Effect of Exposure to Electromagnetic Fields on Brain Function and Behaviour in Mice



Somerville College

Department of Pharmacology

Trinity 2017

A thesis submitted for the degree of Doctor of Philosophy

Louise Lundberg



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Abstract

There is a need for improved understanding of interactions between electromagnetic fields and biological tissues. In this thesis, the effects of exposure to 50 Hz magnetic fields, associated with power generation and use, and 1800 MHz fields associated with mobile phones were investigated with particular focus on the plastic processes that are involved in cognitive function.

After repeated, daily exposure of young adult C57Bl/6J mice to an 1800 MHz field at 3 W/kg, very subtle changes in expression of genes involved in synaptic plasticity were found ($p<0.05$). Spatial memory as measured in the water maze was not significantly affected by exposure. Exposure at 0.3 W/kg did not significantly affect any of the endpoints ($p>0.05$). Indications of a greater sensitivity to exposure at 3 W/kg were seen in a senescence accelerated prone mouse model (SAMP8) compared to a resistant strain (SAMR1). However, only subtle effects of exposure were seen.

Exposure of young C57Bl/6J mice to a 50 Hz field at 100 or 300 μT induced small but significant changes in expression in synaptic plasticity related genes ($p<0.05$). Furthermore, repeated exposure significantly increased microglial density in the dorsal hippocampus ($p<0.05$) and slightly decreased proliferation in the dorsal hippocampus (100 μT, $p<0.05$). Spatial memory was not significantly affected by exposure.

Acute exposure to a 50 Hz magnetic field for 30 minutes at 300 or 580 μT did not affect the adrenal response to a nocturnal white or blue light shock, while exposure at 580 μT in the absence of light significantly decreased *per1* expression in the adrenal glands ($p<0.05$), but not in the liver or dorsal hippocampus. Exposure at 580 μT for 24 hours had only minor transient effects on the rhythmic expression of the core clock genes.

In summary, exposure to 50 Hz or 1800 MHz fields caused subtle and transient changes to some molecular mechanisms and cells involved in cognitive function and circadian rhythm control.

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Acknowledgements

The present thesis is based on experiments carried out at the Department of Pharmacology at the University of Oxford, under the supervision of Professor Daniel C. Anthony, and at the Centre for Radiation, Chemical and Environmental Hazards (CRCE) at Public Health England, Chilton, under the supervision of Dr. Kerry A. Broom and Dr. Zenon J. Sienkiewicz.

I would like to thank you all for giving me the opportunity to work on this project and for sharing your great scientific knowledge. I am immensely grateful for your support, time and encouragement throughout these years.

I am also grateful to Public Health England for funding this work and to Somerville College, Oxford.

Huge thanks to my fellow group members in the Neurobiology and Physiology Group Amélie Gavard and Jutta Järvinen for your invaluable support, great company and for helping me out with experiments in stressful times.

A great thank you to Kevin Whitehill, Chris Ottaway and Graham Holliman for your help, and to other colleagues at Chilton who have provided help and support.

Also thank you to all the people in the Experimental Neuropathology Group at the Department of Pharmacology in Oxford in particular: Yvonne Couch, Iñes Sá Pereira, Jay Roodselaar, Kim Wals and Fay Probert.

Finally, a great thank you to Lars for moving with me to England and making this experience possible, for cheering and supporting throughout, and to mine and his families and friends for all your support.

Louise Lundberg

Abbreviations

3G	Third generation (of mobile phones)
A	Ampere
AD	Alzheimer's Disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APES	3-aminopropyltriethoxysilane
APP	Amyloid precursor protein
BBB	Blood brain barrier
BCA	Bicinchoninic acid assay
BDNF	Brain derived neurotrophic factor
BLBP	Brain lipid binding protein
BMAL1	Brain and Muscle ARNT-Like1
BrdU	5-bromo-2'-deoxyuridine
CA1-3	Cornu ammonis 1-3
CaMK	Calcium/calmodulin-dependent protein kinase
CCD	Charge coupled device
CLOCK	Circadian Locomotor Output Cycles Kaput
CO_2	Carbondioxide
CREB	cAMP response element binding protein
CRCE	Centre for Radiation, Chemical and Environmental effects
CT	Circadian time
CV	Cresyl violet
DAB	3,3'-diaminobenzidine
DCX	Doublecortin

DG	Dentate gyrus
DNA	Deoxyribonucleic acid
EAE	Experimental allergic encephalomyelitis
EC	Entorhinal cortex
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
EF	Electric field
ELISA	Enzyme-linked immunosorbent assay
EMF	Electromagnetic field
EPM	Elevated plus maze
EPSP	Excitatory postsynaptic potential
EtOH	Ethanol
FAD	Flavin adenine dinucleotide
FFPE	Formalin fixed paraffin embedded
GABA	Gamma-aminobutyric acid
GCL	Granule cell layer
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GluA	AMPA receptor subunit
GluN	NMDA receptor subunit
GSM	Global system for mobile communications
h	hour
HE	Hematoxylin and Eosin
HSP	Heat shock protein
HPLC	High performance liquid chromatography

Hz	Hertz
Iba-1	Ionized calcium binding adaptor molecule 1
IF	intermediate frequency field
IHC	Immunohistochemistry
IL	Interleukin
ipRGCs	intrinsic photoreceptive retinal ganglion cells
KA	Kainic acid
LD	Light Dark
LED	light emitting diode
LF	Low frequency
LS	Light shock
LTD	Long-term depression
LTP	Long-term potentiation
m	Meter
MAPK	Mitogen-activated protein kinase
MF	Magnetic field
MS	Multiple Sclerosis
ms	millisecond
NGF	Neurotrophic growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOL	Novel object location
NOR	Novel object recognition
NPY	Neuropeptide Y
NT	Neurotrophin (3, 4/5)
OD	optical density

PACAP	Pituitary adenylate cyclase-activating peptide
PBS	Phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PFC	Prefrontal cortex
PHE	Public Health England
Pp	Perforant path
PSD	Postsynaptic density
PVDF	Polyvinylidene fluoride
RAM	Radial arm maze
RF	Radio frequency
RNA	Ribonucleic acid
RT	Reverse transcription
S	Subiculum
SAM	Senescence Accelerated mouse
SAR	Specific energy absorption rate
SCN	Suprachiasmatic nucleus
SGZ	Subgranular zone
SOD-1	Superoxide dismutase
T	Tesla
TBE	Tris/borate/EDTA
TBST	Tris buffered saline with Tween
TF	Terahertz frequency
TNF	Tumor necrosis factor
TrK A-C	Tropomyosin receptor kinase A-C
TUNEL	Terminal deoxynucleotidyl transferase
UV	ultraviolet

V	Volts
w	week
W	Watt
WB	Western blot
WT	Wild type
ZT	Zeitgeber time

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List of presentations relevant to this thesis

Effects of 50 Hz magnetic fields on neuronal plasticity in the hippocampus of young mice

BioEM 2015

Lundberg, L.

Asilomar, California, USA

Platform presentation

Effects of 50 Hz magnetic fields on neuronal plasticity in the hippocampus of young mice

Lundberg, L; Broom, KA; Anthony, D C; Sienkiewicz, Z.

PHE PhD day 2015

PHE Chilton, UK

Poster presentation

Effects of 50 Hz magnetic fields on neuronal plasticity in the hippocampus of young mice

Lundberg, L; Broom, KA; Anthony, D C; Sienkiewicz, Z.

Harwell Campus Student's Day 2015,

Chilton, UK

Poster presentation

Chapter 1 Introduction

1.1 General introduction

We are constantly exposed to non-ionising electromagnetic fields (EMFs) from natural and man-made sources in our surroundings, e.g., the Earths static magnetic field, low frequency fields associated with electrical power and high frequency fields associated with broadcasting, mobile phones and WiFi (SCENIHR, 2015). The presence of EMFs in our surroundings has increased greatly in the past hundred years and a steep increase has been seen in the past 20 years with the introduction of mobile phones and WiFi to the general public. This has triggered a fear of EMF pollution in the environment for groups of the public, and a need for improved understanding of interactions between EMFs and biological tissues (SCENIHR, 2015). In this project the effects of the two major sources of exposure to the general public were chosen for investigation. These are 50 hertz (Hz) fields, associated with power generation and electrical appliances and 900-2100 MHz fields associated with mobile phones. High level exposures to these fields have known biological effects, however at low exposure levels, as those we encounter in our everyday lives, the effects of exposure are still unknown despite the vast number of studies that have been performed. This thesis focuses on effects on the brain, and specifically cognitive function; for mobile phone use, the brain is the primary area of exposure and for power frequency fields, the brain is particularly sensitive due to its electrical nature (ICNIRP, 2009; SCENIHR, 2015; WHO, 2007).

In relation to effects of EMFs on cognitive function, the sensitivity of the plastic processes involved in learning and memory formation, and the sensitivity of circadian rhythm control to EMF exposure were investigated. Several studies have suggested effects on these processes, but there are only a limited number of studies and the question of whether EMFs at environmentally relevant levels can affect these processes remains unanswered.

1.2 A brief introduction to learning and memory

Cognition can be defined as "the mental action or process of acquiring knowledge and understanding through thought, experience, and the senses" (Oxford Dictionaries). In the healthy brain cognitive function is supported by glial cells, trophic factors, and an effective reorganisation of synaptic proteins and connections that permits optimal function of the neuronal networks (Burke and Barnes, 2010; Patterson, 2015). However, in humans cognitive performance falls with age; this is seen as impairment in memory, verbal skills, ability to reason and visual perception (Hedden and Gabrieli, 2004). Furthermore these processes are known to be highly sensitive to inflammation, circadian rhythm disturbances, chemical pollution and changes involved in neurodegenerative disease, which can then cause transient or long lasting cognitive impairment at an earlier stage (Cunningham et al., 2009; Liu and Lewis, 2014; Smarr et al., 2014).

Learning and memory were chosen as focus of study in this project due to their importance for cognitive function and they can be assessed in rodents by behavioural, cellular and molecular testing.

The concept of memory includes the formation of memories through conscious or unconscious learning, storage, and memory retrieval. The mechanisms involved in short and long-term memory differ. In short-term memory already existing networks are fine tuned, while for memory consolidation and long-term storage of memories further changes are needed, including gene expression and protein synthesis (Kandel, 2001a)

Memories can be described as being explicit or implicit. Implicit memories concern perception and motor skills and are recalled unconsciously, in contrast to explicit memories which are recalled consciously and regard memories of places, things, people or situations. Explicit memory is further divided in episodic and semantic memory. Episodic memory regards personal experiences including their spatial and temporal order, while semantic memory regards

memories about language, social structure, geography among others (Eichenbaum, 2001; Kandel, 2001a).

For practical reasons, studies of the cellular and molecular mechanisms underlying learning and memory are limited in humans. The experimental part of this project was therefore performed in mice. Even though the rodent and human brain differ in structure and complexity, great similarities in the mechanisms and involvement of brain regions are generally found (Morellini, 2013), in addition is spatial memory, which can easily be assessed in rodents, considered a type of episodic memory (Sharma et al., 2010).

1.2.1 Learning and memory in the hippocampus and prefrontal cortex

In the formation of explicit memories, sensory inputs about things and experiences are largely processed in the cerebral cortex. This information is then further processed in the medial temporal lobe where the hippocampus is involved in forming detailed memories by putting them into context. From the hippocampus signals are sent to the cortical areas where they in time are consolidated by formation of networks of related memories, to which new memories can be added later by structural reorganisation. The signalling between the hippocampus and the prefrontal cortex (PFC) has been proposed to be very important for appropriate consolidation of memories, and in the selection of appropriate memories for retrieval in a given situation (Preston and Eichenbaum, 2013). The PFC also plays a central role in working memory (Goldman-Rakic, 1996).

1.2.1.1 The hippocampus

The hippocampus is found deep in the temporal lobe (Figure 1.1). It is one of only two regions of the brain where new neurons are generated throughout life. Also it was the structure where Lomo and Bliss first demonstrated long-term potentiation (LTP), showing that repeated stimulation of neurons leads to increased firing, strengthening the connection between cells (Eichenbaum, 2001).

Mice are known to use their hippocampus in a similar way to humans (Kandel, 2001b), and spatial memory testing of mice is often used to assess effects of experimental manipulations on cognitive function. Studies of human and rodent brains have both shown particular sensitivity of the hippocampus to stress, neurodegenerative diseases and ageing (Miller and O'Callaghan, 2005).

Focusing on the rodent brain, the hippocampus extends from the midline of the brain, over the thalamus and into the temporal lobe. In humans the hippocampus is found deeper in the temporal lobe due to the increased surface area of the brain.

The hippocampus consists of the Cornu Ammonis 1-3 (CA1-3), the dentate gyrus (DG), subiculum, pre-subiculum, parasubiculum and the entorhinal cortex (EC). The two major cell layers are the pyramidal cell layer in the CA and the granule cell layer in the DG (Amaral and Witter, 1989).

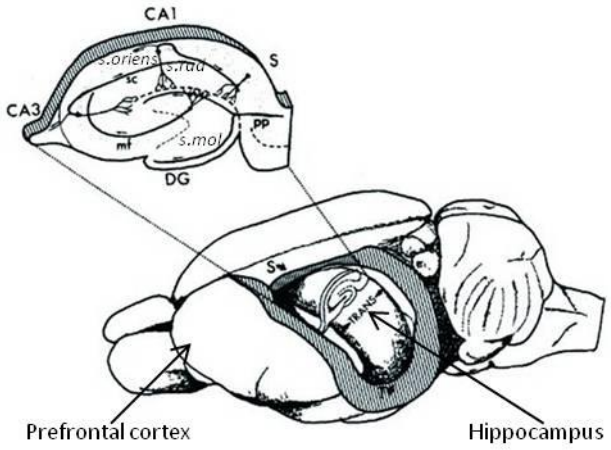


Figure 1.1 An illustration of the rodent brain with indications of the prefrontal cortex and hippocampus. A transverse section through the hippocampus shows the dentate gyrus (DG) and the Cornu Ammonis (CA) 1-3 with the *stratum oriens* and *stratum radiatum* indicated. Signalling from the entorhinal cortex goes through the perforant path (pp) to the *stratum moleculare* of the DG, from here signals are carried through the mossy fibres (mf) to the pyramidal cells in the CA3 which further signals to the CA1 via the Schaffer collaterals, the CA1 pyramidal cells then signals to the subiculum (S). The illustration is modified from (Amaral and Witter, 1989).

The Cornu Ammonis

The Cornu Ammonis (CA) is divided in CA1, 2 and 3 (Figure 1.1). CA3 receives mossy fibre inputs from the DG and the pyramidal cells are bigger here than in the CA1 region. The CA2 is the region found between the CA1 and 3. The subiculum (S) starts after the CA1 where no Schaffer collateral inputs from the CA3 are found. The subiculum, presubiculum and parasubiculum are distinguished by the density of the various cell layers. The region over the CA pyramidal layer is called the *stratum oriens*, while that below the pyramidal cell layer is called the *stratum radiatum* (Amaral and Witter, 1989). There are three major fibre systems associated with the hippocampal formation. The angular bundle carries fibres between the EC and the other hippocampal regions. The fimbria-fornix pathway connects the hippocampus to other brain regions, and the dorsal and ventral commissures connect the hippocampus with the contralateral hemisphere (Raisman et al., 1965).

The dentate gyrus

The dentate gyrus consists of the granule cell layer (GCL) which is four to eight cells wide and extend its dendrites into the *stratum moleculare*. The blade of the c shaped cell layer towards CA1 is referred to as the supra-pyramidal blade and the other the infra-pyramidal blade. The area between the two blades is called the hilus. Besides granule cells there are a number of different types of interneurons in the DG (Amaral and Witter, 1989). Between the *stratum moleculare* and the CA1 *stratum radiatum* the *stratum lacunosum-moleculare* is found, most inputs from the neocortex through the EC terminate here. The input from the EC to the DG is known as the perforant path (pp). Between cells in the DG and CA3 the projecting axons are called mossy fibres, and between the CA3 and CA1 Schaffer collaterals project. The CA1 then further project to the subiculum and the EC (Amaral and Witter, 1989).

Along the long axis of the hippocampus differences in function are known. In rodents, the dorsal hippocampus is involved in learning and memory, and the ventral hippocampus is more involved in affective behaviours (Bannerman et al., 2004).

1.2.1.2 The prefrontal cortex

The PFC is the front most part of the frontal lobe. It is divided medially, laterally and ventrally. 80-90% of the neurons in the PFC are excitatory pyramidal cells and 10-20% are inhibitory interneurons. The structure of the PFC is complex and differs from that of other cortical structures where a conserved structural order of inputs and outputs is seen. However, both inputs and outputs to and from cortical and subcortical structures are found (Heidbreder and Groenewegen, 2003; Riga et al., 2014).

As mentioned before, the PFC is involved in controlling appropriate associations between memories and in the encoding of new memories, and plays a role in working memory and decision making. It is also, in contrast to the hippocampus, involved in both explicit and implicit memory (Preston and Eichenbaum, 2013).

1.2.2 Learning and memory at the excitatory synapse

The plastic properties of the brain underlie its great capacity for memory formation, storage and retrieval, as well as the great ability to adjust to changes and elicit an appropriate response in a given situation. Physical changes in the neurons are known to be the mechanism for memory formation, and the mechanism underlying long-term storage of memories is considered to be changes in synaptic strength (Stuchlik, 2014). In this chapter priority is given to the role of excitatory glutamatergic signalling.

1.2.2.1 The excitatory synapse

Excitatory synapses are most often found between axonal boutons and dendritic spines, and consist of a vesicle rich pre-synaptic active zone, a synaptic cleft and a post-synaptic density. The dendritic spine numbers and dimensions are dynamic, and known to change e.g. in learning and an enriched environment. Polymerisation and phosphorylation of the cytoskeleton is essential for these changes (Bailey et al., 2015). The protein postsynaptic density 95 (PSD95), glutamate receptors and several adhesion molecules play important roles in synapse stability and strength.

Neuronal networks are modulated by addition and elimination of synapses throughout life (Caroni et al., 2012).

1.2.2.2 Glutamate signalling

The most abundant excitatory neurotransmitter is glutamate. Glutamate is released from synaptic vesicles upon stimulation and then taken up mainly by glial cells but also by neurons through glutamate transporters. In the astroglia, glutamate is decarboxylated to glutamine which is then again taken up by neurons where glutamate is synthesized again and stored in vesicles ready for exocytosis (Danbolt, 2001).

There are three forms of ionotropic glutamate receptors; N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainic acid (KA) receptors named after their specific agonists. Upon activation an ion channel is opened allowing passage of cations into the cell. In addition to the ionotropic receptors 8 different metabotropic glutamate receptors have been found, these have mainly modulating roles and are most often found outside the synapse (Malenka and Nicoll, 1999). In this section focus will be on the NMDA and AMPA receptors since they are known to play important roles in synaptic transmission while less is known about that of KA receptors. A high degree of plasticity is seen in relation to the AMPA and NMDA receptors, including subunit composition, posttranslational modification and in synaptic distribution (Collingridge et al., 2004; Lee, 2006).

AMPA receptors

AMPA receptors are tetrameric and four different subunits are known, GluA1-4, encoded by the genes *gria1-4*. GluA1/2 and GluA2/3 are the most common combinations. The receptors are permeable to monovalent cations with a preference for sodium ions. AMPA receptors without GluA2 subunits show calcium permeability (Traynelis et al., 2010; Washburn et al., 1997).

In glutamatergic neurons, the receptor ion channel is opened as a result of a rapid increase in extracellular glutamate at above 100 μM and both fast binding kinetics and a high opening

probability of the receptor is observed (Traynelis et al., 2010). The receptors are rapidly deactivated as glutamate is cleared from the synaptic cleft; persistent stimulation results in desensitization which is also seen at lower glutamate concentrations if this occurs for a longer time. The opening probability and conductance can be modulated by phosphorylation of the receptor subunits (Lee, 2006). The AMPA receptor subunits undergo alternative splicing and can all be found in two different forms, flip and flop, where the flip form desensitise to glutamate at a slower rate than the flop form (Traynelis et al., 2010).

As examples of the plasticity of the AMPA receptors, increased synaptic efficacy, as a result of increased AMPA-mediated response to stimuli, is observed in relation to learning, where also changes in subunit composition are known to take place (Ghafari et al., 2012; Zamanillo et al., 1999).

AMPA receptors are found both in the synaptic membrane, in intracellular vesicles and in the membrane outside the synapse where they can move by lateral diffusion. These plastic changes in receptor density are important for strengthening synaptic connection (Lee and Kirkwood, 2011).

In GluA1 and GluA2 knock-out mouse models the effects of the individual receptor subunits have been studied. No effect of GluA1 knock-out was reported on performance in a spatial memory test, while LTP could not be generated in the CA1 (Zamanillo et al., 1999). In GluA2 knock-out models, a doubling of LTP response was seen in hippocampal slice cultures and a nine fold increase in calcium permeability was seen after stimulation (Jia et al., 1996).

Ageing, disease and some medical treatments have been reported to cause changes to receptors. For example, antimanic and antidepressive treatment have in rats been shown to cause changes in receptor subunit composition and localisation (Paoletti et al., 2013). Stress, both acute and chronic, is known to affect the density and composition of the glutamate

receptors, but in different ways. Acute stress leads to phosphorylation of AMPA receptors, lateral diffusion, and insertion of receptors in the membrane, this enhances LTP and plays a role in memory consolidation after experiences that induce acute stress. After chronic stress impaired glutamatergic signalling and LTP is seen in the hippocampus and prefrontal cortex, coinciding with increased NMDA and AMPA receptor degradation (Timmermans et al., 2013).

NMDA receptors

NMDA receptors are heterotetrameric consisting of GluN1, GluN2 (a-d) and GluN3 (a,b) subunits, encoded by the *grin1-3* genes. The NMDA receptors are permeable to both sodium and calcium ions and require, besides from binding of glutamate, binding of glycine and depolarization of the membrane to remove a magnesium ion which under resting conditions block the ion channel (Traynelis et al., 2010). NMDA receptor subunits combine into either di-heteromers or tri-heteromers which all contain two GluN1 subunits. GluN1 exists in 8 isoforms due to alternative splicing, the expression differs between different cell types and brain regions. 15->50% of the NMDA receptors are tri-heteromeric containing GluN1/GluN2a/GluN2b. The functional properties of the NMDA receptors vary depending on subunit composition and cellular localization, which is known to be plastic (Paoletti et al., 2013; Traynelis et al., 2010).

Compared to AMPA receptors the NMDA receptors have a lower opening probability, lower kinetics, stay sensitive to stimulation for a longer time and they have a very slow glutamate unbinding rate. At the same time they require a glutamate concentration of only 1-10 µM compared to 100-500 µM for the AMPA receptors (Traynelis et al., 2010). The properties of the receptors depend highly on the subunit composition. GluN2a and GluN2b containing receptors exhibit high conductance, whereas GluN2c and GluN2d have lower conductance. GluN1/2a receptors have a higher opening probability than GluN1/2b or 2c or d, which all have a very low opening probability. But, at the same time, the GluN1/2a receptor has the lowest glutamate and glycine sensitivity. The GluN2 subunits differs primarily in the C-terminal domain, which affects the interaction with intracellular molecules. e.g., keeping GluN2b subunits at the synapse

requires binding to PDZ domain containing proteins, whereas GluN2a does not require binding. GluN2b also interact more strongly with CaMKII than GluN2a does (Paoletti et al., 2013).

The receptor composition differs greatly between the hippocampus and prefrontal cortex. In both regions the GluN2b subunit is the predominant GluN2 receptor subunit during development. In the hippocampus a shift towards predominance of the GluN2a subunit is seen by the end of the third postnatal week, and particular importance of this subunit in hippocampus-dependent learning has been reported. However, regional differences are seen, the GluN2b subunit is found in perforant path neurons and CA3 cells, but only at very low levels in mossy fibre neurons. In the prefrontal cortex, on the other hand, the GluN2b subunit dominate even in adult life, where it plays an essential role in working memory function, by sustaining excitability for longer periods (Dumas, 2005; Monaco et al., 2015).

NMDA receptors undergo a similar activity dependent plastic expression as AMPA receptors do. GluN2b receptors are constitutively inserted into the membrane, whereas the exchange of GluN2b with GluN2a depends on synaptic activity (Traynelis et al., 2010). The GluN2b receptor subunit has been found to be particularly important in many of the specialized functions of the prefrontal cortex (Monaco et al., 2015).

Mice with knockout of GluN1 die shortly after birth (Forrest et al., 1994). Therefore models with more localised deletion have been designed. Mice with non-functional CA1 NMDA receptors show impaired spatial learning in the water maze, object recognition, olfactory discrimination and contextual fear discrimination (Rampon et al., 2000; Tsien et al., 1996). Mice with a selective GluN1 knockout from CA3 showed impaired pattern completion in a delayed matching to place version of the water maze, but not impaired spatial learning (Nakazawa et al., 2003). Genetically altered models with decreased ion permeability died shortly after birth, again pointing out the importance of this receptor subunit (Single et al., 2000). In mice with knock-out of GluN2a some impairment of LTP or spatial memory is seen (Sakimura et al., 1995). GluN2b knockout mice die

shortly after birth since this receptor subunit plays an important role in early development (Kutsuwada et al., 1996).

1.2.2.3 Synaptic plasticity

Long-term potentiation (LTP) is the most studied form of synaptic plasticity. This was first described by Bliss and Lomo (Bliss and Lomo, 1973), finding that tetanized stimulation of perforant path cells in the hippocampus of anaesthetised rabbits lead to synaptic changes in the granule cells. Molecularly, LTP involves increased calcium-influx, activation of kinases, e.g., the calcium-calmodulin dependent protein kinase II (CaMKII), phosphorylation of cytoskeletal proteins, increased conductance of AMPA receptors and insertion of both AMPA and NMDA receptors in the postsynaptic membrane. All of this contributing to the strengthening of the contacts between the cells (Lynch, 2004). The early phase of LTP is mediated through modulation of glutamate receptors by phosphorylation and kinase activity, movement of receptors and change in receptor composition, if the late phase of LTP (which require gene transcription and protein synthesis) is blocked the LTP response decline over 3-5 hours (Krug et al., 1984).

Plastic changes can occur for shorter or longer periods. Short-term synaptic plasticity can take place as a result of changed transmitter release or downstream responsiveness (Malenka and Nicoll, 1999). Differences in response to stimulation patterns are known. With a shorter than 20 millisecond inter-stimulus interval a decreased response to a second stimulus is often seen as a result of ion-channel closing and transmitter depletion. At longer intervals (20-500 ms) an enhanced response to a second stimulus is often seen, since the increased calcium concentration at the synapse from the previous stimulation leads to an increased cellular response when the ion channels are opened as a result of the new stimulation. So activation of neurons can lead to synaptic transmission being either strengthened or weakened for milliseconds to weeks or even longer (Malenka and Nicoll, 1999).

1.2.3 Neurotrophic factors in learning and memory

Neurotrophic factors play important roles in synaptic plasticity by promoting growth, survival and death of cells and synapses in a well-controlled manner that ensures optimal synaptic reorganisation to support cognitive function. The neurotrophin family of neurotrophic factors are small (approximately 14 kDa) proteins which are generated as pre-pro-proteins and exist as homodimers. These include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5. There are two common types of receptors for these; the tropomyosin-related kinase A-C (Trk A-C) and p75, simplified they act by promoting cell survival and cell death respectively, simplified because studies have shown that the result of ligand-binding to these receptors is highly dependent on the cellular context and the splice variants of both the receptors and the trophic factors. Three major pathways are involved in the downstream signalling, being: mitogen-activated protein kinases (MAPK) (mostly involved in differentiation and neurite outgrowth), phosphoinositide kinase-3 (cell-survival) and phospho-lipase-γ (synaptic plasticity) (Binder, 2007; Meeker and Williams, 2015).

NGF was the first neurotrophic factor to be described in the 1950s. It binds to the TrkA receptor which is primarily expressed on cholinergic neurons (Binder, 2007). NGF is found in low levels under normal conditions which are then increased as a result of neural activity (Binder, 2007). NT3 is widely expressed in the brain and preferably binds to TrkC receptors but also TrkA and TrkB. It promotes survival and differentiation of newly generated neurons in the hippocampus and is involved in neuronal excitability and signalling (Binder, 2007). NT4/5 differs from the other neurotrophic factors in not being upregulated by seizure activity and that knock-out models develop more normally than seen for knock-out models of the other factors. NT4/5 is expressed at low levels under normal conditions (Binder, 2007).

BDNF is the most abundant growth factor in the CNS. It was first described in 1982 (Barde et al., 1982). Its functions are many and complex, mainly due to the many different splice variants

which are differentially upregulated spatially and temporally under different conditions (Aid et al., 2007; Leal et al., 2014). For example, in rats, *in situ* hybridisation for the 5 most abundant *bdnf* exons has shown differences in expression in the dorsal hippocampus after seizures induced by kainic acid (KA) or pilocarpine. After pilocarpine administration exons II and VI were expressed in the proximal part of the CA1 and CA3 dendrites, exons IV and IX were expressed in the distal parts of the CA1 and CA3 dendrites, and in the inner part of the *s.moleculare*. Exons I and IV was expressed in the soma. In contrast, KA treatment induced dendritic expression only for exons VI and IX, and only in the CA1 dendrites (Chiaruttini et al., 2008).

BDNF binds to both p75 and TrkB receptors, where TrkB is found both in full length and truncated forms. BDNF is widely expressed in the brain and studies have shown that it plays important roles in learning and memory as a major modulator of synaptic plasticity and a stimulator of neurogenesis (Bekinschtein et al., 2008a; Binder, 2007; Chen et al., 2010; Edelmann et al., 2014).

Knock-out models of the various neurotrophic factors have shown important and diverse roles of these in normal development of the nervous system, and in maturation and maintenance of synapses. The expression of the neurotrophic factors, BDNF in particular, is known to be sensitive to modulation by a number of factors and situations. For example, mice exposed to repeated social defeat stress have been reported to have decreased BDNF mRNA levels in the hippocampus, piriform cortex and the amygdala; stress induced by social isolation has been reported to lead to decreased BDNF protein levels in the hypothalamus, prefrontal cortex and hippocampus; and chronic unpredictable stress has been reported to decrease BDNF protein levels in the hippocampus (Bath et al., 2013). In a study investigating effects of restraint stress and exercise, restraint stress was found to decrease hippocampal mRNA levels of BDNF transcripts 1, 2, 3, 4, 6 and 7, but not 8, while exercise increased the mRNA levels of the same transcripts. Exercise prior to restraint stress counteracted the decreased mRNA levels of BDNF induced by the stress, however, this was only seen in the hippocampus, not in the prefrontal

cortex (Ieraci et al., 2015). In patients with Alzheimer's Disease, a selective reduction in BDNF mRNA levels has been reported in the hippocampus, and in patients with Parkinson's Disease, a selective reduction in BDNF protein has been reported in the substantia nigra (Binder, 2007).

1.2.4 Adult hippocampal neurogenesis

Adult hippocampal neurogenesis, the continuous generation of new cells in the subgranular zone (SGZ) of the DG throughout life, contributes with an additional level of plasticity to the hippocampus, the precise role of the new cells is however not yet fully understood (Jessberger 2014). The first studies of adult neurogenesis by Altman and Das (1965) were carried out in rats. Later the phenomenon has been verified in primates and humans (Eriksson et al., 1998; Gould et al., 1999a). In humans changes in the number of proliferating cells in the SGZ have been reported postmortem in patients with Alzheimer's Disease (AD), epilepsy and depression (Jessberger and Gage, 2014).

The SGZ of the hippocampus is found as a two-cell wide zone beneath the granule cell layer into the hilus and as a single cell-wide layer in the GCL, it is highly heterogeneous, containing cells with varying neurogenic potency (Balu and Lucki, 2009). See Figure 1.2.

The maturation process of the newly generated neurons involves proliferation, differentiation, targeting and synaptic integration of the cells (Balu and Lucki, 2009). During the first week of maturation the neurons migrate into the granule cell layer, during the second week axons and dendrites are grown and during the third week the cells establish glutamatergic contacts providing the inputs necessary for survival of the cells (Balu and Lucki, 2009). A very large number of the generated cells die. The surviving neurons are considered mature after 6-8 weeks (Balu and Lucki, 2009). The glutamatergic neurons are known to integrate in the existing tri-synaptic network (Gu et al., 2012).

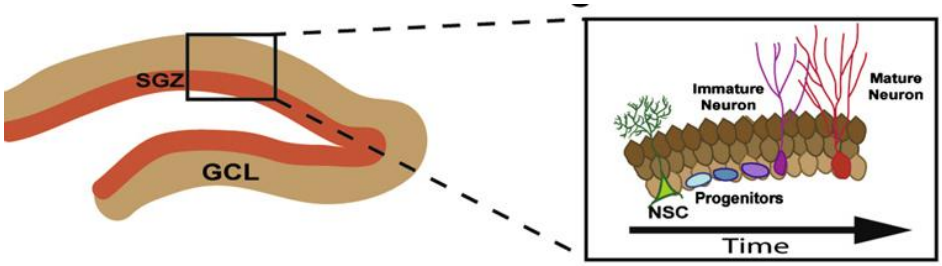


Figure 1.2 Illustration of the subgranular zone of the hippocampus. The subgranular zone (SGZ) is a narrow zone in the innermost part of the dentate gyrus granule cell layer (GCL) extending into the hilus. Cells of varying neurogenic potency are found here. Newly generated cells differentiate and migrate into the GCL where they form contacts and integrate in the existing networks. Figure is modified from (Petrik et al., 2012).

While the precise role of the new cells in learning and memory is not clear, behavioural studies in rodents where neurogenesis has been either enhanced or impaired have suggested a role of the new hippocampal neurons in spatial learning and memory (Zhao et al., 2008). Increased neurogenesis by environmental enrichment and voluntary exercise has a positive impact on learning (Gould et al., 1999b; Koehl and Abrous, 2011; Olson et al., 2006; van Praag et al., 1999a), while decreasing neurogenesis, chemically or by ionizing radiation, has correspondingly been reported to impair learning (Santarelli et al., 2003; Shors et al., 2001; Sienkiewicz et al., 1994; Snyder et al., 2005). Reports have suggested that the role of the new cells for learning and memory is in pattern separation, where they improve the distinction between highly similar patterns of synaptic activation networks (Nakashiba et al., 2012; Sahay et al., 2011).

Adult neurogenesis is sensitive to modulation by both internal and external factors, such as changes in neurotransmitter levels, hormones, growth factors, or exposure to stressors. Environmental stressors such as from predator odors, electric shock, restraint stress and maternal separation is known to inhibit proliferation of new hippocampal neurons (Balu and Lucki, 2009; Cayre et al., 2009; Kempermann et al., 2004). Changes in hippocampal neurogenesis may be found in Alzheimer's disease and depression (Jessberger and Gage, 2014).

1.2.5 Astroglia and microglia

Neurons are connected in highly organised networks that conduct and control the many diverse functions of the brain. The successful function of these neuronal networks depends strongly on the appropriate support from glial cells (Chung et al., 2015a). There are estimated to be at least as many glial cells in the brain as there are neurons (Herculano-Houzel and Lent, 2005). There are three forms of these; oligodendroglia which are involved in myelination of neurons in the central nervous system, astroglia and microglia, the two latter carry out diverse functions which will be considered below.

Astroglia are often considered to have a star shaped morphology with many branches. They form a mesh like structure in the brain, where they are in close contact with neurons but have little contact with each other. Astroglia have many functions; they form the blood brain barrier, maintain homeostasis of glutamate, potassium, energy and pH and protect neurons against oxidative stress (Bélanger and Magistretti, 2009). They also stimulate formation, elimination and plasticity of neuronal synapses and support memory (Ben Achour and Pascual, 2010; Chung et al., 2015a; Zorec et al., 2015).

Microglia are the immune cells of the central nervous system (Perry and Gordon, 1988). In contrast to astroglia they are not in constant contact with neurons but monitor the cells by establishing temporary contacts (Wake et al., 2009). Microglia are furthermore sensitive to peripheral immune signals (Patterson, 2015). Many subtypes of microglia have been described including resting, pro-inflammatory and phagocytotic types. They release a number of factors including cytokines, chemokines and growth factors. The balance between these is very important, and synapses and adult hippocampal neurogenesis are known to be sensitive to change. Resting microglia are also found in the SGZ where they are involved in the regulation of neurogenesis by mediating apoptosis (Aimone et al., 2014; Chung et al., 2015a; Wake et al., 2009).

Inflammation induces activation of both astroglia and microglia, which can be observed as morphological changes, increased secretion of pro-inflammatory cytokines and chemokines, in addition to proliferation of microglia and astroglia. Increased inflammation in the brain is known to affect cognitive function negatively and is seen in ageing and diseases including Alzheimer's Disease (Cunningham et al., 2009; Wyss-Coray and Rogers, 2012).

1.2.6 Ageing and cognitive impairment

Cognitive function is known to decline with age. Impairment in reasoning and encoding of episodic memories are seen earlier in life than impairments of memories of familiar tasks (Hedden and Gabrieli, 2004). Aged adults are known to have less grey matter than young adults, due to a lower density of synapses, rather than cell loss. For example, neocortical synapse density is known to decline steadily between the age of 20 to 100, and is particularly evident in the prefrontal cortex and the medial temporal lobe (Hedden and Gabrieli, 2004). However, there is a high degree of plasticity in the ageing brain with some areas compensating for a decline in another area (Kirova et al., 2015).

The rate of hippocampal proliferation is also known to decline with age (Kuhn et al., 1996), and the levels of the trophic factors does also decrease, correlating with cognitive decline (Budni et al., 2015). Furthermore, changes to glutamate receptor numbers and functional properties of the receptors are known to occur with ageing (Burke and Barnes, 2010).

An age-associated priming of microglia is also known to happen, where the response to damage or inflammation elicits a greater and longer lasting response with increased age compared to younger individuals (Wong, 2013). Also the normal function of the microglia and astroglia is disrupted, providing less support to synapses for example (Diniz et al., 2016; Patterson, 2015).

1.3 Circadian rhythm disturbances and cognitive function

There has been a particular interest in effects of low frequency MFs on circadian rhythms, and interactions that could potentially affect cognitive function have been proposed. This includes sensitivity of the central genes in the control of circadian rhythms, sleep disturbances and disruption of melatonin and cortisol/corticosterone excretion rhythms (Manzella et al., 2015; WHO, 2007).

1.3.1 General introduction

Historically, the rhythm of activity in humans and animals has followed the rise and fall in sunlight during the day, and many biochemical and physiological processes have evolved to show a similar rhythm ensuring the most appropriate conditions for a certain reaction or response to happen. Disturbances to this daily rhythm in relation to ageing, various psychiatric disorders and medical treatments are well described (Duffy et al., 2015; Jones and Benca, 2015). Modern lifestyles cause disturbances by e.g., jetlag, shift work and light pollution from artificial light sources during the night.

The circadian rhythm works at a cycle of approximately 24 hours, during which a great number of hormones and proteins have a controlled cyclic pattern of expression or release. These include cortisol and corticosterone, growth hormone, melatonin and insulin. Also the core body temperature shows a circadian rhythm (Hastings et al., 2008). In constant darkness the internal circadian rhythm is found to be slightly longer than 24 hours in most humans and slightly shorter in wild type mice. The circadian rhythm is sensitive to modulation from zeitgebers, that can delay or advance the rhythm. Light is the most potent of these zeitgebers and entrain the internal circadian rhythm to the external environment through signals from the photoreceptors in the eye. In addition, feeding works as a potent zeitgeber in the liver (Roenneberg and Foster, 1997; Roenneberg and Merrow, 2003).

Activity also show great circadian rhythm. The hormones cortisol/corticosterone and melatonin are important players in this by controlling sleep and wakefulness. The blood levels of both hormones show a strong circadian rhythm with cortisol/corticosterone peaking in the morning and melatonin at night (Selmaoui and Touitou, 2003). The expression of these hormones is highly sensitive to light and increased light results in signalling to the adrenal glands wherefrom the glucocorticoids cortisol and corticosterone are secreted (Ishida et al., 2005). The natural rhythmic change between sleepiness and alertness underlines the biological importance of sleep. Besides from serving as a period of rest from physical work, sleep also supports cognitive function and wellbeing, and sleep disturbances and lack of sleep is known to impair cognitive function (Kreutzmann et al., 2015).

