogen
(Salford, et al. 1993)	915	8-200	0.016-5	albumin and fibrinogen	Permeability increase for albumin but not for fibrinogen
(Persson, et al. 1997)	915	0/CW 4-200	0.016-5	albumin and fibrinogen	Permeability increase for albumin but not for fibrinogen
(Fritze, et al. 1997)	900	217	0.3-1.5	Albumin	Permeability increase at 7.5 W/kg, but not at lower SAR
(Persson, et al. 1999a)	900- 1800	GSM	0.001-0.2	albumin and fibrinogen	Permeability increase for albumin but not for fibrinogen
(Lin 2004)	900	217 Hz (GSM)	7.5	Albumin leakage	
(Phelix 2004)	900	217	?	Albumin	EM
(Prato, et al. 1990)	65	0.4-28 T/s	Clinical MRI examinee.	^{153}Gd-DTPA	MRI increase BBB permeability

Table 2
Non-increased Blood-brain barrier permeability at microwave exposure of rats and mice with frequency

Reference	Microwave Frequency MHz	Modulation Pulses per s "pps"	SAR W/kg	Tracer Studied	Remark
(Gruneau, et al. 1982)	2800	500	240	14C-sucrose	No increase
(Preston, et al. 1979)	2450	CW and pulsed	0.1-30 mW/cm2	14C-sucrose	No permeability increase
(Ward, et al. 1982)	2450	0/CW	2-6 W/kg	14C-sucrose 3H-inulin	No permeability increase
(Williams, et al. 1984b)	2450	CW	13	Na-fluorescine,	Permeability increased but not BBB breakdown
(Williams, et al. 1984a)	2450	CW	13	HRP,	No extravasations of HRP
(Williams, et al. 1984d)	2450	CW	13	14C-Sucrose	No increased leakage
(Williams, et al. 1984c)	2450	CW	4	Na-fluorescine, HRP, 14C-Sucrose	Suppression of BBB permeability at > 40°C
(Lin & Lin 1980)	2450	500 (10 ms pulse)	0.04-240	Evans Blue "EB"	No uptake of EB <240 W/kg
(Ward & Ali 1985)	1700	CW & 1000 (0.5μs pulse)	0.1	^{14}C-Sucrose	No change in uptake
(Tsurita, et al. 2000)	1439	CW	0.25	Evans Blue, albumin	No effect was found.
(Merritt, et al. 1978)	1200	1000 &CW	0.2-2.4	Fluorescine	repeat of Frey 1975
(Preston 1982; Preston & Prefontaine 1980; Preston, et al. 1979)	1300	50-1000	0.3-2 mW/cm^2	mannitol, inulin, and dextran	no U-shape dose/response
(Fritze, et al. 1997)	900	217 Hz (GSM)	0.3-7.5	Albumin	Modest, reversible extravasation
(Lin 2004)	900	217 Hz (GSM)	0.3-1.5	Albumin	No Albumin lextravasation
(Kumlin, et al. 2007)	900	217 Hz (GSM)	0.3-3.0 2 h/d ; 5 d/w	Evans Blue, albumin	improved learning (P = 0.012) and memory (P = 0.01) in exposed rats
(Hirota, et al. 2009)	915	217 Hz (GSM)	0.02-2	albumin	No Albumin extravasation
(Mcquade, et al. 2009)	915	16, 217	0.0018-20	albumin	No Albumin extravasation
(Masuda, et al. 2009)	915	217 Hz	0.02, 0.2, 2.0	albumin	No Albumin extravasation

2 Material and methods

2.1 Exposure in a TEM-cell

A Transverse Electromagnetic transmission line cell (TEM-cell) for the RF exposure of rats was designed by dimensional scaling from previously constructed cells at the National Bureau of Standards (Crawford 1974). TEM-cells are known to generate uniform TEM-fields for standard measurements. The cell is enclosed in a wooden box that supports the outer conductor and central plate. The outer conductor is made of brass-net and is attached to the inner walls of the box. The centre plate, or septum, is constructed of aluminium and is held up by Teflon braces, which are screwed at the inner sidewalls. To allow access to the inside of the cell both ends can be removed. The inside of the cell is ventilated through 18 holes (diam. 18 mm) in the sidewalls and top of the box and the brass-net of 50 mesh allow air to circulate. These holes are also used for examination of the interior during exposure. Probes for monitoring temperature inside the cell or test object are inserted through these holes.

The rats are placed in plastic trays to avoid contact with the central plate and outer conductor. The bottom of the tray is covered with absorbing paper to collect urine and faeces. The rats were exposed to 915 MHz electromagnetic radiation continuous wave and pulse-modulated with different repetition rates. The modulated RF-radiation consists of square wave shaped pulses with durations of 0.57, 4 or 6 ms and various power levels (dBm) during the presence of the pulse. Transmitted and absorbed power was measured at continuous wave exposure with and without rats in the TEM cell. From these measurements the average SAR in the whole rat was calculated to be 1.2 ± 0.4 W/kg per unit (watt) of input power. This value was in good agreement with the theoretical estimate of 1.6 W/kg per watt of input power that was used in the evaluation of the experiment (Crawford 1974; Malmgren, et al. 2012; Martens, et al. 1993; Van Hese, et al. 1992).

2.2 Albumin and fibrinogen immune-histochemistry

Fischer 344 rats by our own breeding of both sexes, weighing 119-555 g (median: 202 g; 25% quartiles: 175g; 75% quartiles 273 g) were used in these experiments. The rats were not anaesthetised, during the exposure. Both controls and exposed animals were sacrificed by perfusion-fixation of the brains under chloralhydrate anaesthesia between 20 minutes and 2 hours after the exposure. The brains were perfused with saline for 3-4 minutes, thereafter fixed in 4% formaldehyde for 5-6 minutes and immersion fixed in 4% formaldehyde for more than 24 hours. Whole coronal sections of the brains (3, 7 and 11 mm from the tip of the frontal pole) were dehydrated and embedded in paraffin and sectioned at 5 m. The chloralhydrate anaesthesia is necessary to avoid stress and blood pressure rise during perfusion-fixation procedure. Also for ethical reason no animals were sacrificed without chloralhydrate anaesthesia.

Albumin was demonstrated with the IgG fraction of rabbit anti rat albumin (Cappel Research Products, Organon Teknika, Västra Frölunda, Sweden) diluted 1:16,000. Fibrinogen was demonstrated with rabbit anti human fibrinogen (Dacopatts AB, Hägersten, Sweden), diluted 1:500. Incubation time for both was over night at 4°C.

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), counterstained with Meyer-HTX (Dacopatt 1994). Standard control procedures were used for both albumin and fibrinogen. The numbers of immunopositive extravasates 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 ventricles. These structures are well known for their insufficient blood-brain barrier. Within any part ~~of~~ the choroid plexus of the ventricles there consistently were immune-positive, mostly of a diffuse type, in the strain used in the present experiments.

2.3 Statistics

The degree of albumin leakage was evaluated by the neuro-pathologist “AB” and given ranking number according to the degree of leakage in the following order:

0 AB = normal brain with no sign of immunopositive extravasates
0.5 AB = normal brain with occasional minor immunopositive extravasates
1.0 AB = pathological leakage with one distinct immunopositive extravasate in each of the three brain slices.
1.5 AB = pathological leakage with several distinct immunopositive extravasate in each of the three brain slices.
2 AB = pathological leakage with massive distinct immunopositive extravasate in each of the three brain slices.
3 AB = pathological leakage with overwhelming number of massive immunopositive Extravasate in each of the three brain slices.

The significance for frequency of occurrence of albumin extravasations in exposed and control animals were tested with chi-square- or Fisher's exact probability test. (Lowry 1999).

3 Results and discussion

3.1 Continuous wave (CW) and pulse-modulated 915 MHz microwaves

In the present investigation we exposed male and female Fischer 344 rats in a TEM chamber to microwaves of 915 MHz as continuous wave (CW) and pulse-modulated at various frequencies, with different pulse power and at various time intervals. The CW-pulse power varied from 0.001W to 10 W and the exposure time from 2 min to 960 min. In each experiment we exposed 4 rats and 4 controls randomly placed in excited and non-excited TEM-cells respectively.

The number of pathological rat brains (albumin leakage score ≥1) among all control rats is 62 out of 372 (ratio: 0.17±0.02). These findings of albumin leakage are occasional and rare and are probably due to normal minor disturbances. The frequency of pathological rats among controls in the various groups is not significantly different ($p<0.4$). In **Figure 1** is shown a typical section of the brain from a normal unexposed Fischer rat immunoassayed for albumin. In **Figure 2** the brain from a Fischer 344 rat exposed with GSM-900 is shown, in which there is pathological leakage around vessels, as demonstrated by immune-staining against albumin.

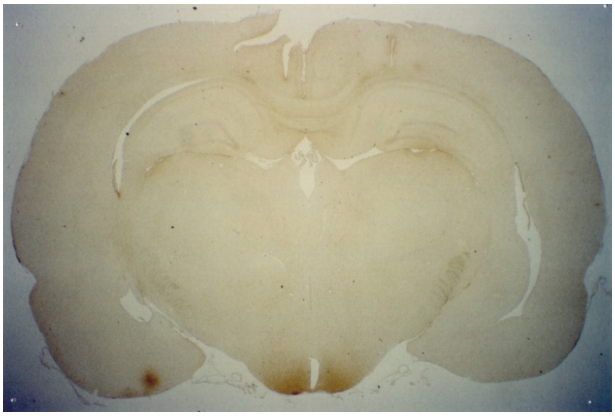

Figure 1
Brain section of non-exposed control rat immunostained for albumin

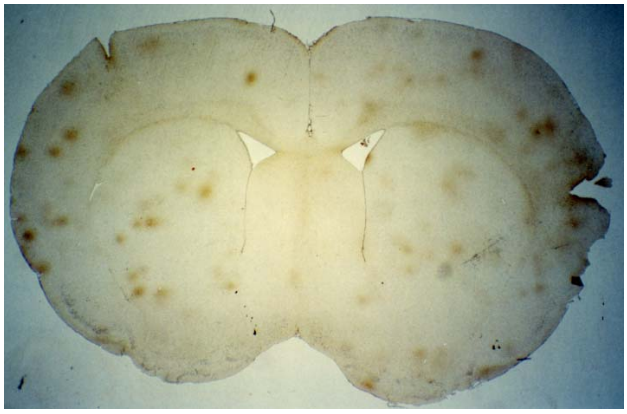

Figure 2
Pathological leakage around vessels in a brain section of an exposed Fischer 344 rat, (brown dots as demonstrated by immune-staining against albumin).

