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BRAIN RESEARCH REVIEWS

Review

Electromagnetic fields and the blood–brain barrier

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ABSTRACT

The mammalian blood–brain barrier (BBB) consists of endothelial cells, linked by tight junctions, and the adjoining pericytes and extracellular matrix. It helps maintain a highly stable extracellular environment necessary for accurate synaptic transmission and protects nervous tissue from injury. An increase in its normally low permeability for hydrophilic and charged molecules could potentially be detrimental. Methods to assess the permeability of the BBB include histological staining for marker molecules in brain sections and measurement of the concentration of marker molecules in blood and brain tissue. Their advantages and disadvantages are discussed. Exposure to levels of radiofrequency electromagnetic fields (EMF) that increase brain temperature by more than 1 °C can reversibly increase the permeability of the BBB for macromolecules. The balance of experimental evidence does not support an effect of 'non-thermal' radiofrequency fields with microwave and mobile phone frequencies on BBB permeability. Evidence for an effect of the EMF generated by magnetic resonance imaging on permeability is conflicting and conclusions are hampered by potential confounders and simultaneous exposure to different types and frequencies of EMF. The literature on effects of low frequency EMF, which do not cause tissue heating, is sparse and does not yet permit any conclusions on permeability changes. Studies on the potential effect of EMF exposure on permeability of the BBB in humans are virtually absent. Future permeability studies should focus on low frequency effects and effects in humans. Care should be taken to avoid the methodological limitations of earlier studies and to determine the pathophysiological relevance of any changes found.

Contents

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Abbreviations: BBB, blood–brain barrier; ELF, extremely low frequency electromagnetic field(s); EMF, electromagnetic field(s); FITC, fluorescein isothiocyanate; GSM, global system for mobile communications; HRP, horseradish peroxidase; MRI, magnetic resonance imaging; PDC, personal digital cellular; RF, radiofrequency electromagnetic field(s); SAR, specific absorption rate; UMTS, universal mobile telecommunications system

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1. Introduction

Electromagnetic fields (EMF) are a pervasive environmental presence in modern society. Extremely low frequency fields (ELF) are generated as a byproduct of electricity production, transport or use. Radiofrequency (RF) fields are used for radar tracking, wireless communication (mobile phones, radio and television), in industrial or kitchen heating, or as a medical diagnostic or treatment tool (magnetic resonance imaging (MRI), RF tissue heating). Static magnetic fields are also necessary in MRI and are used in some industrial processes and scientific research (Feychting et al., 2005). External EMF can induce electric fields in the body that can potentially alter biological functions. The main interaction mechanism for ELF is stimulation of nerve cells and fibres by the induced electric field (Reilly, 1998). The main interaction mechanism for RF fields is the deposition of energy in the form of heat (Habash et al., 2003), although 'non-thermal' mechanisms have also been hypothesised (Repacholi, 1998). Both ELF and RF could potentially result in altered functioning of the brain (Hermann and Hossmann, 1997; Hossmann and Hermann, 2003). The possible link between exposure to EMF and permeability of the separation between blood and nervous tissue in the brain, the blood–brain barrier (BBB), has been the topic of a number of previous reviews. The earliest reviews deal with conflicting evidence and technical issues of the various methods to assess permeability (Albert, 1979b; Blasberg, 1979; Justesen, 1980; Michaelson, 1986; Segal and Magin, 1982; Williams et al., 1984c). Subsequent reviews mainly give overviews of the results of experimental studies and discuss their possible pathophysiological significance (D'Andrea et al., 2003; Hermann and Hossmann, 1997; Hossmann and Hermann, 2003; Johnston and D'Andrea, 2007; Lin, 2005; Nittby et al., 2008). The present review supplements these by including a number of older publications not previously discussed, by adding the results of more recent experimental studies (including attempts to replicate or improve earlier studies that report effects), by using a more systematic approach for assessing the evidence and by discussing the findings in the context of a critical appraisal of methodology and effects of other stimuli on BBB permeability. Other neuropathological findings, such as the occurrence of 'dark neurons', are only discussed if they were conducted in combination with permeability studies. An overview of the frequencies of EMF used in studies on BBB permeability is given in Fig. 1.

An initial Pubmed search was conducted using combinations of the search terms [blood AND brain AND barrier] with [electromagnetic OR non-ionising OR non-ionizing OR (mobile phone) OR gsm OR umts OR microwave OR radar OR (power frequency) OR (low frequency) OR (static field) OR (static magnetic)]. This search was last updated in February 2010. For all full English language experimental papers and reviews retrieved that dealt with the relation between EMF exposure and permeability of the BBB, the reference list was examined to locate experimental papers and reviews that were not indexed by Pubmed. A new Pubmed search was then conducted for all authors of the experimental papers previously identified, and the reference list of any additional papers examined. Conference abstracts were not used, since they are often not peer-reviewed and contain insufficient information to judge relevance and quality. All full experimental papers with EMF exposure levels that did not (or were unlikely to) produce a thermal effect were assessed on quality and completeness using a standardised checklist. The items on the checklist were derived from published quality criteria in relevant methodological reviews (for experimental studies: Greenebaum, 2003; Health Council of the Netherlands, 2005; Johnston, 2010 ; Khmet et al., 2004; Klimisch et al., 1997; WHO, 1998). It is important to note that the quality checklist does not attach weights to individual items (which would be highly subjective), and reflects general scientific criteria and not the advantages and disadvantages of specific methods to assess BBB permeability. For these, the reader is referred to the methodological

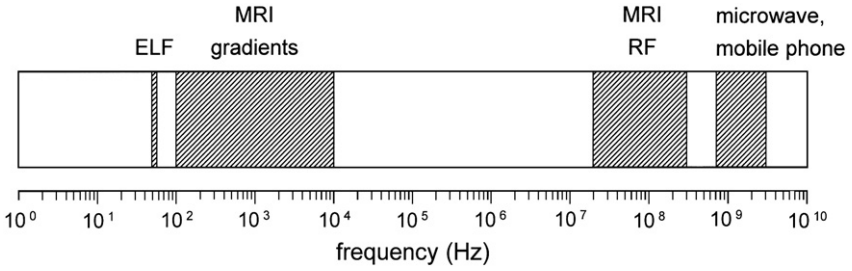

Fig. 1 – Overview of the frequency ranges of electromagnetic fields used in experiments on permeability of the blood–brain barrier. For MRI gradient fields, the frequencies listed are the pulse frequencies. For mobile phones, the frequencies listed are those of the carrier signal. Modulation of the carrier signal occurs at lower frequencies.

discussion in Section 4 and pertinent discussion of experimental papers in Section 5.

2. Anatomy of the blood–brain barrier

The extracellular fluid in the central nervous system (spinal cord and brain) of vertebrates is shielded from that in the rest of the body by the BBB surrounding blood vessels and by the blood–cerebrospinal fluid barrier of the choroid plexus. The physical barrier consists of transmembrane proteins that form tight junction complexes between endothelial cells in the blood vessel wall and between epithelial cells of the choroid plexus, combined with a relative paucity of endocytotic vesicles in the endothelial cytoplasm (Begley and Brightman, 2003). BBB tightness is structurally aided by connective tissue cells called pericytes and by the extracellular matrix of the basement membrane. Astrocytes may be involved in developing and maintaining the BBB without being structurally involved in barrier function (Hawkins and Davis, 2005). Together with surrounding neurons, these cellular and extracellular components form a complex and interdependent 'neurovascular unit' (Hawkins and Egleton, 2008) (Fig. 2). The BBB is absent in a small number of brain areas whose function depends on unrestricted access to the blood: the median eminence, organum vasculosum and subfornical organ in the hypothalamus; the area postrema and nucleus tractus solitarius in the brain stem; the posterior pituitary, subcommissural organ and pineal gland. Limited entry of larger hydrophilic molecules is also possible via the nasal epithelium and cranial nerve roots (Banks, 2004).

The mammalian BBB and blood–cerebrospinal fluid barrier are present from the embryonic stage onward. Although the protein concentration ratios between cerebrospinal fluid and plasma decrease over time, this development is mainly due to gradual changes in the rate of transcellular transfer and distribution volume, rather than increasing barrier tightness (Johansson et al., 2008). Unlike the adult brain, the developing brain also contains specialised barriers in the outer cell layers of the brain–ventricle and brain–subarachnoid interface (Saunders et al., 1999b).