1.3.2 The circadian clock

The circadian rhythm is controlled by a "master clock" found in the suprachiasmatic nucleus (SCN), a small region of the brain found just above the optic chiasm. The SCN receives input from the eye through the retino-hypothalamic tract and orchestrate the clocks in all the body's cells. This happens by activation and deactivation of transcription and translation of the core clock genes, irrespective of light input. However, the clocks are synchronized in response to light (Roenneberg and Merrow, 2003). From experiments in mice lacking both rods and cones it has been found that the light input to the SCN is independent of these two cell types and happens through direct stimulation of intrinsic photoreceptive retinal ganglion cells (ipRGCs) and the photopigment melanopsin. This leads to release of glutamate and pituitary adenylate cyclase-activating peptide (PACAP) in the SCN which in turn leads to increased intracellular calcium concentration and phosphorylation of cAMP response element binding protein (CREB), permitting the phosphorylated CREB to bind to genes with a cAMP response element which activates a number of signalling pathways (Hastings et al., 2014).

In mammals, three forms of the clock gene coding for PERIOD, *per1, per2 and per3* and two forms of the cryptochrome gene *cry1 and cry2* exist. The transcription of both *per* and *cry* is controlled by a dimer of the two other central core clock proteins, CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle ARNT-Like1), both are helix-loop-helix transcription factors which binds to an E-box element in *cry* and *per*. This takes place from early in the light phase and during the subjective day. Translation of the proteins takes place in the cytoplasm by the end of the subjective day, the proteins associate to form a dimer before entering the nucleus where they in turn suppress CLOCK and BMAL activity, this suppression decreases as the CRY and PER proteins degrade. This transcriptional-translational feedback loop has a self-sustained cycling of approximately 24 hours forming the master clock (Hastings et al., 2014). Neuropeptide Y (NPY) and GABAergic signalling from the SCN neurons to target tissues cascade the rhythm of the master clock to the other cells in the body and synchronises their clocks. Downstream of the central clock are a number of genes activated in a rhythmic manner, controlling metabolism, neuronal firing and cell signalling (Hastings et al., 2014). Clock genes have been reported to be sensitive to modulation. For example, in chronically stressed rats, changes to clock gene expression has been reported in a number of brain regions including the hippocampus (Christiansen et al., 2016).

Clocks are found throughout the body, these oscillate themselves but are synchronized to the master clock through electrical, hormonal and metabolic signalling to ensure synchronised oscillations within tissues (Albrecht, 2012). Under normal 12 hour light-dark conditions *per* and *cry* expression in the SCN peaks in the light phase, whereas *bmal1* peaks 12 hours later. In most other tissues and brain regions this phasic expression is kept but is delayed by several hours (Guilding and Piggins, 2007).

The liver is the main metabolic regulator, and circadian rhythm is seen in the expression of many proteins. Disruption of the circadian rhythm has been found to cause obesity and metabolic

syndrome. Certain diet compositions, e.g. high fat, can disrupt the circadian rhythm (Hastings et al., 2008).

1.3.2.1 Entrainment of the circadian clock by light

Entrainment of the master clock by light occurs by photic stimulation of melanopsin-containing ipRGCs which signal directly to the SCN. In experiments time is often described relative to the onset of the zeitgeber, e.g. light, activity or feeding and is expressed as ZT, zeitgeber time, or CT, circadian time where CT12 is defined as the time of onset of activity of noctural animals in the absense of a zeitgeber. The rhythmic expression of the clock genes in the SCN can be shifted by changes to the light conditions, and studies have shown that the functional impact of such changes differ greatly depending on the timing and strength of the change (Schwartz et al., 2011).

per1 and *per2* exhibit a phase-dependent increase in mRNA expression, peaking around 1–2 hours after light exposure, with *per2* peaking later than *per1* (Albrecht et al., 1997; Shearman et al., 1997). The resulting alterations in PER1/2 proteins alter the phase of the circadian clock. The time at which these increases occur is important to the direction of the shift, with light at dawn inducing an advance via the earlier onset of *Per1* expression, and light at dusk inducing a delay, by delaying *per2* offset (Schwartz et al., 2011). The shift in clock gene expression correlates with shifted activity rhythms (Albrecht, 2012). Studies using mutant *per1* and *per2* mice have been found not to show such reset of activity rhythms (Albrecht et al., 2001). More than 500 light responsive genes have been identified in the SCN by microarray analysis, 90% were found to be down regulated while 10% were up regulated after 2 hour light shock at CT16 (Jagannath et al., 2013).

1.3.3 Circadian rhythm and cognitive function

Studies have shown that the clock controls 5-10% of the transcriptome. The influence of circadian rhythm can be seen in neurogenesis (Rakai et al., 2014) and studies have shown

differences in performance in learning and memory tests during the light and dark periods of the day and found disruption of circadian rhythms to negatively impact these processes (Smarr et al., 2014). Effects have been seen on cognitive function, for example, Ruby et al. (2008) found that disruption of circadian rhythm in hamsters using light resulted in impaired recognition memory (Ruby et al., 2008).

1.3.4 Circadian rhythm disturbances

Disruption and disturbances of the circadian rhythm have been studied in many different ways e.g., in human patients with sleeping disorders, or in terms of correlation between various diseases or medical treatments and disturbances to the circadian rhythm. In mice effects of knock-out of the core clock genes and effects of changes to the light/dark cycle have been studied. A very important role of an intact circadian rhythm on health has been found. For example correlations between clock genes and sleep disorders, cancer, feeding abnormalities and drug addiction have been described (Lamont et al., 2007; Rakai et al., 2014; Roenneberg and Merrow, 2003).

1.4 A brief introduction to electromagnetic fields

Non-optical, non-ionising fields and radiation are defined as EMFs having frequencies of between 0 Hz and 300 GHz (SCENIHR, 2015). EMFs are subdivided in static fields at 0 Hz, low frequency fields (LF) at 0.1Hz-1 kHz, intermediate frequency fields (IF) at 1 kHz- 10 MHz, radio frequency fields (RF) at 10 MHz- 300 GHz. Terahertz frequency fields (TF) occur at 300 GHz- 10 THz and in the upper end infrared radiation (780 nm – 1mm) and visible light (380-780 nm). Ultraviolet (UV) radiation (100-400 nm) covers both ionising and non-ionising wavelengths (SCENIHR, 2015).

1.4.1 Known interaction mechanisms

The interaction of EMFs with biological tissues depends on wavelength and strength of the field. At frequencies lower than 10 MHz, fields induce currents and electrical fields inside the body while for fields at higher frequencies, where this is not possible, the main mechanism of interaction is transfer of energy leading to heating. Other mechanisms have been proposed, including interactions with magnetite crystals, although their relevance to health is not yet clear (WHO, 2007).

At low frequencies, the major interaction is with nervous tissue. The electrical nature of neuronal signalling makes it sensitive to external fields when the induced internal field is strong enough. Alternating fields, at sufficiently high levels can lead to flickering light perception, known as the phosphene response by stimulation of excitable cells in the retina, and electric fields (EF) can cause the hair on the skin to vibrate and induce tingling sensations. LF electrical fields lead to charging of the body surface and grounded objects, LF magnetic fields (MF) leads to induction of electrical fields in the body, increasing with frequency and field strength. The highest induced electric field is found at the surface of the body and the lowest (zero) in the centre of the body. To stimulate nerves the external field needs to be above 10 mT or 100 kV/m (WHO, 2007).

At the higher frequencies the major interaction with biological tissues are on dielectric molecules ultimately leading to heating. This is for example seen with water molecules in tissues, where exposure to an oscillating EMF leads to vibration and rotation of molecules where collisions then lead to heating of the tissue. The degree of energy absorption and heating caused by exposure depends on the frequency of the field and the type of tissue, the higher frequency, the less energy is absorbed in the tissue (ICNIRP, 2009). Heating and even burns can be experienced in close proximity to high power field emitters such as mobile phone masts.

In addition, EMFs of up to a few MHz can affect electron spin in radical pairs. Due to the fast paced nature of high frequency fields these are not expected to have such effects. The unpaired electrons in radical pairs can spin in a parallel or antiparallel manner, where a parallel spin is less likely to get reduced than an antiparallel spinning pair, extending the life of the radical. It has been found that high level exposure (>1 mT) leads to decreased formation of radical pairs while low level exposures leads to an increase. Theoretical calculations have shown this can change reaction yield by 15-30%. Much attention has been on cryptochrome when it comes to the interaction between EMFs and radical pairs. Cryptochromes have been found to be magnetosensitive in certain species such as plants, *drosophila* and butterflies where the protein also acts as a photoreceptor. Cryptochromes are also found in rodent and humans but the protein structure is different and magnetosensitivity has not been proven (WHO, 2007).

1.5 Reported effects of electromagnetic field exposure on cognitive function

The brain has been a major area of focus regarding health effects of EMF exposure due to its important orchestrating role of both cognition and of motor and sensory functions, but also its electrical nature. Mobile phone use in the close proximity to the head has been a major source of concern. From the existing data no firm conclusions on effects of EMFs at environmental levels on cognitive function can be drawn (SCENIHR, 2015; WHO, 2007).

1.5.1 Reported effects of power frequency magnetic field exposure on learning and memory in rodents

Rodent studies have investigated the effects of power frequency MFs on learning and memory, but few effects have been reported. In the most recent SCENIHR report (2015) it was concluded that MFs may affect spatial memory in rodents. Effects of both acute and long-term exposure have been studied, and many studies have attempted to identify the cellular and molecular mechanisms involved in the reported responses to exposure. However, only a few studies have examined any of these, and there is often variation in the experimental designs complicating comparisons (SCENIHR, 2015; WHO, 2007). A summary will be given below.

1.5.1.1 Reported effects of power frequency magnetic fields on performance in cognitive tests

To study the effects of MFs on cognition in rodents, many groups have used behavioural memory tests including the hippocampus-dependent spatial water maze, Y-maze and radial-arm maze (RAM), and the non-spatial novel object recognition test (NOR). See Table A1.1 in appendix 1 for details of the studies.

Several studies have reported impairment of spatial learning as a result of exposure. A study from 2007 found this when rats were exposed for 20 minutes after training (at 8 mT but not 2 mT) in the water maze, but not when they were exposed before training (Jadidi et al., 2007). Long-term exposure for 28 days at 8 mT has also been found to impair learning (Duan et al.,

2013). In contrast to what Jadidi et al. reported (2007), Sienkiewicz et al. (1998 a,b) reported transient dose dependent impairments in RAM performance after exposure to 0.75-7.5 mT when exposure was done for 45 minutes immediately before training. Impairments have also been found at lower magnetic flux densities, for example after 8 weeks of exposure to 2 mT (Deng et al., 2013) and after 12 weeks of exposure to 1 mT (Cui et al., 2012). Lai et al. (1998) found that exposure at 1 mT before training sessions in the water maze impaired memory, but not learning. Also in the spatial version of the Y-maze test impaired memory has been shown after 4 weeks of exposure at 2 mT (Fu et al., 2008).

In contrast, a few studies have found improvement of learning and memory: Two studies reported improvement in water maze performance after 4 weeks of exposure at 2 mT 4 hours per day but not 1 hour per day (He et al., 2011; Liu et al., 2008). One study where exposure at 0.1 mT was done during the water maze test has shown improved performance in female mice, but not males (Kavaliers et al., 1996). A connection between exposure induced hippocampal neurogenesis and improved water maze performance at the time of cell maturation has been reported in a series of studies using exposures at 1 mT for 6-12 days (Leone et al., 2014; Podda et al., 2014).

Four studies using lower level fields found no effect of long-term exposure in the water maze: using 0.1 mT 4h/day for 3 months, 0.1 mT for 90 days continuously, 0.5 mT for 14 or 28 days or 0.1 mT for 24 weeks (Cui et al., 2012; Lai et al., 2016; Li et al., 2014a, 2014b). Sienkiewicz et al. (1998b) also found no effect of exposure at 0.0075 and 0.075 mT in the RAM.

In a non-spatial NOR test Sienkiewicz et al. (2001) found no effect of acute 45 minute exposure to 0.0075, 0.075 or 0.75 mT when applied between the familiarization trial and test trial where a new object was introduced. Long-term exposure for 2 weeks, but not 1 week, at 0.2 mT has been shown to impair performance (Mostafa et al., 2002). However, another study comparing

differences between exposure for 1 and 3 weeks at 0.4, 0.6 and 1 mT found that only exposure at 1 mT for 1 week impaired performance in the NOR test (Zhao et al., 2015).

1.5.1.2 Reported effects of power frequency magnetic fields on synaptic plasticity

Only a few studies investigating effects of MFs on synaptic plasticity have been performed and positive findings have not been replicated by independent groups, suggesting more studies are needed to uncover any possible effects of exposure on plasticity of synapses. (See Table A1.2 in appendix 1 for details).

Enhanced electrophysiological response to stimulation has been reported in brain slice cultures exposed prenatally, postnatally (Balassa et al., 2013; Sun et al., 2016) and in adult life (Komaki et al., 2014; Varró et al., 2009).

There appear to be no studies of effects of exposure on the synaptic density or of effects in the post synaptic density *in vivo*. One study has investigated effects of exposure at 0.5 mT for 14 and 28 days on dendritic spines, and found that exposure affected both the morphology of the spines and reduced the density (Xiong et al., 2013).

Neurotransmitters

In mice, long-term high level exposure has been reported to lead to increased levels of glutamate and GABA in the hippocampus and hypothalamus but not striatum or cortex (Duan et al., 2014; Salunke et al., 2014). In a shorter term study significant changes in neurotransmitter concentrations were also found in various brain regions, glutamate was only found to be changed in the thalamus where an increase was seen, GABA was also increased in the thalamus and the striatum, but decreased in the hippocampus, cortex and cerebellum (Chung et al., 2015b). All three studies use higher exposure levels (1 mT, 8 mT and 1.8-2 mT respectively) than the public exposure limits.

Studies of cholinergic, serotonergic and dopaminergic signalling has also been performed (See WHO (2007) for details).

Glutamate receptors

Two studies investigating glutamate receptor subunit levels in rodents have been published. Duan et al. mentioned above in the section on memory testing, investigated the effect of exposure for 28 days to a 8 mT field on the protein level of GluN2b in mice, finding this to be increased (Duan et al., 2014). Li et al. found an increase in both AMPA and NMDA receptor subunit protein levels after long-term exposure at 0.5 mT in the prefrontal cortex, hippocampus and entorhinal cortex, showing a smaller response with time (Li et al., 2014a). Interestingly after exposures at a lower magnetic flux density, 0.05 or 0.1 mT for 90 days, decreased glutamate receptor binding was shown after exposure (Manikonda et al., 2007).

Growth factors

Effects of exposure to MFs on growth factors and related proteins have not been studied much and not directly in relation to learning and memory, findings in other models or regions will be mentioned below as they might serve as indicators of effects in regions with impact on learning and memory.

In a study performed on developing rat pups, the effect of exposure to 60 Hz at 0.002, 0.2 or 1 mT on changes in gene expression of an axonal growth marker (gap43), or BDNF, NGF, NT3 and NT4 in the forebrain showed no effect of exposure (Harry et al., 2000).

In a study otherwise concerning effects of exposure on Huntington's chorea it was reported that in healthy control rats exposure for 21 days at 0.7 mT leads to increased protein levels of BDNF and glial derived neurotrophic factor, in both cases of more than 100% (Tasset et al., 2012).

1.5.1.3 Reported effects of power frequency magnetic field exposure on neurogenesis

Some studies suggest an effect of exposure on adult hippocampal neurogenesis, effects that would affect the plasticity in the hippocampus. See Table 1.3 in appedix 1 for details.

One group has studied effects of MFs on neurogenesis and published a number of very thorough studies finding enhancing effects of 50 Hz fields at 1 mT on various phases of neurogenesis. No attempt to replicate these results have yet been undertaken. The first study was done in neurospheres prepared from postnatal day 1 mouse brain cortex (Piacentini et al., 2008). The neurospheres were exposed for up to 12 days to a 1 mT field. After exposure increased proliferation and differentiation was seen. Increased calcium ion influx through Ca_{v1}-channels was observed. In later studies it was investigated whether the same was true *in vivo*. After 7 days exposure of mice to a 1 mT field a 63% increase in number of 5'-bromo-2'-deoxyuridine (BrdU)-labelled proliferating cells was found, with the majority being of neuronal lineage. This effect persisted after a 4 week delay where the number of BrdU-labelled cells expressing mature neuronal markers was 64% higher in the exposed mice. Electrophysiological studies showed that the new neurons were functionally integrated in the hippocampal network. Expression of genes involved in differentiation of neurons was investigated and several of these were found to be increased (Cuccurazzu et al., 2010). In a later study these increases were found to correlate with improved performance in spatial learning and memory and recognition memory 1 month after the end of 7 days of exposure (Leone et al., 2014). The same paper also verified the boosting effect of exposure in cells on both proliferation and differentiation plus the involvement of Ca_{v1}-channels in this effect. Gene expression studies again revealed changes in expression of basic helix loop helix differentiation factors both *in vitro* and *in vivo* caused by epigenetic modulation. In the most recent study (Podda et al., 2014) exposure was performed after BrdU injections to affect survival of the labelled neurons. This was found to enhance the survival of the new-born neurons both in terms of number of BrdU labelled cells and BrdU-labelled cells co-expressing

neuronal markers (doublecortin and NeuN) and in terms of number of TUNEL stained cells. In the water maze and NOR memory tests improvement in performance was seen at this time.

1.5.1.4 Reported effects of power frequency magnetic fields on neurodegeneration

A number of studies have investigated effects of exposure on transgenic models of neurodegenerative disease or on processes known to be involved in neurodegeneration such as oxidative stress. See Table A1.4 in appendix 1 for details.

Improved spatial memory after MF exposure has been reported in two studies using mouse models of Alzheimer's Disease. The first study used a combined injection model; with D-galactose intra-peritoneally, to induce premature ageing, and β-amyloid intra-hippocampally, to induce neurodegeneration, and thereby induce a complex model of Alzheimer's Disease (Liu et al., 2015). The use of D-galactose to induce premature ageing has previously been demonstrated in kidneys by (Aydın et al., 2012). The mice were exposed to the MF continuously for 2 months at 0.4 mT (Liu et al., 2015). In the second study, the 3xTg mouse model of Alzheimer's Disease was used, and the mice were exposed for 20 hours per day at 0.5 mT for 3 months (Hu et al., 2016). A third study reported no effect of exposure at 1 mT continuously for 16 months. In the third study the APP23 transgenic mouse model was used, this model has a seven fold overexpression of human APP but no fibrillary tangles, in contrast to the 3xTg model (Liebl et al., 2015). The improvements in spatial memory were correlated with decreased phosphorylation of tau, decreased apoptosis and oxidative stress and increased expression of glutamate receptor subunits and post synaptic density proteins (Hu et al., 2016) and transient damage to hippocampal neurons (Liu et al., 2015), while no effects on microglia, astroglia, oxidative stress or the amyloid precursor protein were detected by Liebl et al. (2015), who did not see spatial memory improvement after the exposure. In two studies where aluminium overload was used to induce neurodegeneration, long-term exposure at 0.1 mT did not affect spatial learning and memory, neuronal integrity or amyloid load (Zhang et al., 2014), while long-term exposure at 2

mT caused impaired water maze performance and increased phosphorylation of tau and oxidative stress (Deng et al., 2013).

No effects of exposure were detected in two studies of superoxide dismutase (SOD)-1 mice, as a model of amyotrophic lateral sclerosis (ALS) (Liebl et al., 2015; Poulletier de Gannes et al., 2009). While improvement was detected after long-term exposure in a model of Huntington's chorea, including decreased oxidative stress and increase expression of growth factors (Tasset et al., 2012).

Effects of MF exposure on oxidative stress has been studied extensively in healthy rodents, but the understanding is inadequate for any conclusions to be drawn. (Mattsson and Simkó, 2012) and SCENIHR (2015) provides reviews of the more recent studies.

There are not many studies of effects of exposure on astroglia and microglia in healthy animals, only one recent study has reported that long-term exposure at 3 mT causes apoptosis in the hippocampus with a majority of the affected cells being glia (Teimori et al., 2016).

1.5.1.5 Summary of reported effects of power frequency magnetic field exposure

There are great differences in the protocols used to study effects of power frequency MFs on cognitive function. Some of the presented studies suggest that MFs can modify molecules and processes involved in cognitive function, which calls for further investigation.

More studies of effects of exposures at magnetic flux densities below 1 mT on performance in learning and memory tests are needed since both improvements and impairments in learning and memory have been described, although the majority of studies report no effect of exposure.

Many of the studies of effects of exposure on synaptic plasticity and neuronal signalling indicate that these processes might be sensitive to exposure even at exposure levels below 1 mT, but no firm conclusions can be drawn from the studies presented above, since the exposure levels, models and protocols differ.

Studies of effects of exposure on hippocampal neurogenesis have only been done at 1 mT, where increased proliferation, differentiation and survival was found, correlating with improved memory. This would be an interesting effect to study at exposure levels below 1 mT.

Also the studies of neurodegeneration are limited and the results are conflicting, however, some of these have suggested that MF exposure can protect against oxidative stress, hyper-phosphorylation or β-amyloid aggregation in studies of neurodegeneration.

In general, only few studies have been performed, reporting changes that could have both enhancing and impairing effects on cognitive function. More research is needed to understand the mechanisms and functional impact of the changes.

1.5.2 Proposed effects of power frequency magnetic field exposure on circadian rhythms

A connection between circadian rhythm disturbances, health and cognitive function is well established. A number of studies have suggested that the ubiquitous power frequency MFs in our surroundings affect circadian rhythms, this includes sleep quality and the rhythmic melatonin and cortisol levels (Akerstedt et al., 1999; Lewczuk et al., 2014). Moreover cryptochromes, the central players in circadian rhythm control, have been proposed as a possible magnetosensitive candidate molecule (Fedele et al., 2014a).

1.5.2.1 Reported effects of power frequency magnetic fields on clock genes

No studies of MF effects on clock gene expression *in vivo* appear to have been published. However, Manzella et al. (2015) have reported effects of exposure in human fibroblasts after exposure at 0.1 mT for 1 hour. The cells were kept in the dark and starved and no clock gene oscillations were seen in sham exposed cells. MF exposure was found to induce rhythmic expression of the core clock genes *bmal1, per2, per3, cry1 and cry2* with expression peaks at 8 hours and 32-36 hours post exposure. The expression amplitude decreased over time. It was found that in cells with induced clock gene expression by serum shock MF exposure did not alter the rhythm but did cause significant increases in expression of all genes (Manzella et al., 2015).

1.5.2.2 Proposed effects of power frequency magnetic fields on cryptochromes

One of the core clock genes, the cryptochromes, are thought to be particularly interesting in regard to magnetosensitivity with possible effects on circadian rhythms and cognitive function. The radical pair mechanism is thought to be the central mechanism for interaction between MFs and the cryptochrome molecule. WHO has called it the most plausible mechanism of magnetosensation (WHO, 2007). It was first proposed by Schulten et al. in the 1970s, suggesting that a MF can affect the spin of a radical pair, and it has later been shown that this can happen with MFs up to 20 MHz (Evans et al., 2013, 2015; Schulten and Weller, 1978).

The cryptochromes have been described in plants, insects, birds and mammals. They have N-terminal sequence-similarities with photolyases, a family of proteins which uses an electron transfer mechanism for DNA-repair after UV-light damage. There is great variation in C-terminal structure between species (Liedvogel and Mouritsen, 2010). In *drosophila* two types of cryptochromes are found. CRYI is a blue-light receptor and is found in the retina, and CRYII which is found in the SCN and is involved in circadian rhythm control. The vertebrate cryptochrome is of the CRYII type (Vanderstraeten et al., 2015).

The cryptochromes have been found to be magnetosensitive in *Drosophila* (Gegear et al., 2008, 2010). CRY dependent disruption of circadian rhythms in *Drosophila* have been reported after MF exposure in combination with blue light exposure (Yoshii et al., 2009). Also disruption of spatial orientation has been reported (Fedele et al., 2014a). In species with magnetosensitive CRY the radical pair effect occurs in the flavin adenine dinucleotide (FAD) where energy absorption leads to electron transfer which either causes an antiparallel or parallel electron spin which can then be affected by the external MF possibly affecting the time and rate of the reaction (Vanderstraeten et al., 2015). Other flavin containing proteins are known but none of these have been found to be magnetosensitive (Messiha et al., 2015).

In mammals there is no compelling evidence that CRY works as a photopigment (Owens et al., 2012). It has been speculated that the reduction in the FAD molecule required for magnetosensitivity could be achieved by factors other than light (Vanderstraeten et al., 2015). Human CRY2, but not CRY1 expressed in *Drosophila* has been reported to be sensitive to MF exposure, in terms of changes in activity of the flies (Fedele et al., 2014a). Investigation of mammalian magnetosensitivity in mouse SCN slice cultures under blue light in terms of changes to *per2* expression, showed no effect of exposure. The group concluded that the cellular environment plays an important role. More studies are needed to understand if changes to the cellular environment can make the mammalian CRY magnetosensitive.

1.5.2.3 Reported effects of power frequency magnetic fields on sleep

In addition to the proposed sensitivity of cryptochromes to MFs, studies of effects on sleep, activity and cortisol and melatonin levels have also been performed.

Two studies have investigated effects of power frequency MF exposure during sleep in humans (Akerstedt et al., 1999; Graham and Cook, 1999). Using electroencephalography (EEG) continuous exposure at a magnetic flux density of 1 μT lead to disturbances of sleep in healthy volunteers, measured by total sleep time, sleep efficiency, slow wave sleep and slow wave activity and subjective sleep quality (Akerstedt et al., 1999). Similarly it has been found that intermittent, but not continuous, exposure at 28 μT cause shorter sleep time, decreased sleep quality and efficiency (Graham and Cook, 1999). A number of studies have evaluated effects of exposure on sleep quality using other methods of evaluation. By self-assessment small effects of residential MF exposure were found on self reported sleep disorder in women (Li et al., 2002). In another study worse sleep quality and sleep duration was reported in workers that were exposed more than 4 hours daily at 6 μT compared to workers spending between 1.5-4 working hours at this exposure level (Liu et al., 2014). Another study investigated sleep duration and quality at 1-1.5 μT using an actigraph during a 5 day period. No effect of exposures on these parameters were seen (Tworoger et al., 2004).

One study has investigated effects of exposures at 60 Hz on sleep in rats. The rats were exposed continuously for 30 days at 100 μT, and sleep quality was monitored by implanted electrodes in the brain, no effect of exposure was found (Dyche et al., 2012).

The current knowledge of effects of MFs on sleep is limited and interpretation of findings is difficult since many other factors affect sleep.

Circulating melatonin and cortisol/corticosterone levels show a strong circadian rhythm. MF induced changes to circadian rhythm control might affect these, and on the other hand effects of exposure on these hormones could affect sleep and in turn cognitive function. Many studies

have been performed to investigate such an effect, based on an early report of reduced melatonin secretion after exposure but no conclusions can be drawn due to variation in methodology and conflicting results (Lewczuk et al., 2014; Vanderstraeten et al., 2012).

1.5.3 Reported effects of radio frequency field exposure associated with mobile phone use on learning and memory in rodents

The use of mobile phones by the general public is a relatively new phenomenon, the knowledge of effects of exposure on cognitive function is therefore still limited and only a few studies have looked at effects on the plastic processes involved in learning and memory, while more focus has been on damaging effects of exposure on neurons, and effects in models of neurodegenerative disease.

There is great inconsistency in both the methods used and the results of studies investigating the effect of mobile phone like-RF exposure on cognitive function in rodents (SCENIHR, 2015). The use of commercial mobile phones as exposure sources and the lack of proper dosimetry in a number of studies further complicates the interpretation and understanding of effects of exposure (SCENIHR, 2015). These and other studies with dosimetric or other deficiencies are not included in the summaries below.

1.5.3.1 Reported effects of radiofrequency fields on performance in cognitive tests

Not many well-performed, well-controlled studies of effects of 900-2100 MHz exposure on learning and memory in healthy young and adult rodent are available. Impaired spatial memory was found after long-term exposures in a number of studies (Deshmukh et al., 2013, 2015; Megha et al., 2012; Tang et al., 2015a), it should be noted that three of these studies are from the same group. Others have reported no effect of exposure using various protocols and tests of spatial memory (Ammari et al., 2008a; Dubreuil et al., 2002, 2003; Klose et al., 2014; Sienkiewicz et al., 2000; Yamaguchi et al., 2003). See Table A2.1 in appendix 2 for details.

In the non-spatial NOR test, one group has reported no effect of exposure 45 minutes before or between trials on object recognition (Dubreuil et al., 2003). While recently another group reported increased interest in the novel object 3-8 days after 30 minutes exposure (Wang et al., 2017).

There is great variation in the test regimes used in the studies mentioned above. For example a number of studies use head-mainly exposures which model the exposure of human mobile phone use better than whole body exposures, but it does at the same time require restraint of the rodents during exposure which can cause stress and therefore prevent detection of possible RF induced changes (Ammari et al., 2008a; Dubreuil et al., 2002, 2003; Tang et al., 2015a). The exposure periods in the studies varied from 45 minutes to 19 months of exposure, and also the SARs used varied greatly (see Table A2.1 in appendix 2 for details). There appear to be no correlation between increasing SAR or exposure length and memory impairment, and more studies are needed to draw any clear conclusions of effects of exposure on learning and memory.

1.5.3.2 Reported effects of radiofrequency fields on synaptic plasticity

Very little is known about effects of 900-2100 MHz RF fields on the structural, cellular and molecular processes involved in learning and memory.

Electric activity

The number of studies investigating effects of exposure on neuronal activity as a functional measure of changes to synaptic function in young adult and adult rodents is limited (Table A2.2 in appendix 2). ICNIRP (2009) points out that there are many external factors that might affect the results of electrophysiological experiments and the use of EEG, including interference between fields and electrodes, and antagonism from the use of anaesthesia and restraint on neuronal activity. No conclusions could be drawn based on the studies that were available at that time. Since then a few studies have been published. Prochnow et al. (2011) showed that exposure at 2 GHz to the head of rats continuously for 2 hours had no effect on long-term potentiation or long-term depression at a SAR of 2 W/kg, but exposure at 10 W/kg decreased both parameters. Recently Wang et al. (2017) reported increased excitability of pyramidal cells in both the hippocampal CA1 and the medial prefrontal cortex 3 days after 30 minutes exposure at 3.3 W/kg. In studies where neuronal activity was investigated by EEG very different

experimental protocols were used. Vorobyov et al. (2010) found increased beta-wave activity in wake EEG in rats exposed at 915 MHz for 3x10 minutes per day for 3 days over a 5 day period. Hidisoglu et al. (2016) found that the latency for visual evoked potentials was decreased after 7 days of exposure, while it was increased after 10 weeks of exposure. In a study by Crouzier et al (2007), a transient decrease in duration of REM sleep was seen.

These studies suggest that RF fields might be able to affect neuronal activity under some circumstances. However, there is not sufficient evidence to make clear conclusions.

c-fos

The immediate early gene c-fos has been used as a marker to investigate effects of RF exposure on neuronal excitability and activity (Table A2.2 in appendix 2). Three studies have looked at the effects of acute exposure, in two of these the restraint of the animals was found to increase c-fos mRNA and protein levels (Finnie, 2005; Stagg et al., 2001), while in the last study no effect of exposure was seen unless exposure was increased to thermal levels (Morrissey et al., 1999). In a fourth study the effect of 2 years of exposure at 4 W/kg for 1 hours per day was investigated, and this also did not affect expression (Finnie et al., 2007).

Structural changes

Only one structural study appears to have investigated the effects of exposure on dendrites and synapses (Wang et al., 2017). See Table A2.2 in appendix 2 for details. This reported exposure-induced changes to dendritic spine density, dendritic diameter and spine length in the hippocampus and mPFC three days after exposure at 3.3 W/kg for 30 minutes. Different responses to exposure were found in the two brain regions. In the hippocampus the spine density on second order basal dendrites was found to be increased by 69%, while there was no change to spine density on first order basal dendrites. In the mPFC exposure increased spine length slightly by 10% while spine density was not affected (Wang et al., 2017).

Neurotransmitters

Glutamate is the most abundant excitatory neurotransmitter in the mammalian brain. No studies of effects of exposure on hippocampal glutamate levels appear to have been published. However, a couple of studies have shown age-dependent, transient and region specific changes in the cortex, midbrain, cerebellum and medulla (Khadrawy et al., 2009; Noor et al., 2011). See Table A2.2 in appendix 2 for details.

In terms of effects on glutamate receptors, one study has reported that acute RF exposure at 6 W/kg greatly impacted on protein levels of the NMDA receptor subunits and receptor activation, especially in the cortex but also in the striatum and hippocampus. Great decreases in receptor expression in the postsynaptic density were seen (30-90%), while the changes in the total homogenate were smaller (10-20%), indicating that exposure causes the receptors to move out of the synapse (Mausset-Bonnefont et al., 2004).

Some studies have investigated the effects of exposure on other neurotransmitters with no clear field-dependent effects reported (SCENIHR, 2015).

Growth factors

Only one study appears to have looked at changes in expression of growth factors after RF exposure (Maskey and Kim, 2014). This showed a significant decrease in both BDNF and the glial derived neurotrophic factor in the auditory system after long-term exposure.

1.5.3.3 Reported effects of radiofrequency fields on adult neurogenesis

Addition of immature cells to the SGZ of the hippocampus throughout life contributes with plasticity to this region. The influence of RF fields on adult neurogenesis has only been investigated in a few studies. See Table A2.3 in appendix 2 for details.

Kim et al. (2015) looked at the effects of exposure on proliferation after exposure to 848.5 MHz for 2 weeks at 2 W/kg in a reverberation chamber. BrdU was injected daily for the last 5 days of

exposure and the number of BrdU-labelled cells was counted in the SGZ and SVZ. There was no effect of exposure in either region.

Two papers have used the proliferating cell nuclear antigen (PCNA) as an endogenous marker of proliferation and studied effects of long-term, high dose RF exposure. Son et al. (2015) exposed mice at an SAR of 5W/kg for 60 days and found no effect of exposure. Kim et al. (2008), exposed rats in a head-mainly exposure system at a SAR of 7.8 W/kg for 6 and 12 months. No differences between exposed and sham exposed mice were seen on the number of PCNA expression cells in the SGZ or the SVZ.

A couple of studies have investigated effect of exposure during development. One group found significantly fewer neurons in the hippocampus at 4 weeks of age in rats exposed prenatally which could be a result of impaired neurogenesis (Bas et al., 2009; Odaci et al., 2008). While another study found that exposure of 7 days old rat pups increase the number of BrdU labelled cells in the hippocampus, however at the same time endogenous proliferation markers was decreased indicating a more complex effect of exposure than just stimulation of proliferation. Exposure of 21 day old rats had no effect (Xu et al., 2017).

1.5.3.4 Reported effects of radiofrequency fields on neurodegeneration

Effects of RF exposure on neurodegeneration has been studied both in healthy rodents and in transgenic and experimental models of neurodegenerative diseases.

In healthy rodents, long-term exposures have been reported to lead to up-regulation of pro-inflammatory cytokines (IL6 and TNFα), oxidative stress (Hidisoglu et al., 2016; Megha et al., 2012) and increased apoptosis (Maskey et al., 2012). A couple of studies of autophagy related genes have reported increases after long-term exposure in the cortex, but not in the brain stem (Kim et al., 2017a, 2017b). In contrast to the indications of harmful effects of exposure other studies have found no effects of exposure on oxidative stress, apoptosis, pro-inflammatory cytokines and cell loss (Bouji et al., 2012; Kim et al., 2008; Kumlin et al., 2007; Maaroufi et al.,

2014; Poulletier de Gannes et al., 2009). Reduced apoptosis has been reported by Dasdag et al. (2009). See Table A2.4 in appendix 2 for further details.

Glia cells perform many supportive roles for optimal neuronal functioning, however activation of glia can cause damage to the tissue. Effects of exposure on astroglial reactivity has been reported. A number of studies have used an acute exposure protocol, looking at the response after 15 minutes of exposure at an SAR of 6 W/kg. This SAR might cause heating of tissues. Significant increases in glial fibrillary acidic protein (GFAP) were found 2-3 days after exposure in various brain regions, including the hippocampus by several groups (Barthélémy et al., 2016; Brillaud et al., 2007; Mausset-Bonnefont et al., 2004). No effect was found by Bouij et al. (2012). Other studies have looked at long-term exposure. At SARs of 4-7.8 W/kg where heating might occur conflicting findings were reported after long-term exposure. Court-Kowalski et al. (2015), Maskey et al. (2012) and Ammari et al. (2008b, 2010) reported exposure induced increases in astroglial staining density, while Kim et al. (2008) and Son et al. (2015) found no effect of exposure. Also long-term exposure at SARs thought not to cause any overall or large thermal effect has been reported to affect astroglia. Maskey et al. (2012) found increased density of GFAP in the CA1 but not in the DG after 1 month of exposure. Ammari et al. (2010) found increased staining in the DG 3 days after 2 months of exposure, but not 10 days after, and Maskey et al. (2010) found increases in staining in the CA1, CA3, hilus and GCL after 3 months of exposure, all at SARs of 1.5-1.6 W/kg. See Table A2.4 in appendix 2 for details.

Effects of exposure on microglial numbers has been much less studied. Two papers have been published, both reporting no effect of long-term exposure (2 months or 2 years) (Finnie et al., 2010; Son et al., 2015).

The blood brain barrier (BBB) protects the brain from damaging substances in the blood while allowing passage of oxygen and nutrients. Altered permeability is considered to be harmful to the brain. Several studies of effects of RF exposure have been carried out. An early study using a

commercial mobile phone showed increased permeability of the BBB after exposure (Salford et al., 2003) three independent studies where more controlled exposure systems were used have tried to replicate this without success (Masuda et al., 2009; McQuade et al., 2009). However Sirav et al. (2011, 2016) and Tang et al. (2015) also found increased permeability in rats.

Reported effects of radiofrequency fields on the ageing and degenerating brain

While only few epidemiological studies of associations between mobile phone frequency field exposure and neurodegenerative diseases have been performed, a number of studies using transgenic and experimental rodent models of neurodegenerative disease have revealed interesting effects of exposure on disease progression (see Table A2.4 in appendix 2).

A series of studies have demonstrated protective effects of RF fields towards development and progression of AD pathology. Findings include normalisation of cognitive performance in spatial memory tests, decreased β-amyloid deposition, increased soluble β-amyloid (Arendash et al., 2012; Arendash et al., 2010) and normalisation of mitochondrial function (Dragicevic et al., 2011). Later, similar beneficial effects of exposure have been demonstrated in a different transgenic mouse model (Jeong et al., 2015). However using the same mouse model, the same group found no effect of exposure on cognitive function or amyloid load (Son et al., 2016). A study in healthy rats reported increased β-amyloid-40 concentration and oxidative stress in the brain after long-term exposure (Dasdag et al., 2012).

Using the experimental allergic encephalomyelitis (EAE) model of multiple sclerosis (MS) exposure for 3 weeks did not affect disease onset or progression (Anane et al., 2003).

In regards to epilepsy, a couple of studies have shown that in an acute disease model, using sub-convulsive doses of the GABA-A receptor antagonist picrotoxin, 2 hours of RF exposure induce seizures and increase c-fos expression in a number of brain regions 90 minutes, 1 day and 3 days after exposure (Carballo-Quintas et al., 2011; Lopez-Martin et al., 2006).