We have in total investigated 635 rats exposed with 915 MHz microwaves at various modulation frequencies as well as 371 controls. The frequency of pathological rats is significantly increased ($p<0.0001$) from 62/371 (ratio: 0.17±0.02) for control rats to 244/635 (ratio: 0.39±0.03) in all exposed rats. All results are displayed in **Appendix** grouped in ascending SAR level. The exposure was 915 MHz microwaves either pulse modulated (PW) at 217 Hz with 0.57 ms pulse width, at 50 Hz with 6.6 ms pulse width or continuous wave (CW). The frequency of pathological rats (< 0.2) among controls in the various groups is not significantly different ($p<0.4$). The frequency of pathological rats was 170/481 (0.35±0.03)

among rats exposed to pulse modulated (PW) and 74/149 (0.50±0.07) among rats exposed to continuous wave exposure (CW). These results are both highly significantly different to their corresponding controls ($p<0.0001$) and the frequency of pathological rats after exposure to pulsed radiation (PW) is significantly less ($p<0.002$) than after exposure to continuous radiation (CW). The number of pathological leakages in exposed animals is more frequent, and also more severe, per animal compared to the controls. This is a highly interesting observation as the current opinion is that pulse modulated electromagnetic fields are more potent in causing biological effects.

Table 3a
Summary of results of distribution of score values and pathological ratio = N(Score≥1)/N(Score<1) from controls and from (SAR 0.1-10 mW/kg) rats exposed with 915 MHz microwaves continuous wave and pulse modulated (PM) at 4, 8, 16, 50 and 217 Hz.

SAR =0	**Controls**							
Score	**Continuous W**	**PM 4 Hz**	**PM 8 Hz**	**PM 16 Hz**	**PM 50 Hz**	**PM 217 Hz**	**All**	**All PM**
0	66	1	13	10	69	61	223	154
0,5	24	1	5	5	31	20	86	62
1	19	2	1	2	17	7	49	29
1,5	2	0	0	0	1	4	7	5
2	1	0	0	0	3	2	6	5
3	0	0	0	0	0	0	0	0
Tot	112	4	19	17	121	94	**371**	626
SUM<1	92	2	18	15	100	81	309	216
Sum>=1	22	2	1	2	21	13	62	39
Path. Ratio	0,23	0,5	0,05	0,12	0,17	0,14	0,17	0,06
SD	0,05	0,43	0,05	0,09	0,04	0,04	0,02	0,01
Chi2 p	0,53	0,22	0,31	0,83	0,98	0,6		0,72

SAR=	**0.1 – 1**	**mW/kg**						
Score	**Continuous W**	**PM 4 Hz**	**PM 8 Hz**	**PM 16 Hz**	**PM 50 Hz**	**PM 217 Hz**	**All**	**All PM**
0		4	2	0	5	12	23	23
0,5		4	5	2	5	21	37	37
1		4	8	3	9	7	31	31
1,5		0	2	0	2	3	7	7
2		0	1	1	2	8	12	12
3		0	0	0	0	1	1	1
Tot		12	18	6	23	52	**111**	111
SUM<1		8	7	2	10	33	60	60
Sum>=1		4	11	4	13	19	51	51
Path. Ratio		0,33	0,61	0,67	0,57	0,37	0,46	0,46
SD		0,19	0,23	0,43	0,2	0,1	0,08	0,08
Chi2 p		0.2194	<0,001	0.002	<0,001	<0.0001	<0.0001	<0,001

Continue next page

Table 3a
Continued

SAR=	**1 - 10 mW/kg**							
Score	**Continuous W**	**PM 4 Hz**	**PM 8 Hz**	**PM 16 Hz**	**PM 50 Hz**	**PM 217 Hz**	**All**	**All PM**
0	12		14	8	12	26	72	60
0,5	11		6	7	11	9	44	33
1	16		6	4	22	6	54	38
1,5	4		0	0	3	0	7	3
2	0		0	1	0	0	1	1
3	0		0	0	0	0	0	0
Tot	43		26	20	48	41	178	135
SUM<1	23		20	15	23	35	116	93
Sum>=1	20		6	5	25	6	62	42
Path. Ratio	0,47		0,23	0,25	0,52	0,15	0,35	0,31
SD	0,13		0,1	0,13	0,13	0,06	0,05	0,05
Chi2 p	<0.0001		0.5953	0.3251	<0.0001	0.8124	<0.0001	0.0013

Table 3b
Summary of results (SAR 100- 1000 mW/kg) from exposure with 915 MHz microwaves continuous wave and modulated at 4, 8, 16, 50 and 217 Hz.

SAR=	**10 - 100 mW/kg**							
Score	**Continous W**	**PM 4 Hz**	**PM 8 Hz**	**PM 16 Hz**	**PM 50 Hz**	**PM 217 Hz**	**All**	**All PM**
0	14		8	8	43	25	98	84
0,5	4		2	5	14	12	37	33
1	13		2	5	25	21	66	53
1,5	0		0	0	8	2	10	10
2	1		0	0	1	2	4	3
3	0		0	0	0	0	0	0
Tot	32		12	18	91	62	**215**	183
SUM<1	18		10	13	57	37	135	117
Sum>=1	14		2	5	34	25	80	66
Path. Ratio	0,44		0,17	0,28	0,37	0,4	0,37	0,36
SD	0,14		0,13	0,14	0,08	0,1	0,05	0,05
Chi2 p	0.0011		0.9357	0.39	<.0.0001	0.0007	<.0.0001	<0.0001
SAR=	**≥100 mW/kg**							
Score	**Continuous W**	**PM 4 Hz**	**PM 8 Hz**	**PM 16 Hz**	**PM 50 Hz**	**PM 217 Hz**	**All**	**All PM**
0	20			4	16	12	52	32
0,5	13			0	9	4	26	13
1	29			2	5	4	40	11
1,5	5			0	0	0	5	0
2	5			0	1	0	6	1
3	1			0	1	0	2	1
Tot	73			6	32	20	**131**	58
SUM<1	33			4	25	16	78	45
Sum>=1	40			2	7	4	53	13
Ratio	0,55			0,33	0,22	0,2	0,4	0,22
SD	0,11			0,27	0,09	0,11	0,07	0,07
Chi2 p	<.0.0001			0.4917	0.3161	0.839	<0.0001	0.4932

Table 3c
Summary of results (SAR 0- 8333 mW/kg) from exposure with 915 MHz microwaves continuous wave (CW) and modulated at 4, 8, 16, 50 and 217 Hz. All 1006 rats: 371 Controls; 635 exposed 0.1 - 1000 mW/kg

All SAR 0.2-8333 mW/kg

Mod Frequency (Hz)	**Cont.W.**	**4**	**8**	**16**	**50**	**217**	**All Hz**	**All PW**	**Control**
Score: 0	46	4	24	20	76	75	245	199	223
Score: 0,5	28	4	13	14	39	46	144	116	86
Score: 1	58	4	16	14	61	38	191	133	49
Score: 1,5	9	0	2	0	13	5	29	20	7
Score: 2	6	0	1	2	4	10	23	17	6
Score: 3	1	0	0	0	1	1	3	2	0
Tot	148	12	56	50	194	175	635	487	371
SUM<1	74	8	37	34	115	121	389	315	309
Sum>=1	74	4	19	16	79	54	246	172	62
Path Ratio	0,50	0,33	0,34	0,32	0,41	0,31	0,39	0,35	0,17
SD	0,07	0,19	0,09	0,09	0,05	0,05	0,03	0,03	0,02
Chi2 p	**<0.0001**	0.2194	**0.03**	0.0158	**<0.0001**	**0.0003**	**<0.0001**	**<0.0001**	
Average Score	0,68	0,50	0,49	0,50	0,57	0,52	0,57	0,54	0,31
SD	0,07	0,20	0,09	0,10	0,05	0,05	0,03	0,03	0,03
OR(Ave.)=E/C	2,19	1,61	1,58	1,61	1,85	1,69	1,84	1,73	1,00
SD	0,31	0,68	0,34	0,36	0,25	0,24	0,21	0,20	0,14

In order to consider the magnitude of the score values, the weighted average of score values (WAS) was evaluated for each SAR interval and for each modulation frequency.

$$\textit{Weighted average score values (WAS)} = \textit{Sum [Score} \times \textit{N(Score)} \,/\, \textit{N(all)]}$$

$$\textit{WAS} = \textit{Sum}[0 \times N(0) + 0.5 \times N(0.5) + 1 \times N(1) + 1.5 \times N(1.5) + + 2 \times N(2) + 3 \times N(2)] \,/\, N(all)$$

As quantity for the extent of BBB-leakage of albumin in the rats, we used the observed ratio between the weighted score of the exposed rats and control rats, calculated as:

$$\textit{Observed Ratio (OR)} = \textit{WAS(exposed)} / \textit{WAS (controls)}$$

In the **Figures 3 a-f** are displayed the observed ratios of weighted average score values for BBB-leakage of albumin in rats exposed to various SAR values of 915 MHz microwaves was evaluated for different modulation frequencies.

In Figure 3a is given the observed ratios of weighted average of score values for BBB-albumin leakage in rats exposed to 915 MHz continuous (CW) microwaves at various SAR values versus total number of controls (371) or the CW group controls (112). The p values of Chi2 test of the distribution of score values of each exposure interval versus the distribution of scores in all the 371 controls (black squares) and the 112 group controls (red circles) respectively are given in the figure.

In Figure 3b is given the observed ratios of weighted average of score values for BBB-albumin leakage in rats exposed to 4 Hz modulated 915 MHz microwaves at various SAR values versus total number of controls (371) or the 4 Hz group controls(4). The p values of Chi2 test of the distribution of score values of each exposure interval versus the distribution

of scores in all the 371 controls (black squares) and the 4 group controls (red circles) respectively are given in the figure.