3. Physiology of the blood–brain barrier

The main functions of the BBB are to create the highly stable extracellular environment necessary for accurate synaptic transmission, to protect nervous tissue from injury and possibly to enhance metabolic efficiency by excluding non-essential molecules (Begley and Brightman, 2003; Justesen, 1980). The BBB is not an absolute barrier, but it allows for a more restricted exchange of cells and molecules between the blood and the brain parenchyma. It has sometimes been compared to a "gently leaking wooden boat" (D. Begley, in: Franke, 2003). Substrate-specific transport routes across the BBB include carrier-mediated influx and efflux, receptor-mediated vesicular transport and receptor-mediated entry by immune cells. Non-specific transport routes include passive diffusion, tight junction modulation and adsorptive-mediated vesicular transport (Begley and Brightman, 2003). The relative impermeability of the endothelium in brain vessels to albumin may be enhanced by the virtual absence of albumin binding protein, which mediates albumin transcytosis in vessels outside the brain (Stewart, 2000). Transcellular and paracellular transport can occur not only via the blood vessel wall, but also via cranial and spinal nerve roots (Begley and Brightman, 2003). Interestingly, the ganglia of the autonomic nervous system (sympathetic, parasympathetic and enteric) lack a blood–nervous tissue barrier. This may mean they have less stringent metabolic needs than the

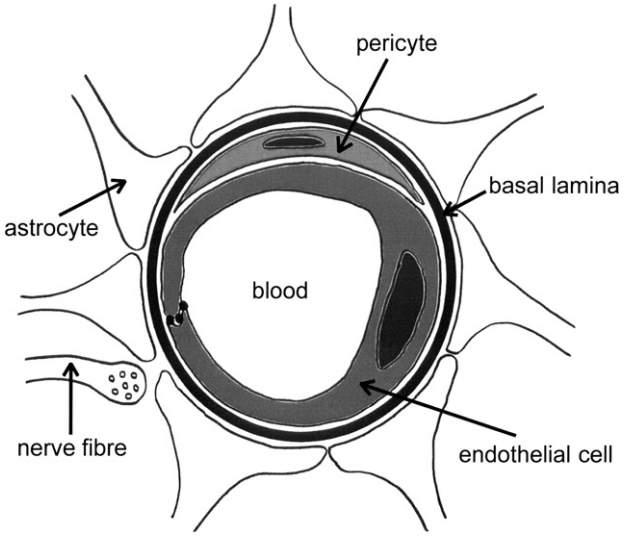

Fig. 2 – Schematic overview of the components of the blood–brain barrier (redrawn after: Abbott, 2005; Hawkins and Davis, 2005).

central nervous system, but it also means they are more vulnerable to damage by blood-borne toxic substances (Kiernan, 1996).

Lipophilic compounds have unrestricted access to the brain by passive diffusion through the endothelial cell membranes. Hydrophilic or charged molecules which are essential for brain metabolism, such as ions, glucose, amino acids and nucleic acid constituents, quickly pass the BBB through specialised channels or carriers. Water molecules can pass the BBB through specialised protein channels called aquaporins, which play an important role in brain fluid homeostasis (Begley and Brightman, 2003). Peripheral hormones and regulatory peptides that exert their action in the brain usually have specialised saturable transport systems across the BBB (Banks, 2010). The transport of hydrophilic molecules such as peptides and proteins that do not have a specific transport system is much slower than that of lipophilic molecules. Nevertheless, the amounts that cross the BBB can often be enough to cause a receptor-mediated effect in neurons (Kastin and Pan, 2003). Sucrose, a compound often used as a tracer in BBB permeability studies, still shows a brain uptake of about 1% of that for more lipophilic compounds such as propanolol or nicotine in the same time period. This is partly due to the relatively large surface area available for diffusion in the brain vasculature (approximately 20 m^2 in humans) (Fagerholm, 2007). On the other hand, cerebrospinal fluid is constantly secreted and reabsorbed via the subarachnoid space and the resulting circulation keeps the concentration of larger hydrophilic molecules in the cerebrospinal fluid artificially low. A range of both hydrophilic and lipophilic compounds can also be removed from the brain by active efflux pumps in endothelial and choroid plexus cells (Saunders et al., 1999a).

The BBB in the developing brain is as efficient a barrier as the adult BBB to large hydrophilic molecules such as proteins. The permeability of the immature BBB to smaller hydrophilic compounds such as the tracers sucrose and inulin, however, is greater than that of the adult BBB. The blood–cerebrospinal fluid barrier of the choroid plexus is more permeable to proteins in the developing than in the mature brain, but the higher concentrations of proteins in the cerebrospinal fluid are excluded from the extracellular space surrounding neurons by specialised junctions that disappear in adulthood (Saunders et al., 1999b).

4. Methods to assess permeability of the blood–brain barrier

The hydrodynamic diameter of most tracer molecules used to assess BBB permeability is larger than the pore size between endothelial cells (1.2 to 1.6 nm) (Blasberg, 1979). Increased passage to the brain must therefore mean either increased transcellular transport or increased permeability of tight junctions. Two categories of methods are used to assess changes in BBB permeability: staining for tracer molecules in sections of the brain ('histological methods') and comparison of the concentrations of the tracer and a reference molecule in blood, brain tissue or both ('physiological methods'). Both have their technical advantages and disadvantages, which will be discussed below. It also is useful to point out that they are not equivalent in the type of permeability they measure. 'Physiological' tracers usually have a smaller molecular mass than 'histological' tracers. The degree of leakage may be greater and the time course more prolonged for these smaller tracers (Habgood et al., 2007). The methods do not by themselves distinguish between substrate-specific transport and non-specific passage through the blood brain barrier. Tracers have to be used that pass the BBB at a minimal rate with normal BBB tightness (Kastin and Pan, 2003). A summary of methods to assess BBB permeability can be found in Table 1.

4.1. *Histological methods*

Histological methods to asses BBB permeability involve the preparation of brain sections after the experiment, which are then stained for the presence of the permeability marker in the brain's extravascular space. The permeability marker is either naturally present in the blood or artificially injected during the experiment. Their main advantage is that they can distinguish between focal changes in permeability around blood vessels and more diffuse tissue permeation. Disadvantages are that the results often non- or semi-quantitative (absent/present, categorical visual score or number of positive vessels) and do not give any information on the rate of transport across the BBB. Since even 'non-thermal' levels of microwave exposure can increase cerebral blood flow, small increases in extravasation could also be a secondary effect of local changes in blood flow, intraluminal pressure or total perfusion surface area (Oscar et al., 1981).

Horseradish peroxidase (HRP) is an enzyme with a relatively large molecular mass (44 kDa). The dark, electron-dense reaction products of added substrates can be detected both under the light microscope and electron microscope. Its use as a marker for BBB permeability is problematic since HRP can be toxic and have effects on permeability itself, either directly or indirectly via the release of inflammatory mediators. Since the reaction product can diffuse over time, it can give an inaccurate picture of the location of any increased permeability (Habgood et al., 2007; Preston et al., 1989). In addition, cross-reactivity with endogenous peroxidase in brain tissue could generate false-positive results (Michaelson, 1986).

Na-fluorescein has been used in both histological and physiological studies of BBB permeability. It has a much smaller molecular mass than HRP (376 Da). A resulting problem with this tracer is that it easily diffuses from regions devoid of a BBB. When cerebral blood flow is increased or renal excretion is decreased (for example at hyperthermic levels of RF exposure), Na-fluorescein diffusion from these regions to adjacent areas increases, and diffusion may continue if fixation is not optimal or freezing does not occur quickly enough after fixation (Williams et al., 1984c). While Na-fluorescein can bind to plasma proteins, a considerable fraction remains free (Wolman et al., 1981). An alternative is to couple fluorescein or its derivative fluorescein isothiocyanate (FITC) to a compound with a much larger molecular mass such as dextran (Kuribayashi et al., 2005; Masuda et al., 2007b). Na-fluorescein and fluorescein-labeled dextran also have a tendency to linger in cerebral vessel wall after saline flush, though this property would not be expected to differ between the controls and the exposed animals (Williams et al., 1984c).

Table 1 – Overview of methods used to assess permeability of the blood–brain barrier.

Tracer	Molecular mass (kDa)	Advantages	Disadvantages
A. Histological methods			
HRP	44	Standard technique; light and electron microscopy	Flow sensitivity; toxicity; cross-reactivity; diffusion of reaction product
Na-fluorescein	0.376	Direct visualisation	Flow sensitivity; variable plasma binding; vessel wall binding; tissue diffusion
FITC-dextran	variable[1]	Direct visualisation	Flow sensitivity; vessel wall binding
Evans blue, trypan blue	0.961	Direct visualisation	Flow sensitivity; variable plasma binding vessel wall binding
Albumin	65	Endogenous protein	Flow sensitivity; uncertainty of blood origin
B. Physiological methods (examples of tracers often used are given in brackets)			
Intra-arterial bolus			
(mannitol)	0.182	Quantitative	Flow sensitivity; short time course; relative measure; anaesthesia
(inulin)	5		
Intravenous bolus/time integral		Quantitative; sensitive; flow-independent	Sensitive to retrograde diffusion; averages several animals
(sucrose)	0.342		
Intravascular infusion		Quantitative; sensitive; flow-independent	Sensitive to retrograde diffusion; averages several animals
(sucrose)	0.342		

Notes:
Details can be found in Section 4.
1. Molecular mass between 4 and 250 kDa, depending on length of the polymer (Hawkins and Egleton, 2008).