A number of studies have investigated effects of exposure in aged rodents, or performed long-term exposure for 6-24 months, thereby looking at effects of exposure on ageing. Arendash et al. (2010) reported that long-term exposure of healthy young and aged wild type mice enhanced learning and memory after 5-8 months of exposure and decreased oxidative stress in the hippocampus but not in the cortex. In a later study this group showed enhanced working memory performance in the Y-maze, but no effect of exposure in the spatial RAM in 21-26 months old mice (Arendash et al., 2012). In contrast, no effect on astroglia, c-fos, apoptosis or memory has been reported after long-term exposure (Court-Kowalski et al., 2015; Finnie et al., 2007; Kim et al., 2008; Klose et al., 2014). One study has investigated differences in sensitivity to acute (15 minutes) or repeated (26 days) exposure in young adult versus aged adults rats finding no major difference in terms of cytokine levels, astroglial reactivity or learning and memory in spatial and non-spatial tests (Bouji et al., 2012, 2016).

1.5.3.5 Summary of reported effects of radiofrequency field exposure

While many studies of effects of RF exposure on performance in learning and memory tests have been performed, very little is known about effects of exposure on the plastic processes involved in this. Furthermore, many studies are not reproducible due to use of improper exposure sources and inadequate dosimetry. The results of other studies may be influenced by heating caused by high exposure levels and stress due to restraint during exposure.

The reported effects of exposure on performance in learning and memory tests are conflicting and very little can be concluded on effects of RF exposure on synaptic plasticity and neuronal signalling from the available data, but several studies do suggest changes that potentially could affect cognitive function, and more studies are therefore needed. Studies indicate no trophic effect of exposure on neuronal progenitor cells in the hippocampus. However, there are very few studies and none of them look at effects of exposure on differentiation, migration and survival of the new granule cells during and after exposure.

Both neurodegenerative and neuroprotective effects of RF exposure have been reported. However, there are still only few studies looking at the various endpoints regarding such effects and more research is needed. Only a few models of neurodegenerative diseases have been used to investigate sensitivity of the degenerating brain to RF exposure, the reported beneficial effects of exposure are intriguing since they suggest a possible reduction of the disease burden as a result of mobile phone use and a potential use of exposures for treatment, but this requires further investigation.

Aim of study

The current understanding of effects of low level EMFs on cognitive function is limited. The aim in this thesis was to improve this understanding with particular focus on the two major sources of exposure to the public, power frequency MFs and RF fields associated with mobile phone use.

To improve the understanding of the impact of EMFs on cognitive function, well-controlled exposure systems, with well-described dosimetry, are required. The use of low exposure levels, relevant to exposures experienced by the public, are needed in particular to properly evaluate the potential impact of EMF on human health and wellbeing. Furthermore, studies that combine investigations of molecular, cellular and functional effects are needed for a better understanding of whether, or how, EMFs might affect cognitive function.

In this thesis, using such a multimodal approach (gene expression studies using qPCR; protein expression studies using western blotting and immunohistochemistry; functional learning and memory testing; and behavioural studies), the following hypotheses were tested:

- That molecules and cells involved in plastic process underlying cognitive function are sensitive to exposure to MF or RF fields and if this affect cognitive function.
- That ageing affects the sensitivity of the plastic processes underlying cognitive function to RF field exposure.
- That power frequency MFs can disturb circadian rhythm control, since changes to this might affect cognitive function.

Chapter 2 Methods and Materials

2.1 Animal work

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and all protocols were approved by the local Animal Welfare Ethical Review Board (PHE, Chilton). Mice were maintained on a 12 hour light-dark cycle unless otherwise described and fed water and standard food *ad libitum*. All mice were supplied from Envigo (Bicester, UK). Male C57BL/6J, senescence accelerated mouse prone 8 (SAMP8) and senescence accelerated mouse resistant 1 (SAMR1) were used. For enrichment of the housing environment mice were group-housed in cages containing a running wheel and cardboard tubes.

2.1.1 Electromagnetic exposures

2.1.1.1 Radio frequency field exposure system

For Global System for Mobile Communication (GSM) signal exposures at 1800 MHz, the SxV-exposure system from IT'IS, Zurich, was used (Figure 2.1). The system consists of 3 identical reverberation chambers with inner dimensions of 70 x 65 x 100 cm. Each chamber has room for 6 single cages and allows whole body exposures with whole body SARs of 3 W/kg, and 0.3 W/kg. Sham exposures at 0 W/kg were performed simultaneously under the same conditions, with the reverberation chamber energised, but without any field being generated.

All chambers have two orthogonal stirrers with operating speeds ensuring the greatest complexity and homogeneity of the field. The exposure system consists of a signal generator, a signal amplifier, a control unit, a computer and a data acquisition unit: EASY4 (Exposure acquisition system 4) which processes the information from probes placed in the chambers. During exposures the field, temperature, humidity, light and airflow are monitored. The average weight of the mice was used by the system to calculate the appropriate exposure parameters to obtain the SARs used in this study. Animal weights were updated weekly.

The dosimetry was performed by IT'IS Zurich, using the FDTD simulation platform SEMCAD X, described in the dosimetry report for the GERoNiMO project prepared by Capstick et al. (2014). The overall homogeneity of the field inside the exposure region was 0.61 dB with all points within ± 2.0 dB, the field isotropy was 0.88 dB.

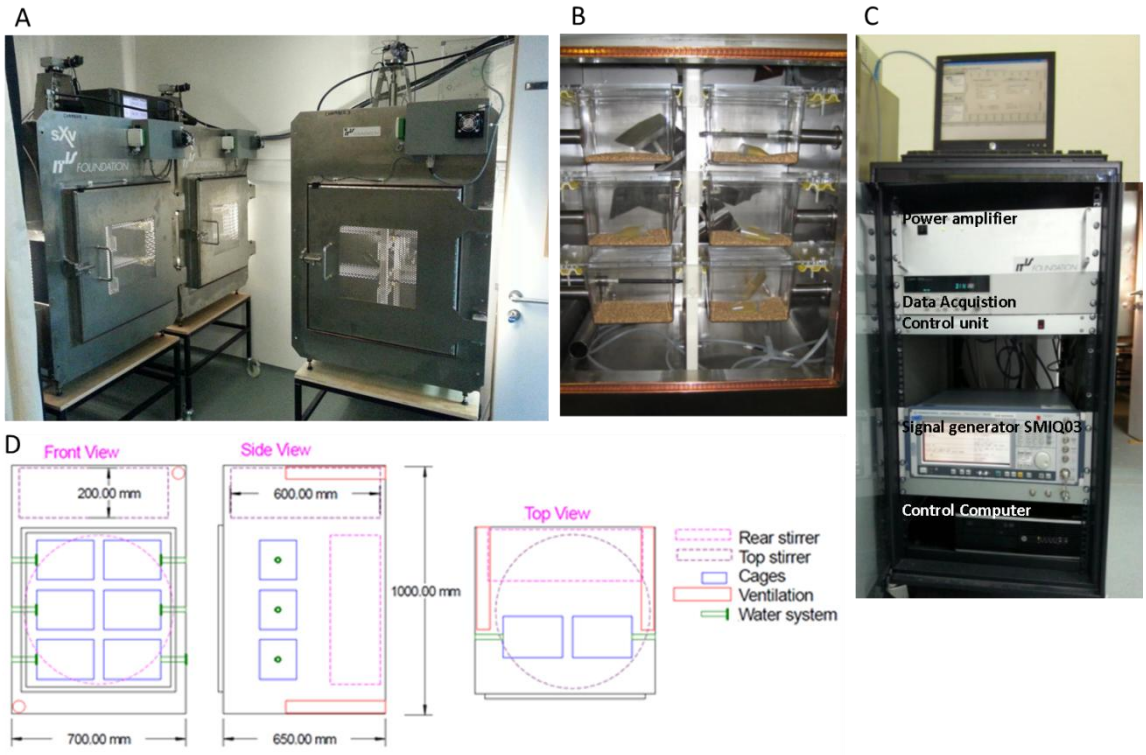


Figure 2.1 The IT'IS SxV-1800 reverberation chambers. The RF exposure system consists of three reverberation chambers (A), each with 6 single mouse housing cages (B). Photo (C) shows the control unit with a computer, a signal generator, a power amplifier and a data acquisition control unit. The schematic presentation of the chambers (D) shows the cage from the front, side and top, with dimensions, cage positions (blue), the two orthogonal stirrers in purple and pink and the ventilation in red. The water system, in green, was not installed in this experiment. Photos and illustrations from (Capstick et al, 2014).

2.1.1.2 Power frequency magnetic field exposure system for exposures up to 300 μT

Magnetic field exposures at 50 Hz up to 300 μT were performed in an exposure system consisting of two pairs of Helmholtz coils (Figure 2.2A). The MF was generated using a function generator (HP 3324A) and a power amplifier (Kepco BOP-50-2 M) connected to the coils. The coils were made of a pair of horizontal coils with a diameter of 54 cm and a pair of vertical coils with a diameter of 99 cm. Each coil was made of 150 turns of 1 mm (diameter) copper wire wound around a frame of Tufnol resin (Clarendon Laboratory, University of Oxford). The average background static MF in the laboratory (30 μT) was maintained stable using a custom-made software and a PC connected to a 3-axis fluxgate magnetometer (MAG-03 MC, Bartington Instruments, Witney, UK) placed inside and aligned with the coils. The 50 Hz electric field exposure was kept at a minimum by an earthed screen wound around the coils. Vibration was minimised by felt pads between the table and the exposure system. The average background 50 Hz MF was 0.1-0.2 μT. Sham exposures were performed in the same system, with all components energised, but without any alternating magnetic field being generated. A cylindrical cage (diameter of 45 cm, height 38 cm) was placed in the middle of the coils and mice were supplied with food and water *ad libitum*.

The MF in the coil at the position of the cage was mapped at 100 μT and 300 μT using a 3-axis fluxgate magnetometer (Bartington Instruments, Mag-03MCI000) coupled to a system used by the NIR Services group at PHE for commercial surveys. This consisted of a magnetometer coupled to a power supply unit, a homemade analogue to digital converter and a PC running customized software measured the output of the magnetometer in terms of X, Y, Z axis and produced the resultant field. Measurements were made at both 100 μT and 300 μT and taken every 3 cm on the diameter of the circular floor, from left to right (Figure 2.2, B top panel) and back to front (Figure 2.2, B bottom panel). The field was measured at three positions in height, the floor level of the cage, 6 cm above where the head of the mice would be when rearing, and

12 cm above, which was higher than where the mice would be found. In Figure 2.2B, the graph shows measurements within the cage and outside of the cage. The measurements are presented as magnetic flux density in percentage of that measured in the centre of the horizontal coils at the level of the cage floor.

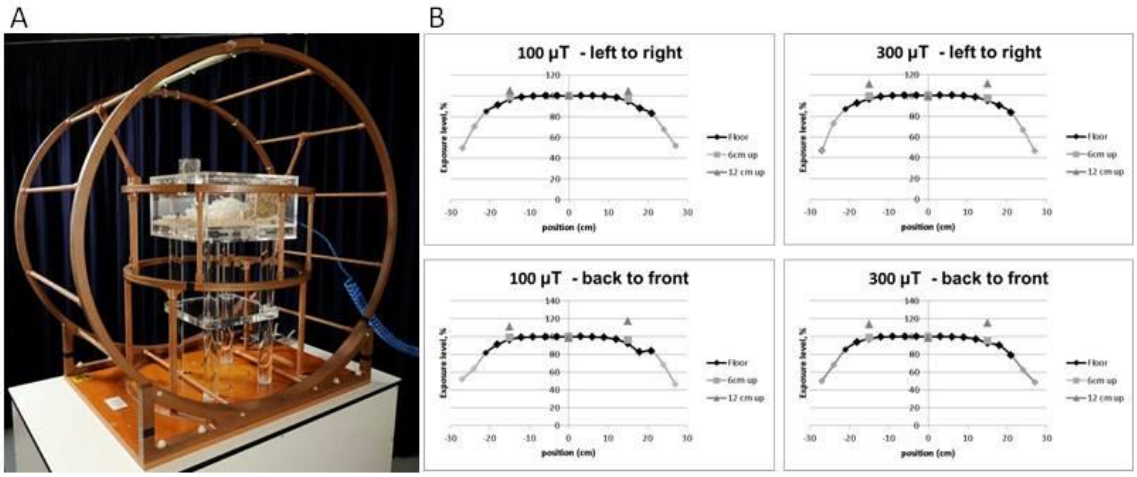


Figure 2.2 Magnetic field exposure system for exposures up to 300 µT. The 50 Hz MF exposure system consists of two pairs of Helmholtz coils (A), the horizontal coils generate the 50 Hz field while the vertical coils keep the background static field constant at 30 µT. Figure B shows the homogeneity of the MF at 100 µT (left panel) or 300 µT (right panel), in two dimensions, left to right of the coils (top panel) or back to front (bottom panel). This is expressed every 3 cm (x-axis) as variation from the level measured at the centre of the two pairs of coils in percent (y-axis).

2.1.1.3 Power frequency magnetic field exposure system for exposures up to 580 µT

Exposures at a magnetic flux density of 580 µT were performed in an exposure system consisting of a pair of Helmholtz coils (Figure 2.3A). The system was designed and built at CRCE for this project, and the intention was to construct a system capable of producing a stable, homogenous 50 Hz field at 600 µT. The MF was generated using a function generator (Keysight Technologies, Waveform Generator 33502B,) and two power amplifiers (Kepco Inc, Bipolar Operational Power Supply/Amplifiers, BOP 50-8M) connected to the coils, and controlled via a personal computer (PC). Each coil had an external diameter of 0.85 m and was made of 180 turns of 1 mm bifilar copper wire wound around a frame of Tufnol resin. Each BOP was connected to one of the bifilar

pairs so either a null field or a x2 field could be made. The 50 Hz electric field exposure was kept at a minimum by an earthed screen wound around the coils. The coils were calibrated using bespoke software that adjusted the output of the amplifiers while measuring the field generated using a 3-axis fluxgate magnetometer (Bartington Instruments, MAG-03MC1000). The long-term, average background 50 Hz MF was measured to be 0.1-0.2 μT. Mice were housed alone or in groups in cylindrical "hatbox" cages in the middle of the coils and were supplied with food and water *ad libitum*.

The homogeneity of the MF was measured at the horizontal level of each coil and at the centre of the coils using instrumentation used by the NIR Services group at CRCE for commercial surveys. A Bartington MAG-03MC1000 magnetometer was coupled to a power supply unit and a homemade analogue to digital converter. A PC running customized software measured the output of the magnetometer in terms of X, Y and Z axes and produced the resultant field. Measurements were made at 100 μT and 580 μT every 10 cm on the diameter of the circular floor, from left to right (Figure 3.2B, top) and back to front (Figure 2.3B, bottom) at each of the three horizontal levels. The measurements are presented as magnetic flux density in percentage of that measured in the centre of the coils. The field was found to be highly homogenous within a diameter of 40 cm from the centre of the coils, which corresponded with the size of the hatboxes that housed the mice.

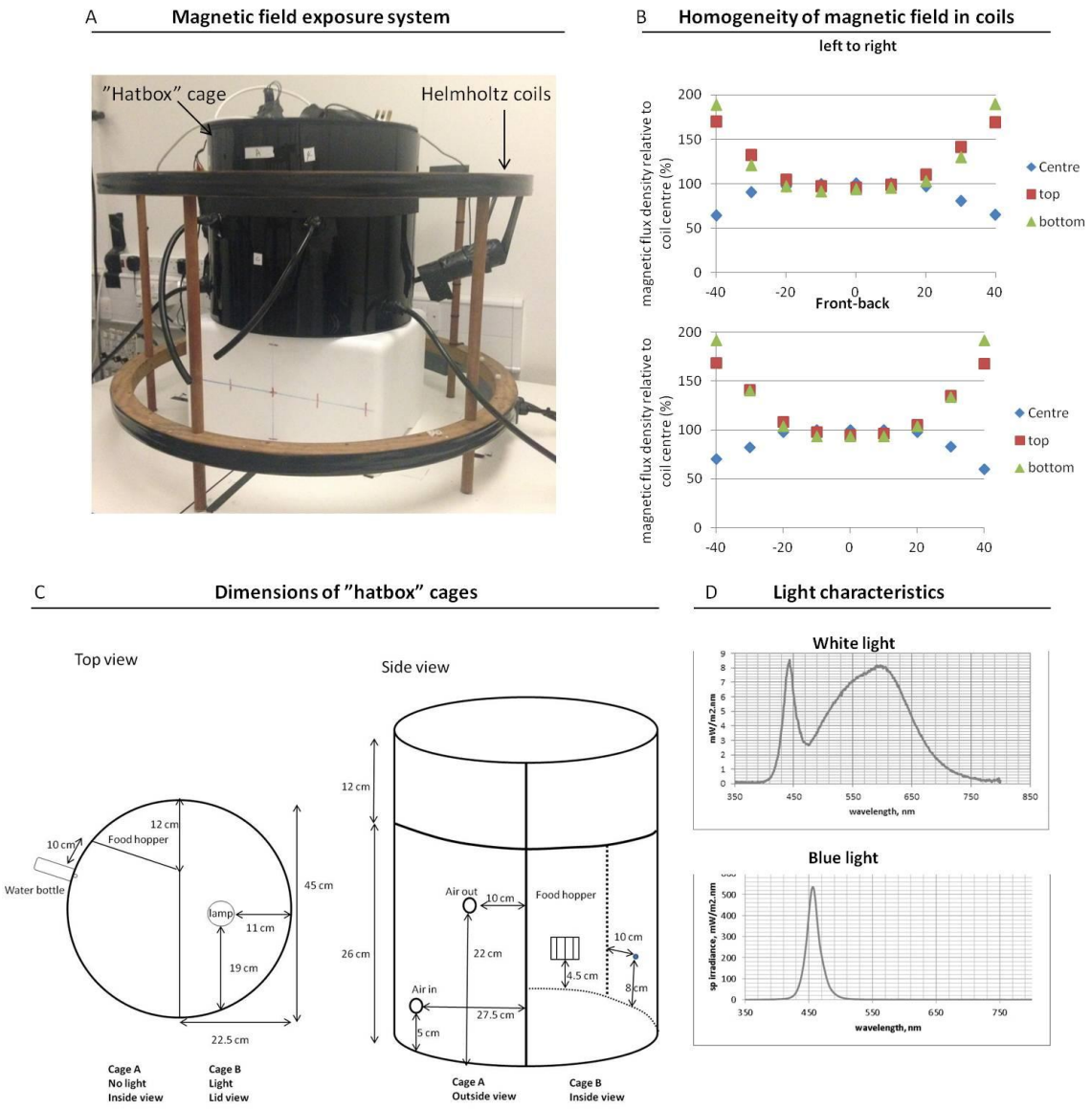


Figure 2.3 Magnetic field exposure system for exposures up to 580 µT and cage, light and MF characteristics. Mice were housed in a hatbox cage placed in the middle of a pair of horizontal Helmholtz coils (A). The magnetic flux density was measured in the coils (B) and the percent variability relative to that in the centre of the coils is presented, measured in two directions: left to right and front to back every 10 cm. This was done at three planes: the centre of the coils (blue), at the level of the top coil (red) and at the level of the bottom coil (green). The hatbox cages were designed to minimise any external stress and were light proof. Each cage was 38 cm high with a diameter of 45 cm and consisted of two compartments, made by a wall at the midline of the cage (C). On one side a lamp was mounted in the lid, while the other side serve as a dark control. Two different lamps were used for light shock experiments, figure (D) shows the spectral distributions, the white light was broad spectrum with a blue peak at 445 nm, while the blue lamp showed a distinct peak at 457 nm.

2.1.2 Equipment for light and day rhythm manipulation experiments

2.1.2.1 Hatbox cages

The MF ± light shock experiments presented in Chapter 5 were carried out in two identical hatbox cages. These cages were cylindrical in shape (diameter of 45 cm, height 38 cm) and made of lightproof, black polymethyl methacrylate material. Each cage consisted of two compartments, made by a wall at the midline of the cage (Figure 2.3C). On one side, a lamp was mounted into the ceiling, while the other side served as a dark control. Food and water was available *ad libitum*. To ensure a proper airflow in the boxes, air was provided at 2 L/minute and an outlet was placed near the top of the cage. One hatbox was placed inside the Helmholtz coils while another served as a sham control. This hatbox was in the same room as the other to ensure consistent environmental conditions, but was placed away from the coils at a distance where the stray field from the coils was <6 μT.

2.1.2.2 Light characteristics

For white light exposures, a broad spectrum light emitting diode (LED) light was used (Figure 2.3D, top) with an intensity of 150 lux at the centre of the floor of the compartment.

For blue light exposures, a narrowband blue LED light (Interlux, GU10) with a peak at 457 nm was used at an intensity of 700 lux at the floor in the centre of the compartment (Figure 2.3D, bottom).

2.1.2.3 Reversal of day rhythm

For reversing the day rhythm, mice were housed in a ventilated cabinet fitted with a light adjustment kit (Scanbur, Karlslunde, Denmark) where the light gradually comes on, reaching 80 lux and gradually goes off again 12 hours later. One day was allowed for adjustment per hour reversal of the day rhythm.

2.1.2.4 Recording of activity and sleep

The behaviour of the mice was recorded using a digital infrared charge-coupled device (CCD) camera (Sony, NC1358WA) connected to a digital video recorder (Samsung, SRD-476D). Sleep (latency and duration) was measured manually as immobility, where the mice had closed eyes. The distance moved by the mice was measured using Ethovision XT10 (Noldus).

2.1.3 Labelling of proliferating cells

To label dividing cells the thymidine analogue 5-bromo-2'uridine (BrdU) (Sigma, B5002) was injected intraperitoneally at a dose of 75 mg/kg daily for 4 consecutive days immediately before exposure to 50 Hz MFs or 1800 MHz RF fields.

2.1.4 Behavioural testing

2.1.4.1 Open field and novel object localisation tests

A 28x42 cm box with frosted sides was used for the novel object localisation (NOL) test (Figure 2.4). First, the mice were allowed to habituate for 10 minutes. Movement was tracked using Ethovision XT 10 (Noldus) to test for differences in exploration and locomotor activity. Exploration was investigated using the nose-point tracking feature in a 2 cm zone around the objects. 24 hours later, the NOL test was performed. In the familiarization trial (trial 1) mice were allowed to freely explore the two objects placed at two corners of the arena for 5 minutes. Two different experiments were performed; in the first, large glass marbles (4 cm diameter) were used and in the second test, purple asymmetric plastic bricks were used (2.5x3 cm). After a 15 minute delay, the test (trial 2) was performed. One object was returned to its former location, while the other object was placed in a new location (counterbalanced across subjects). The arena and objects were cleaned with a 70% ethanol solution and dried between subjects to eliminate odour cues. In trial 1, exploration time was exploring the two objects. In trial 2 the fraction of time spent exploring the novel object was investigated. Mice were successful if they spent significantly more time than chance (50%) exploring the object in the novel location. The

cumulative duration spent exploring the two objects, the frequency of visits to the two objects, and fraction of the time used exploring spent at the novel location were investigated.

2.1.4.2 Water maze

A circular black plastic pool was filled with water (18-21˚C) made opaque by the addition of non-toxic white paint (Figure 2.4). The arena was divided into quadrants designated 1-4 where a circular platform was placed 0.5 cm beneath the surface of the water in the centre of one quadrant. 3D distal cues were placed around the room for the mice to orientate themselves. The movement of the mice was tracked and analysed using Ethovision XT 10 (Noldus). A 1 minute pre-trial was performed to familiarize the mice with the pool and assess motor performance of the mice. This was followed by 7 acquisition trials over a period of 4 days, each consisting of 4 swims for 1 minute or until the platform was found. Each swim started at a new position (Quadrant 1,2,3,4) while the position of the platform remained fixed. Finally, a probe trial investigating the reference memory was performed, where the platform was removed and the mouse was left to swim for 1 minute.

Spatial learning was analysed in terms of the distance moved and escape latency over the first 7 trials. To evaluate reference memory, the time spent in the platform quadrant, platform zone crossings and the platform zone latency was investigated.

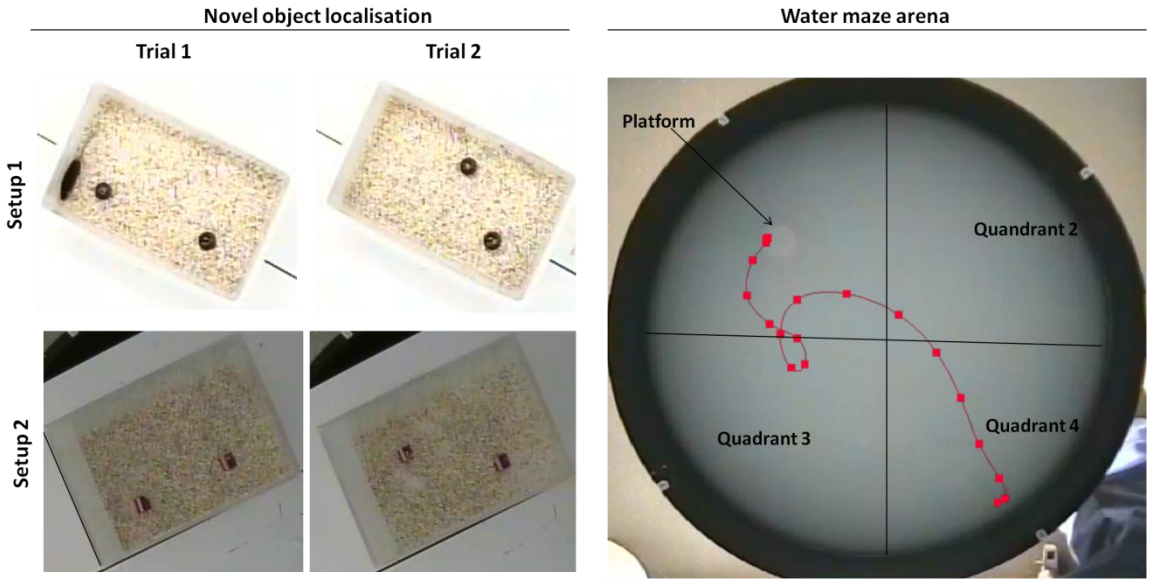


Figure 2.4 Arenas used for memory testing. For NOL testing a box with clean bedding was used as an arena (left panel). In the first test, marbles were used, and in the second, interlocking plastic bricks was used. In both tests, the position of one object was changed from trial 1 to trial 2 (left). Water maze testing (right panel) was done in a circular black plastic pool filled with water made opaque by the addition of non-toxic white paint. A circular platform was placed 0.5 cm beneath the surface of the water in the centre of one quadrant .

2.1.5 Termination

Mice were terminally anaesthetised for cardiac puncture using carbon dioxide (CO_2) overdose and for formalin fixation and brain dissection using an intraperitoneal injection of sodium pentobarbital.

2.1.6 Blinding procedures

Animals were randomly assigned to the treatment groups at the start of the experiments. There was limited capacity to blind these studies as it was essential for animals to be identified to ensure consistency of treatment. However, the brains used for immuno-histochemistry were blinded before starting the tissue processing, and tubes with tissue samples (ELISA, qPCR, WB) were only identified by the mouse number, to make the observer unaware of the treatment

2.2 Sample preparation and tissue processing

2.2.1 Serum preparation for corticosterone measurements

Serum was prepared following cardiac puncture using a 25G needle. Blood was collected and allowed to clot for 30-60 minutes at room temperature. The blood was spun at 1500 g for 10 minutes and the supernatant was transferred to a clean tube and spun again for 10 minutes. The serum was stored at -80°C.

2.2.2 Tissue collection for gene and protein analysis and immunohistochemistry

2.2.2.1 Adrenal glands

Adrenal glands were dissected and placed in RNAlater at 4° C for 24 hours before being stored at -80°C until further processing.

2.2.2.2 Liver and spleen

Liver and spleen samples were frozen directly on dry ice and stored at -80°C until gene- or protein analysis.

2.2.2.3 Brain

Dissected brain samples were frozen on dry ice and stored at -80° C until gene or protein analysis was done. The prefrontal cortex and dorsal hippocampus were dissected using a fine scalpel and spatula. Suprachiasmatic nuclei (SCN) punches were made using a flat tip 21G needle on a frozen 2 mm coronal brain slice that was made using an acrylic brain slice matrix with 1mm spaces (Zivic labs, BSMAA001-1) at the position of the optic chiasm just behind bregma, modified from the method desribed in (Jagannath et al., 2013).

2.2.3 Formalin fixing and paraffin embedding of tissue for immunohistochemistry

For immunohistochemistry (IHC), formalin fixed paraffin embedded (FFPE) brains were used. Mice were transcardially perfused with 50 ml heparinised (Sigma, H3393, 5U/ml) 0.9% saline followed by 50 ml 10 % formalin (Sigma, HT501640). The brains were post-fixed in formalin

overnight and then dehydrated in 100% ethanol for three days. The brains were processed using the Tissue-Tek® VIP® Vacuum infiltration processor (program: 70% ethanol (EtOH) 4 hours, 80, 90, 95, 100% EtOH each for 2 hours, 50% EtOH/Histochoice 1 hour, 25/75% EtOH/Histochoice 1 hour, Histochoice 2 hours x2, Paraffin 1 hour x4) and finally paraffin embedded using a Leica embedding centre (EG 1140).

2.2.4 Microtome sectioning

Paraffin embedded brains were coronally sectioned using a microtome (Sakura Accu-Cut SRM) in 7 µm thick sections and mounted on microscope slides coated with 3% APES (3-aminopropyl-triethoxysilane, Sigma, A3648) or Flex IHC Microscope slides (DAKO, K8020).

2.3 Histology techniques

2.3.1 DAB-staining of paraffin embedded brain sections using the DAKO Autostainer Link 48

Heat-induced antigen retrieval of formalin fixed, paraffin embedded sections was performed in the PT link (DAKO, PT101) containing Target Retrieval solution (either 50x Tris/EDTA buffer pH=9, DAKO, K8004 or 50x citrate buffer pH=6.1, DAKO, K8005. After this the slides were moved to wash buffer (DAKO, K8007, TBST, pH=7.6) at room temperature. Staining was performed using the DAKO Autostainer Link 48 (DAKO, AS480) and the DAKO EnVision FLEX+ detection system. Peroxidase-blocking reagent (DAKO, SM801) was used, for some antibodies, the EnVision FLEX+ Mouse LINKER (DAKO, K8021) was used to enhance reactivity. Primary antibodies were diluted using Envision flex antibody diluent (DAKO, DM830). Following staining, slides were dehydrated in graded ethanol, cleared in xylene and cover slipped using DPX mountant. See Table 2.1 for specifications of the antibodies used.

2.3.1.1 Hematoxylin counter staining

Sections stained for ionized calcium binding adaptor molecule 1 (Iba-1) were counterstained using hematoxylin by briefly immersing 3,3'-diaminobenzidine (DAB) stained sections in Mayer's

hematoxylin (Sigma-Aldrich, MHS16) followed by washing in warm running tap water for 7 minutes, Scott's tap water for 7 minutes, and distilled water for 1 minute before dehydration, clearing and cover slipping as described above.

2.3.1.2 Cresyl violet counter staining

Sections stained for β-Amyloid were counterstained 1-5 minutes in 0.5% cresyl violet, followed by a brief wash in acidified water before dehydration, clearing and cover slipping as described above.

2.3.2 Double fluorescence staining

Double staining for 5-bromo-2'deoxyuridine (BrdU) and doublecortin (DCX) was carried out on serial sections to investigate proliferation and early differentiation in the subgranular zone (SGZ) of the dentate gyrus (DG). The slides with serial sections were dewaxed for 3x 5 minutes in xylene, dehydrated in graded ethanol and washed for 5 minutes in TBS. This was followed by 30 minutes DNA hydrolysis treatment in 2N HCl at 37°C and 10 minutes neutralisation in 0.1M borate buffer (pH 8.5). Slides were then washed in tris-buffered saline with tween (TBST) for 3x10 minutes and 1x in TBS before drawing around the sections using a hydrophobic pen. Blocking was done in 10% serum, from secondary antibodies host species, for 30 minutes in immunostaining trays (Cell path). Incubation with primary antibodies against BrdU and DCX was performed in 1% donkey serum overnight at 4°C. No-primary-antibody-controls were included as a negative control. Slides were washed for 3x 10 minutes in TBST followed by incubation with secondary fluorophore-conjugated antibodies in 1% serum for 3 hours in the dark. This was followed by 3x 10 minute washes in TBS and cover slipping using Vectashield hardmount medium. See Table 2.1 for specifications of the antibodies used.

Table 2.1 List of antibodies used for immunohistochemistry

Antibody	Target	Manufacturer	Cat.no	concentration	Secondary antibody	Antigen retrieval
4G8	β-amyloid	Biolegend	800701	1:5000	Autostainer	Tris/EDTA buffer 65˚C 30 min
c-fos	Immediate early gene	Santa Cruz	SC-253	1:3000	Autostainer	Tris/EDTA buffer 95˚C 20 min
GFAP	Astroglia	DAKO	Z0334	1:2000	Autostainer	Citrate buffer 65˚C 30 min
Iba-1	Microglia	Abcam	Ab178846	1:9000	Autostainer	Citrate buffer 65˚C 30 min
Synaptophysin	Presynaptic synapses	Abcam	ab8049	1:100	Autostainer	Citrate buffer 65˚C 30 min
Double fluorescence staining						
BrdU	Labelled dividing cells	Abcam	Ab6326	1:400	Dk anti-rat 488	Citrate buffer 65˚C 30 min , 2M HCl 37˚C 30 min
DCX	Early neuronal marker	Santa Cruz	SC-8066	1:500	Dk anti-rabbit 555	
Donkey anti-rat Alexa 488	rat	abcam	Ab150153	1:250		
Donkey anti-rabbit Alexa 555	rabbit	Invitrogen	A21429	1:250		

2.4 Analysis of staining

For staining analysis micrographs of DAB stained sections were captured at 5 or 10 times magnification using a Leica DC 2000 microscope coupled to a digital camera (Leica DFC 450 C) and a computer running Leica QWin Standart software (V.3.5.1).

2.4.1 Densitometric measurements of synaptophysin staining

Regional staining density was measured on gray scale micrographs of hippocampal sections stained for synaptophysin, using ImageJ (1.47v, NIH).

2.4.2 Quantification of astroglia and microglia

GFAP and Iba-1 positive cells were manually counted on micrographs captured at 10x magnification using the cell counting tool in ImageJ (1.47v, NIH). Quantification was done in the hippocampal subfields; the hilus, granule cell layer, *stratum moleculare* and the *stratum radiatum* and *stratum oriens* of the CA1 region. The numbers were expressed per area and the total number of cells was the sum of the cells in all these areas.

2.4.3 Quantification of c-fos positive nuclei

c-fos positive cells were manually counted in the dentate granule cell layer on micrographs captured at 10x magnification using the cell counting tool in ImageJ (1.47v, NIH).

2.4.4 Quantification of β-amyloid staining

The number of β-amyloid containing plaques or aggregates were manually counted on two sections per brain at 10x magnification.

2.4.5 Quantification of BrdU-labelled cells and BrdU/DCX double labelled cells

Sections were inspected at a 40x magnification using a Nikon Eclipse Ti microscope and the NIS-elements AR software. All BrdU-labelled cells or DCX+ cells in the GCL and SGZ (2 cell wide zone beneath the GCL) were manually counted on every 6^{th} section from -1.40 to -1.88 from bregma. For evaluation of changes in differentiation, the fate of 50 BrdU labelled cells from each brain was evaluated as being positive or negative for DCX staining.

2.5 Gene expression analysis

2.5.1 RNA extraction

RNA extraction from the adrenal glands, liver, hippocampus and prefrontal cortex was performed using the RNeasy minikit (Qiagen, 74106) following the manufacturer's protocols

(Qiagen, 74106). Before extraction, tissues were lysed in RLT lysis buffer from the RNeasy kit with 1% beta-mercaptoethanol (Sigma, M6250), and homogenised using a pestle homogenizer (VWR, 47748-370). The lysate was then homogenised using the Qiashredder system (Qiagen, M3148). For adrenal glands, hippocampus, prefrontal cortex, the RNA was eluted in 25 µl water, except for the liver where 40 µl water was used.

SCN punches were extracted for RNA using the RNeasy micro Kit (Qiagen, 74004) following the manufacturer's protocol after homogenisation by the phenol/chloroform method. Tissue was homogenised in Trizol using a pestle homogenizer. After 5 minutes incubation, the homogenate was centrifuged for 10 minutes at 12,000 g at 4^{o} C. Next, 0.2 ml chloroform was added, and the solutions were mixed by shaking for 20 seconds. After 3 minutes incubation, the samples were spun for 15 minutes at 12,000 g at 4^{o} C. The aqueous phase was collected in a clean tube and mixed with 100 µl 100% ethanol 1:1 before following the extraction protocol from the RNeasy micro kit. The RNA was eluted in 14 µl water.

2.5.1.1 RNA purity and integrity

RNA concentration was measured using a NanoDrop Lite spectrophotometer (Thermoscientific) and sample purity was evaluated from the 280/260 wavelength ratio. Ratios of 1.8-2.2 were considered acceptable.

To test the RNA integrity, samples were run on a 1.5% agarose gel in tris/borate/EDTA (TBE) buffer (Sigma, T4415) with 10 µl ethidium bromide (Applied Chem., A1152) or GelRed Nucleic Acid Stain (Biotium, 41003). 2 µl 6x sample buffer (ThermoScientific, R0611) was added to 4 µl sample. Product sizes were identified using a ladder (Thermoscientific, SM1344). The gel was run for 50 minutes at 100 V. The integrity of the 18S and 28S bands were then investigated under UV light using the Chemidoc XRS+ or Syngene U:genius 3.

2.5.2 cDNA synthesis

cDNA conversion of RNA was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) following the manufacturer's protocol. RNA was diluted to a volume of 10 µl and was mixed with 10 µl master mix containing MultiScribe Reverse Transcriptase (1.0 µl), 10x RT random primers (2.0 µl), 25x dNTP mix (0.8 µl, 100mM) and 10x RT buffer (2 µl) and then incubated at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes using a BioRad iCycler A and stored at -20°C.

2.5.3 qPCR

qPCR was carried out using PerfeCTa SYBR Green FastMix (Quanta, 95072) and a Roche LightCycler 480, see Table 2.2 for cycle program. The ΔΔCt method was used to estimate differences in expression level between groups (Livak and Schmittgen, 2001). Gene expression was normalised to the geometric mean of expression of the two (out of three) most stably expressed reference genes. Reference genes from Primerdesign were used (*Htatsf1, Csnk2a2, gapdh, β-actin, Fbxo38*). Primers were used at a concentration of 300 nM, except for *grin2b* where 600 nM was used. 25 ng template was used for all tissues except the SCN where 10 ng was used. Table 2.3 for primer sequences.