In Figure 3c is given the observed ratios of weighted average of score values for BBB-albumin leakage in rats exposed to 8 Hz modulated 915 MHz microwaves at various SAR values versus total number of controls (371) or the 8 Hz group controls(19). The p values of Chi2 test of the distribution of score values of each exposure interval versus the distribution of scores in all the 371 controls (black squares) and the 19 group controls (red circles) respectively are given in the figure.

In Figure 3d is given the observed ratios of weighted average of score values for BBB-albumin leakage in rats exposed to 16 Hz modulated 915 MHz microwaves at various SAR values versus total number of controls (371) or the 16 Hz group controls (17). The p values of Chi2 test of the distribution of score values of each exposure interval versus the distribution of scores in all the 371 controls (black squares) and the 17 group controls (red circles) respectively are given in the figure.

In Figure 3e is given the observed ratios of weighted average of score values for BBB-albumin leakage in rats exposed to 50 Hz modulated 915 MHz microwaves at various SAR values versus total number of controls (371) or the 50 Hz group controls (121). The p values of Chi2 test of the distribution of score values of each exposure interval versus the distribution of scores in all the 371 controls (black squares) and the 121 group controls (red circles) respectively are given in the figure.

In Figure 3e is given the observed ratios of weighted average of score values for BBB-albumin leakage in rats exposed to 217 Hz modulated 915 MHz microwaves at various SAR values versus total number of controls (371) or the 217 Hz group controls (98). The p values of Chi2 test of the distribution of score values of each exposure interval versus the distribution of scores in all the 371 controls (black squares) and the 98 group controls (red circles) respectively are given in the figure.

In order to study the Chi2-distribution of score values varies at different SAR level the rats were categorized into 5 categories with various score values:

- Normal brain with no or minor immunopositive extravasates (score 0);
- Brain with minor immunopositive extravasates (score 0.5);
- Brain with one distinct immunopositive extravasate in each of the three brain slices (score 1);
- Brain with some distinct immunopositive extravasates in each of the three brain slices (score 1.5).
- Brain with many distinct immunopositive extravasates in each of the three brain slices (score ≥2).

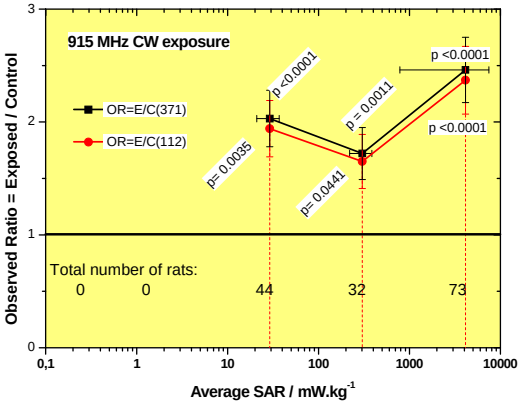

Figure 3a. Continuous wave 915 MHz

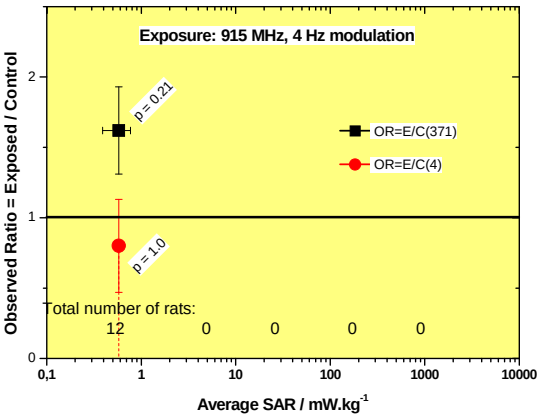

Figure 3b. 4 Hz modulated 915 MHz

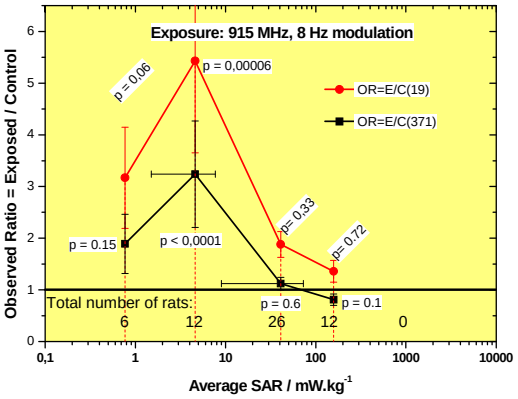

Figure 3c. 8 Hz modulated 915 MHz

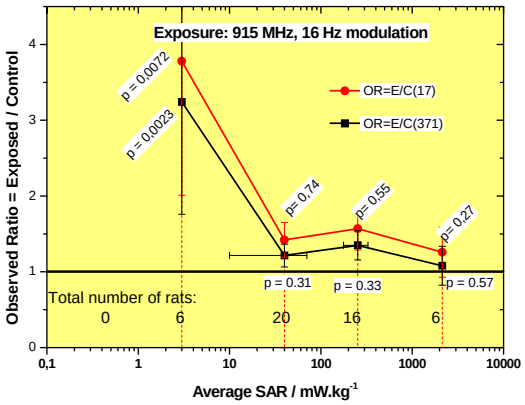

Figure 3d. 16 Hz modulated 915 MHz

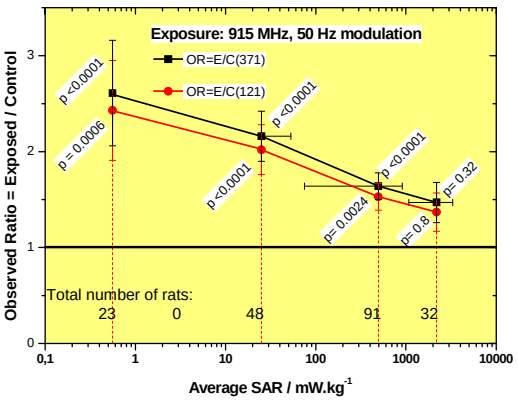

Figure 3e. 50 Hz modulated 915 MHz

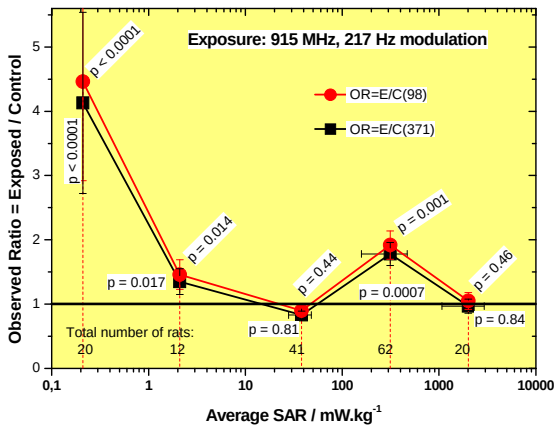

Figure 3f. 217 Hz modulated 915 MHz

In **Figures 3 a-f** are given the Observed Ratios both relative to all controls (black squares) and relative to the group control (red circles). The only group that differs is 4 Hz due to the few controls (N=4) in that group.

3.2 GSM-900 modulated microwaves in TEM-cells,

The results for exposure of Fischer 344 rats in TEM-cells with modulated 900 MHz microwaves from a real GSM-900 mobile telephone are given in **Table 4.** The output power was controlled from a computer to study the effect of various SAR values. So far only the highest SAR level of 200 mW/kg has reached enough number of animals to get a significant effect. At the lowest SAR value was 0.2 mW/kg at which level there seems to be no albumin leakage.

Table 4
Summary of average score of BBB albumin leakage from exposure with real GSM-900 modulated microwaves in TEM-cells

GSM-900 TEM cell	**SAR mW/kg**	**N**	**Average**	**SD**	**E/C(SD)**	**SD**	**T-test**
All Exposed		24	1.04	0.59	1.47	1.60	0.07
All Controls		24	0.71	0.66			
Exposed 33 dBm	200	13	1.04	0.56	1.94	2.42	0.03
Controls 0 dBm	0	14	0.54	0.60			
Exposed 31 dBm	130	4	1.25	0.96	1.07	1.26	0.92
Controls 0 dBm	0	3	1.17	1.04			
Exposed 25 dBm	17	3	0.83	0.76	1.67	2.26	0.57
Controls 0 dBm	0	3	0.50	0.50			
Exposed 3 dBm	0.2	4	1.00	0.00	0.89	0.38	0.64
Controls 0 dBm	0	4	1.13	0.48			

3.3 GSM-900 modulated microwaves in anechoic chamber,

The results from exposure of 16 Fischer 344 rats in an anechoic chamber with 900 MHz GSM modulated microwaves from a real GSM-900 mobile telephone compared to 13 controls was not significant different. The albumin leakage score for exposed was 1.3 and for controls 1.2. The E/C-ratio of was 1.1.

4. Discussion and Conclusion.

4.1 Modulation frequency dependence

The average score values of albumin leakage in Fischer 344 rats (controls and exposed with 915 MHz microwaves at various modulation frequencies) are displayed in **Figure 4.** There is no pronounced difference between the various modulations frequencies other than the effect of CW seems to be most effective in opening the BBB. This is surprising since the common opinion is that modulated microwaves would be more biologically effective.

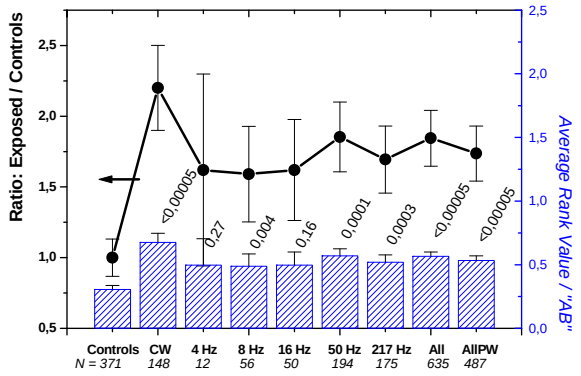

Figure 4
The histogram show the average rank values (”AB”) for BBB albumin leakage in Fischer 344 rats exposed to 915 MHz microwaves modulated at various frequencies (Hz). The p values represent the significance in difference from the controls. The line represents the ratios of the average rank values between exposed and controls. The number of rats in each group is displayed in the lowest line.