Dyes such as trypan blue and Evans blue bind tightly but reversibly to albumin in vivo, resulting in a tracer with a molecular mass of 68.5 kDa, if their concentration is sufficiently low. In many in vivo experiments, though, it is not clear whether this ideal situation is reached and dissociation of dye and protein may have occurred. Staining of brain tissue could therefore represent either protein-bound or unbound dye (Fortin, 2003; Moos and Mollgard, 1993; Saunders et al., 2008). Especially when the experimental treatment causes clotting, interruption of blood flow or damage to the blood vessel wall, part of the dye may stay trapped in the vessel wall and be misinterpreted as extravasation (Habgood et al., 2007; Hawkins and Egleton, 2008).

Directly staining brain sections for endogenous plasma proteins such as albumin avoids most of the problems associated with artificially injected dyes or HRP. It is not toxic at physiological concentrations, does not itself influence permeability, has a fixed molecular mass of 65 kDa, and would therefore be preferable to previously mentioned histological methods. The main disadvantage is that one is never entirely sure whether the albumin detected is derived from the blood or from the cerebrospinal fluid. However, focal deposits surrounding blood vessels would most likely indicate extravasation. For light microscopic visualisation of albumin, the immunohistochemical staining is usually enhanced by secondary antibodies. This makes the method very sensitive for detecting extravasations, but makes quantification in terms of protein mass or concentration difficult (Franke, 2003; Lin, 2005).

Sometimes the presence of albumin or other tracers is not determined in histological sections, but in homogenates of dissected brain regions after the blood vessels have been flushed with saline. When homogenised brain samples are used, it is important to have some idea about the degree of blood contamination of the tissue or adhesion of the marker molecule to the blood vessel wall (Saunders et al., 2008). An alternative is to quantify the intensity of a fluorescent permeability marker though a cranial window after extravasation from blood vessels in the pia mater (Masuda et al., 2007a).

A more general disadvantage of histological methods is that measurements in brain sections or homogenates can only be done at one time-point in each animal. At longer time delays after EMF exposure, it is difficult to estimate when and for how long extravasation has occurred (Hawkins and Egleton, 2008).

Recently, the time course of osmotic BBB opening was investigated by quantifying the entry of a contrast agent in structural magnetic resonance images (Blanchette et al., 2009). Unfortunately this method is not suitable to investigate effects of EMF on permeability, since the imaging method itself requires exposure to strong static, low frequency and RF fields.

4.2. *Physiological methods*

Generally speaking, the advantages and disadvantages of physiological methods are a mirror image of those for histological methods: precise localisation in the brain is not possible, but more precise quantification of the size and rate of transport is, if certain conditions are met. Properties of useful tracers include permeation of the BBB at slow to moderate rates (i.e. a rate of transport limited by permeability, not blood flow), low lipophilicity, no ionisation near physiological pH (i.e. small pH differences between blood and brain tissue do not affect dissociation), a constant size of tissue distribution space, no carrier-mediated or facilitated transport and no reversible binding to blood or vascular components (or the free concentration should be known). If tracers with different molecular masses show a different permeability, any change

in permeability is probably due to intercellular, rather than intracellular, transport, which depends on gap size (Blasberg, 1979).

Two techniques use intra-arterial bolus injection of at least two compounds, a tracer and a reference molecule. The use of several tracers with different molecular masses (for example mannitol, 182 Da, and inulin, 5000 Da) gives extra information on the size of any intercellular gaps (Williams et al., 1984c). A general disadvantage of the intra-arterial bolus methods is that they only detect changes over a short time course (one capillary passage), with limited sensitivity and possibly inconsistent mixing with blood. In the absence of a clear change in BBB permeability, they are not very suitable for measuring the uptake of slowly penetrating molecules such as many peptides (Hawkins and Egleton, 2008; Kastin and Pan, 2003). With the first variant, the 'indicator diffusion' method developed by *Crone* (Crone, 1963), both tracer and reference are sampled in the venous outflow of the brain. The non-diffusable reference gives the fraction of arterially injected tracer passing through vascular bed in the measurement period. The diffusable tracer gives the fraction extracted by crossing the BBB. Problems are that the method only gives an average of whole perfused area without any information about regional differences in perfusion and extraction, and it presumes that blood flow is constant during the experiment, which will usually not be the case (Oscar et al., 1981). In the second variant, developed by *Oldendorf* (Oldendorf, 1970), tracer and reference are sampled in brain tissue. The reference is highly diffusable even when the BBB is intact, and the ratio of tracer and reference in brain tissue is compared to the ratio injected in the blood, giving a brain uptake index. Problems are that if the reference (usually tritiated water) is not completely extracted in single passage, the ratio in blood will not remain constant. In other words, the brain uptake index is only a relative value, since the absolute permeability of the reference is unknown. If the experimental treatment changes the brain uptake index, this could reflect changes in uptake of the tracer, the reference or both. The absence of any change in brain uptake index could reflect either no change in BBB permeability or changes of equal magnitude and direction in the uptake of both tracer and reference. Any increase in cerebral blood flow or vascular volume caused by the treatment would further reduce extraction of the reference from blood and artificially increase the brain uptake index. A modified version of the Oldendorf technique uses a second, non-diffusable reference molecule and corrects for incomplete extraction of the first, diffusable reference. However, the modified method still depends on assumptions that are unlikely to be met and are difficult to measure (Blasberg, 1979; Michaelson, 1986; Oscar et al., 1981; Williams et al., 1984c,d).

A second type of method uses an intravenous bolus injection and time integral measurement of the concentration of the tracer in arterial blood and measurement at the end of the experiment of the concentration of the tracer in the brain. It is sometimes called a 'dual compartment method', since it relies on measurements of the tracer in both blood and brain, Usually, the tracer is radioactively labeled sucrose (molecular mass: 342 Da) (Rapoport et al., 1979). Advantages compared to the intra-arterial bolus method are increased sensitivity and the averaging of changes over multiple capillary passages. Use of the plasma-time integral method eliminates the biasing effect of transient changes in blood flow and can be used in awake and unrestrained rats. Its disadvantages are that it gives an aggregate measure over several animals, and therefore requires a highly reproducible effect. It also requires that the amount of tracer trapped in the vascular compartment is known and corrected for. In addition, any diffusion of the tracer from the brain back to the blood or to other compartments such as the cerebrospinal fluid will introduce errors (Blasberg, 1979; Williams et al., 1984c).

A third type of method uses intravascular infusion of the tracer with constant plasma levels and measurement of brain levels. An advantage compared to intravenous bolus injection is the easier calculation of the time integral blood concentration and distribution volume of the tracer. The disadvantages are similar to those of intravenous bolus injection: it represents an average over several animals, it requires a highly reproducible effect and back diffusion to blood or other compartments introduces errors (Blasberg, 1979).

Occasionally, changes in the cerebrospinal fluid concentration of a marker are taken as an indication for altered BBB permeability (Burchard et al., 1998), but such changes could also reflect entry at the choroid plexuses or circumventricular organs or changes in distribution volume (Saunders et al., 2008). Additional physiological methods to assess BBB permeability exist, but these have not been used in combination with EMF exposure (Blasberg, 1979; Hawkins and Egleton, 2008).

In conclusion, the method using intravenous bolus injection with time integral measurement of the tracer concentration in plasma and the method using intravascular infusion of the tracer measure permeability independently from changes in cerebral blood flow or distribution volume, and would therefore be preferable to the intra-arterial bolus injection method and the isolated measurement of cerebrospinal fluid concentration.