Table 2.2 Program used for qPCR

Step	**Settings**
Enzyme activation	95°C - 00.00.30
Amplification (40 cycles)	95°C - 00.00.05
	60°C - 00.00.30

Table 2.3 Primer details

Name	**Accession no**	**Forward sequence** (5'-3') **Reverse sequence** (5'-3')	**Supplier**
Per1	NM_011065	TCCTCCTCCTACACTGCCTCT TGGCTGACGACGGATCTTT	IDT
Per2	NM_011066.3	CTCAGCCTCCCACTCTATATGT AGGACCAGCAGCACAGATAG	Primerdesign
Cry1	NM_007771	GATCCACCATTTAGCCAGACAC ACAGCCACATCCAACTTCCA	Primerdesign
Cry2	NM_009963	CAAGCACTTGGAACGGAAGG GAAGAGGCGGCAGGAGAG	Primerdesign
Clock	NM_007715	TTCCTTCCTTAGAGACGAGACT CTAAATGCTACCCTGAGGATAGAG	Primerdesign
Arntl1/ Bmal1	NM_007489.4	CCACCTCAGAGCCATTGATACA GAGCAGGTTTAGTTCCACTTTGTCT	IDT
Grin1	NM_008169.3	CCAGACTAAAGATAGTGACAA ACCATTGACTGTGAACTC	IDT
Grin2a	NM_008170	CACCTATGACCTCTACCT TAGACCACTTCGCCTATC	IDT
Grin2b	NM_008171.3	TTGCCATCATCACCACTGCT CCATTTTCCCACCCTCTCCC	IDT
Gria1	NM_008171.3	TGGTGGTGGACTGTGAAT AGGTTGGCGAGGATGTAG	IDT
Gria2	NM_001083806.1	GATGGTCAACACTCGAAGAGAA TCATAAGTCAGGGCCGAAGT	IDT
bdnf	NM_007540	GGATATTGCGAAGGGTTATTAGATT GGAAGGTAATGTGTCTTGTTTGAA	Primerdesign
Ntrk2	NM_008745	TCTATGCCGTGGTGGTGATT GGAATGTCTCGCCAACTTGA	Primerdesign
P75	NM_033217	GATACGGTGACCACTGTGATG ATGGAGCAATAGACAGGAATGAG	Primerdesign
Dlg4	NM_007864	GGTGACGCAGATGGAAGTG CCAAACTTGTCGGGGAACTC	Primerdesign

2.6 Measurements of protein levels

2.6.1 Corticosterone enzyme immunoassay

Serum corticosterone levels were measured using an enzyme immunoassay kit (Enzo Life-Sciences, ADI-900-097), following the manufacturers protocol. Samples were initially mixed with serum displacement reagent and diluted 40x in assay buffer. Corticosterone standards (32-20,000 pg/ml) and samples were pipetted into the anti-sheep IgG coated plate. An alkaline phosphatase-corticosterone conjugate and a sheep polyclonal antibody were added and incubated at RT for 2 hours at 300 rpm. Excess reagent was washed away (3x) and a substrate (p-nitrophenyl phosphate) was added (room temperature for 1 hour). The reaction was stopped using a trisodium phosphate solution and the optical density was measured at 430 nm using a Bio-tek synergy HT plate reader and KC4 software. All samples were run in duplicate (CV*< 10 %)* and all assays were repeated.

2.6.2 Western blotting

2.6.2.1 Protein extraction

Tissue was homogenized in 100 µl phosphate buffered saline (PBS) on ice by pestle and mortar. 100 µl lysis buffer (containing 1x protease inhibitor cocktail and phosphatase inhibitors, 100 nM okadaic acid and 1mM sodium orthovanadate) was added (See appendix 3 for protocols). The lysate was centrifuged through a Qiashredder column for 3 minutes at 14000xg. Protein concentrations were determined in duplicates using the Pierce bicinchoninic acid assay (BCA) protein assay kit (Thermo Fisher, 23225) following the manufacturer's protocol.

2.6.2.2 Western blotting

Samples were diluted in PBS to contain 20 µg protein, mixed with 5x sample buffer (see appendix 3 for protocols) and heated to 95˚C for 10 minutes. Samples were run on a 4-20% mini protean TGX gel with a Precision Plus Protein Dual Color Standard (Biorad, 161-0374) at 150 V for 40 minutes. The Bio-rad Transblot Turbo and polyvinylidene fluoride (PVDF) transfer packs

(Bio-rad, 170-4156) were used for protein transfer. Membranes were blocked in 10% skimmed milk powder for 1 hour at room temperature and then incubated with the primary antibody, diluted in TBST at room temperature, see concentration and time in Table 2.4. This was followed by 3x10 minute washes in TBST, incubation with secondary HRP-conjugated antibody for 1 hour at room temperature. Before being developed, the membranes were washed 3x in TBST. Protein complexes were developed on X-ray film using the Immobilon western ECL kit (EMD Millipore, WBKL0500) and developing and fixing agents from Carestream Dental (190 0943 and 190 1875).

Table 2.4 Antibodies used for western blots

Primary antibody	**Species**	**Manufacturer**	**Cat.no**	**Dilution and incubation time**
GluA1	rabbit	Millipore	ABN241	1000 (2h)
GluA2	rabbit	Millipore	AB1768-I	1000 (2h)
GluN1	rabbit	Millipore	AB98654R	1000 (2h)
GluN2a	rabbit	Chemicon	AB1555	5000 (2h)
PSD95	mouse	Millipore	MAB1598	10,000 (1h)
α-actin	mouse	Millipore	MAB1501	40,000 (0.5h)
Secondary antibodies				
Anti-mouse IgG-HRP	goat	Santa cruz	sc-2005	1:10,000 (1h)
Anti-rabbit IgG-HRP	donkey	Santa cruz	sc-2313	1:10,000 (1h)

2.7 Statistical analysis

Data were presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism (version 5.01). Normality testing was done using the D'Agostino-Pearson test, and the statistical analysis was chosen based on whether data followed a normal distribution. For comparison of two experimental groups either the Mann-Whitney test or an un-paired t-test was used. For comparison of more than two experimental groups either the Kruskall-Wallis test with Dunn's *post hoc* test, or a one-way ANOVA with Bonferroni's *post hoc* test was used. For analysis of effects of two factors, a two-way ANOVA with Bonferroni's *post hoc* test was used. Finally a repeated measures one-way ANOVA was used for comparison of experimental groups when repeated measurements were done.

Chapter 3 A study of the effects of radio frequency fields on cognitive function

3.1 General background

The work described in this chapter seeks to discover if the reported effects of radio frequency (RF) fields associated with mobile phones on cognitive function in adult animals are caused by field exposure or if they might be caused by other factors that may occur during exposure such as restraint stress or heating. In addition, the sensitivity of older animals to exposure was investigated in an ageing mouse model.

When using RF fields, it is essential to use well-controlled exposure systems with appropriate dosimetry to describe and quantify the amount of energy absorbed. Such systems are also necessary to reduce variation between animals as much as possible and this becomes very important if the effects of exposure might be small. For the experiments presented in this chapter, a high quality exposure system with well described dosimetry was used. The sXv-1800 exposure system operates at 1800 MHz and produces a typical 3G mobile phone signal that shifts between listening and talking modes to model the changes in signals experienced by humans in real-life.

There are great differences in mobile phone use between members of the public. This includes differences in the duration of daily use, the position of the phone, differences in usage (data, talking, listening), the age and generation of the phone, and the connection to the basestation. It is therefore very difficult to give a useful value for the average exposure level for the public. The INTERPHONE study has reported average maximal SARs in the brains of study participant to vary from 0.01-1.7 W/kg depending on the phone. The majority of the values were below 1 W/kg (Cardis et al., 2011). Exposures from other sources (distant to the body) are also hard to estimate for people as they depend on distance from the source and traffic on the system.

Generally these exposures are very low, and SARs from 0.1-4 μW/kg have been reported (Sagar et al., 2016).

3.1.1 Experimental approach

A long-term repeated exposure protocol was chosen to reflect the repeated exposure experienced in the everyday lives of the public. Exposures were undertaken in the IT'IS SxV-1800 reverberation chambers. Three exposure groups were included; a sham exposed group serving as control group, a low level group receiving a whole-body specific energy absorption rate (SAR) of 0.3 W/kg, and a high level exposure group exposed at a whole body SAR of 3 W/kg. These SARs should not cause substantial whole-body heating of the mice. Exposures were performed for 30 minutes per day based on similar protocols used in the literature. The dorsal hippocampus was chosen as the main region of interest due to its central role in learning and memory and its highly plastic nature. The prefrontal cortex was also included for some endpoints due to its importance in memory.

To investigate the effects of RF exposure on cognitive function, a variety of endpoints was chosen, to obtain a broader picture of possible interactions. These included: performance in learning and memory tests, hippocampal neurogenesis, astroglial and microglial density, and synaptic density and plasticity, as shown in Figure 3.1.

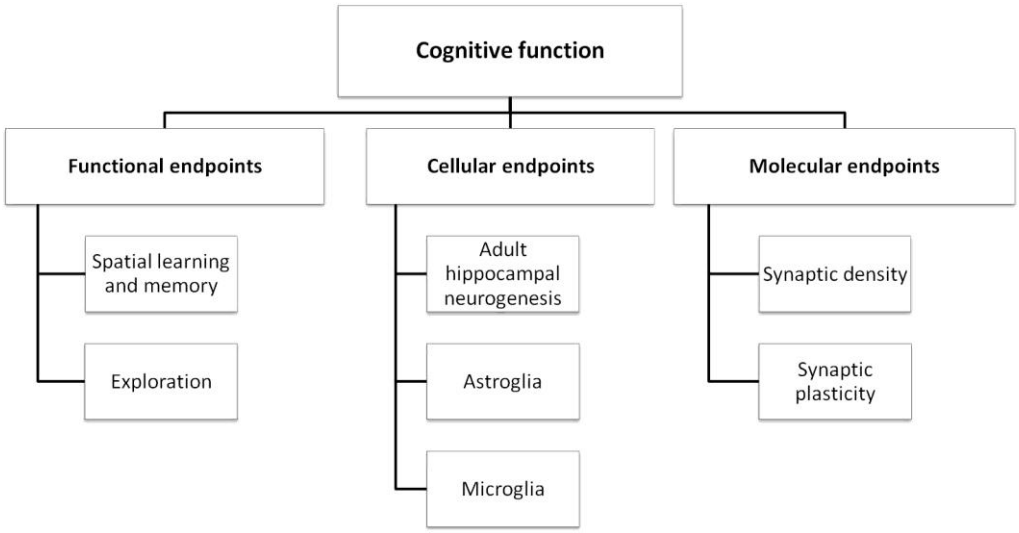


Figure 3.1 Schematic presentation of the functional, cellular and molecular endpoints studied.

3.1.2 Mouse models

Effects of exposure were evaluated in three different mouse models; young adult C57Bl/6J and a senescence-accelerated model, with the prone strain, SAMP8, and the resistant (control) strain, SAMR1.

Young adult C57Bl/6J mice were used at 8 weeks of age at the start of exposure. This is the most commonly used inbred mouse strain (Jax), and it has a median lifespan of 20-30 months (Envigo).

The senescence accelerated mouse (SAM) strains were developed by Dr Toshio Takeda from Kyoto University through the selective inbreeding of the AKR/J strain of mice (Takeda et al., 1997a). The strains have been characterised and the SAMP8 strain in particular, shows accelerated ageing of the brain, with an average lifespan of 9.7 months, while the SAMR1 strain shows normal ageing and an average lifespan of 16.3 months (Cheng et al., 2014; Miyamoto, 1997; Takeda et al., 1997b). The SAMP8 strain has been suggested as a model to study the progression from normal ageing to Alzheimer's disease (AD), since it shows pathological changes in the brain that are often associated with sporadic AD (Cheng et al., 2014; Pallas et al., 2008).

The earliest pathological finding in SAMP8 mice is spongy degeneration from the age of 1 month in the brainstem and spinal cord. Later neuronal loss, increased inflammation, increased amyloid and tau deposition (pathological hallmarks of AD) are seen throughout the brain including the hippocampus (Cheng et al., 2014).

Cognitive decline in SAMP8 mice has been studied extensively using hippocampal-dependent learning and memory tests. Impairments have been shown from the age of 2 months in the water maze with further age-dependent decline in performance in many different tests (Cheng et al., 2014).

In this work, 6 month old SAMP8 and SAMR1 mice were chosen for studies of age-dependent sensitivity to RF field exposure. Cellular and molecular endpoints were studied at 7 months. At this age, a reversible impairment in spatial and non-spatial learning and memory has been reported (Dong et al., 2015; Ma et al., 2016), in addition to synaptic loss (Cheng et al., 2011; Dobarro et al., 2013) and increased inflammation and glial cell numbers (Jin et al., 2016; Yi et al., 2016).

3.2. Experimental protocols

A long-term repeated exposure regime was used. Mice were exposed for 30 minutes a day for 5 days a week for 4 weeks. This was either followed by studies of (A) synaptic plasticity, (B) neurogenesis, synaptic density and changes to glial cell numbers or (C) cognitive function (Figure 3.2).

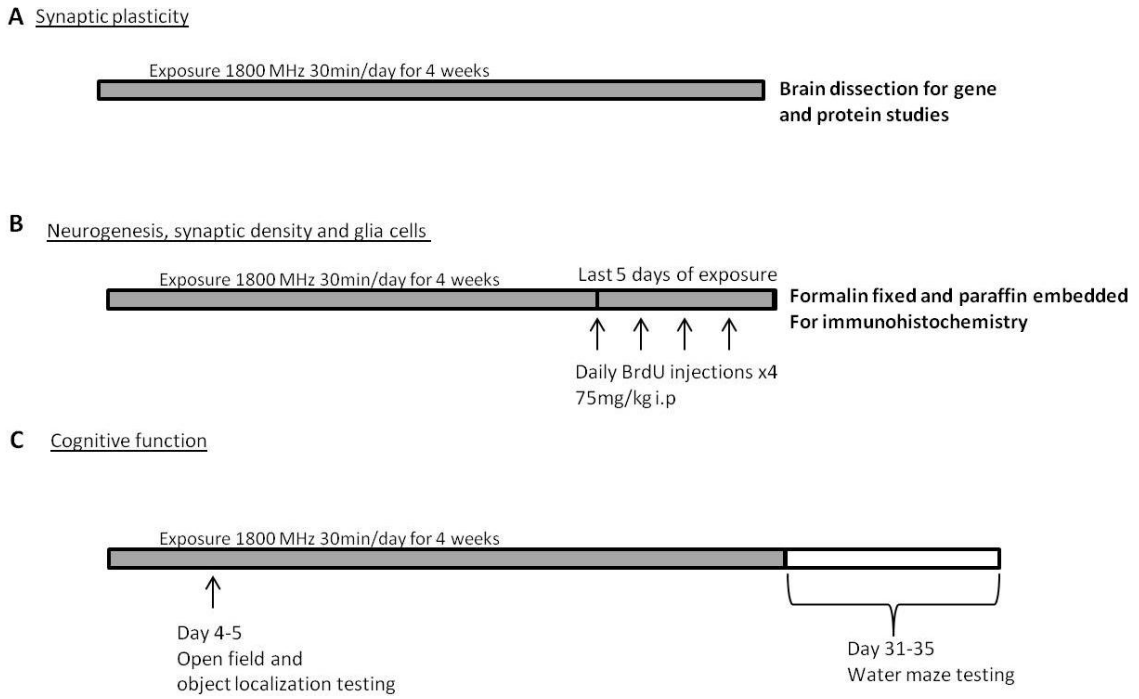


Figure 3.2 Experimental protocols. The same exposure regime of 4 weeks of exposure to an 1800 MHz field, 5 days a week, 30 minutes per day was used for all endpoints. For studies of (A) synaptic plasticity and density, the dorsal hippocampus and prefrontal cortex were isolated for gene and protein expression analyses. For studies of (B) adult hippocampal neurogenesis glial density and synaptic density, mice were injected once per day with BrdU (75mg/kg) on day 24-27 of the 28 day protocol and the brains were formalin fixed for immunohistochemical analysis. For analysis of the functional effect of exposures on learning and memory (C), mice were tested in the open field and novel object localisation test on day 4-5 of exposure, and after the end of exposure they were trained in the water maze task on day 31-35.

3.3 Results

3.3.1. Effects of RF exposure on young adult C57Bl/6J mice

In young adult male C57Bl/6J mice, the effects of three levels of RF exposure were investigated, 0.3 W/kg and 3 W/kg in addition to a sham exposure (0 W/kg). This section starts with presentation of results from experiments investigating effects of exposure on synaptic plasticity and density (molecular endpoints), followed by a study of proliferation and differentiation in the subgranular zone (SGZ) in the hippocampus, and studies of astroglia and microglia numbers (cellular endpoints). Finally, results of behavioural testing in the novel object localisation and water maze tests will be presented (functional endpoints).

3.3.1.1 Molecular endpoints

3.3.1.1.1 Minor changes to genes related to synaptic plasticity were detected after RF exposure

To investigate the effects of 4 weeks of RF exposure on the expression of central genes involved in synaptic plasticity, the brain was dissected on the last day of exposure. Ribonucleic acid (RNA) was extracted from the dorsal hippocampus and prefrontal cortex, and gene expression studies were carried out by qPCR. It was investigated whether RF exposure affected the expression of the AMPA receptor subunits *gria1* and *gria2*, the NMDA receptor subunits *grin1, grin2a* and *grin2b*, and the BDNF signalling molecules *bdnf* and *ntrk2* (Figure 3.3)

Dorsal hippocampus

RF exposure at an SAR of 3 W/kg decreased *grin2a* expression by 22% in the dorsal hippocampus (Figure 3.3A). This decrease was found to be statistically significant compared to the control group using an unpaired t-test ($p<0.05$) but not using a one-way ANOVA. See Table 3.1 for results of the statistical analysis. A similar dose-dependent pattern of reduced expression of the *grin2b* subunit was found after exposure at 3 W/kg compared to the sham group, but this was not significant. Both AMPA receptor subunits *gria1* and *gria2* were expressed at similar levels in all three exposure groups, the same was true for the NMDA receptor subunit *grin1* (Figure. 3.3Ai

and ii). No significant differences in expression of the BDNF signalling genes *bdnf* and *ntrk2* were observed between the sham and either of the exposure groups, although the expression of *bdnf* was slightly decreased in both exposure groups compared to the sham exposed group (Figure 3.3Ci).

Prefrontal cortex

Expression of the same plasticity related genes was investigated in the prefrontal cortex (Figure 3.3Bi,ii and Cii). A small but significant increase of 15% in *ntrk2* expression was found in the 3 W/kg exposure group compared to the sham exposed group ($p<0.01$), whereas the expression level of *bdnf* did not differ significantly between the sham group and the two exposure groups (Figure 3.3Cii). The expression levels of both the AMPA and NMDA receptor subunits were found to be similar in all three exposure groups, and there were no significant differences in expression levels between the sham and RF exposed groups (Figure 3.3B). (See Table 3.1 for results of statistical analyses).

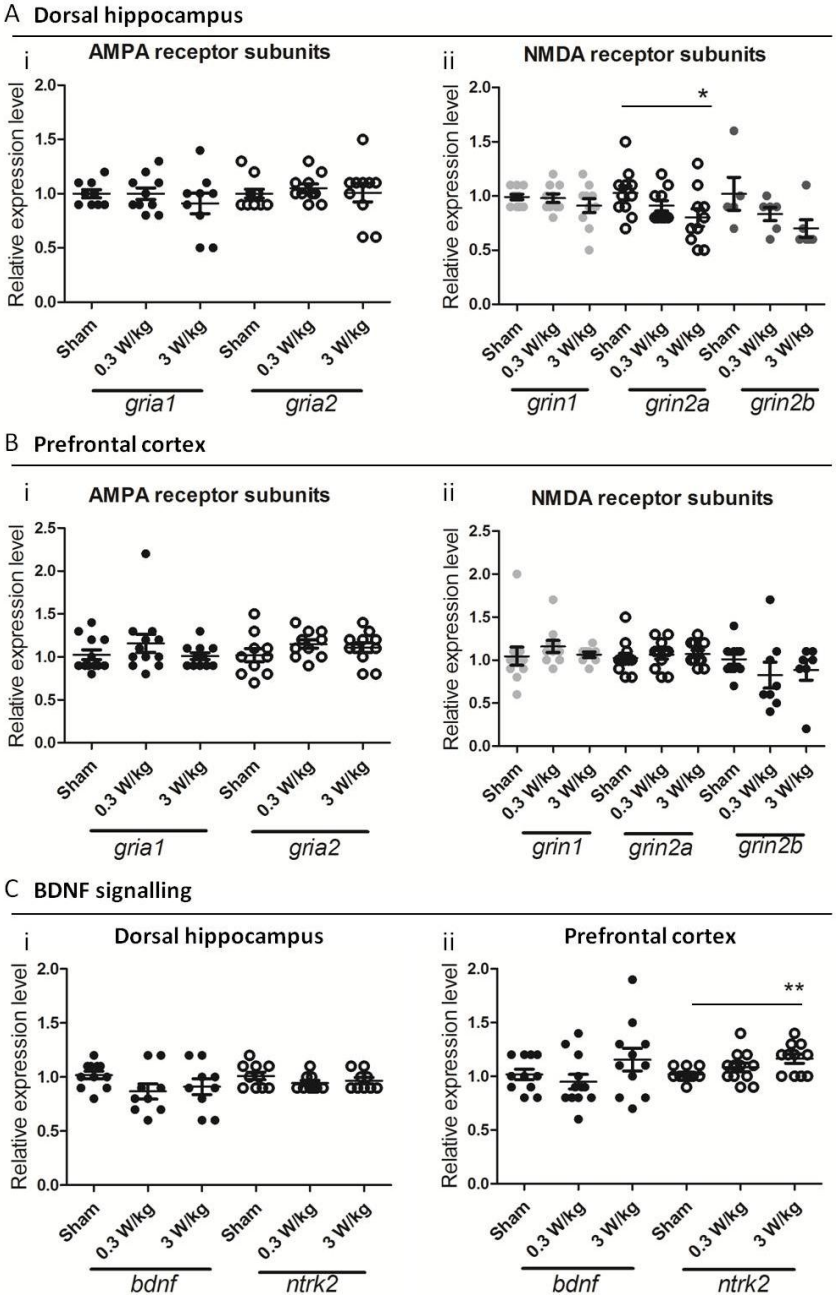


Figure 3.3 Minor effects of RF exposure were observed on the expression of synaptic plasticity related genes in the dorsal hippocampus and prefrontal cortex. Expression of the AMPA receptor subunits *gria1* and *gria2*, the NMDA receptor subunits *grin1, grin2a* and *grin2b* and *bdnf* and its receptor *ntrk2* were investigated in 12 week old male C57Bl/6Js after 4 weeks of RF exposure 30 minutes per day at SARs of 0, 0.3 or 3 W/kg in the dorsal hippocampus (A,Ci) and the prefrontal cortex (B,Cii). In the dorsal hippocampus, the *grin2a* expression was significantly decreased in the 3 W/kg group compared to the sham exposed group. No other significant differences in gene expression were seen between the exposure groups in the dorsal hippocampus. In the prefrontal cortex, *ntrk2* expression was significantly increased in the 3 W/kg group compared to the sham exposed group. Data are presented as mean ± SEM. n=7-12. Statistical analysis was carried out using a one-way ANOVA or unpaired t-test, *p< 0.05, **p< 0.01.

Table 3.1 Details of statistical analysis of gene expression after RF exposure of C57Bl/6J mice

	gene	Difference between means of sham and 0.3W/kg group	Difference between means of sham and 3W/kg group	Group sizes (n)	Gaussian distribution?	One-way ANOVA or non-parametric Kruskall-Wallis,	*Post hoc* test, compared to sham group	Un-paired t-test or Mann-Whitney test, compared to sham group
Dorsal hippocampus	*Grin1*	< 10%	< 10%	10-11	yes	p=0.40		
	Grin2a	11%↓	22%↓	10-11	yes	p=0.07		0.3W/kg:p=0.17 **3W/kg: *p=0.04**
	Grin2b	20%↓	32%↓	5-6	N too small	p=0.10		0.3W/kg: p=0.26 3W/kg: p=0.08
	Gria1	< 10%	< 10%	9-10	yes	p=0.56		
	Gria2	< 10%	< 10%	10-11	no	p=0.53		
	bdnf	13%↓	10%↓	9-11	yes	p=0.18		
	Ntrk2	< 10%	< 10%	9-10	yes	p=0.30		
Prefrontal cortex	*Grin1*	12%↑	< 10%	8-11	no	p=0.44		
	Grin2a	< 10%	< 10%	11-12	no	p=0.54		
	Grin2b	19%↓	13%↓	7-10	N too small	p=0.32		
	Gria1	13%↑	< 10%	11-12	no	p=0.44		
	Gria2	13%↑	< 10%	10-11	yes	p=0.34		
	Bdnf	< 10%	13%↑	11-12	yes	p=0.18		
	Ntrk2	< 10%	15%↑	10--12	yes	**p=0.03***	**3W/kg: *p<0.05**	0.3W/kg: p=0.17 **3W/kg: **p=0.0056**

3.3.1.1.2 No significant changes in levels of proteins involved in synaptic plasticity were found after RF exposure

In order to determine if RF exposure affects protein levels of glutamate receptor subunits, western blots for the AMPA receptor subunits, GluA1 and GluA2 and the NMDA receptor subunits GluN1 and GluN2a were made using protein lysate from the dorsal hippocampus of the same mice used for the gene expression analysis presented above.

RF exposure did not affect the glutamate receptor protein levels significantly (Figure 3.4). However, there was a considerable variation in all groups. Western blots for GluA1 showed three bands, one specific band at 110 kDa as expected for GluA1 and two unspecific bands at approximately 70 and 15 kDa. When the relative density of the 110 kDa band was measured, slightly higher average protein levels were found in both RF exposed groups compared to the sham exposed group, but the increase was not statistically significant (See Table 3.2 for results of statistical analysis). Western blots for GluA2 showed one band at 108 kDa as expected, and the protein level was 30% lower in both exposure groups compared to the sham group, but again, this difference was not found to be significant (Figure 3.4A). Western blots for GluN1 showed a specific band, at 120 kDa. The measured level of GluN1 was found to vary in the 0.3 W/kg group, but the average protein level in both exposure groups was slightly lower than in the sham exposed group (Figure 3.4B). For the expression of GluN2a, a specific band at 180 kDa was found. Only 2-3 mice were included so interpretation is cautious, but, indications of higher levels of protein (25-40%) in the two exposure groups compared to the sham exposed group were seen (See Table 3.2 for results of the statistical analyses).

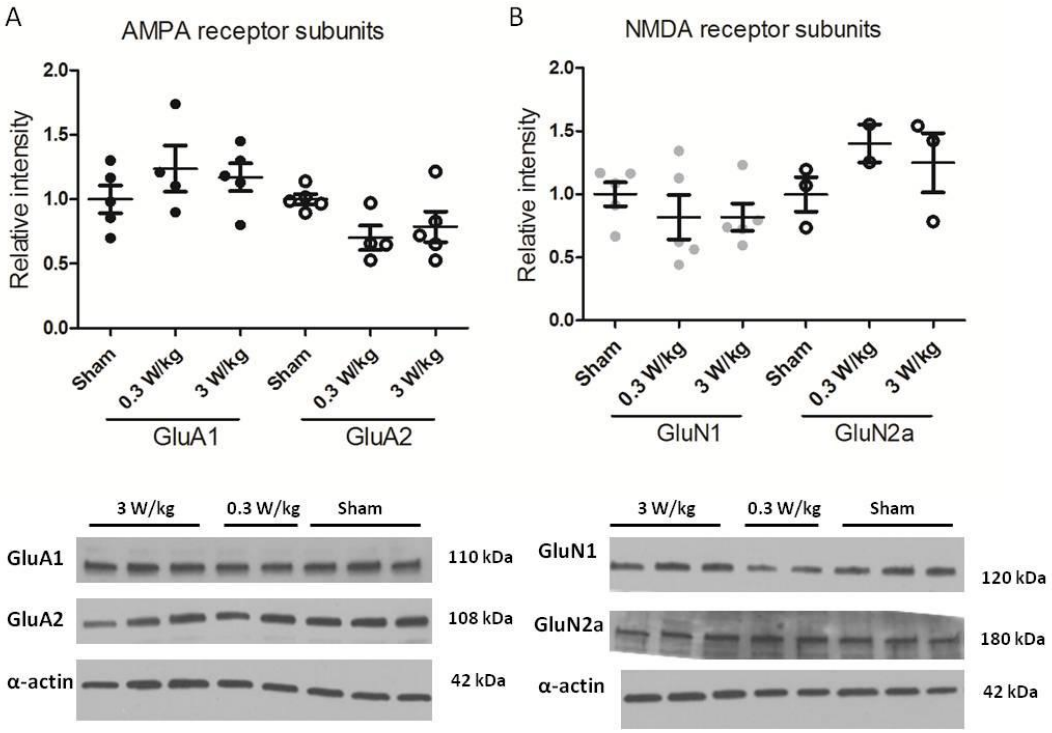


Figure 3.4 No effects of RF exposure were observed on protein levels of glutamate receptor subunits in the dorsal hippocampus. Protein levels of GluA1, GluA2, GluN1, and GluN2a were measured in the dorsal hippocampus of 12 week old male C57Bl/6Js after 4 weeks of RF exposure 30 minutes per day at SARs of 0, 0.3 or 3 W/kg by western blotting. Examples of western blots are shown in the lowest panel with α-actin used as loading control for normalisation of band density. n=2-5. Data are presented as mean ± SEM. Statistical analysis was carried out using a Kruskall-Wallis test with Dunn's post hoc test or the Mann-Whitney test. No significant changes in protein levels were seen in any of the proteins after exposure

Table 3.2 Details of statistical analysis of protein levels of glutamate receptor subunits after RF exposure in C57Bl/6J mice

	Protein	Difference between means of sham and 0.3W/kg group	Difference between means of sham and 3W/kg group	Group sizes (n)	Gaussian distribution?	One-way ANOVA or non-parametric Kruskall-Wallis, p=	*Post hoc* test, compared to sham group	Mann-Whitney test, compared to sham group
Dorsal hippocampus	*GluA1*	24%↑	17%↑	4-5	N too small	p=0.53		0.3W/kg: p=0.41 3W/kg: p=0.42
	GluA2	30%↓	22%↓	4-5	N too small	p=0.10		0.3W/kg: p=0.06 3W/kg: p=0.15
	GluN1	18%↓	18%↓	5	N too small	p=0.51		0.3W/kg: p=0.42 3W/kg: p=0.42
	GluN2a	40%↑	25%↑	2-3	N too small	N too small		3W/kg: p=0.40

3.3.1.1.3 No changes in synaptic markers were observed after RF exposure

The effects of RF exposure on synapse integrity were investigated in terms of density of the pre-synaptic marker synaptophysin (Figure 3.5A-D) and mRNA and protein levels of the marker of the post-synaptic density PSD95 (Figure 3.5E-H). c-fos immunoreactive cells in the dentate gyrus (DG) were quantified as a measure of neuronal activity (Figure 3.5I-J).

The density of synaptophysin staining was measured in five selected subfields of the hippocampus; the *s.oriens* and *s.radiatum* of the CA1, the *s.lacunosum-moleculare*, the *s.moleculare* of the DG, the hilus and in the CA3 *s.radiatum* as shown in Figure 3.5A. Regional differences in staining density were seen, with lightest staining in the *s.lacunosum-moleculare.* The relative synaptophysin density was very similar across the three exposure groups in all hippocampal regions and there were no significant differences between the sham group and the two exposure groups (Figure 3.5D). See Table 3.3 for results of the statistical analyses.

Expression of the gene coding for PSD95, *dlg4,* was investigated in both the dorsal hippocampus and the prefrontal cortex, and the protein level was measured in the dorsal hippocampus (Figure 3.5E-H). The gene expression levels of *dlg4* were very similar in all three exposure groups in both brain regions, with no significant differences. On the western blot a single band was seen at 95 kDa, the expected size for the PSD95 protein, but no significant differences were detected between the three groups (See Table 3.3 for results of the statistical analyses).

The number of c-fos immunoreactive cells in the granule cell layer (GCL) was quantified in groups of 3-4 mice (Figure 3.5J). Large variation was found within the three exposure groups, and no significant differences in numbers of cells were seen between the sham group and the two exposure groups (See Table 3.3 for results of statistical analysis).

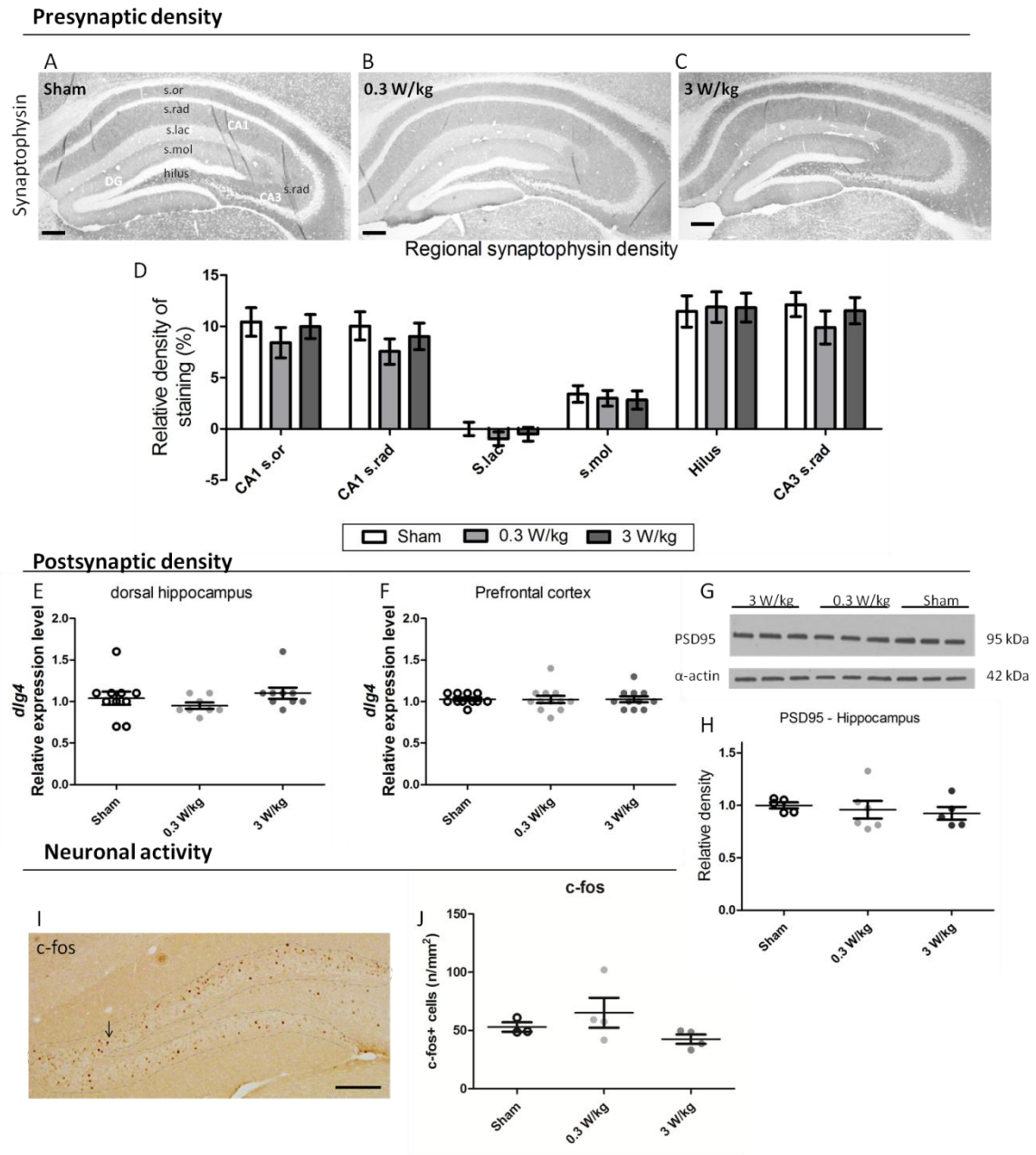


Figure 3.5 No effects of RF exposure were observed on synaptic density or neuronal activity. 12 week old male C57Bl/6J mice were exposed to 0.3 W/kg, 3 W/kg or sham exposure for 30 minutes per day, 5 days a week for 4 weeks. Regional density of synaptophysin was measured in 6 hippocampal subfields as a measure of pre-synaptic density (A-D). PSD95 gene (*dlg4*, dorsal hippocampus and prefrontal cortex) and protein levels (dorsal hippocampus) were investigated as a marker of the post-synaptic density (E-H). Changes to neuronal activity was investigated in terms of numbers of cells reactive for the immediate-early factor c-fos (I-J). Similar regional synaptophysin densities were seen in all three exposure groups, with the lowest density found in the *s.lacunosum-moleculare* and the highest density in the hilus. In (D) the percent change in the density relative to the average level found in the *s.lacunosum-moleculare* in the sham group is shown for each subfield in the three exposure groups (n=6). No significant differences

between exposure groups were found. There were also no significant changes in gene expression of *dlg4* in the dorsal hippocampus or prefrontal cortex (n=10-12) (E,F), or on the level of PSD95 protein in the dorsal hippocampus (n=4-6) (H). c-fos positive nuclei (stained brown as shown by arrow in (I) were counted in the DG and expressed as number per area (n=3-4) (J), Scale:200 µm. No significant difference between exposure groups was found. Data are presented as mean ± SEM. Statistical analysis was carried out using the non-parametric Kruskall-Wallis test.

Table 3.3 Details of statistical analyses of synaptic markers after RF exposure in C57Bl/6J mice

		Difference between means of sham and 0.3W/kg group	**Difference between means of sham and 3W/kg group**	**Group sizes (n)**	**Gaussian distribution?**	**One-way ANOVA or non-paramtric Kruskall-Wallis,**	***Post hoc* test, compared to sham group**
Synaptophysin	*s.lacunosum-moleculare*	-	-	5-6	N too small	p=0.32	
	CA1 s.oriens	19%↓	<10%↓	5-6	N too small	p=0.76	
	CA1 s.radiatum	25%↓	10%↓	5-6	N too small	p=0.51	
	s.moleculare	12%↓	17%↓	5-6	N too small	p=0.60	
	Hilus	<10%↑	<10%↑	5-6	N too small	p=0.94	
	CA3 s.radiatum	18%↓	<10%↓	5-6	N too small	p=0.45	
PSD95	*Dlg4 dorsal hippocampus*	<10%↓	<10%↑	8-10	no	p=0.18	
	Dlg4 prefrontal cortex	<10%↓	0%	11-12	no	p=0.89	
	PSD95 dorsal hippocampus	<10%↓	<10%↓	5-6	N too small	p=0.48	
c-fos	*Dentate gyrus*	23%↓	20%↑	3-4	N too small	p=0.16	

In summary, minor changes in the molecular endpoints were found after RF exposure of young adult C57Bl/6J. Exposure at 3 W/kg reduced *grin2a* expression by 22% in the dorsal hippocampus and increased *ntrk2* expression by 15% in the prefrontal cortex. Group sizes were too small to detect any significant differences in protein levels after exposure, however indications of changes were seen. No effects of exposure were detected on synaptophysin density, PSD95 gene or protein levels or in the number of granule cells expressing c-fos.

3.3.1.2 Cellular endpoints

3.3.1.2.1 No changes in adult hippocampal neurogenesis were observed after RF exposure

The effect of RF exposure on adult hippocampal neurogenesis was investigated in terms of changes in proliferation and differentiation of neuronal progenitor cells in the subgranular zone (SGZ) and granule cell layer (GCL) in groups of 6 mice. A group of mice (n=3) housed in a cage enriched with a running wheel was included as positive control.

The mice were injected with 75 mg/kg BrdU 4 times in the fourth week of exposure. 24 hours after the last BrdU injection the brains were removed, formalin fixed and paraffin embedded. Serial sections were double immunofluorescence-stained for BrdU and doublecortin (DCX). The number of BrdU-labelled cells in the SGZ and GCL was counted on every sixth section from -1.40 to -1.88 from bregma. BrdU-labelled cells were seen throughout the brain. In the DG, the highest density was found in the SGZ and the innermost part of the GCL (Figure 3.6A,B). The DCX-positive cells were found in the innermost part of the GCL extending dendrites into the molecular layer.

BrdU-labelled cells were counted for an assessment of proliferation, a large variation was observed within groups (Figure 3.6C). Only two brains were included within the 0.3 W/kg group due to section artefacts, and this group was excluded from the statistical analysis. A lower average number of cells was counted in the 3 W/kg group compared to in the sham group. In

the positive control (enriched) group, two of the mice had more than 3000 BrdU+ cells in the SGZ and GCL, which was more than any of the mice in the sham group, while only 2303 cells were found in the third mouse. No statistically significant differences in cell numbers were found between the three groups when using a non-parametric one-way ANOVA ($p=0.14$).

For an assessment of cell differentiation, the fate of 50 BrdU-labelled cells in each mouse brain was evaluated as being DCX positive or negative. Approximately 70-80% of the BrdU-labelled cells in the SGZ and GCL were found to be DCX positive in all four groups (Figure 3.6D). No significant differences between the groups were found for the cell fate using a non-parametric one-way ANOVA ($p=0.29$).