The observed ratio of weighted average score values (WAS) for BBB albumin leakage of exposed versus all controls, categorized according to frequency in the different SAR intervals are given in **Figures 5 a-e**.

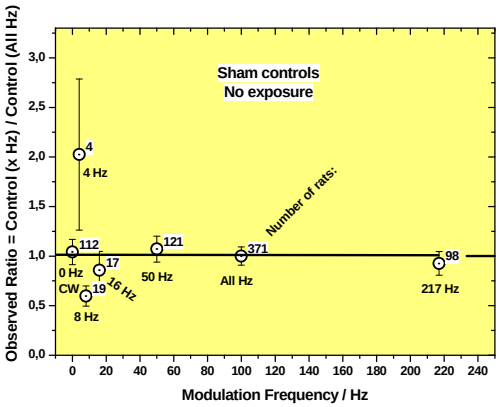

Figure 5a. OR of weighted score average of BBB-leakage for sham controls for groups at various modulation frequency,

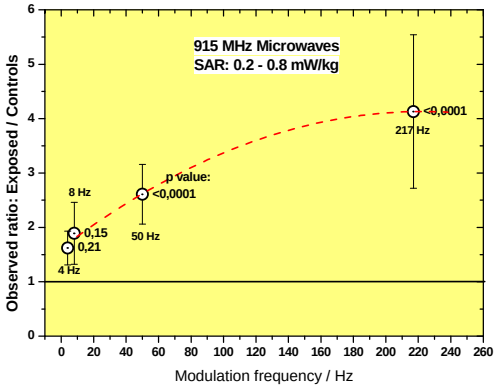

Figure 5b OR of weighted score average of BBB-leakage for sham controls for groups exposed in the SAR interval 0.2-0.8 mW/kg at various modulation frequencies.

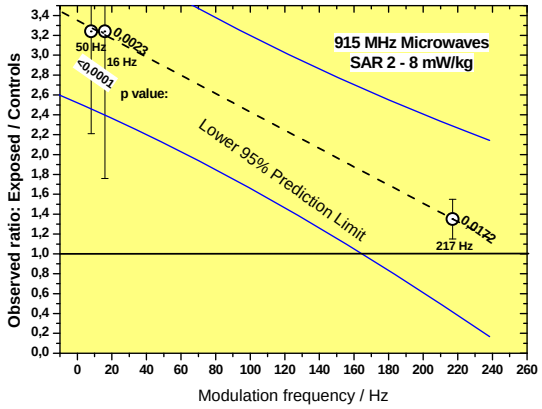

Figure 5c. OR of weighted score average of BBB-leakage for sham controls for groups exposed in the SAR interval 2-8 mW/kg at various modulation frequencies.

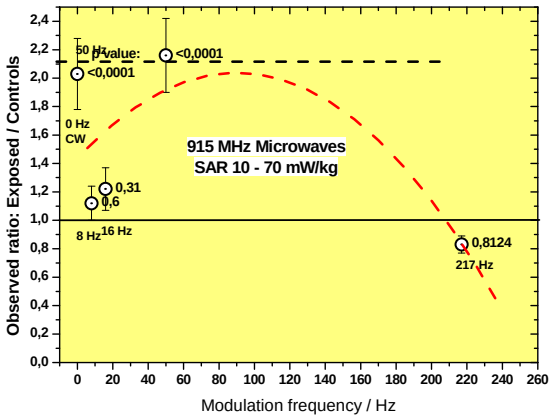

Figure 5d. OR of weighted score average of BBB-leakage for sham controls for groups exposed in the SAR interval 10-70 mW/kg at various modulation frequency.

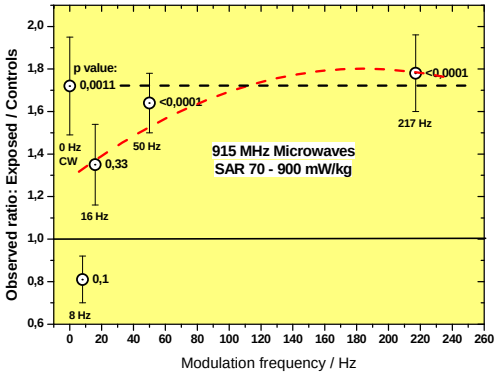

Figure 5d. OR of weighted score average of BBB-leakage for sham controls for groups exposed in the SAR interval 70-900 mW/kg at various modulation frequency.

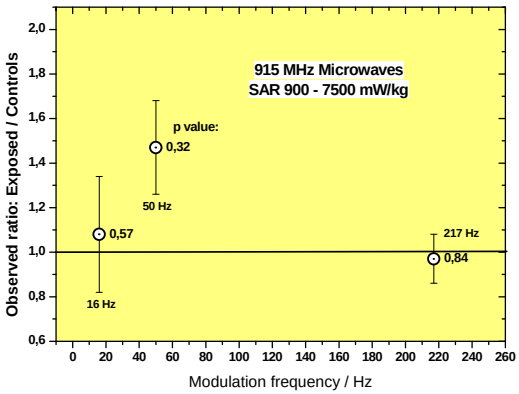

Figure 5d. OR of weighted score average of BBB-leakage for sham controls for groups exposed in the SAR interval 900 mW/kg at various modulation frequency.

As shown in **Figure 5a** there is no significant difference in the observed ratios in the controls for the controls for various modulation frequencies. As shown in **Figure 5b,** the results of OR in the groups exposed to the lowest SAR values (0.2-0.8 $mW.kg^{-1}$) the OR=4 for 217 Hz which is the highest value observed in any group of the whole study. In this low SAR interval the effects are unlikely caused by "thermal effects". But most likely the effects are due to direct electromagnetic interactions at the molecular level. As shown in **Figures 5c,d** the OR in the groups exposed to higher SAR values (2-70 $mW.kg^{-1}$) the variation of OR with the modulation frequency is reversed. The highest values of OR (2-3) are observed at the low modulation frequencies and is close to 1 at 217 Hz. At still higher SAR values (0.9-7.5 Wkg^{-1}) there seem to be no significant dependence of the BBB-leakage with the modulation frequency.

4.2 SAR dependence

The observed ratios of weighted average of score values for BBB-albumin leakage in rats exposed to CW and frequency modulated 915 MHz microwaves at various SAR values versus total number of controls (371) are displayed in **Figure 6.** The SAR dependence is very similar to that previously reported for the microwave alteration of the blood-brain barrier system of rats (Oscar & Hawkins 1977). In **Figure 7** and **Figure 8** is shown polynomial fits of the observed ratios of weighted average recorded at different average SAR values. In **Figure 7** the blue curve represents a 3rd order and the red curve a 4th order polynomial fit, and in **Figure 8** the red curve a 2nd order polynomial fit.

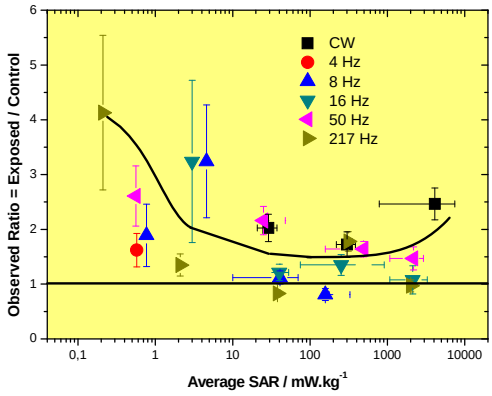

Figure 6.
The observed ratios of weighted average of score values for BBB-albumin leakage in rats exposed to CW and frequency modulated 915 MHz microwaves at various SAR values versus total number of controls (371).

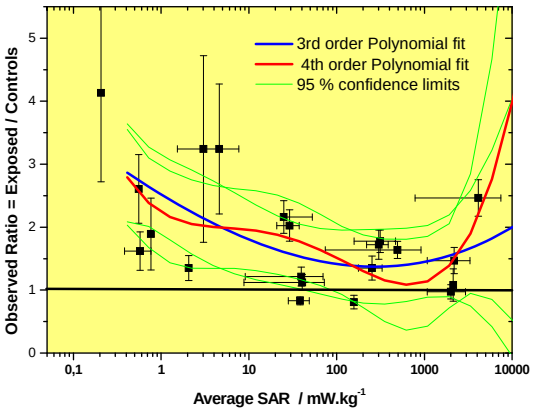

Figure 7.
Polynomial fit of the observed ratios of weighted average recorded at different average SAR values. The blue curve represents a 3rd order and the red curve a 4th order polynomial fit.

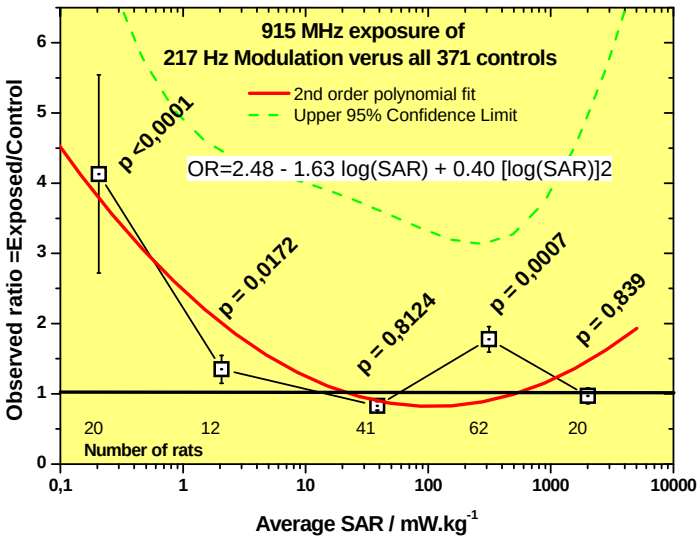

Figure 8.
Second order Polynomial Fit of observed ratio for The weighted average score of BBB albumin leakage due to 217 HZ modulated 915 MHz exposure at varying SAR ($mW.kg^{-1}$) resulting in the equation:
$OR=2.48 - 1.63\times\log(SAR) + 0.40\times[\log(SAR)]^2$

The observed ratios in **Figures 7 and 8** are displayed at the average SAR values for each decade interval. By fitting the values to polynomial of various order a "bath-tube" shaped SAR dependence it is clearly demonstrated. The opening of the BBB is most effective at SAR values in the range of 0.1-0.5 $mW.kg^{-1}$ and less effective in the range of 50-500 $mW.kg^{-1}$. In this low SAR range thermal effects are unlikely. Thus there seems to be a non-thermal mechanism involved triggering the opening of the BBB.