5. Effects of EMF exposure on permeability

5.1. *Thermal EMF exposure*

Environmental heat in excess of the mammalian thermoregulatory capacity can increase the permeability of the BBB to macromolecules (Shivers and Wijsman, 1998). Neuronal albumin uptake in various brain regions was recently shown to be dose-dependently related to brain temperature, with effects becoming apparent with temperature increases of 1 °C or more (Kiyatkin and Sharma, 2009). Since sufficiently strong RF fields can lead to tissue heating, it seems logical to ask whether this could be a mechanism leading to increased BBB permeability. Exposure of the rat head at microwave frequencies (at 2.5–3.2 GHz) that leads to a brain temperature above 40 °C can increase BBB permeability to HRP (Moriyama et al., 1991; Sutton, 1979), Evans blue (Lin and Lin, 1982; Neilly and Lin, 1986; Ohmoto et al., 1996) and ^{84}rubidium (Goldman et al., 1984). The latency and degree of increased permeability depend on the degree of temperature rise and hence on the specific absorption rate (SAR, power absorbed per unit mass) of RF energy, on exposure duration and on the rate of heat distribution and dissipation by the body (Lin and Lin, 1982;

Sutton, 1979). Artificial cooling of the brain during EMF exposure prevents the increase in BBB permeability (Moriyama et al., 1991; Neilly and Lin, 1986; Sutton, 1979). It has been questioned whether the increased permeability following thermal RF exposure is a direct effect of the temperature rise on the BBB, since higher temperatures also increase cerebral blood flow and metabolic rate and reduce renal fluid excretion. In fact vesicular uptake of HRP and sucrose in endothelial cells is decreased at thermal levels, at least until the temperature reaches a level where actual injury or death of the endothelial cell can occur (43 °C) (Williams et al., 1984c). Using the blood flow-independent intravenous injection-time integral method, 2.5 GHz microwave exposure resulting in core body temperatures up to 42 °C was also accompanied by reduced BBB permeability to sucrose or albumin (Preston, 1982). However, the time course for the temperature rise was slower in this study than in those that did find increased permeability (Lin and Lin, 1982; Sutton, 1979). Whatever the mechanism, controlled increase of BBB permeability by microwave exposure could also have practical applications. The uptake of hydrophilic drugs by the brain can be increased by microwave-induced hyperthermia, as has been shown for the acetylcholine antagonist methylatropine (Quock et al., 1986), the dopamine antagonist domperidone (Quock et al., 1987) and the chemotherapeutic compound methotrexate (Lin et al., 1998). On the other hand, exposure to microwaves at thermal levels may make the brain more vulnerable for infections. Following microwave exposure at 2.5 GHz with SARs between 24 and 98 W/kg, increased BBB permeability to HRP was accompanied by increased lethality of Japanese enecephalitis virus (Lange and Sedmak, 1991).

5.2. *'Non-thermal' EMF exposure*

The distinction between thermal and 'non-thermal' effects of RF exposure is less clear than it seems. Even small, local temperature increases of tenths of degrees Celsius, which are difficult to measure or control for, can result in biological effects such as increased protein synthesis or, more importantly, cerebral blood flow (Foster and Glaser, 2007; Katz-Brull et al., 2006). For the purposes of this review, I will define 'non-thermal' exposure as EMF exposure that does not cause a measured increase in brain temperature of more than 1 °C, or is unlikely to cause such a temperature increase in view of the calculated brain SAR or measured external field strength. It could be argued that the expression of RF exposure in terms of SAR is irrelevant for 'non-thermal' effects. However, the SAR is also a measure of the extent to which the external RF field generates an internal electric field in the body, that is: SAR is a function of the strength of the induced electric field (Habash et al., 2003).

A summary of the effects for different types of EMF exposure will be given in the text. For technical details and easier comparison of the individual studies, the reader is referred to Table 2. The table only includes those studies in which permeability was quantitatively assessed and the results statistically analysed.

5.2.1. *Microwave exposure*

Formally, the term 'microwaves' can encompass EMF with frequencies between 300 MHz and 300 GHz (Pozar, 2005). However, the earliest studies of the relation between 'non-thermal' EMF exposure and BBB permeability used microwave frequencies between 1 and 3 GHz of the type generated by microwave ovens and radar for air traffic control. Initially, two studies using Na-fluorescein as a tracer (Frey et al., 1975) or the Oldendorf method (Oscar and Hawkins, 1977) found increased BBB permeability after 20 to 30 min of head exposure to EMF with an external field power density of 2 to 24 W/m^2 and unknown SAR. Pulsed exposure seemed to be more efficient than continuous wave exposure. One possible mechanism for the effect would be increased cerebral blood flow, which was subsequently reported during 'non-thermal' microwave exposure (Oscar et al., 1981). However, a number of independent studies with similar incident power densities and exposure times subsequently failed find increased BBB permeability. Some of these used the same cerebral flow-dependent tracer methods (Lin and Lin, 1980; Merritt et al., 1978; Preston et al., 1979; Williams et al., 1984a; Williams et al., 1984b), while others used the cerebral flow-independent intravenous bolus–time integral method (Gruenau et al., 1982; Preston and Prefontaine, 1980; Ward and Ali, 1985; Ward et al., 1982; Williams et al., 1984d). No differences were found between pulsed or continuous exposure. External field power densities lay in the range of 1 to 750 W/m^2 and calculated head SARs lay in the range of 0.08 to 11.5 W/kg. It should be noted that a significant effect of microwave exposure was found when the data of one of these studies (Merritt et al., 1978) were re-analysed with more appropriate statistical methods. However, despite the relatively low power densities used, body temperature in that study may have been elevated by more than 1 °C due to resonant absorption (Justesen and Baird, 1979). Two additional histological studies in rats and hamsters with an incident power density of 100 W/m^2 and a calculated SAR of 2.5 W/kg found HRP staining in endothelial cells and adjacent nerve tissue, but this was not quantified (Albert, 1979a; Albert and Kerns, 1981). A study with similar incident power density and a calculated SAR of up to 2 W/kg found increased uptake of rhodamine-ferritin in endothelial cells, but BBB permeability was not assessed (Neubauer et al., 1990). In an elegant behavioural pharmacology paradigm, a 45 min exposure of rats to 2.5 GHz pulse-modulated EMF at a whole body SAR of 2 W/kg failed to potentiate the effect of a peripherally injected muscarinic acetylcholine antagonist with poor BBB permeability. No extravasation of Evans blue was found in a separate group of EMF-exposed rats (Cosquer et al., 2005).

Taken together, experimental evidence in laboratory animals indicates that acute, 'non-thermal' microwave exposure does not increase BBB permeability.

5.2.2. *Mobile phone exposure*

BBB permeability has mainly been investigated after exposure to two types of mobile phone signal: the 900 MHz band of the Global System for Mobile communications (GSM) and the 1.4 to 1.5 GHz band of the Japanese Personal Digital Cellular (PDC) system. In a series of experiments with exposure in the 900 MHz band at different whole body SAR levels (0.02–8.3 W/kg) and pulse frequencies, a Swedish group found increased BBB permeability to albumin in the rat brain for some treatment combinations, without any obvious dose–response relation (Persson et al., 1997, preliminary findings in Salford et

al., 1993 and Salford et al., 1994). Unfortunately, analysis was qualitative only ("one larger or several leakages"=positive), there was no mention of observer blinding and exposure duration varied from 2 to 960 min. A follow-up study found a higher score for the occurrence of 'dark neurons' 7 weeks after a 2-hour exposure at 0.002 to 0.2 W/kg, but the results of the albumin immunohistochemistry in the same study were not analysed quantitatively (Salford et al., 2003). A subsequent report by the same group with blinded analysis found increased BBB permeability for albumin 2 weeks (but not 4 weeks) after a 2-hour exposure at whole body SARs of 0.00012 to 0.12 W/kg, with no apparent dose–response relation. Albumin extravasation was accompanied by increased prevalence of albumin uptake in neurons. An increased occurrence of 'dark neurons' was seen 4 weeks after exposure (Eberhardt et al., 2008). In contrast, no increase in albumin extravasation or neuronal damage was found 5 to 7 weeks after the last of 55 weekly 2-hour exposures at SARs of 0.0006 to 0.06 W/kg (Grafstrom et al., 2008). A study reporting increased BBB permeability for manganese ions following mobile phone exposure at 900 MHz (Vojtisek et al., 2005) is of limited use, since the magnetic flux density, power density and SAR were unknown, quantitative data were not reported, and manganese is actively transported (Yokel, 2009).