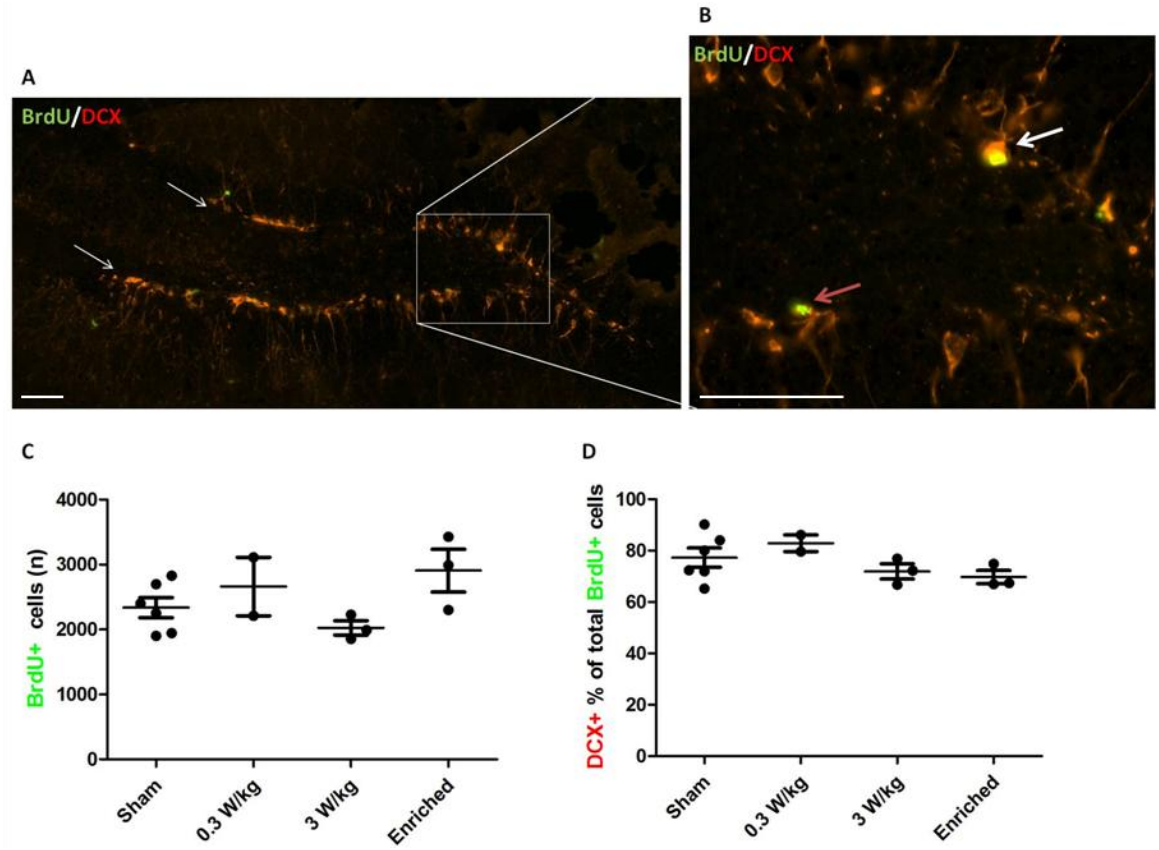


Figure 3.6 RF exposure did not affect adult hippocampal neurogenesis. Adult hippocampal neurogenesis was investigated in terms of changes in proliferation (by BrdU-labelling) (green) and neuronal differentiation (red) (by BrdU/doublecortin (DCX) double-fluorescence staining) in the subgranular zone in the hippocampus, after exposure of 12 week old male C57Bl/6J mice to 0.3 W/kg, 3 W/kg or sham exposure for 30 minutes per day, 5 days a week for 4 weeks (n=2-6). A group of mice housed in an environment enriched with a running wheel was included as a positive control (n=3). In the hippocampus DCX+ cells were found in the subgranular zone (SGZ) and innermost part of the granule cell layer (GCL) (indicated by white arrows in (A)), while BrdU-labelled cells were seen in all parts of the hippocampus, with a higher density in the SGZ and GCL. Quantification was carried out under 40 times magnification on brain sections from -1.40 to -1.88 mm from bregma. All BrdU-labelled cells were counted, while co-localisation of BrdU and DCX was evaluated in 50 BrdU-labelled cells. An example of co-localisation of the two markers is indicated by the white arrow in (B), while an example of a BrdU-labelled cell not co-expressing DCX is indicated by the red arrow in (B). No significant differences in number of BrdU-labelled cells (C) or the percentage of BrdU-labelled cells co-expressing DCX were found between exposure groups. Scale: 100 µm. Data are presented as mean ± SEM. Statistical analysis was carried out using a non-parametric one-way ANOVA, no statistically significant differences were found.

3.3.1.2.2 No changes in microglial or astroglial numbers were observed after RF exposure

Microglia and astroglia were quantified in hippocampal sections from the same groups of 6 mice investigated for changes in neurogenesis, using Iba-1 and GFAP as their respective markers (Figure 3.7 and 3.8).

There was an almost significant increase in the total number of Iba-1 positive cells in the 3 W/kg group when compared to the sham exposed group ($p=0.06$, t-test) (Figure 3.7B). Moreover, the number of Iba-1 positive cells was counted in 5 subfields of the hippocampus, the *s.moleculare*, GCL and hilus of the DG, and the *s.radiatum* and *s.oriens* of the CA1 region (Figure 3.7A,C). For the regional distribution of Iba-1 positive microglia, the density was found to be fairly uniform between the regions (Figure 3.7C). There was very little variation between groups in the *s.moleculare* and *s.radiatum*, in contrast to the greater variation observed in the GCL, hilus and *s.oriens*. Using a non-parametric one-way ANOVA for comparison in each individual region, no significant effect of RF exposure was found when the exposure groups were compared to the sham group (see Table 3.4 for results of statistical analyses). Qualitatively, the morphology of the Iba-1 positive cells was similar between the three exposure groups.

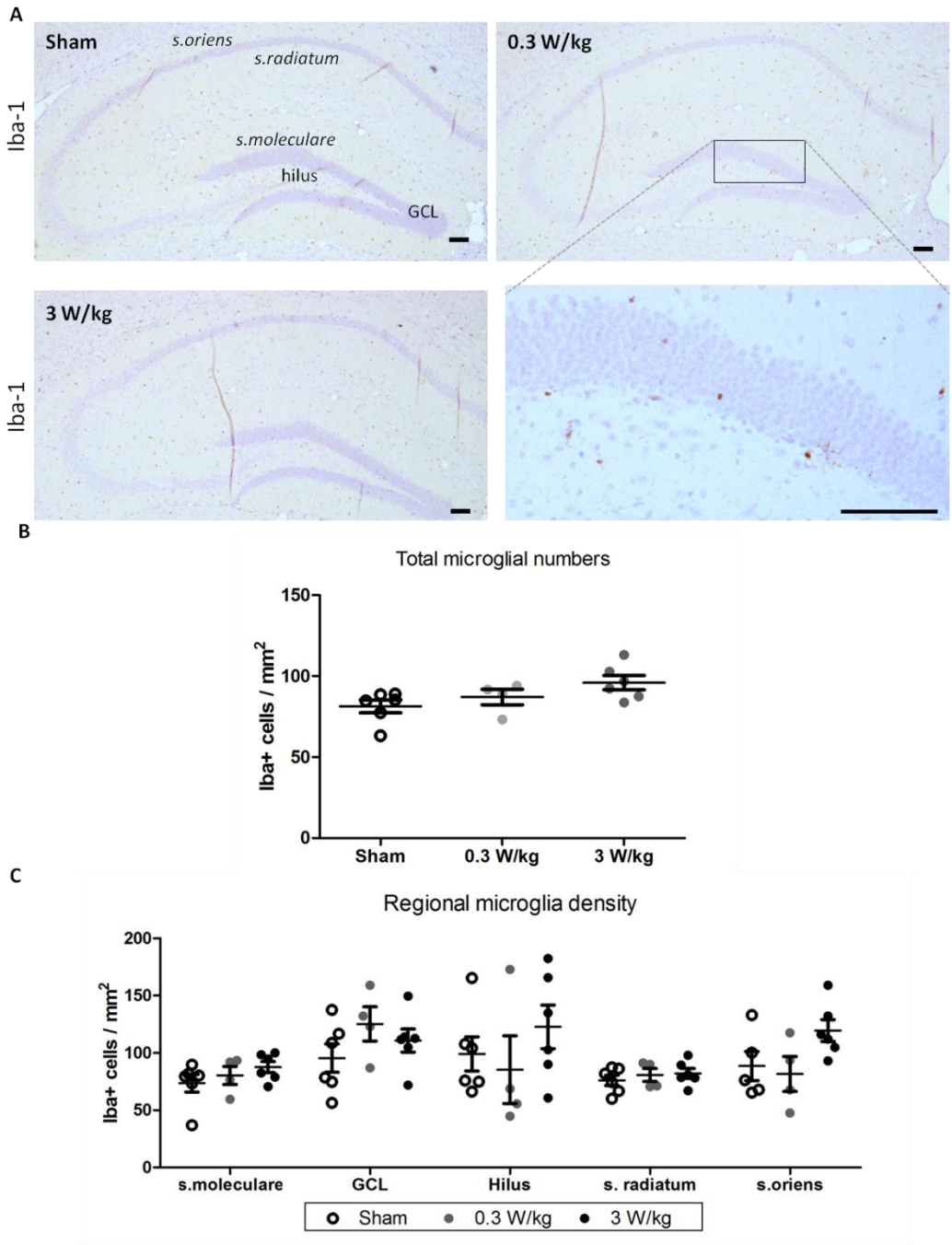


Figure 3.7 There were no effects of RF exposure on distribution of microglia in the hippocampus. Iba-1+ microglia (in brown) were counted in the DG (*s.moleculare,* granule cell layer (GCL) and hilus) and CA1 (*s.radiatum* and *s.oriens*) in the hippocampus of 12 week old male C57Bl/6J mice exposed to 0.3 W/kg, 3 W/kg or sham exposure for 30 minutes per day, 5 days a week for 4 weeks (A). Sections were counterstained with hematoxylin for orientation (purple). Similar distribution and numbers were found in the total area (B) and in the individual regions (C). A magnification of the GCL is shown in (A). Scale: 100 µm. Data are presented as mean ± SEM. Statistical analysis was carried out using one-way ANOVA for comparison of exposure groups, no statistical differences were found.

GFAP positive cells were observed in all regions of the hippocampus except the pyramidal layer (Figure 3.8A,B). The number of GFAP positive cells were counted in the *s.moleculare* and hilus of the DG, and the *s.radiatum* and *s.oriens* of the CA1 region. The total number of GFAP positive cells in the hippocampus was similar in all three exposure groups and no statistically significant differences between the groups were found when using non-parametric one-way ANOVA (Figure 3.8B) (See Table 3.4 for results of the statistical analysis). Qualitatively, the morphology of the GFAP positive cells was similar between three exposure groups.

The hilus had the highest density of astroglia in all three exposure groups, with average numbers of 578-646 cells per mm^2. In the hilus and the *s.moleculare* and in the *s.radiatum* and *s.oriens* of the CA1, a density of approximately 400 cells/mm^2 was found with very little variation between the exposure groups (Figure 3.8C). Statistical analysis was performed using non-parametric one-way ANOVA, no significant differences between the groups were found in any of the regions after exposure. See Table 3.4 for results of the statistical analyses.

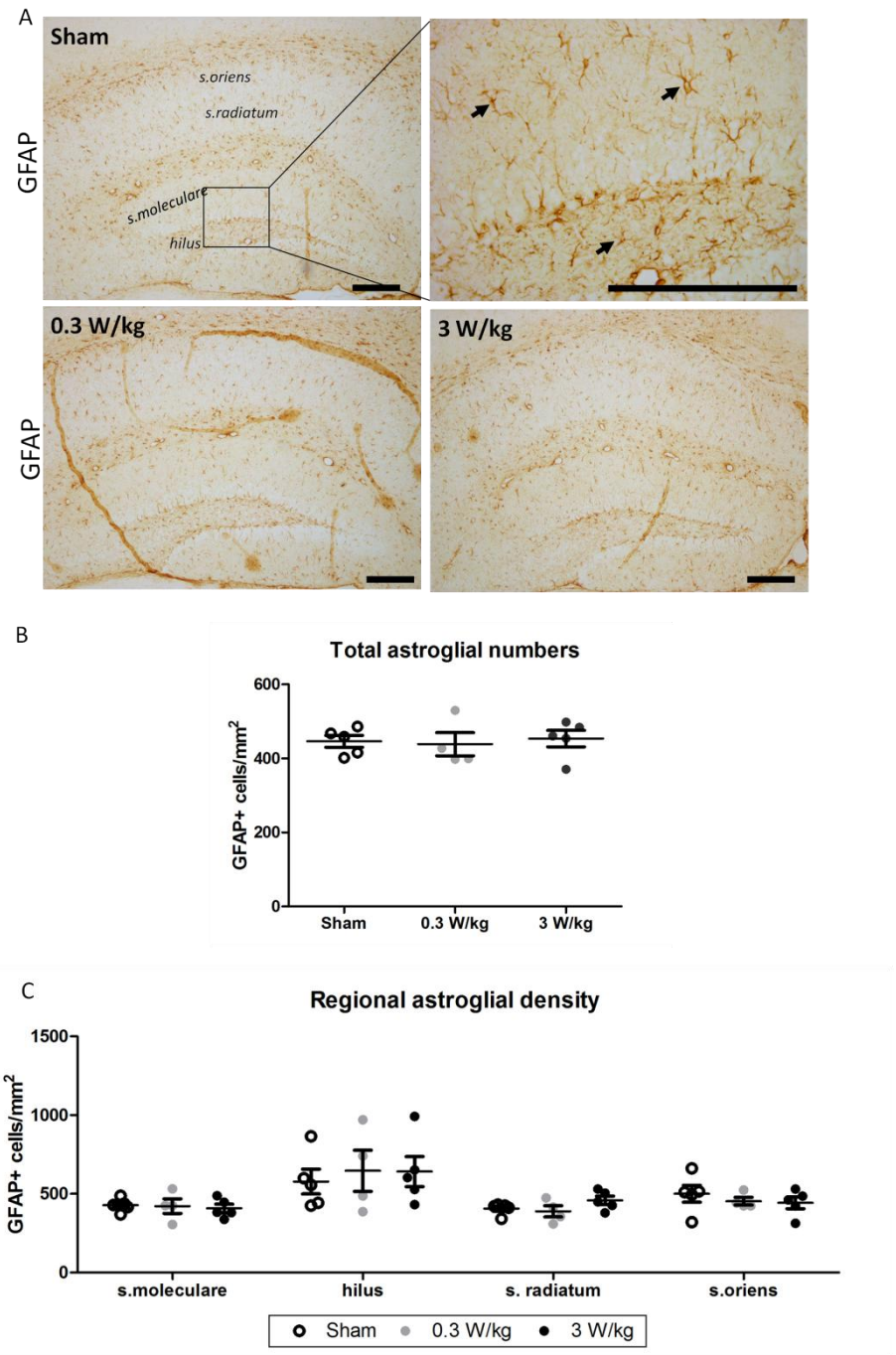


Figure 3.8 No effects of RF exposure on distribution of astroglia in hippocampus. GFAP+ astroglia were counted in the DG (*s.moleculare* and hilus) and CA1 (*s.radiatum* and *s.oriens*) in the hippocampus of 12 week old male C57Bl/6J mice exposed to 0.3 W/kg, 3 W/kg or sham exposure for 30 minutes per day, 5 days a week for 4 weeks. Similar morphology (A), total numbers (B) and regional distribution (C) were found. The morphology of the GFAP+ astroglia can be seen at 40x magnification in the picture in the top right corner of (A). Scale: 200 µm. Data are presented as mean ± SEM. Statistical analysis was carried out using non-parametric one-way ANOVA, no statistical differences were found between exposure groups.

Table 3.4 Details of statistical analysis of glial numbers after RF exposure in C57Bl/6J mice.

		Difference between means of sham and 0.3W/kg group	**Difference between means of sham and 3W/kg group**	**Group sizes (n)**	**Gaussian distribution?**	**One-way ANOVA or non-parametric Kruskall-Wallis,**	***Post hoc* test, compared to sham group**	**Mann-Whitney test, compared to sham group**
Microglia	Total	<10%↑	18%↑	4-6	N too small	p=0.09		0.3W/kg: p=0.17 3W/kg: p=0.06
	CA1 s.oriens	<10%↓	35%↑	4-6	N too small	p=0.16		3W/kg: p=0.13
	CA1 s.radiatum	<10%↑	<10%↑	4-6	N too small	p=0.62		
	s.moleculare	<10%↑	19%↑	4-6	N too small	p=0.34		
	Hilus	14%↓	24%↑	4-6	N too small	p=0.33		
	GCL	31%↑	16%↑	4-6	N too small	p=0.29		0.3W/kg: p=0.17
Astroglia	Total	<10%↓	<10%↑	4-5	N too small	p=0.77		
	CA1 s.oriens	<10%↓	12%↓	4-5	N too small	p=0.52		
	CA1 s.radiatum	<10%↓	13%↑	4-5	N too small	p=0.19		
	s.moleculare	<10%↓	<10%↓	4-5	N too small	p=0.87		
	Hilus	11%↑	10%↑	4-5	N too small	p=0.79		

In summary, no statistically significant changes in the cellular endpoints were found after repeated RF exposure of young adult C57Bl/6J mice for 4 weeks. A near significant increase in the total number of microglia of 18% was observed in the 3 W/kg group compared to the sham group. Exposure did not affect the total or regional number of astroglia, or proliferation or neuronal differentiation in the SGZ.

3.3.1.3 Functional endpoints

3.3.1.3.1 No changes in hippocampus-dependent learning and memory were found after RF exposure

The effects of RF exposure on hippocampus-dependent memory were investigated in two different tests. After 5 days of exposure, mice were tested in the novel object localisation (NOL) test (Figure 3.9) and after 4 weeks of exposure they were trained and tested in the water maze test (Figure 3.10).

Open field and novel object localisation tests

On days 4 and 5 of exposure, groups of six mice were assessed in the NOL test to investigate early RF induced changes in exploration and spatial memory.

Mice were introduced to the NOL arena for 10 minutes on day 4 of exposure. The arena was dimly lit with dimensions of 28x42 cm with high frosted sides and clean bedding. The behaviour of the mice was investigated for differences in activity or exploratory behaviour in terms of distance moved, number of rears and latency to first rear (Figure 3.9B,C,D).

The average distance moved during the 10 minute habituation trial was not found to be significantly different between groups using a non-parametric one-way ANOVA. However, using a Mann-Whitney test for further investigation of the longer distance moved by the mice in the 3 W/kg exposure group compared to the sham group, a statistically significant difference was found ($p<0.05$)(see Table 3.5 for details). Using a non-parametric one-way ANOVA the latency to first rear was found not to differ significantly between groups, with average latencies between

1.4 and 2.4 minutes (Figure 3.9C). In the 0.3 W/kg exposure group, two mice showed much longer latencies of more than 5 minutes, while the remaining four mice behaved very similarly to the mice in the other exposure groups. The number of rears was similar between groups (Figure 3.9D).

On the second day the mice were introduced to the two objects in a 5 minute exploration trial (trial 1), next the 3 minute memory trial (trial 2) was performed 15 minutes later in which one of the objects was moved to a novel location. However, no preference for the novel object over the known object was seen in any of the exposure groups, and the effect of exposure on spatial memory could therefore not be evaluated (Figure 3.9E-G). Object preference was evaluated by the cumulative time spent at a distance of <2 cm from the object (Figure 3.9E), the fraction of time spent exploring the moved object relative to the total exploration time (Figure 3.9F) and the frequency of visits to the object zones (<2 cm from the object)(Figure 3.9G).

Using two-way ANOVAs for statistical analysis, no significant differences between the exposure groups were found. (see Table 3.6 for details of the statistical analyses). Although not significant, some of the mice in the 3 W/kg group spent more time exploring the two objects and visited the object zones more frequently than the mice in the two other groups, however this was the case for both the novel and the known object.

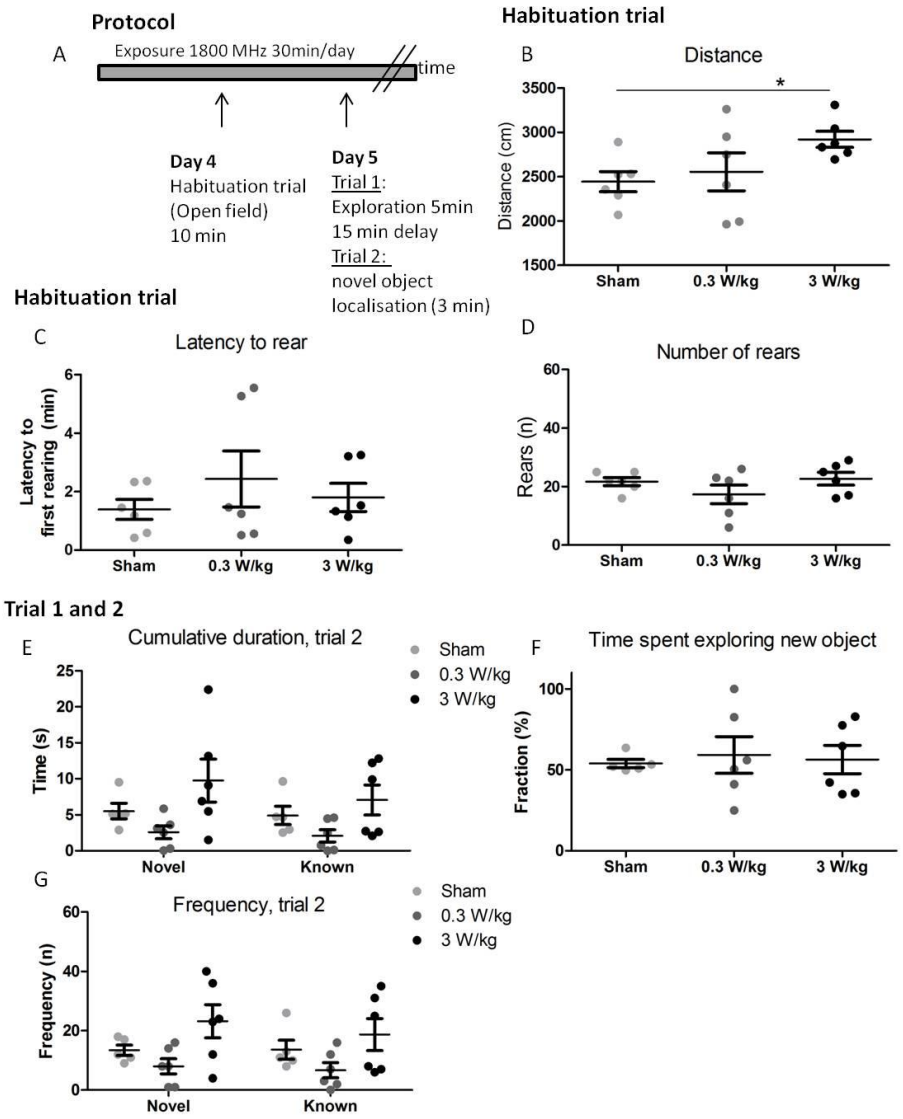


Figure 3.9 The novel object localisation test was used to evaluate effects of RF exposure. 12 week old male C57Bl/6J mice exposed to 0.3 W/kg, 3 W/kg or sham exposure for 30 minutes per day, 5 days a week for 4 weeks were tested in the NOL test on days 4-5 of exposure as a test of spatial memory (A). Locomotor function and exploratory behaviour was evaluated in the 10 minute habituation trial on day 4 (B-D). There were no significant differences between groups exposed at 0 (sham), 0.3 W/kg or 3 W/kg on distance moved (B), latency to first rear (C) or the total number of rears (D) when using a one-way ANOVA for comparison. However a comparison of the distance moved by the sham and 3 W/kg groups by the Mann-Whitney test revealed a statistical significant difference. In trial 2 no discrimination was seen between the objects at the known or novel positions, evaluated in terms of cumulative time spent exploring the objects (E), fraction of time spent exploring the moved object relative to the total exploration time (F), frequency of visits (G). Data are presented as mean ± SEM. N=6. Statistical significance was evaluated by non-parametric one-way ANOVA or the Mann-Whitney test for comparisons of groups to the sham group or two-way ANOVA for investigation of object preference. *p<0.05.

Table 3.5 Details of statistical analyses of open field test after RF exposure in C57Bl/6J mice

	Difference between means of sham and 0.3W/kg group	**Difference between means of sham and 3W/kg group**	**Group sizes (n)**	**Gaussian distribution?**	**One-way ANOVA or non-parametric Kruskall-Wallis,**	**Mann-Whitney test, compared to sham group**
Distance	<10%↑	20%↑	6	N too small	p=0.10	0.3 W/kg: p=0.82 **3 W/kg: p=0.03***
Latency	75%↑	30%↑	6	N too small	p=0.85	0.3 W/kg: p=0.70 3 W/kg: p=0.70
Rearings	20%↓	<10%↑	6	N too small	p=0.42	

Table 3. 6 Details of statistical analyses of NOL test after RF exposure in C57Bl/6J mice

	Difference between means of sham and 0.3W/kg group	**Difference between means of sham and 3W/kg group**	**Group sizes (n)**	**Gaussian distribution?**	**One-way ANOVA or non-parametric Kruskall-Wallis,**	**Mann-Whitney test, compared to sham group**
Fraction time spent exploring object	<10%↑	<10%↑	5-6	n too small	p=0.99	
Two-way ANOVA for exposure group and object						
	Interaction	**Effect of exposure group:**	**Effect of object**	**Group sizes (n)**	**Percent difference compared to sham group, known object**	
Trial 2 cumulative duration	F(2,28)=0.25, p=0.78	F(2,28)=6.25, **p=0.0057**** Bonferronis *post hoc* test: Sham vs 0.3 W/kg: n.s. for both objects. Sham vs 3 W/kg: n.s. for both objects	F(1,28)=0.76, p=0.39	n=5-6	Mean compared to time used at known object in sham group: <u>Sham:</u> Novel: 12 %↑ <u>0.3 W/kg:</u> Known:58%↓, Novel: 48%↓ <u>3 W/kg:</u> Known: 44%↑, Novel: 99%↑	
Trial 2 frequency	Interaction: F(2,28)=0.18, p=0.83	Exposure group: F(2,28)=6.29, **p=0.0055**** Bonferronis *post hoc* test: Sham vs 0.3 W/kg: n.s. for both objects. Sham vs 3 W/kg: n.s. for both objects	Object F(1,28)=0.34, p=0.57	n=5-6	Mean compared to frequency of visits to known object in sham group: <u>Sham:</u> Novel: 1%↓ <u>0.3 W/kg:</u> Known:41%↓, Novel: 51%↓ <u>3 W/kg:</u> Known: 37%↑, Novel: 70%↑	

Water maze

For the water maze test, mice were trained to find a submerged platform in 7 trials over 4 days after the completion of the 4 week RF exposure regime for investigation of lasting effects of exposure on learning and memory.

Before starting training, mice were tested in a pre-trial to ensure that there were no differences in motor activity between groups and that all mice were fit to swim. The average distance swum during the 1 minute trial was 1000 cm ± 100 cm in all three exposure groups, and in all groups the time spent in each of the four quadrants was similar (data not shown).

During training, in all three exposure groups, the mice learned to locate the platform during the first three or four trials as can be seen by the platform latency (Figure 3.10A) and the distance swum before finding the platform (Figure 3.10B). Examples of swimming paths can be seen in Figure 3.10D, showing the path of one mouse in the first and last trials. The platform is located in the upper left quadrant (Q1). The effects of RF exposure and training on platform latency and distance swum were evaluated using repeated measures two-way ANOVAs for effects of exposure and trial. No significant effect of exposure was found on either parameter, while a significant improvement in performance was seen over trials (see Table 3.7 for statistical details). The mean swimming velocity was also investigated (Figure 3.10C). Occasions where mice were immobile were excluded. Statistical analysis using a two-way repeated measures ANOVA revealed no effect of exposure, while a significant decrease in velocity was seen over the trials (see Table 3.7 for statistical details).

After the seven acquisition trials, reference memory was tested in a probe-trial where the platform was removed and the mice were allowed to swim freely for 1 minute (Figure 3.10E-I). A typical swimming path is shown in Figure 3.10I, where a clear preference for the area surrounding the platform zone was seen. The mice in all three groups swam a similar distance of 1000 cm ± 100 cm during the trial, with no signs of motor impairment or thigmotaxis (Figure

3.10E). The platform zone latency and the number of platform zone crossings were also similar between exposure groups (Figure 3.10F,G). Statistical analysis of the three parameters revealed no significant differences between the three exposure groups when using a one-way ANOVA. As shown in Figure 3.10H, the mice in all groups spent approximately 50% of the time in the quadrant where the platform had previously been located (Q1). Statistical evaluation using a two-way ANOVA showed that the preference for the platform quadrant over the three other quadrants was significant in all three exposure groups, while no significant difference in time spent in either of the quadrants was found between the sham exposed group and the two RF exposed groups (See Table 3.7 for details).

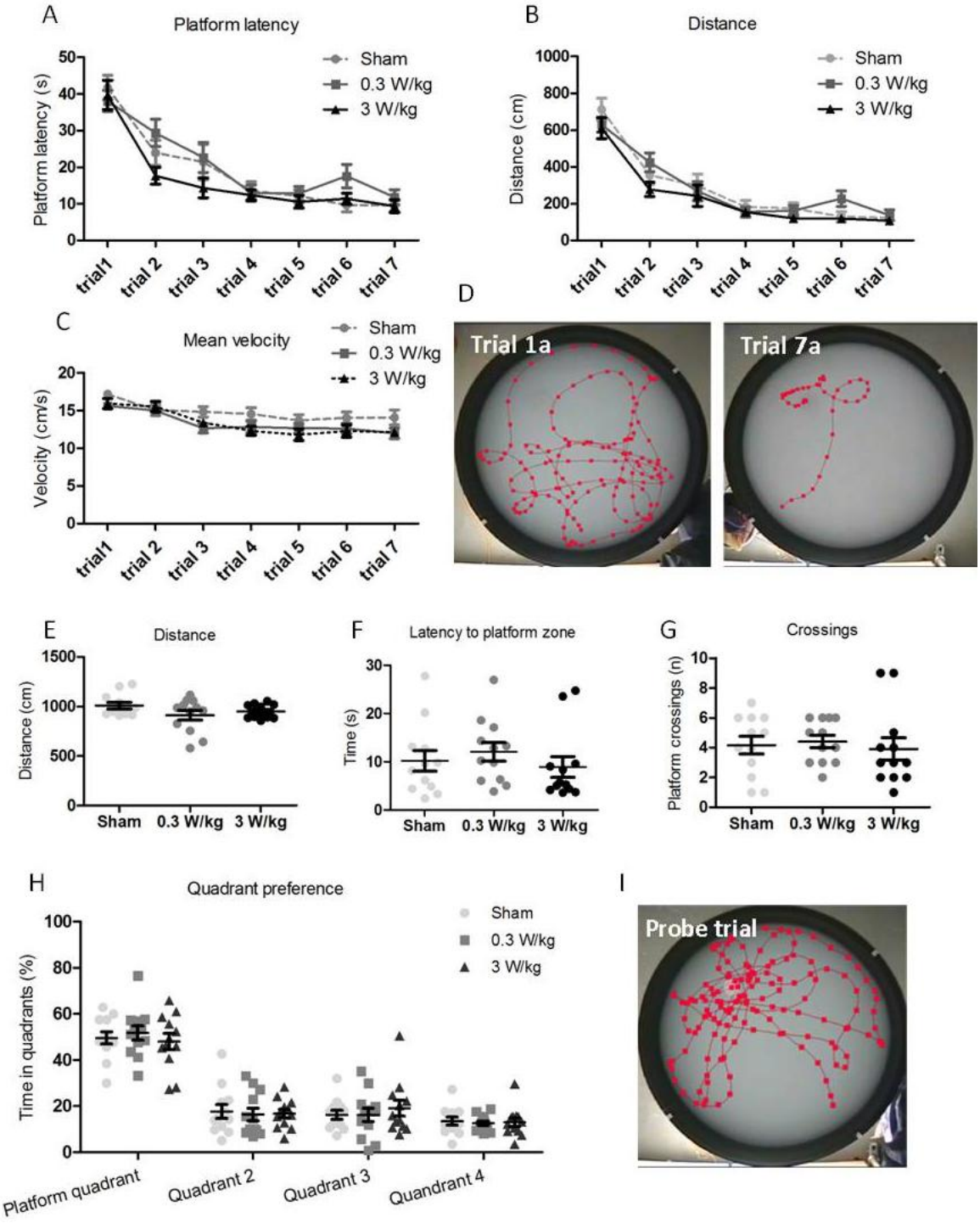


Figure 3.10 No effect of RF exposure was observed on learning and memory in the water maze test. 12 week old male C57Bl/6J mice exposed to 0.3 W/kg, 3 W/kg or sham exposure for 30 minutes per day, 5 days a week for 4 weeks were tested in a water maze test consisting of 7 learning trials, each trial consisting of 4 swims, to find the hidden platform, and one memory test where no platform was present. The movement of the mice was tracked using Ethovision XT 10 software (Noldus). In the learning trials, platform latency (A), distance swum (B) and the mean swimming velocity (C) was evaluated. For the probe test, the distance swum (E), platform zone latency (F), number of

platform zone crossings (G) and quadrant preference (H) was compared between experimental groups. No significant effect of exposure was seen for any of the endpoints. (D) and (I) show representative swim tracks in the first and last learning trials, and in the probe trial respectively. Statistical analysis for the learning trials was carried out using a repeated measures two-way ANOVA. For the probe trial, statistical comparisons were done by one-way ANOVA, except for quadrant preference where a two-way ANOVA was used. Data are presented as mean ± SEM. No statistically significant differences were found between the sham groups and the two RF exposed groups.

Table 3.7 Details of statistical analyses of water maze data after RF exposure in C57Bl/6J mice

	Repeated measures two-way ANOVA	**Interaction**	**RF exposure**	**Trial**	**Bonferroni's *post hoc* test**
Training	**Platform latency**	F(18,264)=1.19 p=0.27	F(3,264)=1.05 p=0.38	F(6,264)=64.8 **p<0.0001*****	**Comparison to trial 1:** <u>Trial 2:</u> 0.3 W/kg **p<0.05*** Sham and 3 W/kg **p<0.001***** <u>Trial 3-7:</u> **p<0.001***** in all three exposure groups
	Distance	F(12,198)=0.97 p=0.48	F(2,198)=1.85 p=0.17	F(6,198)=77.9 **p<0.0001*****	**Comparison to trial 1:** <u>Trial 2-7:</u> **p<0.001***** in all three exposure groups.
	Velocity	F(12,198)=1.22 p=0.27	F(2,198)=2.69 p=0.08	F(6,198)=24.1 **p<0.0001*****	**Comparison to trial 1:** <u>Trial 2:</u> sham **p<0.01**** <u>Trial 3:</u> Sham **p<0.01**** 0.3W/kg and 3 W/kg: **p<0.001***** <u>Trial 4-7:</u> **p<0.001***** in all three exposure groups

Probe-trial	**One-way ANOVA**	**Compared to sham**	**Group size and normality test**	**One-way ANOVA or Kruskall-Wallis test, p=**	
	Distance	0.3 W/kg: 10%↓ 3 W/kg: 6%↓	N=12 Gaussian distribution	0.17	
	Platform latency	0.3 W/kg: 18%↑ 3 W/kg: 13%↓	N=12 Does not follow a Gaussian distribution	0.21	
	Crossings	0.3 W/kg: 6%↑ 3 W/kg: 6%↓	N=12 Gaussian distribution	0.84	
	Two-way ANOVA	**Interaction**	**RF exposure**	**Quadrant**	**Bonferroni's *post hoc* test**
	Quadrant preference	F(6,132)=0.34, p=0.91	F(2,132)=0 p=0.99	F(3,132)=132.63 **P<0.0001*****	Comparison to platform quadrant: Quadrant 2-4: **p<0.001*** in all three exposure groups

In summary, RF exposure did not affect learning or memory in the water maze test. The protocol for the NOL test did not produce the expected results in the sham exposed animals so requires further optimisation. Mice exposed at 3 W/kg for 4 days moved a significantly longer distance in the open field test than sham exposed mice.

3.3.2. Effects of RF exposure in a mouse model of accelerated senescence

In this section, results of experiments investigating the effects of RF exposure at 3 W/kg in a senescence accelerated mouse model (SAMP8) compared to a senescence accelerated resistant mouse model (SAMR1) will be presented. These include investigations of effects of exposure on synaptic plasticity and synaptic density (molecular endpoints), and a study of proliferation and differentiation in the SGZ in the hippocampus and studies of astroglia and microglia numbers (cellular endpoints). Functional endpoints were not investigated in this model.

3.3.2.1 Molecular endpoints

3.3.2.1.1 Subtle changes in gene expression were found after RF exposure

Expression of the AMPA receptor subunits (*gria1, gria2),* NMDA receptor subunits *(grin1, grin2a, grin2b* and BDNF signalling molecules *(bdnf, ntrk2* and *p75)* were investigated in 7 month old male SAMP8 and SAMR1 mice exposed to an 1800 MHz field at a whole body SAR of 3 W/kg or sham exposed for 30 minutes per day, 5 days a week for 4 weeks (n=6). *p75* was included due to its role in BDNF-induced death signalling.

Statistical analysis was carried out using two-way ANOVA for investigation of the effect of strain and exposure on gene expression. The results concerning strain effects are presented in the section below and is followed by a section on the exposure effects.

Characterisation of plasticity related genes in SAMP8 and SAMR1 mice

Sham exposures were used to characterise the baseline expression of plasticity related genes in the two strains. In the dorsal hippocampus, the level of *gria1* was lower by 33% in the SAMP8 mice compared to the SAMR1 mice, this was found to be significant using a two-way ANOVA with Bonferroni's *post hoc* test for comparison of the two sham groups ($p<0.01$), *gria2* expression was not affected (Figure 3.11A). The expression levels of *grin1* (Figure 3.11B) and *bdnf* (Figure 3.11E) were lower in the SAMP8 mice both by approximately 25% but not

significantly. There were no differences in expression of *grin2a, grin2b, ntrk2* or *p75* between the two strains. See Table 3.8 for results of the statistical analyses.

In the prefrontal cortex, the level of *grin1* was significantly lower in the SAMP8 mice compared to the sham exposed SAMR1 mice by 22%. No significant differences in expression of any of the other genes were observed between the sham groups in the two strains (Figure 3.11C,D,F). However, non-significant differences were seen in the baseline level of *grin2b*, which was lower in the SAMP8 mice than in the SAMR1 mice, and in *bdnf* which was higher in the SAMP8 than in the SAMR1 mice. See Table 3.8 for the results of the statistical analyses.

Small effects of RF exposure on plasticity genes in SAMP8 and SAMR1 mice

No significant effect of RF exposure was found on the hippocampal expression of either the AMPA receptor subunits, the NMDA receptor subunits *grin2a* and *grin2b*, or the BDNF signalling genes, *bdnf*, *ntrk2* and *p75,* using two-way ANOVA (Figure 3.11A,B,E).

There was a significant interaction between strain and exposure on *grin1* expression ($p<0.05$) with a 47% increase in expression in the SAMP8 mice after exposure, whereas there was no change in gene expression after exposure in the SAMR1 mice (Figure 3.11B). The increase was investigated further by an unpaired t-test comparing the sham and 3W/kg groups in the SAMP8 srtain, and found to be significant ($p<0.01$). See Table 3.8 for results of the statistical analysis.

In the prefrontal cortex, the statistical analysis by two-way ANOVA revealed a significant difference in *bdnf* expression between the strains in terms of differences in response to exposure, where an increase in expression was seen in the SAMP8 mice, while no change was seen in the SAMR1 mice (Figure 3.11F). RF exposure did not affect gene expression of any of the other genes significantly in either strain (Figure 3.11C,D,F). See Table 3.8 for details of the statistical analyses.