In **Figure 8** the observed ratios for 217 Hz modulated 915 MHz (simulating GSM-900) versus the logarithm of the average SAR values (mW/kg) in each decade group is fitted to a 2nd degree polynom:

$$OR=2.48 - 1.63\times log(SAR) + 0.40\times[log(SAR)]^2$$

The highest and most significant OR values are at the low SAR values (≤ 2 $mW.kg^{-1}$) while at very high SAR values >1000 mW/kg the OR value is not significant different from 1. The bath-tube shape of the curve might be explained by Weibull statistics (Weibull, G. W. 1981; Weibull, W. 1951).

The shape of the OR versus $x={}^{10}\log(SAR)$ can be described by a "Hazard function"

$$H(x) = OR-1$$

The shape of the Hazard function can be described by a parameter s>0 which is equal to the slope of the Waybill distribution (Weibull, G. W. 1981; Weibull, W. 1951).

S = 0,5 for x< 1 which results in a decreasing "Hazard function H(x,s)"
S=1 for 1<x<100 which results in a constant "Hazard function" H(x,s) = 0
S>1 results in an increasing Hazard function H(x,s)>0

The shape or the extension of the Hazard function also depends on another parameter: $\lambda > 0$

The Hazard function is assumed to be equal to the "Failure rate" of the Weibull distribution:

$$OR - 1 = H(x;s;\lambda) = \frac{s}{\lambda}\cdot\left(\frac{x}{\lambda}\right)^{s-1}$$

$$where \quad x = {}^{10}\log(SAR)$$

Thus the observed ratio of the failure rate of the BBB can be given as OR= H+1. In **Figure 9** is given the OR estimated from the Hazard function with various values of s and λ. The red curve is fitted from the experimental data shown in **Figure 8**.

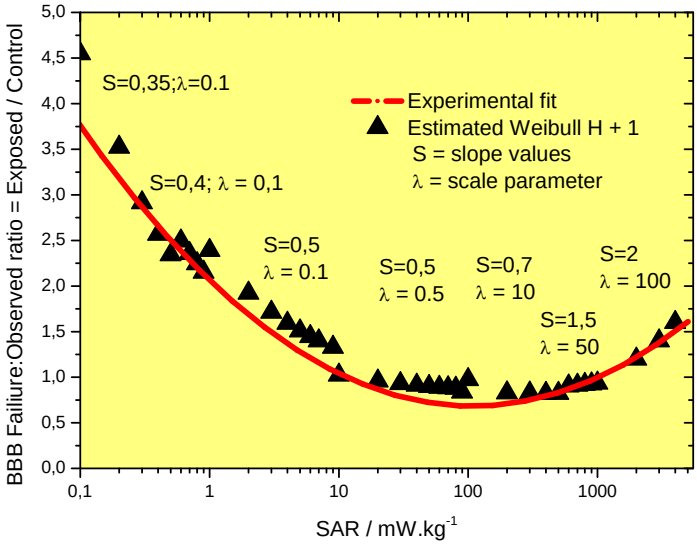

Figure 9
The OR estimated from the Hazard function (OR=H+1) with various s and λ values and curve fitted from the experimental data

4.2 Specific Absorbed Energy (SAE) dependence

In the area of ionizing-radiation (X- and gamma rays) the biological effect are related to the absorbed dose, which is defined as the absorbed radiation energy per mass unit i.e. joule per kg (J/kg). Thus it is of interest to correlate the biological effects of non-ionizing radiation i.e. microwaves to the absorbed energy. Thus in the present study the specific absorbed energy (SAE) was calculated by multiplying the SAR values (W/kg) with the corresponding exposure time in seconds. The weighted observed ratios (OR) in the various intervals of SAE for the rats exposed to 217 Hz modulated 915 MHz microwaves are displayed in **Figure 10**. The shape of OR versus log(SAE) follows the same pattern as OR versus log(SAR) in **Figure 10**. The OR values were significantly increased also at the highest SAE values of 2 and 20 kJ/kg, But at medium SAE values 20 200 J/kg no significant increase of the OR was recorded. This might indicate that different mechanisms are involved in the biological effects at high and low energy.

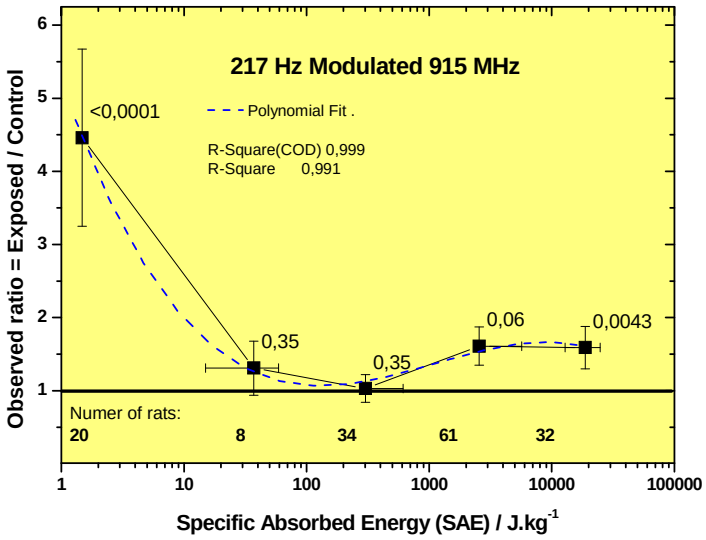

Figure 10
Observed Ratio of weighted rank values versus Specific Absorbed Energy (J/kg). The blue curve represents a 3rd order polynomial fit.

4.3 Exposure time dependence

Changes in BBB permeability in Fischer 344 rats exposed to continuous microwaves at various time intervals are presented in **Figure 11** as weighted rank difference between exposed and matched sham exposed animals. There seems to be no difference in the degree of opening the BBB with the time of exposure that might indicate a switch mechanism that is turned on by the microwaves but turned off again by the brain.

4.4 Lag time dependence

Differences in BBB permeability in Fischer 344 rats exposed to microwaves at various ~~lag~~ time lags between end of exposure and perfusion fixation is shown in **Figure 11.** There seems to be a biphasic decrease in time of the BBB-permeability. The fast component has a halftime of about 20 min and the slow component has a halftime of about 2000 min, which means that some fraction never closes or might close very slowly.

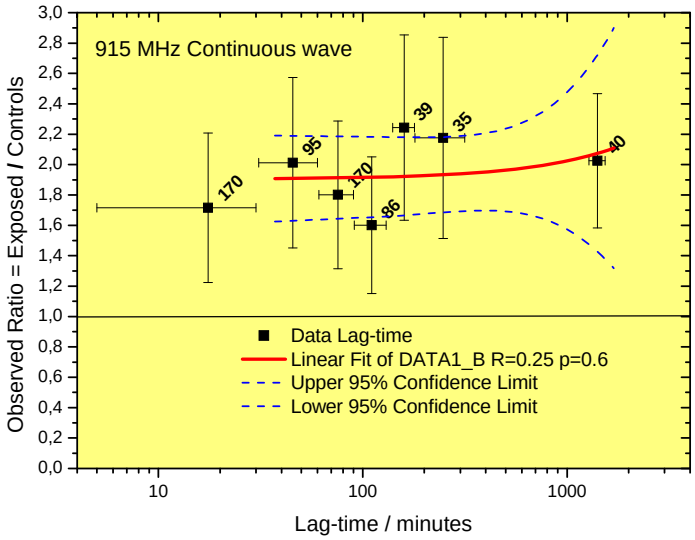

Figure 11.
Observed ratios of weighted average weighted rank values for BBB-permeability in Fischer 344 rats exposed to Continuous Wave 915 MHz microwaves analyzed at various lag-time intervals between 2 h-exposure and perfusion fixation. The numbers of rats in each group are displayed on the bars.

4.5 Sex dependence

The weighted average of all AB-rank values in 249 female Fisher rats of our own breed was 0.29±0.03 (SEM) and in 114 males it was 0.36±0.05 (SEM). Those values were, however, not statistically different p=0.18. The average of all AB-rank values in 62 female Fisher rats imported from Germany was 1.20± 0.13 (SEM) and in 41 males ~~it~~ was 1.35±0.19 (SEM). Those values were also not statistically different p=0.52.

4.6 BBB effects of global system for mobile communication (GSM)

The effect of global system for mobile communication (GSM) microwave exposure on the permeability of the blood-brain barrier is of special interest. Thus GSM pulse sequence of 217 Hz modulations with 0.52 ms pulse width was studied with pulse modulated 915 MHz microwaves (**Table 3**), as well as real GSM exposure (**Table 4**).

5. Further BBB-studies performed by the Lund group

5.1 BBB leakage at 50 days after exposure and Neuronal damage

A study was performed to study the BBB leakage of albumin at 50-days after exposure during 2 h for GSM-900 microwaves at SAR values 200, 20, and 2 mW/kg. The results of BBB-leakage in the exposed rats indicated no significant leakage of albumin, probably due to the strange results of the 8 controls with massive distinct immunopositive extravasate (rank 2 AB) in 2 rats. In contrary to the in-significant albumin leakage we found highly significant ($p < 0.002$) evidence for neuronal damage in the cortex, hippocampus, and basal ganglia in the brains of exposed rats (Salford et al., 2003). Therefore the results of the rats in the various groups exposed to various SAR-value, have been compared to the 96 controls of the 217 Hz group in the primary 915 MHz study. The results of the BBB leakage of albumin at 50-days after exposure have been re-evaluated and the results are given in **Table 5.**

Table 5
The results of the BBB leakage of albumin at 50-days after 2 hours exposure to various SAR values compared to the unexposed 8 rats as well as to controls of the 217 Hz group in the primary study reported above.