Permeability studies by other research groups using mobile phone signals have generally shown no evidence for increased permeability at 'non-thermal' exposure levels. Immediately or 7 days following exposure at 900 MHz of rats to a head SAR of 0.3 to 1.5 W/kg or mice to whole body SARs of 0.25 to 4 W/kg, research groups in Germany and Australia found no effect on the absolute number of vessels with albumin extravasation. The German group did find a reversible increase at a head SAR of 7.5 W/kg, but the parametric statistics used were probably inappropriate for the low number of counts registered. Exposure durations varied from 1 to 4 h per day, either acutely or chronically for up to 104 weeks. The exposed rodents included adults, juveniles and fetuses in utero (Finnie and Blumbergs, 2004; Finnie et al., 2001, 2002, 2006a,b; Fritze et al., 1997). Likewise, exposure of rats to whole body SAR of 0.3 W/kg (head SAR 0.6 to 6 W/kg) in the Japanese mobile phone frequency band (1.4 to 1.5 GHz) did not increase the number of albumin extravasations or intensity of brain staining for fluorescently labeled dextran. Either acute exposure for 10 min or chronic exposure (1 to 4 weeks, 5 or 6 days per week, 1 to 1.5 h per day) was used (Kuribayashi et al., 2005; Masuda et al., 2007a,b; Tsurita et al., 2000). Two of these studies measured real-time extravasation of FITC-dextran from pial vessels through a cranial window (Masuda et al., 2007a,b), which may not be entirely representative for permeability in deeper brain vasculature (Hawkins and Egleton, 2008). Recently, three independent studies were published that were specifically designed to reproduce the exposure conditions of the Swedish group and used the same rat strain, the Fischer 344. The choice of this strain may be relevant, since its reactivity to stress differs from that in other rat strains (Shepard and Myers, 2008; Wakizono et al., 2007). The rats were exposed in the 900 MHz band to a whole body SAR of 0.0018 to 20 W/kg or head SAR of 0.14–2 W/kg for 30 min or 2 h and assessed either directly following exposure or after 2 to 7 weeks. No effect on the number of albumin extravasations, the number of 'dark neurons' or other neurodegenerative markers in the brain was found (Masuda et al., 2009; McQuade et al., 2009; Poulletier de Gannes et al., 2009). Besides more quantitative analysis methods, these studies usually added a positive control for increased BBB permeability (heat, cold, toxin, hypertonic injection) and an undisturbed home cage control for possible non-specific effects of the exposure cage such as stress (Table 2). They also used improved methodology in the areas of dosimetry, tissue fixation, albumin staining, neuronal staining, morphology and reduced variability in age and sex.

Despite its drawbacks, two recent studies have used Evans blue, a low molecular mass marker, to assess BBB permeability following GSM exposure in rats. The first found no histological evidence for increased permeability or neuropathology after chronic exposure (5 weeks, 5 days per week, 2 h per day) to 900 MHz EMF with a whole body SARs of 0.3–3 W/kg (Kumlin et al., 2007). The second found increased staining of homogenised brain tissue after a single 20-minute exposure to 900 or 1800 MHz EMF with an external electric field strength of 13 V/m but unknown SAR. The relevance of this finding is unclear, since exposure occurred under anaesthesia and the effect was found in male but not in female rats (Sirav and Seyhan, 2009). Another study in rats measured the concentration of fluorescently labeled albumin in the cerebrospinal fluid after intravenous injection. This would reflect the balance between blood–cerebrospinal fluid barrier permeability and cerebrospinal fluid volume and resorption. No effect of a 30-minute exposure to a 1.5 GHz PDC signal at 10 W/kg was found (Ushiyama et al., 2007). One possible secondary effect of increased BBB permeability is increased expression of aquaporin water channels to increase water clearance from the brain parenchyma. No change in aquaporin expression has so far been reported following exposure to 'non-thermal' levels of mobile phone EMF (Finnie et al., 2009; Kuribayashi et al., 2005). Finally, the concentration in the blood of proteins synthetised in the central nervous system was measured in humans before, during and after a 30-minute exposure of the head to a 900 MHz band GSM signal with local SAR of 1 W/kg. The concentration of one of the markers increased 60 min after exposure (Soderqvist et al., 2009). Since no sham control group was used and other factors may influence blood concentrations of these proteins, better controlled follow-up studies should be performed before any conclusion can be drawn.

Taken together, experimental evidence in laboratory animals indicates that acute or chronic exposure to 'non-thermal' levels of EMF from GSM/PDC-type mobile phones does not increase BBB permeability. No peer-reviewed research has so far been published that assessed BBB permeability in vivo following exposure to EMF of third generation mobile phones such as those using the Universal Mobile Telecommunications System (UMTS).

5.2.3. *MRI exposure*

Magnetic resonance imaging (MRI) uses three types of field to image tissue properties. The static magnetic field aligns the magnetic moments of the nuclear spins of hydrogen nuclei to the direction of the field. The gradient fields are pulsed EMF which superimpose small linear variations in magnetic flux density on the static field that help localise the magnetic

Table 2 – Overview of experimental studies into the effect of 'non-thermal' EMF exposure on BBB permeability.

Frequency [a]	Intensity [b]	Duration [c]	Tracer	Detection [d]	Quantitative	Anaesthesia	Control groups [e]	Quality score (%) [f]	Reference (first author, year)
A. Studies reporting a significant effect of 'non-thermal' EMF on BBB permeability									
1.2 GHz [P]	2–24 W/m^2	30 min [0]	Fluorescein	Staining intensity	Semi	Y	Sham	19	Frey et al., 1975
1.3 GHz [C/P]	3–20 W/m^2	20 min [0.1–24 h]	Mannitol, inulin [g]	Concentration [b]	Y	Y/N	Sham	45	Oscar and Hawkins, 1977
MRI	(all 3 fields)	23 min [0]	HRP	Staining intensity	?	Y	Sham	16	Shivers et al., 1987
MRI	(all 3 fields) [h]	40 min [10 min]	Mannitol	Concentration [b]	Y	Y	Sham	42	Garber et al., 1989
MRI	(all 3 fields)	46 min [1 h]	Gd-DTPA	Concentration [b]	Y	Y	Sham	45	Prato et al., 1990
MRI	(all 3 fields)	46 min [1 h]	Gd-DTPA	Concentration [b]	Y	Y	Sham	52	Prato et al., 1994
915 MHz [C/P]	0.02–8.3 W/kg [B]	2–960 min [0.3–2 h]	Albumin	# positive vessels	N	N	Sham	47	Salford et al., 1993, 1994; Persson et al., 1997
900 MHz [P]	0.01, 0.12 W/kg [B]	2 h [2 wk]	Albumin	# positive vessels	N	N	Sham	43	Eberhardt et al., 2008
900 MHz [C/P]	0.012 W/kg [B]	2 h [7 d]	Albumin	# positive vessels	N	N	Sham	43	Nittby et al., 2009
900, 1800 MHz	14 V/m	20 min [15 min]	Evans blue	Concentration [a]	Y	Y	Sham	50	Sirav and Seyhan, 2009
50 Hz	5 mT	3 h, 30 d [1 d]	Evans blue	Concentration [a]	Y	N	Sham, home	67	Gulturk et al., 2010
B. Studies reporting no significant effect of 'non-thermal' EMF on BBB permeability									
1.2 GHz [P]	20–750 W/m^2	30 min [0]	Fluorescein, Mannitol, inulin	Staining intensity Concentration [b]	Y Y	Y	Sham, positive	45	Merritt et al., 1978
2.5 GHz [C]	1–300 W/m^2	30 min [0]	Mannitol	Concentration [b]	Y	Y	Sham, positive	48	Preston et al., 1979
2.5 GHz [C]	0.08–1.6 W/kg [H]	30 min [25 min]	Sucrose	Concentration [c]	Y	Y	Sham, positive	58	Preston and Prefontaine, 1980
2.5 GHz [P]	0.08–11.5 W/kg [H]	20 min [0]	Fluorescein, Evans blue	Staining intensity	Semi	Y	Sham	53	Lin and Lin, 1980
1 GHz [C]	20–3000 W/m^2	20 min [0.3–5 h]	Albumin	Concentration CSF	Y	N	Sham	42	Chang et al., 1982
2.8 GHz [C/P]	10–400 W/m^2	30 min [30 min]	Sucrose	Concentration [c]	Y	Y	Sham	48	Gruenau et al., 1982
2.5 GHz [C/P]	2–6 W/kg [B]	30 min [10 min]	Sucrose	Concentration [c]	Y	Y	Sham, positive	68	Ward et al., 1982
2.5 GHz [C]	4 W/kg [B]	30–180 min [10 min]	Fluorescein	Concentration [a]	Y	N	Sham, positive	66	Williams et al., 1984a
2.5 GHz [C]	4 W/kg [B]	30–180 min [10 min]	HRP	# positive vessels	Semi	N	Sham, positive	59	Williams et al., 1984b
2.5 GHz [C]	4 W/kg [B]	30–90 min [10 min]	Sucrose	Concentration [c]	Y	N	Sham, positive	71	Williams et al., 1984c
1.7 GHz [C/P]	0.1 W/kg [H]	30 min [5 min]	Sucrose, inulin	Concentration [c]	Y	Y	Sham	58	Ward and Ali, 1985
MRI	all 3 fields	23 min [7 min]	Sucrose	Concentration [c]	Y	Y	Sham, positive	39	Preston et al., 1989
TMS	2 T	10 min, 1–7d [2 h]	Sucrose, urea	Concentration [d]	Y	Y	Sham?	61	Ravnborg et al., 1990
MRI	all 3 fields	2 h [0]	Albumin	Concentration [c]	Y	Y	Sham	39	Liburdy et al., 1992
900 MHz [C/P]	0.3–1.5 W/kg [H] [i]	4 h [0–7d]	Albumin	# positive vessels	Semi	N	Sham, home, positive	62	Fritze et al., 1997
1.5 GHz [P]	2 W/kg [H]	1 h, 5 d, 2–4 wk [1–48 h]	Albumin, Evans blue	# positive vessels	?	N	Sham, home, positive	38	Tsurita et al., 2000