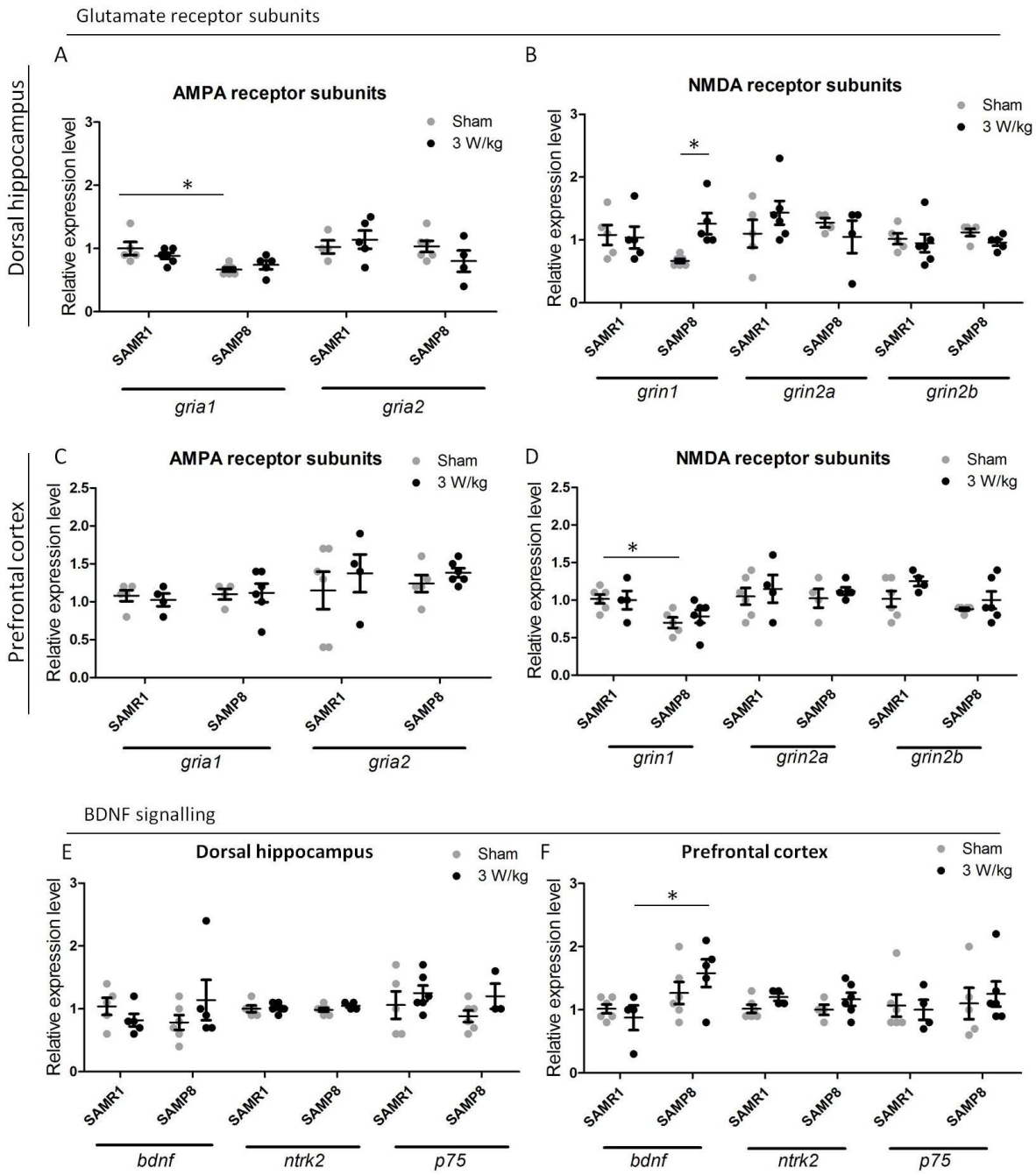


Figure 3.11 Small differences in expression of plasticity related genes were found between SAMP8 and SAMR1 strains after RF exposure. Expression of the AMPA receptor subunits *gria1* and *gria2*, the NMDA receptor subunits *grin1, grin2a* and *grin2b* and *bdnf* and its receptors *ntrk2* and *p75* were investigated in 7 months old SAMP8 and SAMR1 mice after 4 weeks of RF exposure 30 minutes per day at SARs of 0 (sham) or 3 W/kg in the dorsal hippocampus (A,B,E) and the prefrontal cortex (C,D,F). In the dorsal hippocampus, significantly lower *gria1* expression was seen in the SAMP8 strain than in the SAMR1 strain (A). A significant increase in *grin1* expression was observed in the SAMP8 strain after exposure compared to the sham group (B). In the prefrontal cortex a significantly lower baseline level of *grin1* was seen in the SAMP8 mice compared to the SAMR1 mice. After exposure a significantly

higher *bdnf* expression level was seen in the SAMP8 strain compared to the SAMR1 strain (F). Exposure did not affect gene expression of any of the other genes in the dorsal hippocampus or prefrontal cortex. The data are presented as mean ± SEM, n=4-6. Statistical analysis was carried out by two-way ANOVA with Bonferroni's *post hoc* test.* $p < 0.05$.

Table 3.8 Details of statistical analysis of gene expression in RF exposed SAMP8 and SAMR1 mice

	Two-way ANOVA	**interaction**	**exposure**	**strain**	Bonferroni's *post hoc* test	**Percent-wise differences**
Dorsal hippocampus	*Gria1*	F(1,18)=2.14 p=0.16	F(1,18)=0.11, p=0.74	F(1,18)=13.48, **p=0.0014***	comparison of strains: **Sham: p<0.01**,** 3 W/kg: n.s.	Compared to SAMR1 sham: SAMR1 3W/kg: 12%↓, SAMP8 sham: 34%↓ Comparison to SAMP8 sham:, SAMP8 3 W/kg:11%↑
	Gria2	F(1,15)=1.89, p=0.19	F(1,15)=0.22, p=0.65	F(1,15)=1.71, p=0.21		
	Grin1	F(1,17)=5.16, **p=0.04***	F(1,17)=3.93, p=0.06	F(1,17)=0.48, p=0.50	Comparison of exposure groups: SAMR1: n.s., **SAMP8: p<0.05***	Compared to SAMR1 sham: SAMR1 3W/kg: 2%↓, SAMP8 sham: 17%↓ Comparison to SAMP8 sham:, SAMP8 3 W/kg: 51%↑
	Grin2a	F(1,15)=1.84, p=0.19	F(1,15)=0.07, p=0.79	F(1,15)=0.26, p=0.62		
	Grin2b	F(1,17)=0.20, p=0.66	F(1,17)=1.33, p=0.27	F(1,17)=0.3, p=0.59		
	bdnf	F(1,17)=2.43, p=0.14	F(1,17)=0.14, p=0.71	F(1,17)=0.03, p=0.87		
	ntrk2	F(1,17)=0.89, p=0.36	F(1,17)=2.95, p=0.10	F(1,17)=0.81, p=0.38		
	P75	F(1,17)=2.53, p=0.13	F(1,17)=0, p=0.98	F(1,17)=2.37, p=0.14		

		interaction	**exposure**	**strain**	Bonferroni's *post hoc* test	**Percent-wise differences**
Prefrontal cortex	*Gria1*	F(1,15)=0.13, p=0.73	F(1,15)=0.04, p=0.85	F(1,15)=0.31, p=0.59		
	Gria2	F(1,17)=0.05, p=0.83	F(1,17)=1.01, p=0.93	F(1,17)=0.07, p=0.79		
	Grin1	F(1,17)=0.35, p=0.56	F(1,17)=0.16, p=0.70	F(1,17)=10.01, **p=0.006****	comparison of strains: **Sham: p<0.05*,** 3 W/kg: n.s.	Compared to SAMR1 sham: SAMR1 3W/kg: 1%↓, SAMP8 sham: 31%↓ Comparison to SAMP8 sham: SAMP8 3 W/kg:12%↑
	Grin2a	F(1,15)=0.0, p=0.98	F(1,15)=0.64, p=0.43	F(1,15)=0.05, p=0.82		
	Grin2b	F(1,17)=0.35, p=0.56	F(1,17)=3.45, p=0.08	F(1,17)=4.13, p=0.06		
	bdnf	F(1,17)=1.81, p=0.20	F(1,17)=0.26, p=0.62	F(1,17)=7.99, **p=0.012***	comparison of strains: Sham: n.s., **3 W/kg: p<0.05***	Compared to SAMR1 sham: SAMR1 3W/kg: 12%↓, SAMP8 sham: 25%↑, Comparison to SAMP8 sham: SAMP8 3W/kg:25%↑
	ntrk2	F(1,16)=0.01, p=0.92	F(1,16)=4.04, p=0.06	F(1,16)=0.08, p=0.78		
	P75	F(1,17)=0.28, p=0.60	F(1,17)=0.04, p=0.84	F(1,17)=0.48, p=0.50		

3.3.2.1.2 No changes in protein levels of glutamate receptor subunits were observed after RF exposure

Protein levels of the AMPA receptor subunits (GluA1 and GluA2) and the NMDA receptor subunits (GluN1 and GluN2a) were investigated in the hippocampus of 7 month old male SAMP8 and SAMR1 mice exposed to an 1800 MHz field at a whole body SAR of 3 W/kg or sham exposure for 30 minutes per day, 5 days a week for 4 week (n=4-6).

The effects of strain and exposure were evaluated using a two-way ANOVA with Bonferroni's *post hoc* test. A significantly lower level of GluA2 (50%) was seen in the RF exposed SAMP8 mice compared to RF exposed SAMR1 mice. There were no significant differences between the strains after sham exposure nor when the sham and exposed groups were compared for each strain (Figure 3.12A). The same trend of a decreased GluA1 level was observed in the RF exposed SAMP8 mice compared to the RF exposed SAMR1 mice but this was not statistically significant.

A significantly lower level of GluN1 (50%) was found in the sham exposed SAMP8 mice compared to the sham exposed SAMR1 mice (Figure 3.12B). No significant effect of exposure was observed in either strain. No strain or exposure effects were found on GluN2a level. See Table 3.9 for details of statistical analyses.

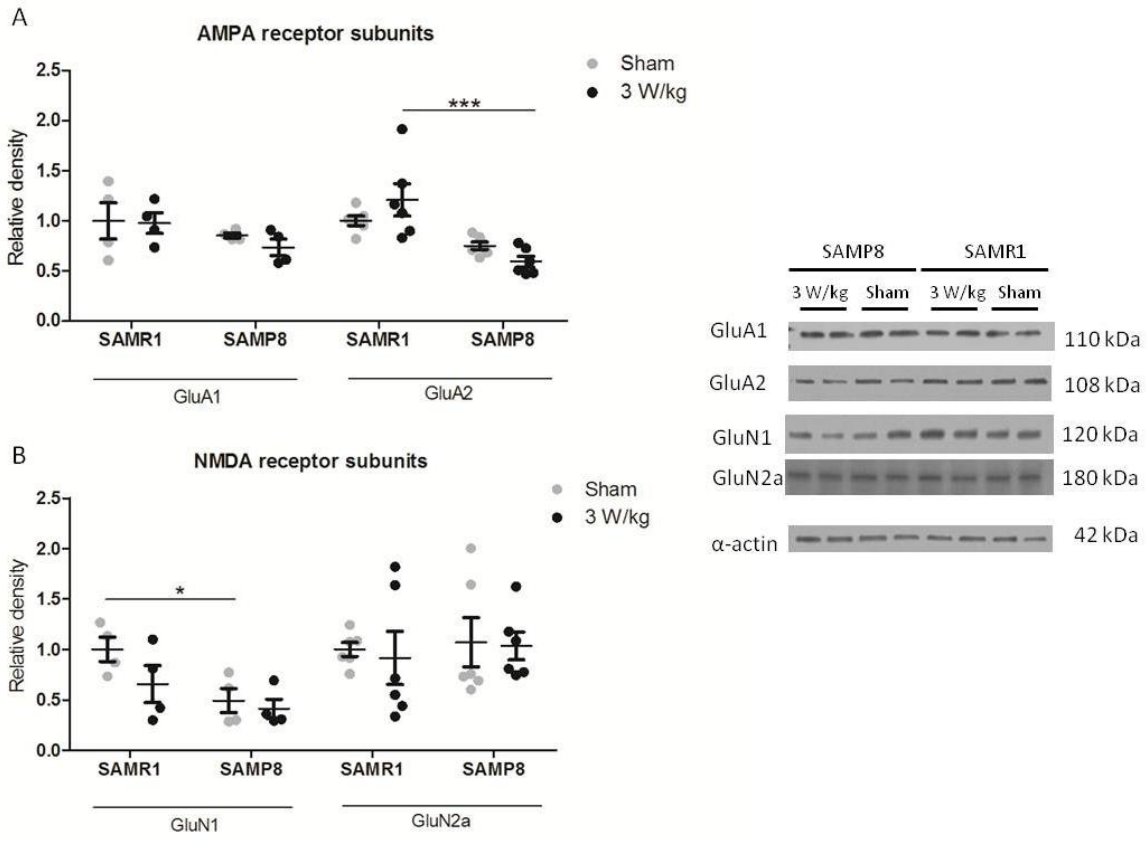


Figure 3.12 Small changes in protein levels of glutamate receptor subunits were observed after RF exposure. By western blotting, hippocampal protein levels of the AMPA receptor subunits (GluA1 and GluA2) and NMDA receptor subunits (GluN1 and GluN2a) were investigated in 7 months old SAMP8 and SAMR1 mice after 4 weeks of RF exposure at 3 W/kg or sham exposure 30 minutes per day. A significantly lower level of GluA2 was measured in the SAMP8 strain compared to the SAMR1 strain after exposure at 3 W/kg, but no difference in GluA1 was observed between strains or after RF exposure (A). In SAMP8 mice a significantly lower GluN1 level was found compared to SAMR1 mice after sham exposure (B), but no differences in GluN2a was observed between strains or after RF exposure. Protein levels are expressed relative to α-actin levels and data are presented as mean ± SEM. n=4-6. Statistical analysis was carried out by two-way ANOVA with Bonferroni's *post hoc* test. *p<0.05, ***p<0.001.

Table 3.9 Details of statistical analysis of protein levels of glutamate receptor subunits in SAMP8 and SAMR1 mice after RF exposure

Two-way ANOVA	**Interaction**	**Exposure**	**Strain**	**Bonferroni's *post hoc* test**
GluA1	F(1,12)=0.18 p=0.68	F(1,12)=0.39 p=0.54	F(1,12)=2.93 p=0.11	
GluA2	F(1,20)=4.14 p=0.055	F(1,20)=0.08 p=0.79	F(1,20)=22.82 *****p<0.0001**	Comparison of strains in both exposure groups: Sham: n.s. **3 W/kg ***p<0.0001**
GluN1	F(1,12)=0.97 p=0.34	F(1,12)=2.5 p=0.14	F(1,12)=7.87 ****p=0.016**	Comparison of strains: **Sham *p<0.05** 3 W/kg n.s.
GluN2a	F(1,20)=0.02 p=0.90	F(1,20)=0.09 p=0.76	F(1,20)=0.24 p=0.63	

3.3.2.1.3 Indications of differences in response to RF field exposure in synaptic markers

The effects of RF exposure on synapse integrity were investigated in terms of density of the pre-synaptic marker synaptophysin, and mRNA and protein levels of the post-synaptic density component PSD95 in SAMP8 and SAMR1 mice.

The density of synaptophysin staining was measured in six selected subfields of the hippocampus: the *s.oriens* and *s.radiatum* of the CA1, the *s.lacunosum-moleculare*, the *s.moleculare* of the DG, the hilus and in the CA3 *s.radiatum* as shown in Figure 3.13. Effects of strain and exposure on staining density were investigated in each hippocampal subfield using two-way ANOVA. There was no significant strain difference when staining density was compared between the sham groups in the two strains. However, slightly lower synaptophysin densities were seen in the CA1 *s.radiatum, s.lacunosum-moleculare*, hilus and CA3 *s.radiatum* of sham exposed SAMP8 mice compared to sham exposed SAMR1 mice. With regards to RF exposure, no

significant effects were found, but there was a significant interaction between strain and exposure in all regions measured, except for the hilus and CA3 *s.radiatum*. This interaction was due to lower synaptophysin densities in RF exposed SAMR1 mice than sham exposed SAMR1 mice, and also due to the higher synaptophysin densities in RF exposed SAMP8 mice than sham exposed SAMP8 mice. Using Bonferroni's *post hoc* test for pairwise comparisons a significant difference between the two exposed groups was seen in the *s.oriens* ($p<0.05$) and a significantly lower synaptophysin density was found in the sham exposed SAMP8 mice compated to the sham exposed SAMR1 mice in the *s.lacunosum-moleculare* ($p<0.05$). See Table 3.10 for details of the statistical analyses.

PSD95 was used as a marker of the post-synaptic density. Gene expression (*dlg4*) was investigated in the dorsal hippocampus and prefrontal cortex (Figure 3.13C,D). No significant effects of exposure or strain were found on gene expression levels in the hippocampus or prefrontal cortex by two-way ANOVA. See Table 3.10 for details of the statistical analyses. The protein level of PSD95 in the hippocampus measured by western blotting, also showed no significant effects of exposure or strain by two-way ANOVA (see Table 3.10 for statistical details) (Figure 3.13E,F).

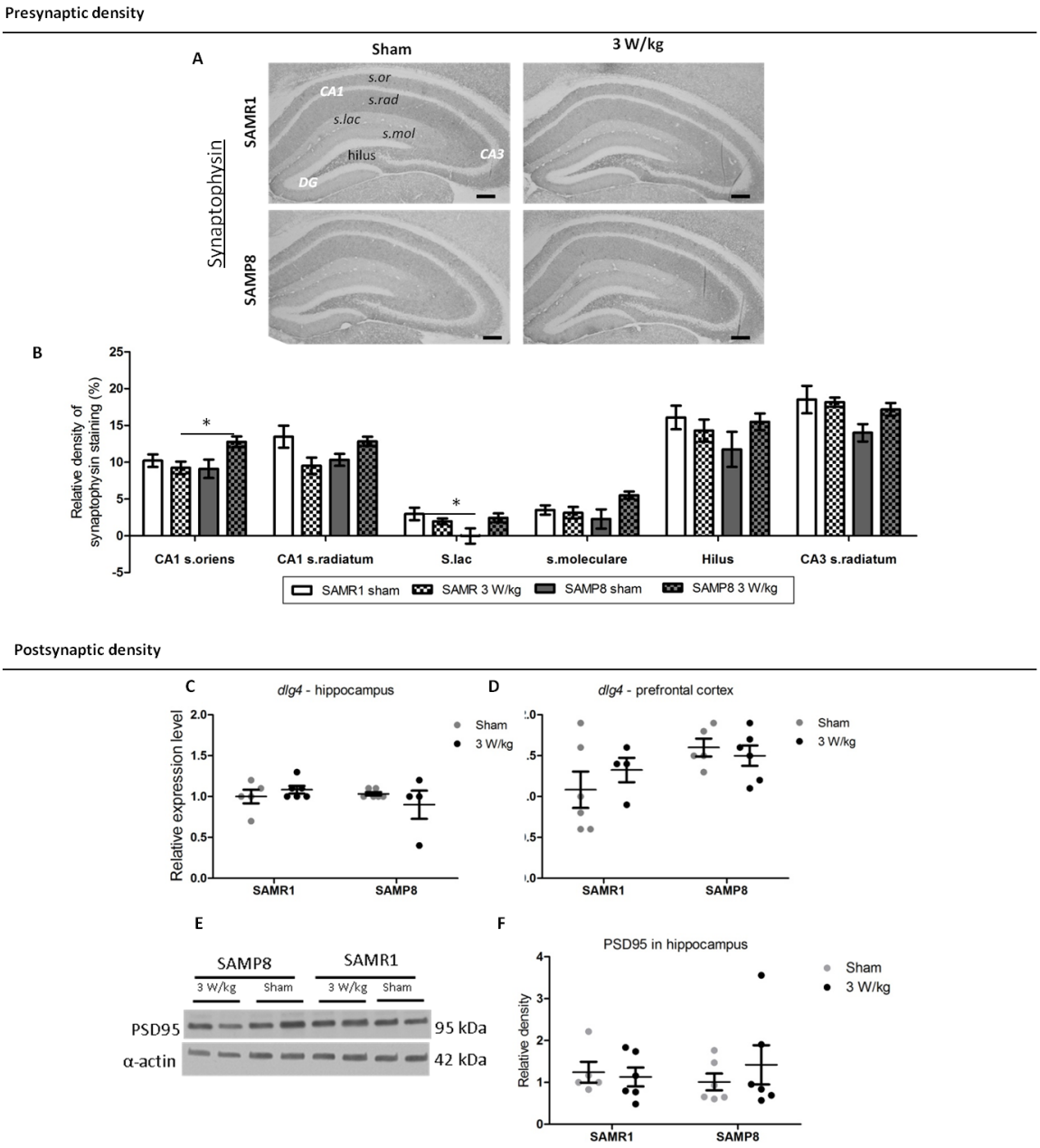


Figure 3.13 Indications of synaptic changes after RF exposure in SAMP8 and SAMR1 mice. 6 months old male SAMP8 and SAMR1 mice were exposed to 3 W/kg or sham exposed for 30 minutes per day, 5 days a week for 4 weeks**.** Regional density of synaptophysin was measured in 6 hippocampal subfields as a measure of pre-synaptic density (A-B). PSD95 gene (*dlg4,* dorsal hippocampus and prefrontal cortex) and protein levels (dorsal hippocampus) were investigated as a measure of the post-synaptic density (C-F). In (B) the percent change in the density (relative to the average level found in the s.*lacunosum-moleculare* in the sham group) is shown for each subfield in the three exposure groups (n=6). There were no significant differences between exposure groups, however a significant interaction between strain and exposure was observed in the CA1, *s.lacunosum-moleculare* and *s.moleculare*. By Bonferroni's *post hoc* test a significant difference was found between the two exposed groups in the *s.oriens*, and

between the two sham groups in *the s.lacunosum-moleculare*. In (C,D,F) gene and protein levels of PSD95 are shown. There were no significant changes in gene expression in the dorsal hippocampus or prefrontal cortex (n=4-6) and also no effect of exposure on the protein level in the dorsal hippocampus (n=5-6) (F). Scale:200 µm (A). Data are presented as mean ± SEM. Statistical analysis was carried out using a two-way ANOVA. *p<0.05.

Table 3.10 Details of statistical analysis of RF effects on synaptic markers in SAMP8 and SAMR1 mice

Two-way ANOVA				
Synaptophysin staining	**Interaction**	**Exposure**	**Strain**	**Bonferroni's *post hoc* test** **SAMR1vs. SAMP8**
CA1 *s.oriens*	F(1,17)=0.66 **p=0.02***	F(1,17)=2.21 p=0.16	F(1,17)=1.8 p=1.2	Sham: n.s. **3W/kg: *p<0.05**
CA1 *s.radiatum*	F(1,17)=8.04 **p=0.01***	F(1,17)=0.41 p=0.53	F(1,17)=0.01 p=0.94	Sham: n.s. 3W/kg: n.s
s.lacunosum-moleculare	F(1,17)=5.34 **p=0.03***	F(1,17)=0.93 p=0.35	F(1,17)=2.82 p=0.11	**Sham: *p<0.05** 3W/kg: n.s.
s.moleculare	F(1,17)=5.39 **p=0.03***	F(1,17)=0.36 p=0.08	F(1,17)=0.56 p=0.47	Sham: n.s. 3W/kg: n.s
Hilus	F(1,17)=2.9 p=0.1	F(1,17)=0.38 p=0.55	F(1,17)=0.93 p=0.35	Sham: n.s. 3W/kg: n.s
CA3 *s.radiatum*	F(1,17)=1.76 p=0.2	F(1,17)=1.15 p=0.30	F(1,17)=4.33 p=0.53	Sham: n.s. 3W/kg: n.s
PSD95 and *dlg4* expression	**interaction**	**exposure**	**strain**	
dlg4 dorsal hippocampus	F (1,17)=0.05, p=0.83	F(1,17)=0.32 p=0.58	F(1,17)=3.59 p=0.08	
PSD95 dorsal hippocampus	F(1,19)=0.69 p=0.42	F(1,19)=0.22 p=0.64	F(1,19)=0.01 p=0.93	
dlg4 prefrontal cortex	F(1,17)=1.03 p=0.32	F(1,17)=0.18, p=0.68	F(1,17)=4.23 p=0.055	

In summary, subtle changes in some molecular endpoints were found in the SAM strains after RF exposure. The hippocampal gene expression of *grin1* was significantly increased after exposure in SAMP8 mice but not in SAMR1 mice. The GluA2 protein level was significantly lower in exposed SAMP8 mice than in exposed SAMR1 mice. In the prefrontal cortex the *bdnf* gene expression was significantly higher in the exposed SAMP8 mice compared to the exposed SAMR1 mice. Furthermore, there was an interaction between strain and exposure on density of synaptophysin staining in the hippocampus, where a decrease in staining density was seen after exposure in the SAMR1 strain, while there was an increase in staining density after exposure in the SAMP8 strain.

Only minor differences between the two strains were seen when the two sham exposed groups were compared. Significantly lower gene expression levels of *gria1* in the dorsal hippocampus, and *grin1* in the prefrontal cortex were found, and the protein level of GluN1 was significantly lower in the hippocampus of SAMP8 mice compared to SAMR1 mice. The synaptophysin staining density appeared slightly lower in the hippocampus of SAMP8 mice compared to SAMR1 mice, while there were no differences in hippocampal *dlg4* or PSD95 levels. In the prefrontal cortex, the level of *dlg4* appeared higher in the SAMP8 strain than in the SAMR1 strain.

3.3.2.2 Cellular endpoints

3.3.2.2.1 No changes to adult hippocampal neurogenesis after RF exposure

To investigate the effect of RF exposure on neurogenesis in SAM mice, the same procedure was followed as for young adult C57Bl/6J mice, as presented in Figure 3.6. Here, 6 month old SAMP8 and SAMR1 mice were exposed for 4 weeks. BrdU-labelled cells were found in most parts of the brain. In the DG, the highest density was seen in the subgranular zone (SGZ) and the inner most part of the granule cell layer (GCL) (Figure 3.14A,B).

The average numbers of BrdU-labelled cells in all four groups were found to be similar (see Figure 3.14C). A two-way ANOVA was used to investigate the effect of strain and exposure on proliferation. A significant effect of strain was found ($p<0.05$), but the Bonferroni's *post hoc* test showed that the difference between the two strains was not significant when comparing the two sham groups or the two exposed groups. See Table 3.11 for details of the statistical analysis.

Only a few doublecortin (DCX) positive cells were seen in the GCL, these were counted to investigate the effects of exposure on immature neurons. There were fewer DCX+ cells in the sham exposed SAMP8 mice compared to sham exposed SAMR1 mice, this strain effect did not quite reach statistical significance using a two-way ANOVA for comparison ($p=0.07$). (See Table 3.11 for details of the statistical analysis). No indication of an effect of exposure was seen (Figure 3.14D).

To investigate the effect of RF exposure on the fate of the BrdU-labelled cells, the proportion of BrdU+ cells co-expressing DCX was determined. In all four experimental groups, an average of 65-75% of BrdU+ cells were found to co-express DCX. No significant differences between strains or exposure groups were found when using a two-way ANOVA (Figure 3.14E), see Table 3.11 for details of the statistical analysis.

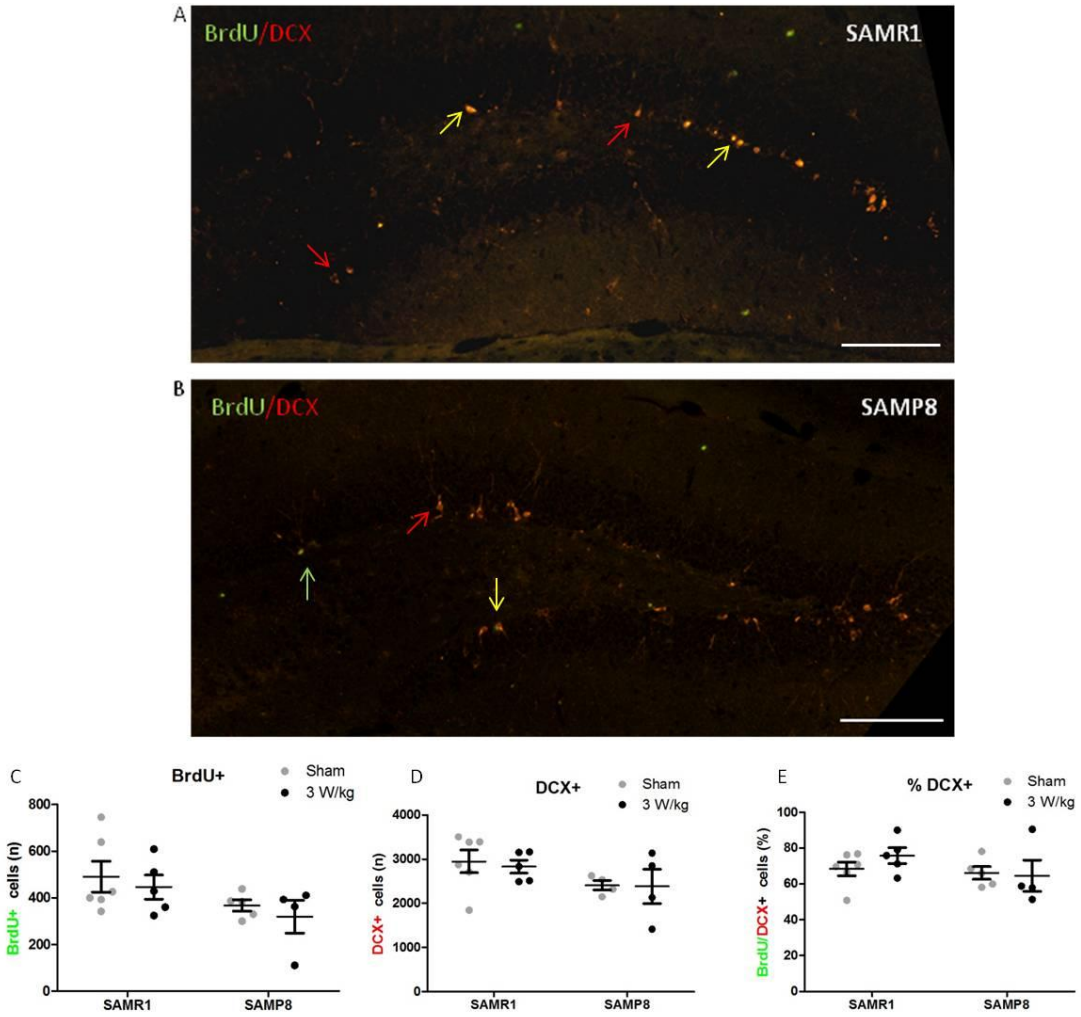


Figure 3.14 No changes in adult hippocampal neurogenesis after RF exposure in SAMP8 and SAMR1 mice. Adult hippocampal neurogenesis was investigated in terms of changes in proliferation (by BrdU-labelling) (green) and neuronal diffentiation (by BrdU/doublecortin (DCX) double-fluorescence staining) (red) in the subgranular zone in the hippocampus, after exposure of 6 months old male SAMP8 and SAMR1 mice to 3 W/kg or sham exposure for 30 minutes per day, 5 days a week for 4 weeks (n=4-6). BrdU was injected daily on day 1-4 of the last week of exposure. In (A) and (B) representative micrographs showing BrdU and DCX staining in the dorsal hippocampus of SAMR1 mice and SAMP8 mice are shown. Quantification was carried out under 40 times magnification on brain sections from -1.40 to -1.78 mm from bregma. The number of BrdU-labelled cells is shown in (C), the number of DCX+ cells is shown in (D) and the percentage BrdU-labelled cells that co-express DCX in the four experimental groups is shown in (E). Scale: 200 µm. Data are presented as mean ± SEM. Statistical analysis was carried out using a two-way ANOVA. No significant differences were found between strains or exposure groups.

Table 3.11 Details of statistical analyses of effects of RF exposure on adult hippocampal neurogenesis in SAMP8 and SAMR1 mice

Two-way ANOVA	**Percent differences**	**Interaction**	**Exposure**	**Strain**	**Bonferroni's *post hoc* test**
BrdU-labelled cells	compared to SAMR1 sham : SAMR1 3 W/kg: 9%↓ SAMP8 sham: 25%↓ Compared to SAMP8 sham: SAMP8 3W/kg: 13% ↓	F(1,16)=0 p=0.97	(F(1,16)=0.66 p=0.43	F(1,16)=4.8 ***p=0.04**	Comparison of strains: Sham: n.s. 3W/kg: n.s.
DCX+ cells	compared to SAMR1 sham : SAMR1 3 W/kg: 4%↓ SAMP8 sham: 18%↓ Compared to SAMP8 sham: SAMP8 3W/kg: 1% ↓	F(1,15)=0.04 p=0.85	F(1,15)=0.08, p=0.78	F(1,15)=3.89, p=0.07	
BrdU/DCX + cells	compared to SAMR1 sham : SAMR1 3 W/kg: 10%↑ SAMP8 sham: 3%↓ Compared to SAMP8 sham: SAMP8 3W/kg: 2% ↓	F(1,16)=0.78 p=0.39	(F(1,16)=0.34, p=0.57	F(1,16)=1.80, p=0.20	

3.3.2.2.2 Effects of RF exposure on microglia and astroglia

The number of microglia, as determined by Iba-1 immunohistochemistry, and astroglia, as determined by GFAP immunohistochemistry, were compared between exposed and sham exposed SAMP8 and SAMR1 mice.

No effect of RF exposure on microglial numbers

The number of microglia was counted in the CA1 (*s.oriens* and *s.radiatum*) and DG (granule cell layer (GCL), *s.moleculare* and hilus) in the hippocampus (Figure 3.15). Effects of exposure and strain on microglial numbers were evaluated using a two-way ANOVA.

Looking at the effect of exposure by 2 way-ANOVA, a significant effect was found as a decrease in the total number of Iba-1+ cells, however there was no significant difference between the sham and exposed groups in either strain when compared by Bonferroni's *post hoc* test ($p<0.05$) (Figure 3.15B). This was also seen in all the hippocampal regions individually, however, this was only significant in the hilus, *s.radiatum* and *s.oriens* (Figure 3.15C) see Table 3.12 for the results of the statistical analyses.

Morphologically, the microglial cells appeared more darkly stained in the SAMR1 mice, but no apparent differences in morphology were seen.

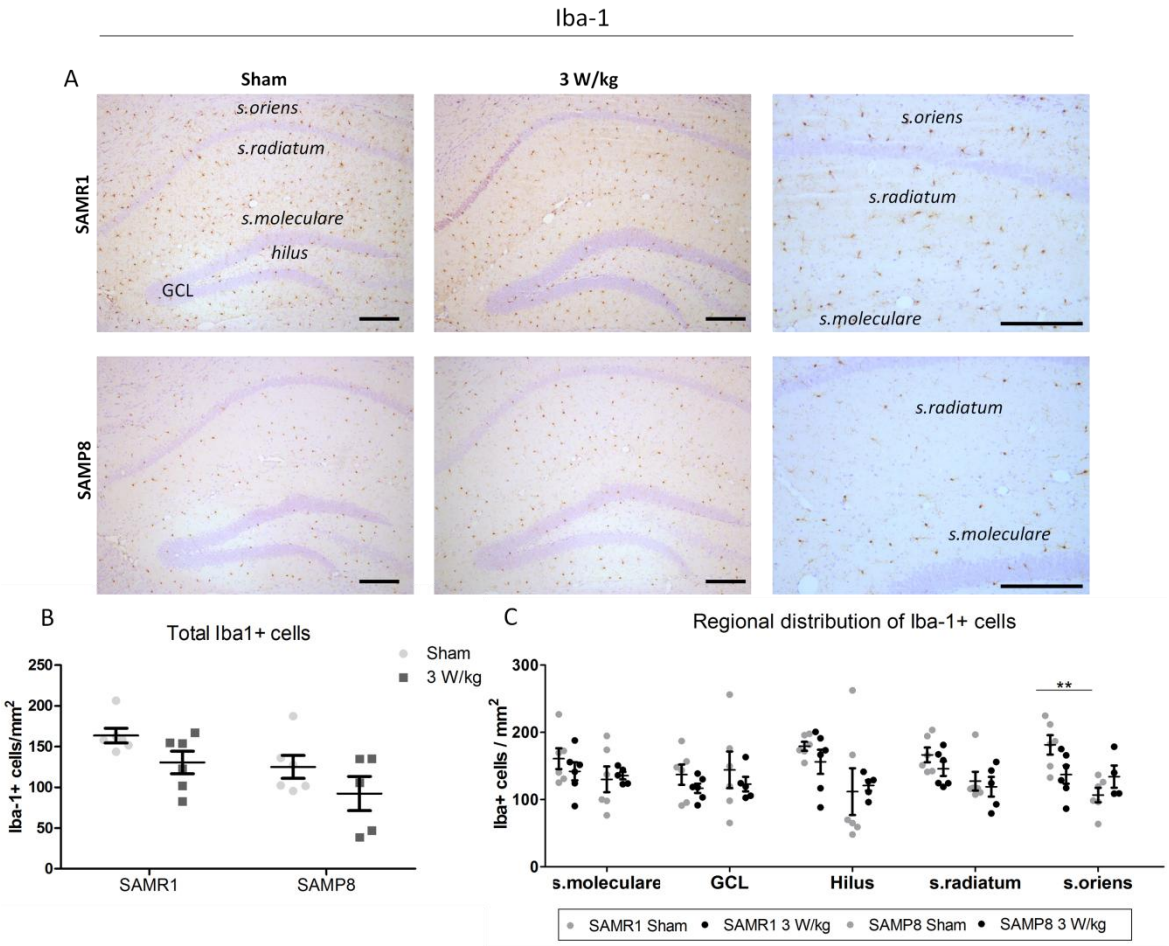


Figure 3.15 No effect of RF exposure on microglial numbers was found in the hippocampus of SAMP8 and SAMR1 mice. Iba-1+ microglia (in brown) were counted in the DG (*s.moleculare,* granule cell layer (GCL) and hilus) and CA1 (*s.radiatum* and *s.oriens*) in the hippocampus of 7 months old male SAMP8 and SAMR1 mice after exposure to 3 W/kg or sham exposure for 30 minutes per day, 5 days a week for 4 weeks (n=5-6). Sections were counterstained with hematoxylin for orientation (purple). Lower numbers of microglia were found in most regions (A,C) and in the total area (B) in the SAMP8 mice compared to the SAMR1 mice after sham exposure. However, this was only significant by two-way ANOVA in the total area, *s.radiatum* and hilus, while in the *s.oriens* a significant difference between the two sham expose groups was found using Bonferroni's *post hoc* test. Scale: 200 μm. Data are presented as mean ± SEM. Statistical analysis was carried out using two-way ANOVA, **p<0.01.

Table 3.12 Details of statistical analyses of effects of RF exposure on microglia numbers in SAMP8 and SAMR1 mice

	Two-way ANOVA			
Region	**interaction**	**strain**	**Exposure**	**Bonferroni's *post hoc* test**
s. radiatum	F(1,19)=0.24 p=0.63	F(1,19)=6.82 ***p=0.02**	F(1,19)=1.32 p=0.26	Comparison of strains: Sham: n.s. 3 W/kg: n.s.
s.oriens	F(1,18)=6.68 p=0.09	F(1,18)=7.84 ***p=0.011**	F(1,18)=0.37 p=0.55	Comparison of strains: **Sham : ** p<0.01** 3 W/kg: n.s.
s.moleculare	F(1,19)=0.67 p=0.42	F(1,19)=1.56 p=0.23	F(1,19)=0.21 p=0.65	
GCL	F(1,19)=0 p=0.98	F(1,19)=0.15 p=0.70	F(1,19)=1.41 p=0.25	
hilus	F(1,19)=0.58 p=0.46	F(1,19)=5.86 ***p=0.03**	F(1,19)=0.11 p=0.74	Comparison of strains: Sham : n.s 3 W/kg: n.s.
Total numbers	F(1,19)=0 p=0.99	F(1,19)=7.02 ***p=0.016**	F(1,19)=5.16 ***p=0.03**	Comparison of strains: Sham : n.s 3 W/kg: n.s. Comparison of exposure groups: SAMP8 : n.s SAMR1: n.s.