SAR Values:	**200 mW.kg^{-1}**	**20 mW.kg^{-1}**	**2 mW.kg^{-1}**	**0 mW.kg^{-1}**	**External Control**
Rank values (AB)					
0	3	2	2	6	64
0,5	2	3	1	0	20
1	0	1	1	0	8
1,5	1	0	1	0	4
2	1	0	0	2	2
2,5	1	2	1	0	0
3	0	0	2	0	0
Number of rats	8	8	8	8	98
CHi2	13,8	13,75	23,15	13,05	0
P-value	0,011	0,008	0,0001	0,011	1
Fisher Exact Probability					
p -versus 98 Controls	0,042	0,044	0,0033	0,13	1
p -versus 0 mW.kg^{-1}	0,35	0,125	0,18	1	0,13
Weighted AB rank values	0,81	0,81	1,13	0,50	0,29
Observed WAB ratio	2.84±0.98	2.84±0.93	3.94±1.2	1.75±0.89	1.00±0.04

The observed ratio of the weighted AB rank-values for BBB-permeability in the rats exposed to various SAR values are displayed in **Figure 12.** It is interesting to note that the shape of the curve is similar to the curves in **Figures 6-10.**

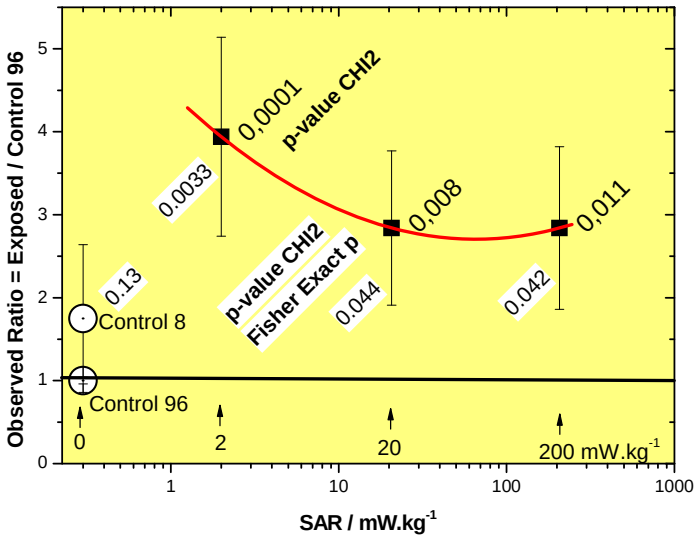

Figure 12
The observed ratio of the weighted AB rank values for the rats exposed to various SAR values.

5.2 BBB leakage 14 or 28 days after exposure

In another study 96 non-anaesthetized rats were either exposed to microwaves or sham exposed in TEM-cells for 2 h, at specific absorption rates of average whole-body Specific Absorption Rates (SAR) of 0.12, 1.2, 12, or 120 mW/kg. The rats were sacrificed after a recovery time of either 14 or 28 d, following exposure and the extravasations of albumin, its uptake into neurons, and occurrence of damaged neurons were assessed. Albumin extravasations and also its uptake into neurons was seen to be enhanced after 14 d (Kruskal Wallis test: $p = 0.02$ and 0.002, respectively), but not after a 28 d recovery period. The occurrence of dark neurons in the rat brains, on the other hand, was enhanced later, after 28 d ($p = 0.02$). Furthermore, in the 28-d brain samples, neuronal albumin uptake was significantly correlated to occurrence of damaged neurons (Spearman $r = 0.41$; $p < 0.01$) (Eberhardt, et al. 2008).

5.3 BBB leakage 7 days after exposure

We have continued our own studies by investigating the effects of GSM mobile phone radiation upon the blood–brain barrier permeability of rats 7 days after one occasion of 2 h of exposure. Forty-eight rats were exposed in TEM-cells for 2 h at non-thermal specific

absorption rates (SARs) of 0 mW/kg, 0.12 mW/kg, 1.2 mW/kg, 12 mW/kg and 120 mW/kg. Albumin extravasations over the BBB, neuronal albumin uptake and neuronal damage were assessed. Albumin extravasations was enhanced in the mobile phone exposed rats as compared to sham controls after this 7-day recovery period (Fisher's exact probability test, p = 0.04 and Kruskal–Wallis, p = 0.012), at the SAR-value of 12 mW/kg (Mann–Whitney, p = 0.007) and with a trend of increased albumin extravasations also at the SAR-values of 0.12 mW/kg and 120 mW/kg. There was a low, but significant correlation between the exposure level (SAR-value) and occurrence of focal albumin extravasation (R = 0.33; p = 0.04) (Nittby, et al. 2009). Those findings are in agreement with the studies where we have observed increased BBB permeability immediately and 14 days after exposure (Eberhardt, et al. 2008; Persson, et al. 2012a; Persson, et al. 1997).

Table 6
Results of BBB albumin leakage at 7 days after 2h exposure at various power levels.

Type of exposure Power; SAR	Number of rats	Frequency of Positive BBB-leakage	P: Fisher Exact Probability Test
Sham	16	0	
0.2 mW; 0.12 mW/kg	8	0.25	0.10
2 mW; 1.2 mW/kg	8	0	1
20 mW; 12 mW/kg	8	0,50	0.01
200 mW; 120 mW/kg	8	0,25	0,10
All exposed	32	0,53	0.04

5.4 Cognitive functions after life-long exposure (55 weeks)

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 2-h period, for totally 55 weeks, at different average whole-body 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 extravasations, 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 parameters was found, when comparing the GSM exposed animals to the sham exposed controls (Grafstrom, et al. 2008).

We have also directed attention to possible implications on cognitive functions by investigating in a rat model the long-term effects of protracted exposure to GSM-900 radiation. Out of a total of 56 rats, 32 were exposed for 2 h each week for 55 weeks to radio-frequency electromagnetic radiation at different SAR levels (0.6 and 60 mW/kg at the initiation of the experimental period) 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 this protracted exposure, GSM-900 exposed rats were compared to sham exposed controls. Effects on exploratory behaviour were evaluated in the open-field test, in which no difference was seen. Effects on cognitive functions were evaluated in the episodic-like memory test. In our study, GSM exposed rats had impaired memory for objects and their temporal order of presentation, compared to sham exposed controls ($P = 0.02$). Detecting the place in which an object was presented was not affected by GSM exposure. Our results suggest significantly reduced memory functions in rats after GSM microwave exposure ($P = 0.02$) (Nittby, et al. 2008b).

5.5 Effects of an external electrical fan at 50 Hz used for ventilation the TEM-cells

In most of the Lund BBB-experiments, the animals were exposed in TEM-cells, ventilated by an external electrical fan at 50 Hz. In a special study, we examined whether the extremely low frequency (ELF) magnetic fields from the fan (50 Hz, 0.3-1.5 μT) might add to the RF effect. Sixty-four rats were divided into 4 groups: RF only, ELF only and RF + ELF exposure plus a sham group. The GSM-900 MHz RF exposure was at the very low, non-thermal, average whole-body SAR level 0.4 mW/kg. 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. A fan was placed next to the outer wall of its respective TEM-cell. However, the air flow of the fan was not directed towards the TEM-cell, but instead turned 90^{o} away, in order just to get the ELF effect of the fan and no additional ventilation. The measured RMS (root mean square) ELF B-field strength from the fan in the TEM-cell was 1.7-1.9 μT in the part closest to the fan, 0.5-0.8 μT in the middle of the TEM-cell and 0.1-0.3 μT on the wall opposing the placement of the fan.

Demonstration of the normally occurring albumin extravasations in the basal hypothalamus is our inbuilt control proving that the staining is reliable. Two full series of staining of the whole material gave negative results for hypothalamus. Not until we changed to avidin, biotin, and antibodies from a third supplier, we received an acceptable staining.

The incidence of pathological brains in each of the exposed group was 25 % in the RF group, 19% in the ELF group and 13% in the RF+ELF group. Twenty-five percent of the RF animals had a pathological albumin leakage, while the ELF and RF + ELF groups with three and two pathological findings, respectively, were not significantly different from the control group. When all exposed animals (RF, RF+ELF, ELF) were compared to the sham group, it was shown that albumin leakage does not reach a significant increase in the exposed group (Fisher $p = 0.06$). When all four groups are compared simultaneously with the Fisher exact probability test (2-sided), we receive a p-value of 0,241.

In spite of the technical difficulties, we could show that in none of the sham animals, there was a pathological albumin leakage in the brain. In the RF animals there was a barely significant leakage p=0.05 (in 25% of the animals), while the ELF and RF+ELF groups with 3 and 2 pathological findings (p=0.10 and p=0.11) respectively, were not significantly different from the control group. We conclude that the use of external fans has had no major influence upon the results (Nittby, et al. 2011).

6. BBB-studies performed by other groups

6.1 Hossmann´s group in Cologne

On request from Bolzano (Motorola) we shared our immune-staining technique for studying BBB in microwave exposed Fischer rats 344 with professor Hossmann and his colleagues in Cologne where we presented and demonstrated our techniques and results. They started a study by using the carousel exposure technique proposed by Motorola. Their results were presented 1997 at the 2nd World Congress for Electricity and Magnetism in Biology and Medicine, in Bologna, Italy (Fritze, et al. 1997; Hossmann & Hermann 1999).

They used a calibrated microwave exposure system in the 900 MHz band. Rats were restrained in a carousel of circularly arranged plastic tubes and sham-exposed or microwave irradiated for a duration of 4 h at specific brain absorption rates (SAR) ranging from 0.3 to 7.5 W/kg. The extravasations of proteins was assessed either at the end of exposure or 7 days later in three to five coronal brain slices by immunohistochemical staining of serum albumin.