900 MHz [P]	4 W/kg [B]	1 h [0]	Albumin	# positive vessels	Semi	N	Sham, home, positive	62	Finnie et al., 2001
900 MHz [P]	0.25–4 W/kg [B]	1 h, 104 wk [2 h]	Albumin	# positive vessels	Semi	N	Sham, home, positive	64	Finnie et al., 2002; Finnie and Blumbergs, 2004
50 Hz	5 mT	8 h, 3 wk [0]	Evans blue	Concentration [a]	Y	N	Sham	32	Oztas et al., 2004b
2.5 GHz [P]	2 W/kg [B]	45 min [0]	Scopolamine	Behaviour	Y	N	Sham, home, positive	69	Cosquer et al., 2005
			Evans blue	# positive vessels	N[j]				
1.4 GHz [P]	0.3 W/kg [B]	1.5 h, 6 d, 1–2 wk [0]	FITC-dextran	Staining intensity	Y	N	Sham, positive	63	Kuribayashi et al., 2005
900 MHz [P]	4 W/kg [B]	1 h, 19 d [?]	Albumin	# positive vessels	N[j]	N	Sham, home, positive	43	Finnie et al., 2006b
1.4 GHz [P]	0.6–4.8 W/kg [H]	10 min [0–20 min]	FITC-dextran, fluorescein	Staining intensity	Y	N	Sham	65	Masuda et al., 2007a
1.4 GHz [P]	2.4 W/kg [H]	1 h, 5 d, 2–4 wk [0–1 d]	FITC-dextran, Fluorescein	Staining intensity	Y	N	Sham, home	71	Masuda et al., 2007b
1.5 GHz [P]	0.5–10.5 W/kg [H]	30 min [0–150 min]	Albumin	Concentration CSF	Y	Y	Sham	48	Ushiyama et al., 2007
900 MHz [P]	0.3–3 W/kg [B]	2 h, 5 d, 5 wk [0]	Evans blue, IgG	# positive vessels	Semi	N	Sham	63	Kumlin et al., 2007
900 MHz [P]	0.0006–0.6 W/kg [B]	2 h, 55 wk [5–7 wk]	Albumin	# positive vessels	N	N	Sham, home	57	Grafstrom et al., 2008
900 MHz [C/P]	0.0018–20 W/kg	30 min [15 min]	Albumin	# positive vessels	Semi	N	Sham, home, positive	69	McQuade et al., 2009
900 MHz [C/P]	0.14–2 W/kg	2 h [2–7 wk]	Albumin	# positive vessels	Semi	N	Sham, home, positive	67	Poulletier de Gannes et al., 2009
915 MHz [C/P]	0.02–2 W/kg	2 h [2–7 wk]	Albumin	# positive vessels	N[j]	N	Sham, home, positive	83	Masuda et al., 2009

Only studies with quantitation and statistical analysis of BBB permeability are listed.
Abbreviations:
CSF, cerebrospinal fluid; FITC, fluorescein isothiocyanate; Gd-DTPA, gadolinium-diethylene triamine pentaacetic acid; HRP, horseradish peroxidase; IgG, immunoglobulin G; MRI, magnetic resonance imaging; TMS, transcranial magnetic stimulation.

[a] [C], continuous wave; [P], pulsed.

[b] For studies reporting a significant effect, the range of effective intensities is given; [H], head-only exposure, local SAR; [B], whole body exposure and SAR.

[c] First number indicates exposure time, number in square brackets indicates duration between end of exposure and permeability measurement.

[d] [a], Absolute concentration in tissue only; [b], Intra-arterial bolus injection and measurement of tracer and reference in brain (bloodflow-dependent); [c] Intravenous bolus injection, time integral measurement of tracer in blood, terminal measurement of tracer in brain (bloodflow-independent); [d], Intravenous infusion, (near)constant tracer concentration in blood, terminal measurement of tracer in brain (bloodflow-independent).

[e] Sham: treatment identical to EMF exposure group but without EMF exposure; home: home cage control group (undisturbed); positive: positive control for increased permeability.

[f] Percentage of maximum possible quality score (see Section 1).

[g] No increased permeability was found for a marker with larger molecular mass, dextran.

[h] The 3 types of MRI field were also tested separately; effect due to gradients.

[i] An increase in permeability was seen at the highest SAR tested: 7.5 W/kg.

[j] No extravasation was found.

resonance signal in the body. The RF field consists of pulses of RF energy that synchronise nuclear spins and change the direction of their magnetic moments, generating a new RF EMF whose speed of decay reflects tissue properties (McGowan, 2008). The pulse frequency of the gradient fields lies in the kHz range and that of the RF field in the MHz range. It is important to note that RF exposure during MRI can reach thermal levels under some circumstances, and the strength of the internal electric fields induced by the gradients can exceed safety limits for occupational exposure in international safety guidelines (Stam, 2008). Experiments on BBB permeability following MRI exposure have all been conducted in rats under general anaesthesia. The rate of change of the gradient field flux density in these experiments lay in the range of 0.1 to 3 T/s. The external field strength or SAR of the RF field was usually unknown, but in those studies where it was estimated the whole body SAR was less than 0.1 W/kg (Prato et al., 1994).

A Canadian group reported increased BBB permeability following a 23- or 46-minute exposure to all three MRI fields, using staining for HRP or the Oldendorf method (Prato et al., 1990; Shivers et al., 1987). In a third study, also using the Oldendorf method, one combination of strengths of gradient and RF exposure increased BBB permeability, but a different combination decreased BBB permeability. Static field exposure alone had no effect (Prato et al., 1994). The control groups were exposed to the same gradient noise as the MRI-treated rats, but gradient-induced vibrations or microwave hearing may also have acted as confounders (see Section 6.1). An independent study tried to disentangle the effects of the three MRI fields by including groups with separate 40-minute exposures to static, gradient or RF fields. Increased BBB permeability was found with combined exposure or gradient field exposure alone, using the Oldendorf method (Garber et al., 1989). It is possible that gradient noise or vibrations acted as confounders, though the highest intensity of combined MRI exposure had no effect. The MRI studies discussed so far used HRP as a histological tracer or the intra-arterial bolus method, both of which are influenced by changes in cerebrovascular flow or volume. Two other studies investigated 23-minute or 2-hour exposure to all three MRI fields and either used the flow-independent intravenous bolus–time integral method (Preston et al., 1989) or radioactivity-labeled albumin method with a constant blood concentration during most of the experiment (Liburdy et al., 1992). Neither of these found a change in BBB permeability. It is unclear whether the discrepancy between studies that do and those that do not report an EMF effect is caused by methodological differences, differences in field strengths and scanning protocol, or both.

Taken together, there is insufficient evidence for an effect of MRI exposure on BBB permeability and insufficient insight into which physical properties of MRI exposure might mediate any effect.

5.2.4. *ELF*

One study has investigated the effect on BBB permeability of the type of pulsed magnetic field used in transcranial magnetic stimulation, which can cause electrical stimulation of neurons. Fifty 4 ms pulses with a peak magnetic flux density of 1.9 T were applied in 15 min and BBB permeability was measured with the intravascular infusion-time integral method. No changes in permeability were found either in anaesthetised or awake, restrained rats (Ravnborg et al., 1990). Another study applied 200 or 400 pulses of 200 kV/m at 1 Hz to awake, unrestrained rats and found microscopic evidence for extravasation of albumin and lanthanum nitrate 1 to 6 h later (Ding et al., 2009). Since pulse duration was not given and permeability was not quantified, no conclusions can be drawn. Two studies have investigated the effect of chronic exposure of rats to 50 Hz EMF with a magnetic flux density of 5 mT. Although the induced electric field was not calculated, this flux density is 10 times higher than the occupational reference level of the International Commission for Non-Ionizing Radiation Protection, which serves to protect against potentially harmful levels of electrical stimulation of the brain (ICNIRP, 1998). The first study found no significant effect on homogenised brain content of Evans blue after 3 weeks of 8-hour per day exposure (Oztas et al., 2004b). The second study found a significant increase in homogenised brain content of Evans blue after 4 weeks of 3-hour per day exposure (Gulturk et al., 2010).

Thus, the four studies published so far do not indicate that acute, pulsed low frequency magnetic field exposure increases BBB permeability, but give equivocal evidence for an effect of chronic exposure to 50 Hz EMF on permeability of the BBB. The positive study with 50 Hz EMF needs to be reproduced, preferably at several different flux densities and with more reliable methods to assess BBB permeability (see Section 4.1).