No effect of RF exposure was detected on astroglial numbers

The number of astroglia was counted in the CA1 (*s.oriens* and *s.radiatum*) and DG (*s.moleculare* and hilus) in the hippocampus to investigate the regional response to RF exposure (Figure 3.16A-C).

Two-way ANOVA was used to investigate differences between strains and exposure groups. Neither the total nor the regional number of astroglia differed significantly between the two strains or between the sham exposed and the RF exposed groups in either strain. However, in the total area and in all regions, except for the hilus, the astroglial density was found to be significantly lower ($p<0.01$ and $p<0.05$ respectively) in the RF exposed SAMP8 mice compared to the RF exposed SAMR1 mice. This was due to small increases in glial density in the SAMR1 mice after exposure and small decreases, or no change, in astroglial density in the SAMP8 mice after exposure (Figure 3.16B,C, and see Table 3.13 for details of the statistical analysis). Qualitatively no differences in astroglial morphology were observed between strains or exposure groups.

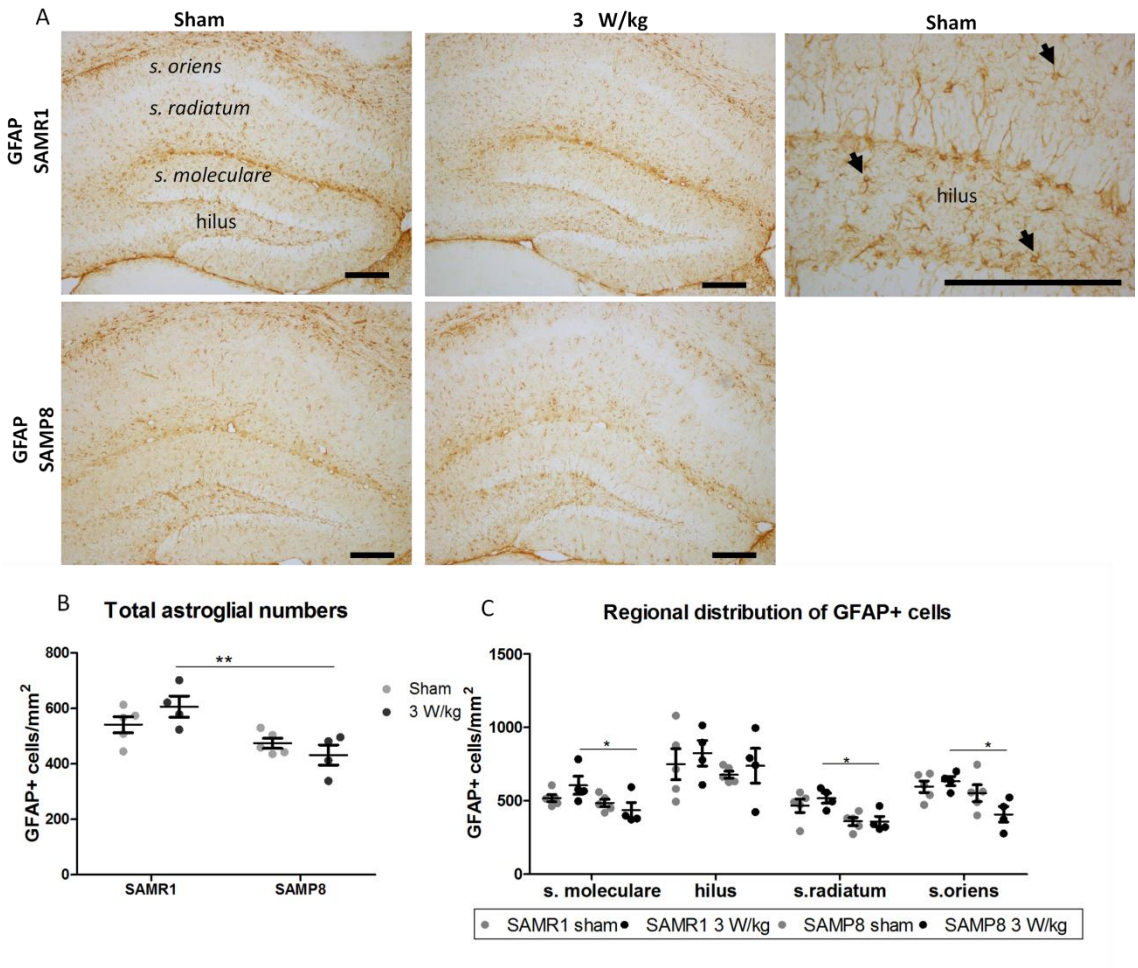


Figure 3.16 No effect of RF exposure on astroglial numbers was found in the hippocampus of SAMP8 and SAMR1 mice. GFAP+ astroglia were counted in the DG (*s.moleculare* and hilus) and CA1 (*s.radiatum* and *s.oriens*) in the hippocampus of 7 months old male SAMP8 and SAMR1 mice after exposure to 3 W/kg or sham exposure for 30 minutes per day, 5 days a week for 4 weeks (n=4-6). Similar regional distribution and numbers were found in the individual regions (A,C) and in total (B), however small increases in astroglial density was observed after RF exposure of SAMR1 mice while a small decrease was seen in the SAMP8 mice after exposure, resulting in significant differences between these two groups in the total area and all the regions except for the hilus. The morphology of the GFAP+ astroglia can be seen in greater detail in the picture in the upper right corner of (A), examples are indicated by the black arrows. Scale: 200 µm. Data are presented as mean ± SEM. Statistical analysis was carried out using two-way ANOVA with Bonferroni's *post hoc* test, *p<0.05. **p<0.01.

Table 3.13 Details of statistical analyses of effects of exposure on astroglial density in SAMP8 and SAMR1 mice.

	Two-way ANOVA			
Region	**interaction**	**strain**	**Exposure**	**Bonferroni's *post hoc* test**
s. radiatum	F(1,14)=0.5 p=0.49	F(1,14)=0.48 p=0.5	F(1,14)=12.92 ****p=0.003**	3 W/kg ***p<0.05**
s.oriens	F(1,14)=3.61 p=0.08	F(1,14)=1.19 p=0.29	F(1,14)=8.01 ***p=0.013**	3 W/kg ***p<0.05**
s.moleculare	F(1,14)=2.79 p=0.12	F(1,14)=0.22 p=0.65	F(1,14)=6.18 ***p=0.026**	3 W/kg ***p<0.05**
hilus	F(1,14)=0.01 p=0.94	F(1,14)=0.58 p=0.46	F(1,14)=0.78 p=0.39	
Total numbers	F(1,14)=3.14 p=0.10	F(1,14)=0.14 p=0.71	F(1,14)=15.98 ***p=0.0013**	3 W/kg ****p<0.05**

In summary, no major effects of RF exposure were found on the cellular endpoints in the SAMP8 or SAMR1 models. A significant difference in microglial numbers was detected between the sham exposed groups in the two strains, with less microglia in the SAMP8 than in the SAMR1 strain. Significant differences in astroglial density were also seen between the two strains after exposure. There was a significantly lower number of BrdU-labelled cells in the sham exposed SAMP8 mice than in the SAMR1 and an indication of the same in terms of doublecortin expressing cells, while there was no change in the fraction of BrdU-labelled cells that co-expressed doublecortin.

3.4 Discussion

The studies in this chapter have investigated whether RF exposure might affect processes central to cognitive function, and if there are differences in the sensitivity to exposure between the young adult C57Bl/6J mouse brain and the senescent mouse brain.

Minor, but statistically significant changes in expression of selected genes associated with synaptic plasticity were seen in both young adult C57Bl/6J mice and 7 month old SAM mice. Furthermore, C57Bl/6J mice were found to be more active after 4 and 5 days of exposure at 3 W/kg. In SAM mice, differences in response to exposure were seen between the two strains for glial density. No significant changes in any of the other endpoints studied were found after RF exposure, although changes that did not quite reach statistical significance were seen in microglia numbers in C57BL/6J mice, and in synaptic density in SAM mice.

3.4.1 RF field induced changes in the brains of young adult C57Bl/6J mice

3.4.1.1 Molecular endpoints

3.4.1.1.1 Effects of RF exposure on synaptic plasticity and density

Effects of RF exposure on synapses in the brain have not been extensively studied. Synaptic plasticity is essential for our ability to learn and remember and it is important to understand possible effects of exposure on these processes.

Glutamate receptors

To the best of my knowledge, the data presented in this chapter are the first investigations of the effects of RF fields at exposures that are relevant for the general public on the most abundantly expressed ionotropic glutamate receptor subunits; the AMPA receptor subunits (*gria1* and *gria2)* and the NMDA receptor subunits *(grin1, grin2a* and *grin2b)* in the hippocampus and prefrontal cortex.

Two identical experiments were performed, finding similar results. The presented results are from the pooled experiments. On mRNA levels, a significant 30% decrease in *grin2a* was found

after exposure at an SAR of 3 W/kg in the hippocampus, but not in the 0.3 W/kg group or in the prefrontal cortex. Protein levels were measured in the hippocampus only, and no changes were found on GluA1, GluA2, GluN1 or GluN2a after exposure. A pattern was seen in all receptor subunits, where both groups that had been exposed to RF fields had either higher (GluA1 and GluN2a) or lower (GluA2 and GluN1) protein levels than in the sham exposed group. However the group sizes were too small to detect any statistically significant differences. In regard to the GluN2a levels, only a few samples were used, and it is therefore difficult to make firm conclusions, but the data suggest an increase rather than a decrease in protein levels (which was seen for the mRNA level). This needs further investigation in larger groups of mice.

Since most NMDA receptors in the hippocampus contain GluN1/GluN2 subunits, it seems unlikely that a change in *grin2a*, and ultimately GluN2a, would not be accompanied by changes in either GluN1, as a result of a loss of NMDA receptors, or in GluN2b altering the physiological properties of the receptor (Paoletti et al., 2013). No change in grin2a/2b ratio was detected in these experiments (data not shown). It is therefore difficult to speculate on the functional impact of the change in gene expression without larger group sizes for measurements of protein levels.

One study has previously investigated the effects of RF exposure on protein levels of NMDA receptors *in vivo*, and this was on protein levels after exposure for 15 minutes at a much higher SAR of 6 W/kg, and in the cortex and striatum, but not the hippocampus. In that study, decreases in postsynaptic levels of both GluN2a and GluN2b were seen (Mausset-Bonnefont et al., 2004). The high SAR might have caused acute heating of the exposed rats and comparison should therefore be cautious. In 2017 one new study has been published, where exposure of rats to 1800 MHz at an SAR of 0.024-0.028 W/kg did not affect protein levels of NR1 (Bodera et al., 2017).

Glutamate is the most abundant excitatory neurotransmitter in the brain, and glutamate receptors are expressed in high numbers in both the hippocampus and prefrontal cortex where they play important roles in the neuronal signalling involved in learning and memory. There is a high degree of plasticity in the expression of these receptors, with insertion of new ones in both old and new synapses during learning, plus a constant reorganisation of synaptic connections and lateral diffusion of the receptors in and out of the postsynaptic density. The receptor subunits have a number of phosphorylation sites which, depending on the combination of sites that are phosphorylated, affect the activity of the receptors in different ways (Collingridge et al., 2004; Lee, 2006). The composition and abundance of the different receptor types differs across the regions of the hippocampus. The functional impact of changes at the mRNA and protein levels can therefore be difficult to predict.

The more general investigation approach chosen in this chapter looking at the overall mRNA and protein levels of the NMDA- and AMPA type ionotropic glutamate receptors should be seen as the initial part in a new line of studies. As previously mentioned, the glutamate receptor expression is plastic at many levels, and where this project has investigated the total mRNA and protein levels, and found minor changes it would be interesting to investigate expression levels regionally, and to look at changes in phosphorylation of the various sites on the receptor subunits involved in regulation of receptor properties.

BDNF signalling

The literature suggests that this is the first study of effects of RF exposure on BDNF signalling in the hippocampus and prefrontal cortex.

BDNF is the most abundantly expressed trophic factor in the hippocampus where it plays many roles in cell growth and synaptic plasticity (Bekinschtein et al., 2008b). In this project, no significant changes to *bdnf* mRNA levels were seen as a result of exposure. A significant increase of 15% was seen for *ntrk2* in the prefrontal cortex but not in the hippocampus.

One study has looked at changes in expression of growth factors, but in the auditory system, after long-term exposure at an SAR of 1.8 W/kg for 3 months (Maskey and Kim, 2014). This study showed a significant decrease in protein levels of both BDNF and the glial derived neurotrophic factor in all the auditory nuclei investigated. This is in contrast to the findings on mRNA level in the dorsal hippocampus and prefrontal cortex presented in this chapter.

BDNF expression is complex, it is transcribed rapidly as a result of neuronal activity and is highly sensitive to modulation e.g by stress and physical exercise (Bath et al., 2013; Ieraci et al., 2015). The lack of change in gene expression does therefore indicate that the exposure did not induce any large overall effects on neuronal activity. However, *bdnf* is known to be stored locally to ensure rapid translation when and where needed, the protein level should therefore be measured. Also investigations of the ratio of pro-protein versus mature protein would be interesting due to their highly different functions.

In this project only the expression of transcript variant 1 was investigated, but nine different promotors are known with different temporal and spatial transcription patterns (Aid et al., 2007; Chen et al., 2010). A more detailed investigation of effects of exposure on these, in addition to investigations of regional expression changes would be interesting due to the many important roles of BDNF on promotion of survival, synapse development and growth, cell death and long-term potentiation (Leal et al., 2015).

Synaptic effects

Effects of exposure on synaptic density were investigated in terms of changes to synaptophysin and PSD95.

No change to the density of synaptophysin was found in the 6 hippocampal subfields after RF exposure. Synaptophysin is a glycoprotein found in the membrane of presynaptic vesicles. It is expressed in all neurons and is one of the most abundant proteins on synaptic vesicles and it is therefore a commonly used presynaptic marker (Wiedenmann and Franke, 1985). Decreases in

synaptophysin have been reported as a result of stress, ageing and neurodegeneration by a number of groups (Canas et al., 2009; Ojo et al., 2012; Wang et al., 2016; Wong et al., 1998). Moreover, increases and recovery has been reported as a result of enriched housing conditions (Frick and Fernandez, 2003; Frick et al., 2003; Nithianantharajah et al., 2004). This makes synaptophysin a very sensitive marker of both harmful and trophic modulation, and the lack of effect of exposure on this indicates that exposure does not affect synaptic density.

PSD95 was used as a marker of the post synaptic density. This protein is actively involved in stabilising the postsynaptic density and bridging glutamate receptors to proteins involved in their downstream signalling, which is important in learning-induced synaptic plasticity, and it is involved in regulation of other synaptic proteins e.g. NMDA receptor subunits and AMPA receptor function (Kim and Sheng, 2004). Total PSD95 mRNA was measured in the dorsal hippocampus and prefrontal cortex, in addition to the protein level in the dorsal hippocampus. No changes in expression levels were seen between exposure and sham groups in any of the regions.

c-fos is an immediate early gene that is expressed in the hippocampal granule cells after depolarisation (Herrera and Robertson, 1996). In this chapter, c-fos was used to investigate whether long-term RF exposure might affect the activity of these granule cells. No change in numbers of cells expressing c-fos were seen after RF exposure.

In four previous studies in the literature the effect of exposure at non-thermal levels was found not to affect c-fos expression, but in three of the studies the level was found to be elevated compared to cage controls that had not been restrained in the exposure system (Finnie, 2005; Finnie et al., 2007; Morrissey et al., 1999; Stagg et al., 2001). It is therefore possible that restraint stress and heating may obscure any possible exposure induced changes. In the current work, using the reverberation chambers, mice were allowed to move freely during exposure minimising the potential for stress in the mice. No changes in c-fos expressing granule cells were

detected in the groups exposed to RF fields, indicating that there was no masked effect of exposure in those experiments where rodents experienced restraint stress. However, the previous studies exposed acutely for a few hours or repeatedly for 2 years, this difference in exposure duration should be kept in mind.

3.4.1.2 Cellular endpoints

3.4.1.2.1 No changes to adult hippocampal neurogenesis after RF exposure

Adult hippocampal neurogenesis has been proposed to play a central role in learning and memory by a continuous supply of immature granule cells, thereby enhancing the plasticity of the region. The rate of neurogenesis has been found to be highly sensitive to modulation by stress and environmental enrichment (Balu and Lucki, 2009).

In this chapter, the effect of RF exposure on proliferation and early differentiation was investigated. Effects of exposure to RF fields have not yet been reported on differentiation in the SGZ.

No significant differences between the experimental groups were found (sham, 0.3W/kg, 3W/kg and having an enriched environment with a running wheel) on the number of proliferating cells, or the proportion of BrdU/DCX labelled cells. Only two brains were included from the group exposed to 0.3 W/kg due to technical difficulties, therefore the effect of this exposure level can therefore not be evaluated.

One study has previously looked at the effect of RF exposure on proliferation in the SGZ in terms of BrdU-labeling in adult rodents (Kim et al, 2015a). Rats were exposed at a whole body SAR of 2 W/kg daily for 1 or 8 hours per day, 5 days a week for 2 weeks. BrdU was injected daily for the last 5 days of exposure and the numbers of BrdU-labelled cells were counted in the SGZ and SVZ, similar to the protocol used for the experiments outlined here. The authors found no effect of exposure on proliferation. Two papers have used proliferating cell nuclear antigen (PCNA) as an

endogenous marker of proliferation to investigate effects of very high SARs of 5 W/kg and 7.8 W/kg and reported no effects on proliferation (Kim et al., 2008; Son et al., 2015).

Thus, neither in the results presented in this chapter, nor in the literature are there indications that RF exposure at environmentally relevant levels affect proliferation in the SGZ. No effect of 4 weeks exposure at an SAR of 3 W/kg was found on proliferation or the number of immature neurons generated during the last 5 days of exposure. No conclusions can be drawn on the effect of exposure at 0.3 W/kg, but the lack of changes at 3 W/kg together with the findings by Kim et al (2015a) suggest that exposure has no significant effects on proliferation in the adult mouse hippocampus.

For investigation of differentiation in the SGZ after exposure, DCX was used as marker for immature neurons (Rao and Shetty, 2004). Its expression peaks from day 4-7 after division (Brown et al., 2003) coinciding with the age of the BrdU labeled cells in this experiment. On average 80% of the BrdU+ cells were found to co-express DCX, which corresponds well with the findings by Rao and Shetty, where it was reported that 76% of the BrdU+ cells expressed DCX following 12 days of BrdU-injections in 4 month old rats.

In the current work, a group of mice was housed in an enriched environment with a running wheel for 4 weeks before BrdU labeling as a positive control. This has previously been reported to stimulate both proliferation and survival of new neurons (van Praag et al., 1999a). However, no effect of the environmental enrichment was seen in this experiment. The mice were group housed and it is possible that the mice may have not had the opportunity to exercise sufficiently on the wheel. Dostes et al (2016) have recently reported a significant difference in the effect of 3 hours/day compared to 24 hours/day access to a running wheel on the number of DCX+ cells. The effects of running in individually housed mice have been shown, so this approach would be chosen for future studies to increase the running time (Dostes et al., 2016)

Studies should be continued to see whether RF exposure affects the survival and functional integration of the newly-generated neurons by extending the survival of mice after BrdU injection to 4-8 weeks.

3.4.1.2.2 Effects of RF exposure on astroglia and microglia

To investigate whether RF exposure at non-thermal levels affect glia cells (which might impact on synaptic function and integrity), the density of astroglia and microglia in the dorsal hippocampus were investigated.

Glia cells are highly sensitive to changes in the cellular environment and respond quickly to any harmful changes. They also support neurons, synapses and neurogenesis (Aimone et al., 2014; Bélanger and Magistretti, 2009; Ben Achour and Pascual, 2010).

When the total numbers of microglia were compared between exposure groups a small, near significant, increase was seen in the group exposed at 3 W/kg compared to the sham exposed group, while no effect of exposure at 0.3 W/kg was seen. In addition, no significant differences between the exposure groups were found when regional changes in the hippocampus were investigated.

Only two papers have reported studies investigating the effects of RF exposure on mouse microglia *in vivo*. Neither of them reported any effects after; 1 hour acute or 2 years repeated exposure at a whole body SAR of 4 W/kg (Finnie et al., 2010), or 5 W/kg for 60 days (Son et al., 2015). These two papers did not contain a description of any morphological changes. The findings presented in this chapter are similar to those found in these two papers, although in the light of the small, near significant increase in total numbers of Iba-1 positive cells found in this chapter further work is needed to identify any physiological impact. This could be measurements of cytokines, morphological analysis to identify whether the increase is of microglia in a particular state or co-labelling studies to investigate whether the increases were seen in relation to blood vessels, certain types of neurons etc.

In contrast to the sparse literature on the effects of RF exposure on microglia, more studies have investigated effects on astroglia, and on inflammation and oxidative stress, reporting complex effects. Three of these studies have reported increased GFAP density after long-term exposure at SARs of 1.5-1.6 W/kg (Ammari et al., 2010; Maskey et al., 2010, 2012). Ammari et al (2010) reported dose dependent differences in the persistency of this increase, for 3 days (1.5 W/kg, 6 W/kg) or 10 days (6 W/kg). The results of the long-term exposure studies also indicated that the brain does not habituate to exposure even after several months of exposure. However, one study found no effect of exposure at 1.6 W/kg (Ammari et al., 2008b).

In the results presented here, very little variation in the total and regional density of GFAP positive astroglia was seen between the exposure groups. The effect was investigated immediately after the end of exposure for 4 weeks. In the studies presented above exposure durations between 1-12 months were used and delays of 0, 3 and 10 days in the studies finding effects. It should however be noted that the previous studies looked at GFAP density in astroglia and not the number of astroglia, so future studies looking at this would also be informative. Similarly for the studies of microglia, cytokine expression and co-labelling studies would also be interesting for investigation of more subtle and/or specific effects of exposure on glial function.

3.4.1.3 Functional endpoints

3.4.1.3.1 No impairment in learning or memory after RF exposure

Two different tests to evaluate the effects of RF exposure on hippocampal-dependent memory were performed. Memory could not be evaluated in the NOL test, while no significant effect of RF exposure was found on learning or memory retrieval in the water maze test.

At the end of the first week of exposures mice were tested in the NOL test, as a quick test to investigate hippocampus dependent memory without aversive stimuli.

In the initial open field test, all mice were found to behave and move normally. However, for comparison of activity in terms of distance moved a significant difference was found between

the sham and 3 W/kg groups. It should be noted that this difference could only be detected using a t-test and not an ANOVA for comparison of the three groups. No differences in rearing were observed, and also no differences in activity or swimming pattern in the water maze were observed 3 weeks later.

In the NOL test, no preference for the object moved to the novel location was seen in any of the groups. The NOL test was done twice in two sets of mice, the same lack of interest in the moved object was seen in both. In the first experiment, large marbles (Ø 4 cm) mounted on stands were used as the objects. In this experiment the mice showed very little interest in the objects, and the low sides on the experimental arena resulted in several mice jumping out of the box. In the second experiment, a box with higher sides was used and objects made from interlocking plastic bricks with a less regular 3D shape were used. The mice showed more interest in the plastic objects than in the marbles (data not shown), but did not explore them more in the novel position than in the familiar position. In the second experiment, the room was lit from the floor to avoid shadows in the arena and limit anxiety related to bright light. An interesting observation was however made, that the mice that had been exposed to 3 W/kg visited the objects more frequently, and for a longer cumulative duration, than the mice in the two other exposure groups, but there was no preference for either object. Two possible reasons for the lack of object discrimination in all groups could be that the mice did not have sufficient spatial cues in the arena to generate a memory, or the use of ethanol for cleaning to avoid stress of odours from previous animals may have created an atypical and strong olfactory cue that confused the mice. Before future studies the impact of ethanol should be tested by comparison of performance in the test when ethanol, other disinfectants or no cleaner is used. Additional visual cues should also be included.

After 4 weeks of exposure the mice were tested in the water maze. This much-used test has allowed investigation of effects of modulation on learning and memory after hippocampal

lesions (Morris, 1984) and exercise (van Praag et al., 1999a) and has been used in several studies showing impaired learning and memory after exposure (Deshmukh et al., 2013, 2015, 2016; Megha et al., 2012; Tang et al., 2015a).

No significant effects of exposure at SARs of 0.3 W/kg or 3 W/kg were found in the learning or memory trials. The experiment was performed twice with no difference between the results. Previously several studies have reported impairments in performance, even using very low SARs (as described in Chapter 1). Several of these studies also reported correlation with increased inflammation or oxidative stress, but not all.

The protocol used in this chapter, with 7 learning trials over 4 days ensures a strong acquisition of the task and the mice reach the minimal escape latency around trial 4, it is therefore possible that by using a more challenging protocol, e.g. with less frequent training sessions and a longer interval between the training and the probe trial, possible small changes in cognitive function could be detected (van Praag et al., 1999a). It should be noted that the studies reporting impaired water maze performance after RF exposure used similar protocols to the ones used in my studies.

3.4.2 RF field induced changes in the brains of SAMP8 and SAMR1 mice

Little is known about the sensitivity of the senescent brain to RF fields compared to the young and the adult brain. Many changes are known to happen in the brain as a result of ageing. This includes loss of synapses, changes to glial activation and increased oxidative stress, and the ageing brain is known to be more sensitive to some forms of modulation than the young (Cunningham et al., 2007; Liu and Lewis, 2014; Smarr et al., 2014).

Studies of the effects of RF exposure on the senescent brain have investigated effects of long-term exposure for 4 months to 2 years. The majority of studies have reported no effect of exposure, mainly on endpoints such as astroglia, c-fos, inflammation and behaviour (Court-Kowalski et al., 2015; Finnie et al., 2007; Kim et al., 2008; Klose et al., 2014). On the other hand, some studies in models of familial AD have shown ameliorating effects of exposure on cognitive function, amyloid deposition and mitochondrial function (Arendash et al., 2010, 2012; Dragicevic et al., 2011; Jeong et al., 2015). As discussed previously, sensitivity of the plastic processes known to underlie cognitive function have not been the subject of much investigation.

The aim of this part of the project was to discover if molecular and cellular processes that play essential roles in cognitive function are more sensitive during senescence than in normal adult mice.

The senescence accelerated mouse, SAMP8, was chosen as a model to study this since it has been reported to show impaired cognitive function and cellular and molecular changes associated with ageing and with sporadic Alzheimer's Disease (AD). The majority of cases of AD are sporadic, with less than 1% being hereditary. So far studies of RF exposure in AD models have only been in transgenic mouse models with mutations in the genes coding for APP, presenilin and tau known from familial AD cases. Sporadic AD is characterised by synaptic loss, inflammation, oxidative stress and this model might be more representative of the changes

associated with most cases of ageing and AD, and it shows more general neurodegeneration rather than just that associated with altered amyloid and tau load (Cheng et al., 2014).

3.4.2.1 Molecular endpoints

3.4.2.1.1 Effects of RF exposure on synaptic plasticity and density

The gene and protein levels of the glutamate receptor subunits and *bdnf* signalling molecules did not differ substantially between the sham exposed groups in the two strains. In the dorsal hippocampus the gene coding for GluA1, *gria1,* and the protein level of GluN1 were lower in the SAMP8 strain than in the SAMR1 strain, while in the prefrontal cortex a significantly lower level of the gene coding for GluN1, *grin1* was found.

A significant interaction between exposure and strain was observed after RF exposure, with a 47% increase in *grin1* expression in the dorsal hippocampus of SAMP8 mice and no change in gene expression in the SAMR1 mice. *bdnf* expression was also significantly higher in the prefrontal cortex of RF exposed SAMP8 mice compared to the RF exposed SAMR1 mice, but not compared to the sham exposed group. Similarly the GluA2 protein level in the dorsal hippocampus was lower in RF the exposed SAMP8 mice than in exposed SAMR1 mice, but these values were not significantly different from the respective sham exposed group, which indicates a difference in sensitivity of the two strains to RF exposure.

Little is known about the expression levels of glutamate receptors and trophic factors in SAMP8 mice. Previous studies have shown no difference in GluN1 expression between strains at 6 months of age (Zhao et al., 2010). At 9 months of age a 50% lower level of GluN1 expression in the hippocampus, but not cortex, has been described in SAMP8 mice, while the GluN2b levels were twice as high in the cortex but not hippocampus (Xu et al., 2016). At 6, 7 and 10 months of age a 30-40% decrease in phosphorylation of the GluN1 subunit has been found (Lin et al., 2014, 2013; Sun et al., 2013; Zhao et al., 2010). Thus, some alterations in glutamatergic signalling through NMDA receptors were expected in the SAMP8 mice at the age of 6-7 months, while

there was no literature on AMPA receptors. The lower GluN1 level in the hippocampus of sham exposed SAMP8 mice compared to SAMR1 mice reported here is in line with the literature and shows that the decreased hippocampal GluN1 level reported by Xu et al (2016) at 9 months of age is seen already at 7 months. However, in general there were only limited strain differences in gene expression levels of glutamate receptor subunits at 7 months of age.

In SAMP8 mice, BDNF levels have been reported to be lower than in age matched SAMR1 mice in the hippocampus at 6 months (Zhao et al., 2011), 8 months (Cosin-Tomas et al., 2014) and 9-11 months (Katayama et al., 2014; Li et al., 2009; Lin et al., 2014, 2013) but not at 4 months of age (Li et al., 2009; Yan et al., 2011). Kaisho et al. (1994) reported a higher hippocampal BDNF level in 10 months old SAMP8 mice compared to 1 and 4 week olds, while Li et al (2009) have reported a 2.5 times higher level at 4 months compared to 10 months (Kaisho et al., 1994; Li et al., 2009). In 6-7 months old SAMP8 mice a significant difference in the ratio of mature to pro-BDNF protein levels has been reported compared to SAMR1 mice, with the greatest change observed on the mature form of the protein (Dobarro et al., 2013). Yan et al (2011) reported that although the BDNF baseline levels in the two strains was similar at 4 months of age, the SAMP8 strain is more sensitive to chronic stress than the SAMR1 strain, measured in terms of decreased BDNF mRNA and protein levels, decreased proliferation in the subgranular zone, and impaired cognitive function. For this project a decreased BDNF level was therefore expected in the hippocampus of SAMP8 mice compared to SAMR1 mice, possibly with a higher sensitivity to stressors. Only gene expression levels were measured, but no differences were detected between the two strains at 7 months of age. After RF exposure, gene expression levels of *ntrk2* and *p75* were also measured in the two strains and no differences were found. There appear to be no literature on strain differences of these two receptors.

With the chosen approach little effect of RF exposure was found on expression of plasticity-related genes, and also there was little difference between the two strains. The literature

suggests that more pronounced changes to glutamate receptor subunit levels and BDNF levels are seen at older age, supplementing the present studies with studies of older SAMP8 and SAMR1 mice would therefore be relevant to improve our understanding of age dependent differences in sensitivity of these genes and proteins.

3.4.2.1.2 Effects of RF exposure on synaptic markers

Altered sensitivity of synapses to RF exposure was investigated in the SAMP8 and SAMR1 strains. This was undertaken by studying regional synaptophysin in the hippocampus, and the total mRNA and protein levels of PSD95 in the dorsal hippocampus, and mRNA levels in the prefrontal cortex.

Synaptophysin levels have previously been reported to be sensitive to modulation in terms of chronic stress, in 6 month old SAMP8 mice but not SAMR1 mice (Wang et al., 2016). Decreased synaptophysin immunoreactivity has also been reported in aged rats by some (Canas et al., 2009; Ojo et al., 2012), while others have reported increases thought to be compensatory (Benice et al., 2006). In the SAMP8 model, significantly lower hippocampal and cortex levels of synaptophysin protein have been reported at 2, 6 and 12 months of age, compared to SAMR1 (Cheng et al., 2011), in that study it was also found that there is no age-related decrease in synaptophysin levels in the SAMR1 mice. Dobarro et al. (2013) also found significantly lower protein levels in 7 months old SAMP8 mice compared to SAMR1 mice.

In the experiment presented in this chapter, no statistically significant differences in regional staining density for synaptophysin were found between the sham exposed SAMP8 and SAMR1 mice, although slightly lower densities were seen in the CA1 (*s.radiatum, s.lacunosum-moleculare*), DG (hilus) and CA3 (*s.radiatum*) in the SAMP8 mice compared to SAMR1 mice. These findings do therefore differ from what has previously been reported. However, in both of the studies of SAM mice mentioned above total hippocampal protein levels were measured

rather than the regional densities, where smaller overall loss of synapses might be below the detection limit.

In regard to RF exposure, no significant effect was found in either strain, but small changes in synaptophysin density were seen going in opposite directions in the two strains. There was a reduction in density in SAMR1 and an increase in density in the SAMP8 strain which suggests a possible difference in response to RF exposure. It would be interesting to investigate this further using a different presynaptic marker (e.g. synapsin or synaptotagmin).

PSD95 was used as a marker of the post synaptic density. No significant differences between strains or exposure groups were detected. However, it should be noted that the gene expression level was 60% higher in the prefrontal cortex in the SAMP8 strain than in the SAMR1 strain. This difference was not statistically significant. Based on the literature it was not clear to what extent PSD95 would differ between SAMP8 and SAMR1 mice at 7 months of age. Differences in PSD95 levels have been reported between the SAMR1 and SAMP8 strains. Lower protein levels have been reported by a number of groups in SAMP8 mice at 10 months (Li et al., 2009; Lin et al., 2014, 2013), while Zhao et al. (2009) found no difference at 11 months of age (Zhao et al., 2009).

3.4.2.1.3 Summary of effects of RF exposure on molecular endpoints

The experiments identified indications of differences between the senescence prone and the senescence resistant strains in response to RF exposure. However, all these differences were small, including increased *bdnf* expression in the prefrontal cortex and increased *grin1* expression in the dorsal hippocampus only in the SAMP8 mice, while no effects of exposure were detected in the SAMR1 mice. Exposure did not affect presynaptic density or PSD95 gene or protein levels significantly, however indications of a strain difference in response to exposure were seen, with increases in synaptophysin density in the SAMP8 strain.

The increases in synaptophysin and *bdnf* indicate a potential beneficial effect of exposure on age-related synaptic changes, which is in line with the beneficial effects of exposure reported by the studies by Arendash and colleagues (2010, 2012) in models of AD, but in contrast with the neurodegenerative effect reported in studies of glia and oxidative stress by Megha et al. (2012), Hidisoglu et al. (2016) and Maskey et al. (2012).

The small differences in response to exposure suggests that there might be age-related differences in the synaptic sensitivity to RF exposure. Studies of this could be continued in older SAMP8 mice.

3.4.2.2 Cellular effects of RF exposure in SAMP8 and SAMR1 mice

3.4.2.2.1 No effects of RF exposure on adult hippocampal neurogenesis were detected

The effect of RF exposure on adult hippocampal neurogenesis was investigated in SAMP8 and SAMR1 mice to explore if the senescent environment would affect the sensitivity of this plastic feature of the hippocampus to RF exposure.

Little is known about age dependent changes in adult neurogenesis in the SAMP8 strain. A compensatory increase in proliferation in SAMP8 mice has been reported, with a higher level at both 5 and 10 months compared to SAMR1 mice, and with lower levels at 10 months than at 5 months in both strains (Gang et al., 2011). At 4 months one study found only a few BrdU-labelled cells in the hippocampus, with a small decrease after chronic restraint stress. No SAMR1 mice were included in that study (Yan et al., 2011).

In this study, the number of BrdU-labelled cells in both the SAMP8 and SAMR1 mice was much lower than that seen in young adult C57Bl/6J mice presented previously in this chapter. There were significantly fewer BrdU-labelled cells in the SAMP8 strain than in the SAMR1 strain when compared using a two-way ANOVA, this could not be detected using Bonferroni's *post hoc* test. The difference was due to large variation within groups especially in the SAMP8 strain. This is in contrast with the strain differences reported in the study by Gang et al. (2011).

The fate of all BrdU-labelled cells was scored as DCX positive or negative, the fraction of double-labelled cells in the two SAMP8 groups was slightly lower than in both the SAMR1 groups, although this difference was not significant. In addition to this, the total number of DCX+ cells in the GCL was counted. No significant difference was seen between strains or exposure groups, while indications of a lower number in the sham exposed SAMP8 strain was seen. In the study by Gang et al (2011) the total number of DCX+ cells and the total level of DCX protein were also quantified in 5 and 10 months old SAMP8 and SAMR1 mice. These authors reported much higher protein levels in the SAMP8 mice, and especially at 5 months of age, observed many more DCX+ cells in the SAMP8 mice than in the SAMR1 mice, while at 10 months of age similar lower numbers were found in both strains.

In terms of the effect of RF exposure on neurogenesis, no studies in ageing rodents or in other neurodegeneration models appear to have been published. In one study, mice were exposed for 12 months resulting in 14 months old mice (Kim et al., 2008). Using young adult C57Bl/6J mice and a high SAR of 7.8 W/kg no differences in proliferation was found in this study for an endogenous proliferation marker in the SGZ and the SVZ.

Far fewer BrdU-labelled and DCX+ cells were seen in both SAM strains compared to the young adult C57Bl/6J mice presented in the earlier part of this chapter. This could be an indication of an age related difference, although this should be investigated in young SAM mice before drawing any conclusions. Irrespective of which, it would be prudent to compare sensitivity to RF fields in SAMP8 mice at a higher age where strain differences can be detected. This timing should be identified by a study of age-related changes.

3.4.2.2.2 Effects of RF exposure on microglia and astroglia

The effect of 4 weeks of RF exposure at an SAR of 3 W/kg on microglia and astroglia was investigated in the DG and CA1 regions in the hippocampus of SAMP8 and SAMR1 mice. No

effects of exposure were found in the individual strains, there was however a significant difference in response to RF exposure between the two strains.

The astroglial density was slightly increased after exposure of SAMR1 mice, while it was slightly decreased after exposure in SAMP8 exposure, resulting in significant differences between these two groups in all hippocampal regions, except for the hilus. There were no apparent differences in the astroglial morphology. In contrast to what has been reported in the literature, there were no differences between the sham exposed groups from the two strains. Higher GFAP levels have been reported at 4-5 months of age in the whole brain in SAMP8 mice compared to SAMR1 mice (Fernández-Gómez et al., 2010), and at 8 and 16 months in the hippocampus (Wu et al., 2005; Yi et al., 2016), but not at 3 months (Wu et al., 2005). It should be kept in mind that in this study, only the number of astroglia was counted.

A limited number of studies have investigated microglial numbers in SAMP8 mice, and an increased number was expected in the hippocampus of 7 months old SAMP8 mice (Currais et al., 2015; Jin et al., 2016; Yi et al., 2016). Surprisingly, a lower number of microglia was observed in the SAMP8 compared to the SAMR1 mice in total, and in several hippocampal regions. Also the microglia appeared more darkly stained in the SAMR1 compared to the SAMP8 strain. No effect of RF exposure was found on the total, or regional number of Iba-1 positive cells in the SAMP8 or SAMR1 mice. Despite the difference in microglia between the two strains, no major effects of this was seen on synaptic plasticity or neurogenesis which are known to be sensitive to changes in the balance between pro and anti-inflammatory factors secreted by the microglia, which indicates that this balance is not affected (Aimone et al., 2014; Chung et al., 2015a; Wake et al., 2009).

3.4.3 Summary of RF field induced effects on processes involved in cognitive function, studied in young adult C57Bl/6J mice and two SAM strains

The studies in this chapter investigated whether RF fields could affect cognitive function in young adult animals and whether older animals might be more sensitive to exposure.

The reverberation chamber system used for exposures provided the clear advantage over many others of not requiring mice to be restrained during exposure and using whole-body SARs known not to cause any major heating effects in mice. In the absence of these stressors, very little effect of RF exposure at 0.3 W/kg or 3 W/kg was detected.