They found that increase in serum albumin extravasations after microwave exposure reached significance only in the group exposed to a very high SAR of 7.5 W/kg but not at lower power density levels. Histological injury was not observed in any of the examined brains and they concluded that microwave exposure in the frequency and intensity range of mobile telephony is unlikely to produce pathologically significant changes of the blood-brain barrier permeability (Fritze, et al. 1997). The extravasations of proteins were assessed either at the end of exposure or 7 days later in three to five coronal brain slices by immunohistochemical staining of serum albumin. The results are displayed in **Table 7**. Their results show an increase in serum albumin extravasations after microwave exposure (p=0.01, Fisher exact probability test) in all groups if cage controls and sham exposed rats are used as controls. Rats exposed to the highest SAR of 7.5 W/kg and the bulked 0.3+1.5 W/kg groups show significant leakage when only sham controls are used in t-test.

Their results displayed in **Table 6** show a significant increase in serum albumin extravasations after microwave exposure (p=0.01) by applying Fisher exact probability test in all groups if cage controls and sham exposed rats are used as controls. Rats exposed to the highest SAR of 7.5 W/kg and the bulked 0.3+1.5 W/kg groups show significant leakage when only sham controls are used in t-test. These results related to exposure with GSM-900 and 217 Hz modulation frequencies at various SAR levels are in good agreement with our results at the corresponding SAR levels (Persson, et al. 2012b; Persson, et al. 1997).

Table 7
Effect of global system for mobile communication (GSM) microwave exposure on blood-brain barrier permeability in rat (Fritze, et al. 1997).

	Cage Control	Sham exposed	Total control	0.3 W/kg	1.5 W/kg	7.5 W/kg	0.9 W/kg
No. rats	20	20	40	10	10	10	20
No. dots	1	4	5	7	6	14	13
% dots	5	20	12,5	70	60	140	65
Rel. SD	5	10	6	26	24	37	18
	t-test	Sham vs.	Exp.	0,1	0,12	0,03	0,02
	Fisher-test	Sham vs.	Exp.	0,08	0,08	0,08	
	t-test	all Control	Exp	0,06	0,06	0,03	
	Fisher-test	all Control	Exp	0,01	0,01	0,01	

The results related to exposure with GSM-900 and 217 Hz modulation frequencies at various SAR levels are displayed in **Figure 13**. As can seen from this figure there is a good agreement between the results of Fritze et al. and our own results (Fritze, et al. 1997). In most of the groups there is a significant difference between exposed and controls. In some groups the ratio between exposed and controls are >1 with no significance, that is probably due to too few exposed animals. We have found that also exposure to GSM-1800 results in BBB openings at various SAR level. When all 61 exposed animals are t-tested against their 103 controls there is a significant difference (p=0.03).

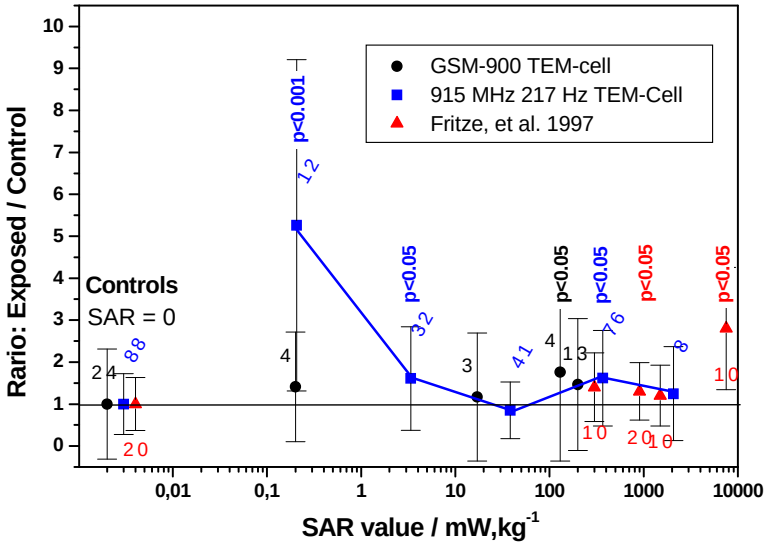

Figure 13
Summary of pathological ratio (BBB-leakage rank ≥1 versus rank <1) between recorded immunostained albumin in Fischer 344 rats exposed to GSM-900 modulated microwaves at various SAR values. The t-test p vales for ratios significant > 1 are also displayed.

6.2 Mason´s group in San Antonio TX

Patric Mason from the Air Force Research Laboratory, at Brooks City-Base, San Antonio, Texas visited our laboratories and learned all about our exposure system and the histopathological techniques.

They then performed a confirmation study at the Air Force Research Laboratory in San Antonio, designed to determine whether the BBB is altered in rats exposed in a transverse electromagnetic (TEM) transmission line cell to 915 MHz energy at parameters similar to those in the Lund University studies. Un-anesthetized rats were exposed for 30 min to either continuous-wave or modulated (16 or 217 Hz) 915 MHz energy at power levels resulting in whole-body specific absorption rates (SARs) of 0.0018 - 20 W/kg. Albumin immune-histo-chemistry was performed on perfused brain tissue sections, to determine the integrity of the BBB. Chi-square analysis revealed no significant increase in albumin extravasations in any of the exposed animals compared to the sham-exposed or home cage control animals (Mcquade, et al. 2009).

But when we were analyzing their data from the group of 33 rats exposed to 16 Hz modulation at 1.8 mW/kg there seems to be a significant increase ($p<0.05$) in albumin extravasations in the exposed animals. This is in agreement with our results of rats exposed to 18 Hz modulated 916 MHz microwaves with SAR values in the mW/kg range ($p<0.01$) (Malmgren, et al. 2012)

6.2 Veyret´s group in Bordeaux,France

In a study including 16 Fischer 344 rats (14 weeks old) were exposed head-only to the GSM-900 signal for 2 h at various brain-averaged SARs (0, 0.14 and 2.0 W/kg) or were used as cage or positive controls. Albumin leakage and neuron degeneration were evaluated 14 and 50 days after exposure. No apoptotic neurons were found 14 days after the last exposure using the TUNEL method. No statistically significant albumin leakage was observed. Neuronal degeneration assessed using cresyl-violet or the more specific marker Fluoro-Jade B, was not significantly different among the tested groups. No apoptotic neurons were detected.

These results agree pretty well with our studies performed by the investigation of the effects of global system for mobile communication (GSM) microwave exposure on the permeability of the blood-brain harrier and signs of neuronal damage in rats using a real GSM programmable mobile phone in the 900 MHz band (Eberhardt, et al. 2008; Grafstrom, et al. 2008).

Ninety-six non-anaesthetized rats were either exposed to microwaves or sham exposed in TEM-cells for 2 h at specific absorption rates of average whole-body Specific Absorption Rates (SAR) of 0.12, 1.2, 12, or 120 mW/kg. The rats were sacrificed after a recovery time of either 14 or 28 d, following exposure and the extravasations of albumin, its uptake into neurons, and occurrence of damaged neurons was assessed. Albumin extravasations' and also its uptake into neurons was seen to be enhanced after 14 d (Kruskal Wallis test: $p = 0.02$ and

0.002, respectively), but not after a 28 d recovery period. The occurrence of dark neurons in the rat brains, on the other hand, was enhanced later, after 28 d ($p = 0.02$). Furthermore, in the 28-d brain samples, neuronal albumin uptake was significantly correlated to occurrence of damaged neurons (Spearman $r = 0.41$; $p < 0.01$) (Eberhardt, et al. 2008).

Out of a total of 56 rats, 32 were exposed with GSM-900 microwaves once weekly in a 2-h period, for totally 55 weeks, at different average whole-body SAR values of 0.6 and 60 mW/kg. Sixteen animals were sham exposed and eight animals were cage controls, which never left the animal house. The brains were evaluated for histo-pathological alterations such as albumin extravasations, 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 parameters was found, when comparing the GSM exposed animals to the sham exposed controls (Grafstrom, et al. 2008).

In the **Table 8** is given the numerical average values of dark neurons stained using cresyl violet staining in different brain areas 50 days after exposure as well as in sham exposed rats (De Gannes, et al. 2009). This show a significant occurrence of dark neurons after exposure to SAR 2 W/kg which is in agreement with our previous study of 3 groups with 8 rats in each exposed for 2 hr to GSM-900 microwaves fields of SAR values 0.2; 0.02 and 0.002 W/kg .Highly significant ($p < 0.002$) evidence was found for neuronal damage in the cortex, hippocampus, and basal ganglia in the brains of exposed rats (Salford, et al. 2003b).

Table 8.
Dark neurons 50 days after exposure (De Gannes, et al. 2009).

	Sham	0.14 W/kg	2 W/kg
F1	0,25	0.5	0.85
F4	0,6	0.25	0,7
M1	0.35	0,6	0.85
M3	0,6	0.85	1,15
M4	0.7	0,15	1.15
CA1	0,35	0.35	0,85
CA2	0.25	0,25	0.65
CA3	0,6	0.65	1
DG	0,5	0.35	1
P1	0,25	0.5	0
P2	0,5	0.5	0,75
P3	0	0	0.25
Testing:	Sham vs. 0.14 W/kg	0,14 W/kg vs. 2 W/kg	Sham vs. 2 W/kg
Paired t-test	1	p = 0.004	p = 0.0003
Sign test	1	0.01	P = 0.01
Wilcoxon	0.96	0.03	P = 0.008

6.3 Pierre Aubineau and Fatma Töre (*Lab. Signal. Interactions Cellulaires, France)*

To examine whether the exposure to 900 MHz GSM microwaves could change the permeability of cerebral and meningeal blood vessels using well-defined experimental

conditions for the estimation of SAR during head exposure as well as for the possible influence of stress during exposure (monitoring of arterial pressure, anaesthetized controls).
81 rats (Charles Rivers, 400-450 g) were used, divided into ten groups:

1. Control
2. Sham-exposed
3. Exposed to 3 W/kg for 2 h
4. Exposed to 1.5 W/kg for 2 h
5. Exposed to 0.75 W/kg for 2 h
6. Exposed to 0.2 W/kg for 2 h
7. Exposed to 3 W/kg for 40 min
8. Exposed to 3 W/kg for 10 min
9. Exposed to 2 W/kg for 2 h under light urethane anaesthesia
10. Positive control for protein extravasations

All rats except controls and exposed under anaesthesia, were daily trained to contention for one week (increasing progressively training duration from 15 min to 2 hours) before the experiment. One day before exposure, left femoral artery and vein were catheterized under anaesthesia (femoral artery for monitoring of arterial blood pressure, femoral vein for tracer and anaesthetic infusion at the end of exposure). BSA-FITC was infused i.v. at the beginning- and 15 minutes before the end of the experiment (total: 25 mg/kg) when rats were exposed for 2 h and 40 min, in one shot when exposed for 10 min.