5.2.5. *In vitro studies*

Properties of the BBB can be mimicked to a degree using in vitro cell or tissue cultures. Systems used include isolated brain capillaries, primary cultures or immortalised lines of endothelial cells or choroid plexus endothelial cells. They provide the added value of being able to investigate molecular and cellular mechanisms in detail under controlled conditions. However, quantitative analysis and extrapolation to the complex and interactive neurovascular unit in vivo is still difficult (Hawkins and Egleton, 2008; Reichel et al., 2003).

Three studies by the same research group have tested the effects of the type of EMF produced by mobile phones. The earliest used a co-culture of rat astrocytes and pig brain capillary endothelial cells and found increased permeability to sucrose following 4 days of exposure to a 1.8 GHz GSM signal with an average SAR of 0.3 W/kg (Schirmacher et al., 2000). However, no increased permeability was found in a follow-up study under the same exposure conditions when different endothelial cell cultures were used. These culture systems had a lower permeability to sucrose in the control group without EMF exposure that better reflected the in vivo situation (Franke et al., 2005a). In a separate study with the same low permeability model, the effect of 3.5 days of exposure to a 1.97 GHz UMTS signal with an average SAR of 1.8 W/kg was tested. Permeability to sucrose or albumin was not changed by EMF exposure, but increased by a positive control (temperature increase to 39 °C) (Franke et al., 2005b). The permeability of a cultured monolayer of endothelial cells to an antiretroviral drug was found to be increased after exposure to a 915 MHz EMF, but the relevance of this finding cannot be judged in the absence of relevant dosimetric information (Kuo and Kuo, 2008).

Taken together, the studies using in vitro models that show the closest resemblance to the BBB in vivo do not indicate that 'non-thermal' exposure to EMF from mobile phones increases permeability. Publications on the effects of other types and frequencies of EMF are not yet available.

6. Discussion

6.1. *Methodological issues*

6.1.1. *General experimental issues*

Three types of disturbing factors could influence the results of experimental studies into the causal relationship between EMF exposure and altered BBB permeability. Firstly, factors that affect only the EMF-exposed group but are unrelated to the direct effect of EMF on BBB permeability may act as a confounder. One example is the vibrations of the active gradient coils in the MRI-scanner, if the sham group is exposed to taped noise outside the scanner. Any such stimulus associated with the EMF exposure (light, sound or touch cues) must be avoided at all cost. Another example is the microwave hearing induced by certain types of pulsed RF field, which is caused by thermoelastic expansion of the skull (Elder and Chou, 2003; Lin and Wang, 2007). It is very difficult to control for, since it is an integral part of the EMF signal, and can only be excluded when the energy density per pulse is clearly below the threshold for thermoelastic expansion (see: "EMF exposure issues"). When the RF field is part of an MRI scan though, the noise or vibrations generated by the gradient coils may dominate any auditory effect of the RF field (Elder and Chou, 2003).

Secondly, factors that affect both the EMF-exposed group and the sham-exposed control group (anaesthesia, restraint, noise) could increase the sensitivity of the BBB for an additional disturbance caused by EMF. This would mean that the effect of EMF may be real, but would be expected to occur at a higher threshold in undisturbed animals. On the other hand, such factors could also prevent the detection of an EMF effect on BBB permeability or even reverse its direction. For example, some forms of anaesthesia such as barbiturates induce hypothermia unless this is controlled for. There are indications in the literature that hypothermia can either increase (Elmas et al., 2001) or decrease (Krantis, 1983) BBB permeability. If anaesthesia-related hypothermia increases permeability in all treatment groups, sufficiently strong RF exposure could mitigate the hypothermia and thereby actually reduce BBB permeability compared to the control group. Anaesthetics may also directly influence membrane properties and thereby permeability (Justesen, 1980). Even in animals that have been 'habituated' to the procedures, confinement in small compartments ('restraint') can induce a rise in core body temperature and blood levels of hormones of the hypothalamo-pituitary adrenal axis. If an increase in body temperature or blood flow mediates increased BBB permeability, a larger increase caused by restraint stress may mask any smaller effect caused by EMF (Stagg et al., 2001). Depending on the frequency and duration of individual sessions, repeated restraint can either sensitise or reduce subsequent responses to stress (Stam, 2007). This may exacerbate the problem in experiments using chronic, daily exposure of rats to EMF in restricted compartments. The addition of a second, undisturbed home cage control group could unmask any effect of the sham procedure on BBB permeability. In those studies that have included such a home cage control, however, there is no significant effect of sham exposure on the number of vessels with albumin extravasation, even though the absolute number of extravasations is sometimes greater in the sham group than in the home cage control group (Finnie et al., 2001, 2002; Fritze et al., 1997; McQuade et al., 2009).

Thirdly, some factors may increase the baseline for BBB permeability in all three groups (EMF-exposed, sham-exposed and home cage control). One example is the occurrence of asymptomatic infections with certain viruses in laboratory rodents (Franke, 2003). Another example would be the use of a particular rodent strain with a different barrier function compared to other strains (Riachi et al., 1991). Such differences may explain why some studies find no extravasations in any group (Masuda et al., 2009), while others find a low number of extravasations in all treatment groups (Finnie et al., 2001; McQuade et al., 2009; Poulletier de Gannes et al., 2009). When animals within each treatment group are exposed and measured at different times of the day, circadian variations in BBB permeability may increase the within-group variance and therefore decrease the likelihood of detecting an effect of the experimental treatment (Prato et al., 1994).

6.1.2. *EMF exposure issues*

One problem in translating exposure sources for humans to experimental studies in rodents is that the frequency of maximum RF energy absorption depends on body size, shape, orientation and composition. Maximum ('resonant') absorption in rats at lies in the frequency range of microwave and mobile phone exposure used in BBB permeability studies (0.5 to 3 GHz), but would scale to about 100 MHz in humans. This factor can in principle be taken into account in SAR calculations, but presents a problem for those studies that only use the external field strength to set exposure levels (Michaelson, 1986). Penetration depth relative to head size is also expected to be greater in laboratory rodents than in humans and their tissue parameters, heat redistribution and dissipation mechanisms differ. Another potential source of inaccuracies in exposure level is the RF exposure cell. Exposure cells that are relatively small compared to the animal's body size may cause an inhomogeneous field distribution in the body and can result in extreme variations in whole body SAR between animals. Differences in weight and size between individual animals could introduce additional variability. It has been suggested that the animal must not fill more than 30% of the exposure cell to give a clear dosimetry and homogeneous field distribution (Franke, 2003).

The majority of studies into the effect of mobile phone type fields on BBB permeability in conscious rodents have used whole body exposure (Table 2). This will result in a more homogeneous distribution of RF energy across the body than head-only exposure, which would result in a SAR distribution that more closely resembles that in the human head during mobile phone use (Christ et al., 2005). On the other hand, head-only exposure in rodents may not be possible without using restraint or anaesthesia. The disadvantages of these alternatives have to

be carefully weighed for each experiment. One interesting hypothesis is that the highly localised peaks in brain SAR ('hot spots') found for mobile phone exposure in humans (Moneda et al., 2003) would contribute to highly localised changes in BBB permeability, for example via local increases in blood flow. Such inhomogeneities have not been found in the rat (Ward et al., 1986), but it may be that the measurement resolution in small laboratory animals is still insufficient.

Microwave hearing (or the equivalent stimulation of auditory pathways in the brain in anaesthetised animals) can occur for pulsed EMF between hundreds of kHz and tens of GHz. It is caused by thermoelastic expansion of the skull which is transferred to the auditory system at or distal to the cochlea (Elder and Chou, 2003; Lin and Wang, 2007; Postow and Swicord, 1986; Roschmann, 1991). The response depends on the energy density of the pulse, rather than average SAR. Thresholds in both humans and rodents lie in the range of 1–50 μJ/cm^2 (Elder and Chou, 2003). This phenomenon can occur during exposure to RF fields in MRI and could theoretically contribute to effects on the BBB where pulsed microwaves are more effective than the continuous wave signal of the same frequency (Frey et al., 1975; Oscar and Hawkins, 1977). However, similar energy densities are found in microwave studies that do not report an effect on BBB (Lin and Lin, 1980; Merritt et al., 1978). It is likely that any role of the microwave hearing phenomenon would be highly dependent on the proximity of the source and on experimental conditions such as background noise, which may vary between different studies.