Rat heads were exposed to 900 MHz GSM microwaves through an antenna shifted laterally by 10° in order to expose one hemisphere slightly more than the other and to avoid preferential exposure of the sagital sinus and inter-hemispheric gap. One typical experiment included one exposed and one sham-exposed rats treated together, from training to immune-histochemistry. At the end of all experimental procedures, rats were killed with an overdose of anaesthetics (via the chronic venous catheter for exposed and sham-exposed rats).

The ascending aorta was rapidly cannulised for perfusion fixation (200 ml of cold heparinised phosphate buffer saline followed by 500 ml of cold 4% paraformaldehyde at a controlled pressure of 140 mmHg). Dura maters and brains were post-fixed overnight by immersion in the same fixative solution at 5°C and placed for 48 h in a 30% solution of sucrose in PBS at 5° C. Dura mater were then directly examined for fluorescence of BSA-FITC. Brains were frozen in isopentane at –80°C and sectioned (coronal sections, 40 µm-thickness, 3 sections every 2 mm for the whole brain) Sections were mounted on polysin slides and treated for indirect immunohistochemistry against BSA in order to increase the intrinsic fluorescence of BSA-FITC (rabbit polyclonal anti-BSA antibody + anti-rabbit IgG biotin conjugate followed by incubation in ABC reagent (Vector Laboratories, ABC kit PK-6100).

Preparations were examined using a Leitz DMRX fluorescence microscope equipped with a cooled CCD video camera and a computer-controlled motorized stage allowing optical tomography for acquisition of sequential digitized images through the depth of the tissue. In the rats placed in the rockets (container), mean arterial blood pressure could vary from 95 to 135 mm Hg, (117 ± 2.7 mm Hg, mean ± SE). In none of the rats was the recorded upper limit

of arterial pressure compatible with a loss of auto regulation of cerebral blood flow which could lead to an opening of the BBB (>170 mm Hg). There was no significant difference between mean arterial pressures of sham-exposed and exposed animals, respectively (Student's t-test).

Control and sham-exposed rats showed no noticeable BSA extravasations while positive controls showed widespread and intense extravasations. Exposure during 2 hours with 900 MHz GSM microwaves at SAR 3 W/kg induced marked BSA-FITC extravasations in the whole brain of conscious and lightly anaesthetised rats. Fluorescence was particularly visible in the more exposed hemisphere under the antenna. Hippocampus, central grey nuclei (putamen, caudate) and frontal cortex were heavily labelled. BSA diffusion was mainly restricted to the immediate vicinity of microvessels.

Exposure during 2 hours with 900 MHz GSM microwaves at SAR 1.5 W/kg and 0.75 W/kg still allowed to detect BSA, but it was restricted to the deep gray matter located under the antenna and to the frontal cortex and in lesser amounts than after exposure at 3 W/kg. At the lowest SAR level tested in this experimental series (0.18 W/kg), a 2-h exposure did not induce visible BSA-FITC extravasations in the brain parenchyma. Exposure during 40-minutes with 900 MHz GSM microwaves at SAR 3 W/kg resulted in extravasations of BSA-FITC comparable to that obtained after a 2-h exposure at the same SAR. However, 10-min exposures at 3 W/kg did not lead to any visible extravasations (Aubineau & Töre 2003).

6.4 JW Finnie (Australia)

In a purpose-designed exposure module with well-defined dosimetry, mice were placed in tubes arranged radially around a dipole antenna. Treated mice received a 60 min far-field, whole body exposure to RF fields with similar pulsing and modulation characteristics to those used for mobile communication (900 MHz fields modulated at a pulse repetition frequency of 217 Hz and a pulse width of 0.6 ms) (Finnie, J.W., et al. 2003; Finnie, J. W., et al. 2001). .

In the short-term study, mice were given a single, 60 minute far-field whole body exposure of 4 W/kg, while exposures in the long-term experiment were of 60 minutes duration on 5 consecutive days per week for 104 weeks. In the long-term study, mice were exposed to increasing energy absorption levels to determine if there was a dose-response relationship: 0.25, 1.0, 2.0 and 4.0 W/kg. Control mice were either sham-exposed or permitted free movement in a cage to evaluate any stress-related module confinement effects. The efficacy of albumin as a vascular tracer was confirmed with a positive control group exposed to a clostridial toxin (*Clostridium perfringens* type D epsilon toxin) known to markedly increase BBB permeability in the brain, resulting in a severe, generalised vasogenic oedema.

In all groups (except the positive control), albumin extravasations was minimal and there was no significant difference in tracer leakage between exposed, sham-exposed and freely moving, cage control groups, whether acutely or long-term exposed. No dose-response effect was detected.

They claim that acute or prolonged exposure of mice to mobile telephone-type RF fields produces negligible brain damage *as assessed by disruption to BBB integrity at the light microscope level using endogenous albumin as a vascular tracer*. Moreover, any increase in

BBB permeability caused by these non-thermal SAR levels is probably subtle and reversible, with prompt restitution of BBB integrity and rapid oedema resolution.

But our statistical testing, using Fisher Exact Probability Test, of number of mice with leaking vessels, displayed in **Table 9**, revealed significant differences between Exposed versus Controls (p=0.03) as well as between exposed versus all unexposed (p=0.04).

Table 9. Summary of number of mice with or without albumin leaking albumin I the brain (Finnie, J. W., et al. 2001).

	Total	No mice No leaking	No mice leaking	Fisher test 2 tailed p value Exp. vs Ctrl	Exp. /Un-exp.
Contro1	10	8	2		
Sham	10	6	4		
Un-exposed	20	14	6		
Exposed	27	10	17	0.03	0,04

6.5 Tsurita (Japan)

Tsurita et al. in Japan exposed rats with 1.439 GHz microwaves from cellular phone at an average SAR of 0.25 W/kg for 2-4 weeks and found no evidence of albumin leakage with the same method that we have used previously. The number of rats (2 control rats and 4 rats each in the exposed groups), were too few to draw any statistical significant conclusion from their results (Tsurita, et al. 1999a; Tsurita, et al. 2000; Tsurita, et al. 1999b).

In our studies, however, we have found that also exposure to 1.8 GHz microwaves results in BBB openings at various SAR level. When all 61 exposed animals are t-tested against their 103 controls there is a significant difference (p=0.03) in BBB albumin leakage.

7. CONCLUSION

The effect of microwaves on the blood brain-barrier (BBB) has been studied in Fischer 344 rats of both sexes. The rats were not anaesthetised during the exposure to microwaves of 915 and 1800 MHz as continuous wave (CW) and pulse-modulated with different pulse power and at various time intervals. The CW-pulse power varied from 0.001W to 10 W and the exposure time from 2 min. to 960 min. In each experiment we exposed 4 rats with 4 controls randomly placed in excited and non-excited TEM-cells respectively. In total we investigated 630 exposed rats at various modulation frequencies and 372 controls. GSM exposure was simulated by 915 MHz microwaves pulse modulated at 217 Hz with 0.57 ms pulse width. Other pulse modulations with 0.57 ms pulse width were 4, 8 and 16 Hz. Another pulse modulation at 50 Hz. with 6.6 ms pulse was used to simulate DUX exposure.

The ratio of pathological rats (albumin leaking score ≥ 1) is 62/372=0.17±0.02 for un-exposed control rats. The ratio of pathological rats was 170/481=0.35±0.03 ($p<0.002$) among

rats exposed to pulse modulated (PW) and 74/149 (0.50±0.07) ($p<0.0001$) among rats exposed to continuous wave exposure (CW).

We found no pronounced difference between the various modulation frequencies. The effect of CW-exposure seems, however, to be more effective in opening the BBB the pulse-modulated microwaves. This is surprising since the common opinion is that modulated microwaves would be more biologically effective. There seems to be no difference in the degree of opening the BBB with the time of exposure that might indicate a switch mechanism that is turned on by the microwaves. There seems to be a biphasic relaxation of closing the opened BBB with a fast component with a halftime of about 20 min. There is also a slow component which has a very long halftime of about 200 000 min which means that some parts of the BBB stay open for a long time.

The effects of global system for mobile communication (GSM) microwave exposure was simulated by modulation of 915 MHz microwaves with a pulse sequence of 217 Hz and 0.52 ms pulse width. We studied 169 rats exposed to various SAR levels as well as 24 rats exposed to real GSM-900 modulated microwaves. BBB-leakage related to exposure with GSM-900 and 217 Hz modulation frequencies were significant at both very low SAR levels and at higher levels. There is a good agreement between our results and those presented by Fritze et al. (1997), whose results show a significant increase ($p=0.01$ Fisher exact probability test) in serum albumin extravasations after microwave exposure in all groups if cage controls and sham exposed rats are used as controls. Rats exposed to the highest SAR of 7.5 $W.kg^{-1}$ and the bulked 0.3+1.5 $W.kg^{-1}$ groups show significant leakage when only sham controls are used in t-test.

By considering the whole spectrum of albumin leaking score values, from no leakage (0) up to extensive leakage (3), weighted average score values (WAS) were estimated for the controls and for the various exposed groups. The observed ratio [OR = WAS(exposed) / WAS (controls)] was estimated for groups exposed to various SAR values. Rats exposed to 915 MHz microwaves modulated at 217 Hz (simulating GSM) exhibited a bath-tube shaped response cure. The largest OR of about 4-3 was recorded for the lowest SAR value of 0.1-0.5 $mW.kg^{-1}$ and the OR decreased to 1 in the range of 50-500 mW/kg to increase further to about 2 above 2000 $mW.kg^{-1}$. At the very low SAR range thermal effects are unlikely. Thus there seems to be a non-thermal mechanism involved triggering the opening of the BBB. This shape of the response curve also explains why other researchers who exposed the animals, above 100 $mW.kg^{-1}$ have difficulties to record significant albumin leakage.

Acknowledgement

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