6.2. *Pathophysiological significance of altered permeability*

6.2.1. *Extent of increased permeability in positive studies*

In those studies that report increased permeability following 'non-thermal' exposure using histological methods, no exact information is available on the number of blood vessel cross sections showing leakage. Increased permeability is either defined as "one larger or several leakages" (Nittby et al., 2009; Persson et al., 1997) or "score of 1 or higher on a scale of 0 to 3" (with 8 leakages scoring "2") (Eberhardt et al., 2008). This would suggest a number of positive vessels per section of less than 10. The number of venules greater than 16 μm diameter per rat brain section lies in the order of 200–300 (Park et al., 2008), and a similar number of arterial cross sections greater than 10 μm can be found in 24 mm^2 areas of the human cortex, basal ganglia and thalamus (van der Zwan et al., 1993). The total number of arterial and venous cross sections of all diameters per rat brain section would thus be estimated to exceed 500. Low-level extravasation of albumin is often found in control animals (Finnie et al., 2001, 2002; Fritze et al., 1997; McQuade et al., 2009; Poulletier de Gannes et al., 2009), and it is unclear why an increased permeability of less than 10 vessels per section would have any pathophysiological significance. In studies using thermal levels of exposure, the increase in permeability is reversed within 30 to 120 min after cessation (Albert and Kerns, 1981; Lin and Lin, 1982). It is therefore unclear why the Swedish studies still found an increase 1 and 2 weeks after 'non-thermal' RF exposure (Eberhardt et al., 2008; Nittby et al., 2009). Such long-term effects were not found in two independent replication studies (Masuda et al., 2009; Poulletier de Gannes et al., 2009).

6.2.2. *Toxicity*

Limited entry of albumin into the brain is a normal event, not so much directly via BBB but by diffusion from other points of entry such as the subarachnoid space and circumventricular organs. The cerebrospinal fluid normally contains albumin at one 200th of the concentration in plasma. Since albumin does not have a specific brain to blood efflux mechanism and is resistant to enzymatic degradation, it can slowly diffuse throughout the brain (Banks, 2004). Albumin may be toxic to brain in higher concentrations, and may cause astrocyte proliferation (Begley and Brightman, 2003; Hassel et al., 1994). The critical local tissue concentration, however, is not known. Since the immunohistochemical detection of albumin is enhanced by antibodies, quantitative estimation of local concentration with this method is difficult (Franke, 2003). A more precise determination of albumin concentration in brain tissue in future experiments is therefore important (Szymas and Hossmann, 1990). Increased prevalence of albumin extravasation 2 weeks after mobile phone EMF exposure was found to correlate with the prevalence of 'dark neurons' 4 weeks after exposure (Eberhardt et al., 2008). This correlation is not necessarily causal, and the increase in 'dark neurons' has not been reproduced by other groups (Kumlin et al., 2007; Masuda et al., 2009; Poulletier de Gannes et al., 2009). Furthermore, 'dark neurons' usually reflect either a reversible contraction of the neuronal cell body, for example following osmotic stimuli, or an artifact produced by post-mortem fixation or processing of brain tissue (Jortner, 2006).

6.2.3. *Permeability changes induced by other stimuli*

Opening of tight junctions via cytoskeletal proteins can be an active process in response to stimuli from the internal environment (Persidsky et al., 2006). Cell types involved in modulating BBB permeability following stimuli include glia, mast cells and neurons (Abbott, 2000). Psychological stress such as that induced in rats by immobilisation or forced swimming can be associated with increased BBB permeability (Esposito et al., 2001; Madrigal et al., 2002), earlier papers summarised in (Bested et al., 2001). The effect of stress is not always found and may depend on the type of stressor used or on the age of the animals (Sinton et al., 2000). Where the same methodology has been used by the same research group, experimental stress increases permeability to a greater degree than EMF exposure (Oztas et al., 2004a,b). It is not yet clear to what degree the effect of stress is mediated by direct interactions between neurons and BBB components or via indirect effects such as mast cell secretion (Esposito et al., 2001), increased body temperature or increased blood pressure or flow (Fishman, 1997). Acute hypertension can reversibly increase BBB permeability. This is a direct effect of mechanical stress on endothelial cells, which may increase both transepithelial (vesicular) and paracellular transport (Johansson, 1980). Pain can also increase BBB permeability via neural pathways or the release of inflammatory mediators (Wolka et al., 2003). Pain is often accompanied by cardiovascular reflexes such as increased blood pressure, and it is possible that these also contribute to increased BBB permeability (Ness and Gebhart, 1990).

The BBB becomes more permeable to macromolecules such as HRP in a variety of inflammatory conditions caused by infections with microorganisms, autoimmune responses,

traumatic injury and exposure to toxic or hypertonic chemicals. Although opening of tight junctions may play a role in some of these conditions, there is also evidence for increased trans-endothelial transport via the formation of vesicles and tubules. There are indications that leukocytes and microorganisms can use both paracellular and transcellular routes to pass the BBB (Lossinsky and Shivers, 2004). Locally released inflammatory mediators such as bradykinin, arachidonic acid, histamine and nucleotides play an important mediating role (Abbott, 2000). In peripheral inflammatory conditions, BBB opening may occur indirectly via the experience of pain (Hawkins and Egleton, 2008). It is not clear to what extent BBB permeability changes act to exacerbate or to inhibit the disease process.

Various medical diagnostic or therapeutic treatments (urea, contrast agents, hypnotics and anaesthetics, antidepressants) increase BBB permeability without apparent long-term adverse effects (Justesen, 1980). Osmotic opening of the BBB is mediated by vasodilatation, dehydration of endothelial cells and contraction of their cytoskeleton (Rapoport, 2000). Osmotic opening can be experimentally induced by intra-arterial injection of hypertonic solutions of mannitol, arabinose, lactamide, saline, urea or radiographic contrast agents. The concentration needed depends on the agent's lipid solubility (higher for greater lipid solubility). Osmotic barrier opening is usually performed under anaesthesia, since it generates a significant level of pain in conscious animals (Fortin, 2003). The degree of increased BBB permeability induced by hypertonic solutions is greater than for other stimuli, because bulk fluid flow following opening of the barrier adds to simple diffusion. Nevertheless, BBB tightness is usually restored within 10 min after injection (Rapoport, 2000). Thus, other stimuli such as stress, pain, inflammation and hypertonic solutions can induce increases in BBB permeability which are often of greater magnitude than those in studies that report an effect of EMF on permeability. It is far from clear whether such changes result in damage that is beyond the body's autoregulatory capacity.

7. Conclusions and recommendations for future studies

The balance of experimental evidence in animals points against an effect of 'non-thermal' RF fields with frequencies between 900 MHz and 3 GHz on permeability of the blood–brain barrier. Given the wide variety of exposure strengths, durations and conditions, time delays, assessment methods and replication efforts, it seems unlikely that additional animal experiments will add useful knowledge. However, there is an almost total lack of experimental studies on EMF and BBB permeability in humans, despite the fact that the technology for this has been available for more than fifteen years (Hara et al., 1988; Knudsen et al., 1994). In view of the highly localised exposure associated with mobile phone use, such studies in humans would be more useful than additional animal studies. Studies using third generation mobile phone systems have so far been rare, and should be explicitly included. Studies into the effect on BBB permeability of EMF generated by MRI have so far been inconclusive, due to lack of adequate dosimetry, possible confounding with gradient noise or vibrations, and simultaneous exposure to multiple field strength and frequencies. These problems will have to be addressed in any follow-up studies. In contrast with RF exposure, only a handful of studies have assessed the effects of ELF on BBB permeability. Carefully controlled animal studies on the effects of chronic exposure to power frequency EMF, using reliable and quantitative methodology to assess permeability, are needed before any conclusions can be drawn.

The advantages and disadvantages of the different techniques used to assess BBB permeability have been discussed in Section 4. Unfortunately, tracers such as Evans blue continue to be used in recent EMF studies, despite the practical problems and uncertainties associated with them. Future studies should preferably use quantitative histology for endogenous plasma proteins such as albumin and blood flow-independent physiological tracer methods. To compensate for the disadvantages of individual methods, to get more information on the mechanism and size of any permeability change and to minimise the chance of false-positives, it would be very helpful if more than one technique were used in a single study.

Any increase in permeability of the BBB to albumin would likely be accompanied by increased permeability for other, smaller and potentially neurotoxic molecules. These would need to be measured, along with a more quantitative assessment of protein extravasation, in future EMF studies. The experimental design, including the use of sham and home cage control groups, should maximise the chance of detecting an effect if it is really there and minimise the chance of false-positives and confounding between EMF and other environmental factors. Appropriate dosimetry should be applied to estimate the effect of the external field on the body in terms of induced electric field or SAR. The effects of signal modulation or different pulse sequences and properties have rarely been addressed and could be more systematically investigated. If a reproducible effect of EMF on BBB permeability is found, the extent and mechanism involved should be investigated, where appropriate using in vitro methods, and its pathophysiological significance in relation to other stimuli determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainresrev.2010.06.001.

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