From the experiments presented in this chapter, where focus was on cellular and molecular mechanisms involved in the plastic processes underlying learning and memory, very little effect of exposure was found. The endpoints were chosen based on their sensitivity to modulation and their broad functions, increasing the chance of detecting any effects of exposure that would affect cognitive function. The experimental findings suggest that repeated RF exposure at environmental relevant levels does not have any major effects on cognitive function. However, small gene expression and cellular changes were found. The main focus of these experiments was on the dorsal hippocampus, however, the gene expression changes detected in the prefrontal cortex should also be supplemented with studies of e.g. performance in prefrontal cortex dependent working memory tests.

Only minor differences in sensitivity to RF exposure were identified in the older mice (SAMR1 and SAMP8 strains), such as the indications of different effects on synaptic density and glia cells. Given the lack of major strain differences at the age of 7 months, these possible age related differences in sensitivity could be investigated further in mice showing more senescence related cognitive decline.

3.5 Conclusion

The studies in this chapter investigated whether the reported effects of radio frequency (RF) fields on cognitive function in adult animals were caused by field exposure or if they could be explained by other conditions that may occur during exposure, such as restraint stress or heating. In addition, whether older animals might be more sensitive than younger animals to exposure was investigated in a senescence accelerated mouse model.

Exposure without such causes of stress and variability did not have any major effects on central molecular or cellular functions involved in cognitive function, in young adult C57Bl/6J or in SAMP8 and SAMR1 strains at 7 months of age.

In young adult C57Bl/6 mice repeated RF exposure at 3 W/kg for 4 weeks decreased hippocampal *grin2a* expression by 22% and increased the expression of *ntrk2* in the prefrontal cortex by 15%. In addition, a near significant 18% increase in the total number of microglia in the dorsal hippocampus was seen, and after four days of exposure exposed mice were found to be significantly more active in the open field test. There were no effects of exposure on presynaptic density, PSD95 expression, astroglial density and in performance in the water maze test. Exposure at 0.3 W/kg did not affect any of the investigated endpoints.

In 7 month old SAMP8 and SAMR1 mice repeated RF exposure at 3 W/kg increased hippocampal *grin1* expression by 47% in SAMP8 mice while there was no effect of exposure on gene expression in the SAMR1 strain. Furthermore, the total microglial density was decreased in both strains after exposure. Significant differences between the RF exposed groups in the two strains were found on several endpoints including 1) *bdnf* expression in the prefrontal cortex where exposure increased expression in the SAMP8 mice but not in the SAMR1 mice, 2) GluA2 expression was decreased after exposure of SAMP8 mice and slightly increased after exposure of SAMR1 mice, 3) synaptophysin density in the *s.oriens* of the CA1 was increased in SAMP8

mice but not SAMR1 mice, 4) astroglial density was decreased after exposure of SAMP8 mice and increased in SAMR1 mice.

Chapter 4 A study of the effects of power frequency magnetic fields on cognitive function

4.1. General introduction

The work described in this chapter seeks to discover if 50 Hz magnetic fields (MF) at magnetic flux densities <1 mT can stimulate molecular and cellular processes involved in cognitive function in young adult animals, as has been proposed for MFs at magnetic flux densities >1 mT. This includes studies of synaptic changes in the hippocampus and prefrontal cortex, effects on hippocampal neurogenesis, glia cells and performance in a spatial memory test.

MFs from electrical appliances are ubiquitous in the environment where the average levels measured in homes are in the range of 0.025 to 0.07 μT, while peak levels close to sources can be several hundred microtesla (WHO, 2007). The exposure limits in the UK are 100 μT for public exposure and 1 mT for occupational exposure (ICNIRP, 1998). The majority of the studies of the effects of 50 Hz MFs on cognitive function have been carried out at magnetic flux densities > 1 mT where various effects have been reported. Far fewer studies have investigated the effects of exposure at magnetic flux densities < 1 mT (SCENIHR, 2015; WHO, 2007).

In relation to the effects of exposure on the plasticity of the brain, it has been reported that exposure at 1 mT affects hippocampal neurogenesis by increasing proliferation, differentiation and survival of new cells that correlates with improved performance in the water maze (Cuccurazzu et al., 2010; Leone et al., 2014; Podda et al., 2014). No other groups have studied effects on hippocampal neurogenesis, while improved memory has been described after exposure at 2 mT (He et al., 2011; Liu et al., 2008). In contrast several studies have reported impairments in spatial memory tasks after varying lengths of exposure at flux densities from 1-8 mT (Cui et al., 2012; Deng et al., 2013; Duan et al., 2014; Jadidi et al., 2007; Sienkiewicz et al., 1998a, 1998b).

When focusing on exposures at magnetic flux densities < 1 mT in healthy rodents, a number of studies on memory have been performed but the reported effects also vary (Cui et al., 2012; Kavaliers et al., 1996; Lai et al., 2016; Li et al., 2014a, 2014b; Sienkiewicz et al., 1998b). The animal model, animal age and exposure duration all varied between studies, with acute exposures lasting 5-45 minutes and long-term exposures of 2-24 weeks. No effect of exposure was found in most of these studies. However, Kavaliers et al. (1996) exposed mice at 0.1 mT during memory testing and saw improved performance of female, but not male, mice, and Sienkiewicz et al. (1998) found that exposure before training impaired performance using a flux density of 7.5 and 0.75 mT, but not at 0.075 or 0.0075 mT.

Besides functional studies of learning and memory, few studies have investigated the effects of MF exposure on cognitive function in terms of changes to central cellular and molecular processes *in vivo*. These have indicated MF induced changes on dendritic spine density (Xiong et al., 2013), glutamatergic signalling (Chung et al., 2015b; Duan et al., 2014; Li et al., 2014a; Manikonda et al., 2007; Salunke et al., 2014) and electrical activity of neurons (Balassa et al., 2013; Komaki et al., 2014; Varró et al., 2009).

Based on the existing literature, differences in sensitivity of the brain to MFs depending on age and magnetic flux density are not clear.

4.1.1 Experimental approach

In this chapter, the effects of exposure at magnetic flux densities of 100 µT and 300 µT on cognitive function were investigated. These magnetic flux densities are based on the exposure limits recommended by ICNIRP (1998). Based on the reports by Cuccurrazzu et al. (2010) investigating proliferation, differentiation and memory after 1 mT exposure, a similar protocol was chosen for studies of the effects of exposure on cellular and molecular processes that are important for cognitive function and synaptic plasticity in particular. Four week old male C57Bl/6J mice were exposed for 4 hours per day for 5 days. As depicted in Figure 4.1, changes in

synaptic plasticity and density, hippocampal neurogenesis and glia cells were investigated, in addition to an assessment of memory in the water maze. In parallel, studies of the effects of acute exposure were carried out to investigate changes in expression of synaptic plasticity genes and memory performance.

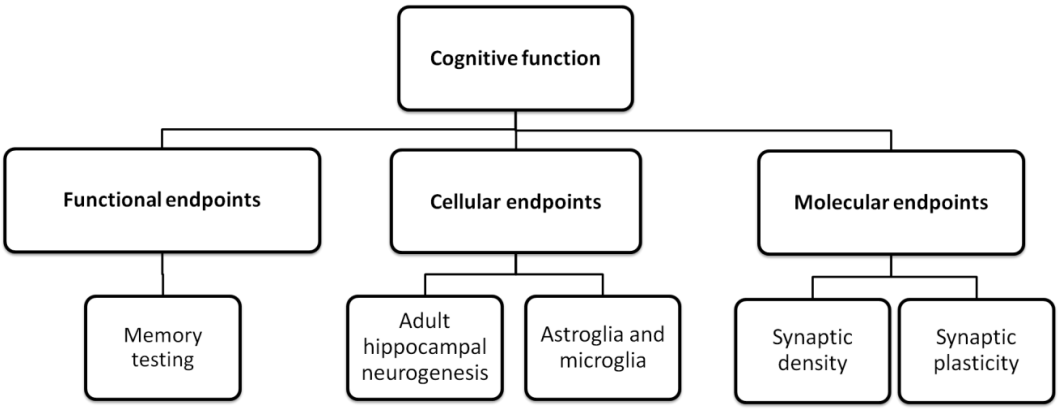


Figure 4.1 Schematic presentation of experimental endpoints.

4.2 Experimental protocols

The experimental protocols used in this chapter are presented in Figure 4.3. A 2 hour acute exposure protocol (protocol 1) and a 5 days repeated exposure protocol (protocol 2) was used to study the effects on; synaptic plasticity (a, immediate, b, persistent), memory (c) neurogenesis (d), and glial cells (d).

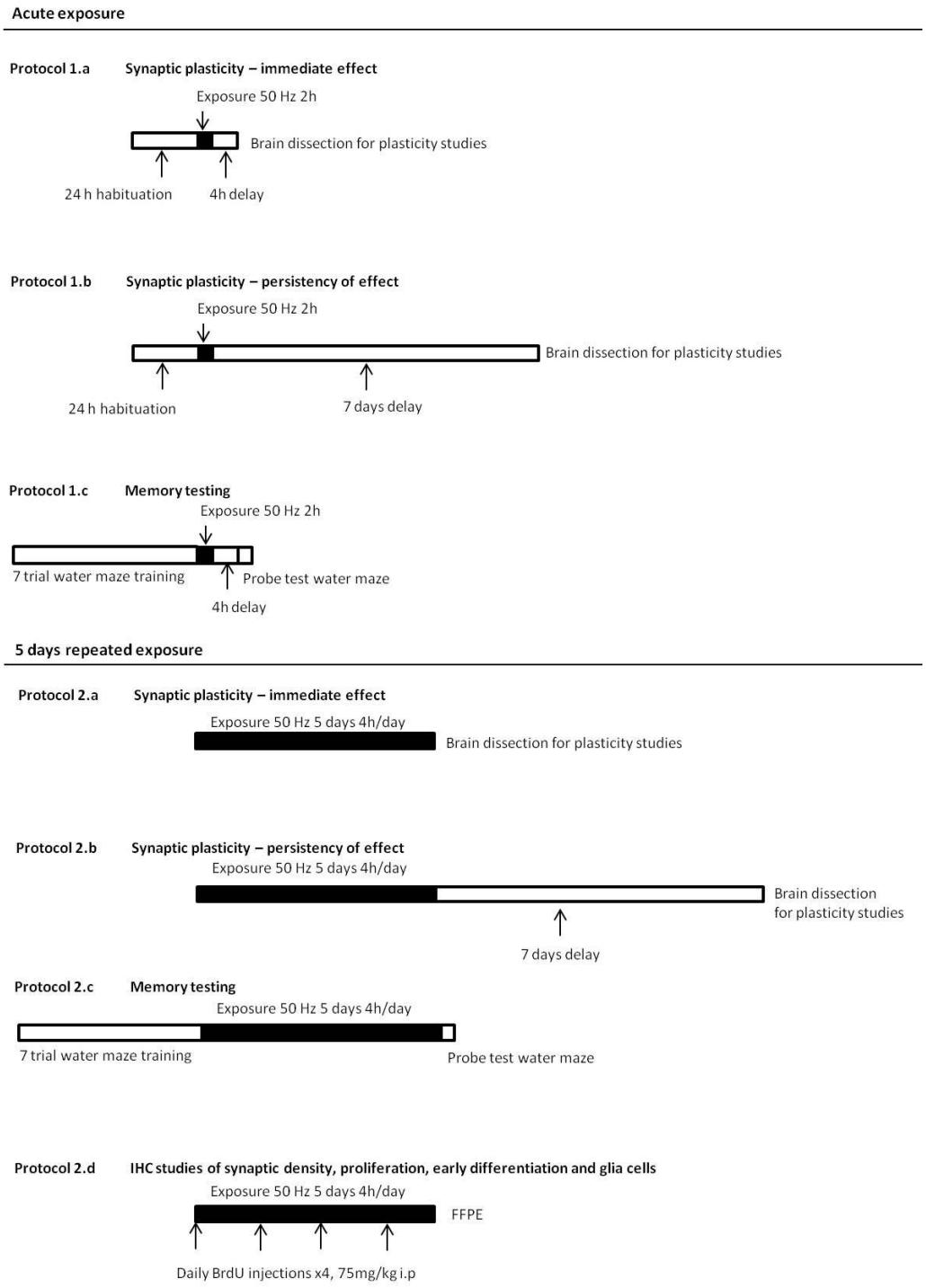


Figure 4.2 Experimental protocols. A 2 hour acute exposure protocol (1) and a 5 days repeated exposure protocol (2) were used to study the effects on; synaptic plasticity (a,b), memory testing (c), neurogenesis (d) and glia cells (d).

4.3 Results

4.3.1 Effects of acute exposure on synaptic plasticity and memory retrieval

4.3.1.1 Molecular endpoints

4.3.1.1.1 Synaptic plasticity

Mice were habituated to the exposure cages for 24 hours before being exposed for 2 hours (protocol 1a). Four hours later, mice were euthanized, perfused with heparinised saline and the dorsal hippocampus and prefrontal cortex was dissected for studies of gene expression using qPCR. Two magnetic flux densities of 100 µT and 300 µT were tested and compared to sham exposure.

Effects of acute exposure on gene expression of synaptic plasticity related genes

After the end of exposure significant changes in gene expression were found in both the dorsal hippocampus and prefrontal cortex (n=6-12) (Figure 4.3). Statistical analysis was first done by one-way ANOVA, and then with the less challenging unpaired t-test for comparisons of groups that appeared different in Figure 4.3. In the dorsal hippocampus a 33% increase in *gria1* was observed in the 100 µT group compared to the sham group ($p<0.05$, one-way ANOVA) (Figure 4.3A). In addition, *bdnf* expression was increased by 15% in the 100 µT group compared to the sham exposed group (Figure 4.3E). Using a one-way ANOVA for multiple comparisons this was not significantly different ($p=0.27$), but it was significant using an unpaired t-test for comparison of the sham and 100 µT groups ($p<0.05$). No significant differences in expression were seen in the remaining genes using a one-way ANOVA; *ntrk2*, *gria2, grin1*, *grin2a* or *grin2b* (Figure 4.3A,B,E). See Table 4.1 for details of the statistical analyses.

In the prefrontal cortex, no effects of exposure were seen in the 100 µT group, while in the 300 µT group a small but significant increase in *gria1* expression was found when an unpaired t-test was used for comparison (Figure 4.3C,D,F*),* of (*gria1*; t-test $p<0.05$, one-way ANOVA $p=0.086$.

Moreover a near significant increase in *grin1* expression was seen (*grin1*; t-test p=0.07). There were no significant changes in expression of *gria2, grin2a, grin2b, bdnf* and *ntrk2* (Figure 4.3C,D,F) .

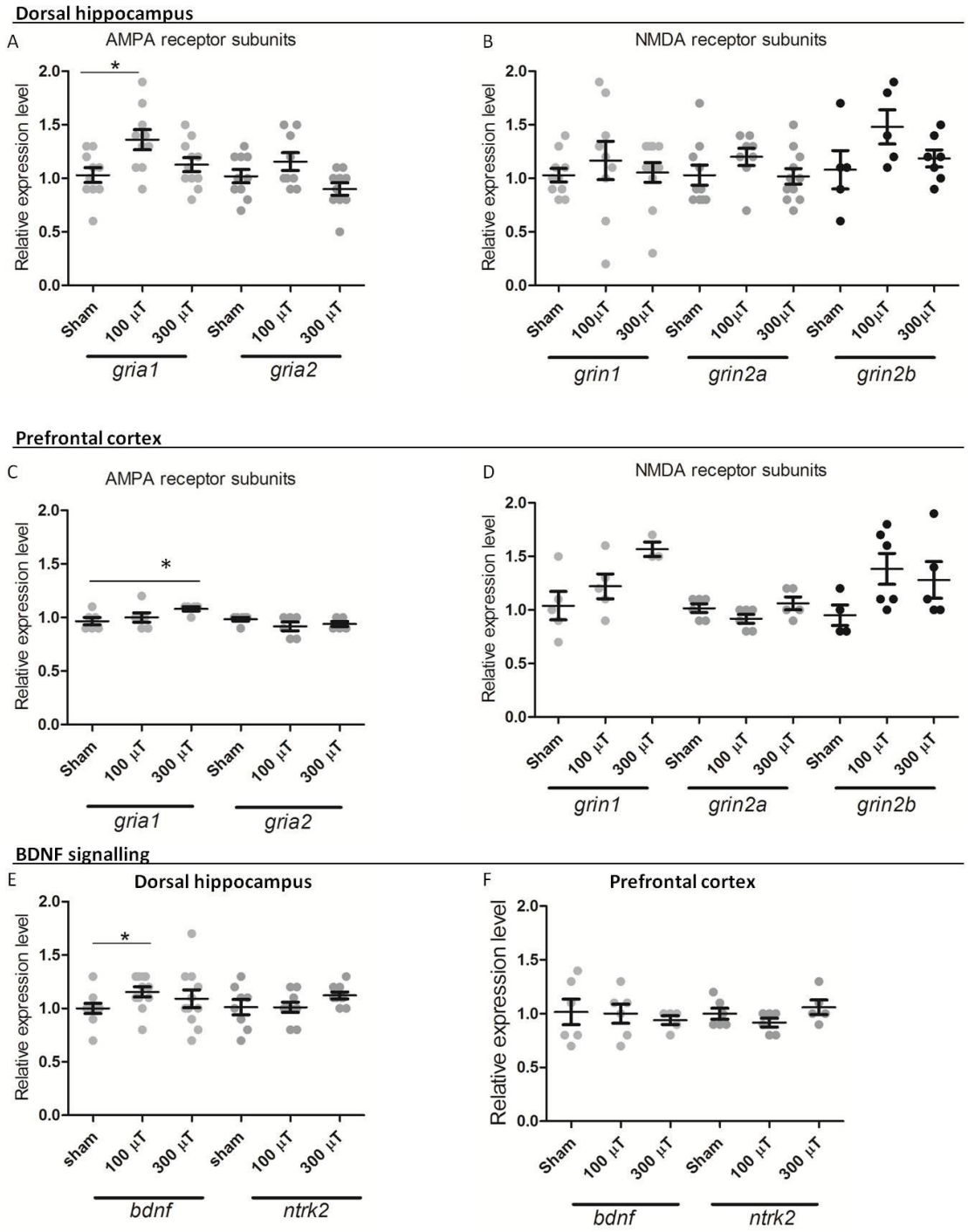


Figure 4.3 Subtle changes in expression of genes involved in synaptic plasticity were observed after 2 hours exposure to a 50 Hz MF. Gene expression of glutamate receptor subunits (*gria1, gria2*, *grin1, grin2a* and *grin2b)* and BDNF signalling molecules (*bdnf* and *ntrk2)* were investigated 4 hours after 2 hours exposure in the dorsal hippocampus (A,B,E), n=12, and the prefrontal cortex (C,D,F) n=6, in mice exposed at magnetic flux densities of 0 µT (sham), 100 µT or 300 µT. In the dorsal hippocampus *gria1* and *bdnf* was significantly increased after exposure (A,E), and in the prefrontal cortex *gria1* expression was increased after exposure (D). Data are presented as means ± SEM. Statistical comparison was done using a one-way ANOVA, or a non-parametric t-test, * $p<0.05$.

Table 4.1 Details of statistical analysis of effects of acute MF exposure on expression of synaptic plasticity related genes

	gene	Difference between means of sham and 100 µT group	Difference between means of sham and 300 µT group	Group sizes (n)	Gaussian distribution?	One-way ANOVA or non-parametric Kruskall-Wallis,	*Post hoc* test, compared to sham group	Un-paired t-test, compared to sham group
Dorsal hippocampus	*Grin1*	25% ↑	<10% ↑	8-11	no	p=0.22		100 µT: p=0.11
	Grin2a	17% ↑	<10% ↓	8-11	no	p=0.20		
	Grin2b	40% ↑	10% ↑	5-7	N too small	p=0.15		100 µT: p=0.09
	Gria1	33% ↑	10% ↑	10-11	yes	**p=0.016***	**100 µT: <0.05*** 300 µT: n.s.	**100 µT: p=0.01***
	Gria2	10% ↑	10%↓	9	yes	p=0.08		100 µT: p=0.34 300 µT: p=0.14
	bdnf	15% ↑	<10% ↑	9-11	yes	p=0.27		**100 µT: p=0.04***
	Ntrk2	0%	10% ↑	8-9	yes	p=0.23		
	Dlg4	<10%↓	13% ↑	6-9	N too small	p=0.37		
Prefrontal cortex	*Grin1*	18% ↑	52% ↑	3-5	N too small	p=0.08		100 µT: p=0.29 300 µT: p=0.07
	Grin2a	<10%↓	<10% ↑	5-6	N too small	p=0.30		
	Grin2b	43% ↑	33% ↑	4-6	N too small	p=0.14		100 µT: p=0.086 300 µT: p=0.17
	Gria1	<10% ↑	12% ↑	5-6	N too small	p=0.09		**300 µT: p=0.04***
	Gria2	<10%↓	<10%↓	5-6	N too small	p=0.30		
	bdnf	<10%↓	<10%↓	5-6	N too small	p=0.86		
	Ntrk2	<10%↓	<10%↓	5-6	N too small	p=0.24		
	Dlg4	0 %	<10% ↑	5-6	N too small	p=0.61		

To investigate the persistency of these effects and a possible delayed effect of exposure, gene expression was investigated seven days after the end of exposure (n=6) (protocol 1b). A significant increase of 70% in *bdnf* ($p<0.05$, one-way ANOVA) was seen in the dorsal hippocampus of mice exposed to 300 µT compared to the sham exposed group (Figure 4.4E). No changes in expression of other genes were found at this time-point in the dorsal hippocampus or prefrontal cortex (Figure 4.4A-F). See Table 4.2 for details of the statistical analyses.

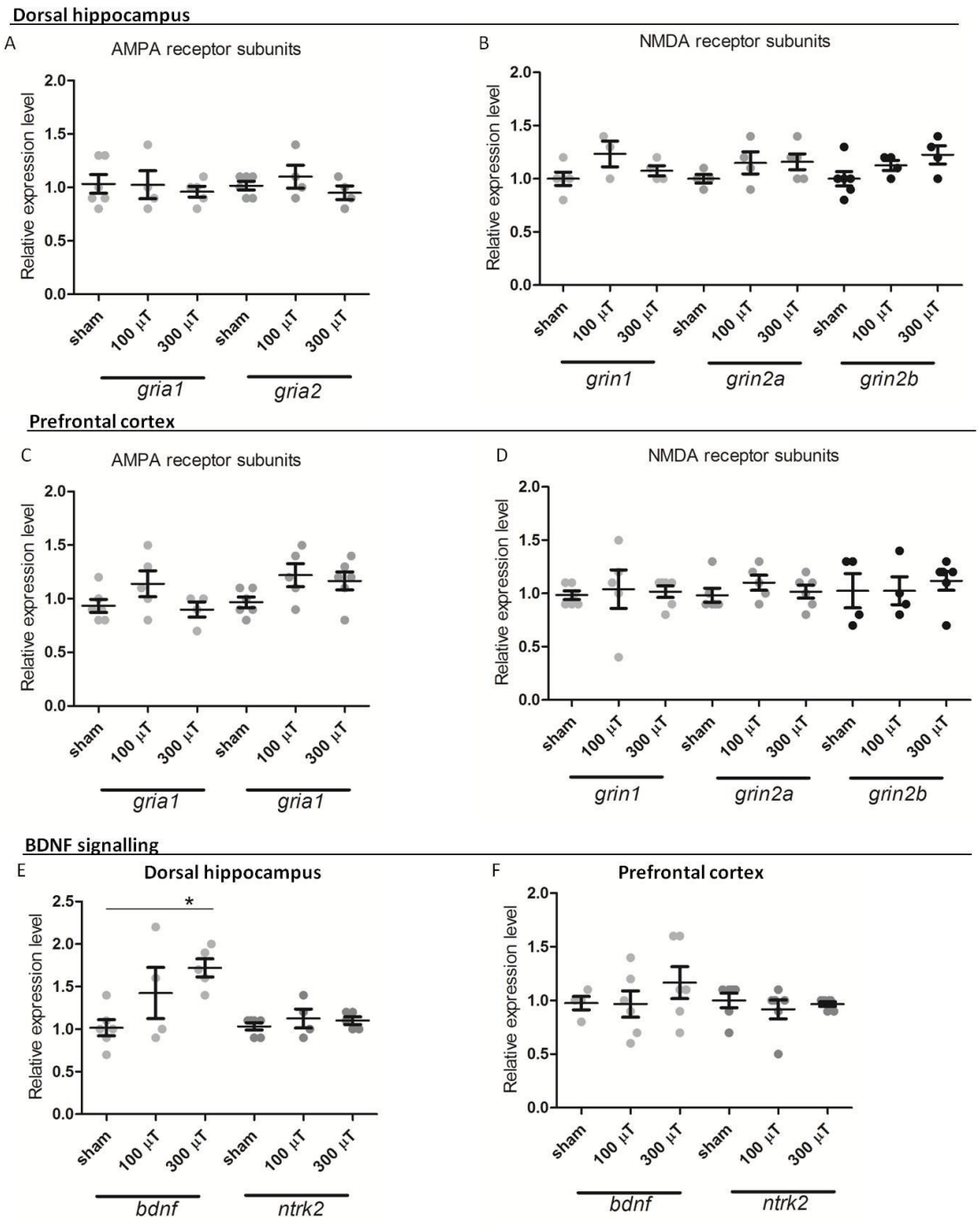


Figure 4.4 Effects of MF exposure on plasticity gene expression 7 days after exposure. Gene expression of glutamate receptor subunits (*gria1, gria2*, *grin1, grin2a* and *grin2b)* and BDNF signalling molecules (*bdnf* and *ntrk2)* were investigated 7 days after 2 hours exposure in the dorsal hippocampus (A,B,E) and the prefrontal cortex (C,D,F) n=4-6, in mice exposed at magnetic flux densities of 0 µT (sham), 100 µT or 300 µT. Only a significant increase in *bdnf* expression was observed, and only in the dorsal hippocampus at 300 µT (E). Data are presented with means ± SEM. Statistical comparison was done using a one-way ANOVA with Dunn's *post hoc* test, * $p<0.05$.

Table 4.2 Details of statistical analysis of MF effects on expression of plasticity related genes seven days after end of acute exposure

	gene	Difference between means of sham and 100 µT group	Difference between means of sham and 300 µT group	Group sizes (n)	Gaussian distribution?	One-way ANOVA or non-parametric Kruskall-Wallis,	*Post hoc* test, compared to sham group	Un-paired t-test, compared to sham group
Dorsal hippocampus	*Grin1*	23% ↑	<10% ↑	3-5	N too small	p=0.21		
	Grin2a	15% ↑	16% ↑	4-5	N too small	p=0.28		
	Grin2b	12% ↑	22% ↑	4-6	N too small	p=0.12		100 µT: p=0.19 300 µT: p=0.098
	Gria1	0%	<10% ↓	4-6	N too small	p=0.98		
	Gria2	<10% ↑	<10% ↓	4-6	N too small	p=0.52		
	bdnf	41% ↑	71% ↑	4-6	N too small	**p=0.04***	100 µT: n.s. **300 µT: p<0.05***	100 µT: n.s. **300 µT:** p=**0.01***
	Ntrk2	<10% ↑	<10% ↑	4-6	N too small	p=0.62		
	Dlg4	10% ↑	<10% ↑	4-6	N too small	p=0.93		
Prefrontal cortex	*Grin1*	<10% ↑	<10% ↑	5-6	N too small	p=0.55		
	Grin2a	<10% ↑	<10% ↑	5-6	N too small	p=0.39		
	Grin2b	0%	<10% ↑	4-6	N too small	p=0.95		
	Gria1	21% ↑	<10% ↓	4-6	N too small	p=0.24		
	Gria2	26% ↑	20% ↑	5-6	N too small	p=0.09		100 µT: p=0.077 300 µT: p=0.075
	bdnf	0 %	19% ↑	4-6	N too small	p=0.54		
	Ntrk2	<10% ↓	<10% ↓	6	N too small	p=0.41		
	Dlg4	<10% ↓	12% ↓	5-6	N too small	p=0.39		

4.3.1.1.2 No effect of acute exposure on dlg4 gene expression

The effect of acute exposure on synapse structure was investigated in terms of changes in the expression of the gene coding for PSD95, *dlg4*, in the hippocampus (Figure 4.5A,B) and prefrontal cortex (Figure 4.5C,D) (n=6). No acute (p_{hipp}=0.37, p_{pfc}=0.60) or delayed (p_{hipp}=0.93, p_{pfc}= 0.39) changes were found after exposure at either 100 µT or 300 µT using a non-parametric ANOVA for comparison.

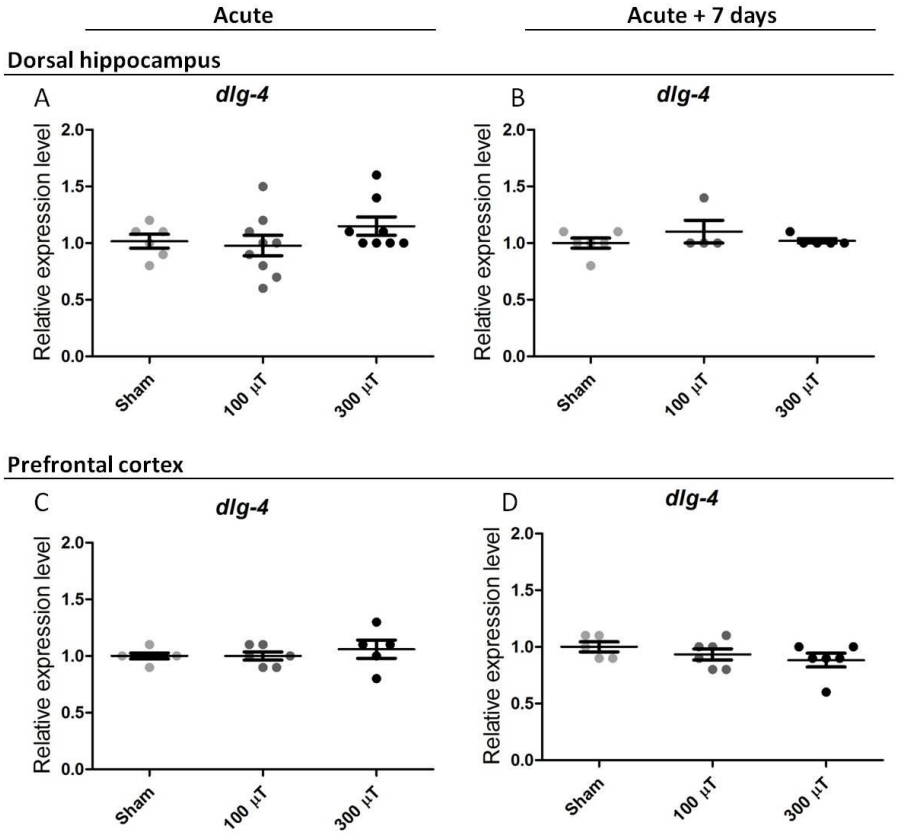


Figure 4.5 No effect of acute MF exposure on *dlg-4* expression. Expression of *dlg-4* was measured in the dorsal hippocampus and prefrontal cortex 4 hours after 2 hours of exposure (A,C) or 7 days after the end of exposure (B,D). n=4-9. Data are presented as mean ± SEM. Statistical comparison was done using a one-way ANOVA with Dunn's *post hoc* test, no changes in expression were seen after exposure in either brain region.

In summary, 2 hours acute MF exposure affected gene expression of glutamate receptor subunits and *bdnf* in a transient and region specific manner.

4.3.1.2 Functional endpoints

4.3.1.2.1 No effect of acute exposure on memory in the water maze test

The effect of acute exposure on memory retrieval was tested in the probe trial of the water maze (protocol 1c, n=6).

Before training, mice were randomly assigned to the exposure groups (sham, 100 μT and 300 μT). The mice explored the pool without the platform for 1 minute, to test motor function. All mice swam a distance of 1000 cm on average, and spent the same duration in each quadrant. The mice were then trained in finding the submerged platform over 7 trials, and learned equally well in terms of distance swum and platform latency (Figure 4.6A,B). An example of a swim path in trial 1 and 7 is shown in Figure 4.6D. See Table 4.3 for details of the statistical analyses.

On the day following training completion, mice were exposed to a MF for 2 hours. Four hours after exposure they were tested in a probe trial for 1 minute (Figure 4.6E-H). All exposure groups swam approximately 1000 cm ± 100 cm and there were no significant differences between the exposure groups (see Figure 4.6I for an example of a swim path). The platform latency (Figure 4.6F), was 10-20 s on average which was the same as before exposure (Figure 4.6A) and there were no significant differences between the exposure groups. A preference was seen for the platform quadrant in all exposure groups (Figure 4.6H) as all the mice spent 40-50% of the time in the platform quadrant, and 15-20% of the time in each of the three other quadrants and no significant differences were seen between exposure groups. In the sham and 100 μT groups, the mice crossed the platform zone (Figure 4.6G) an average of 3 times, whereas in the 300 μT group the average number of crossings was 4. This difference was not significant. See Table 4.3 for details of the statistical analyses.

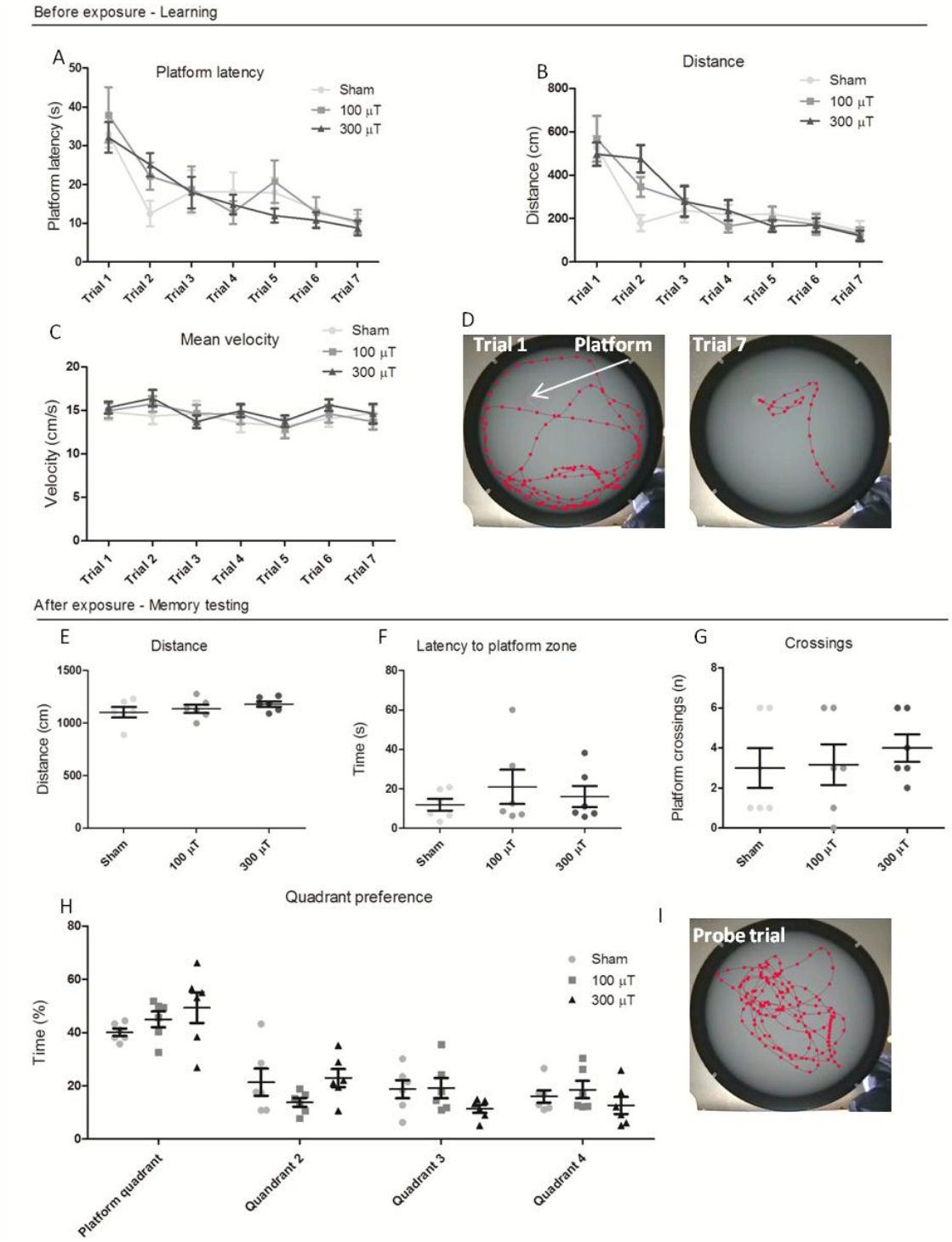


Figure 4.6 No effect of acute MF exposure was observed on memory in the water maze test. Mice were trained in finding the submerged platform over 7 trials before exposure. No significant differences in the platform latency (A), distance swum (B) or mean velocity (C) were found between the three exposure groups. Swimming path examples are shown in (D). On the following day, mice were exposed to a 50 Hz MF at 100 µT, 300 µT or sham for 2 hours. Four hours later, mice were tested in the probe trial. No significant differences in the distance swum (E), the platform latency (F) or the number of platform zone crossings (G) were found. In all exposure groups there was a preference for the platform quadrant, and equal preference for the three other quadrants (H). An example of a probe trial swim

is shown in (I). Data are shown as mean ± SEM. Statistical analysis of learning trials was done using a two-way repeated measures ANOVA, while statistical analysis of data from the probe trial was performed by non-parametric ANOVA, except for quadrant preference which was evaluated by two-way ANOVA. No statistically significant differences between exposure groups, while significant improvements in performance were detected between trials.

Table 4.3 Details of statistical analysis of water maze performance before and after 2 hours MF exposure

Two-way ANOVA, repeated measures				
Before exposure	**Interaction**	**Group effect**	**Trial effect**	**Bonferroni's post hoc test**
Distance	F(12,90)=2.1 **p=0.02***	F(2,90)=0.29 p=0.76	F(6,90)=28.3 **p<0.0001*** **	Trial 2: sham vs. 300 µT **p<0.001*** **
Latency	F(12,90)=1.29 p=0.24	F(2,90)=0.17 p=0.85	F(6,90)=19.74 **p<0.0001*** **	
Velocity	F(12,90)=0.65 p=0.80	F(2,90)=0.32 p=0.74	F(6,90)=2.64 **p=0.02***	
Two-way ANOVA				
After exposure	**Interaction**	**Exposure**	**Quadrant**	
Quadrant preference	F(6,60)=2.09 p=0.07	F(2,60)=0.00 p=1.0	F(3,60)=49.89 **p< 0.0001*** **	
Non-parametric one-way ANOVA				
	Percent difference between sham group and 100 µT group	**Percent difference between sham group and 300 µT group**	**Kruskall-Wallis test, p=**	
Distance	3%↑	6%↑	0.44	
Latency	75%↑	34%↑	0.83	
Platform zone crossings	6%↑	33%↑	0.62	

In summary, 2 hours acute MF exposure 4 hours before testing in the water maze did not affect performance.

4.3.2 Effects of repeated exposure on synaptic plasticity and memory retrieval

The effects of 5 days repeated exposure on cognitive function was first investigated in terms of changes in plasticity gene expression as investigated previously for acute exposure. In addition, changes in the protein levels of these were also assessed by western blotting, as well as effects on synaptic density, microglia, astroglia and hippocampal neurogenesis. Changes in memory were assessed in the water maze test.

4.3.2.1 Molecular endpoints

4.3.2.1.1 Synaptic plasticity

Effects of repeated exposure on gene expression of synaptic plasticity related genes

Mice were exposed for 4 hours per day for 5 days before gene and protein expression analysis (protocol 2a). Changes to the gene expression levels of AMPA receptors (*gria1, gria2), NMDA* receptors *(grin1, grin2a, grin2b)* and BDNF signalling genes *(bdnf* and *ntrk2)* were investigated in the dorsal hippocampus and prefrontal cortex (Figure 4.7) (n=6-12), while the protein levels of GluA1, GluA2, GluN1 and GluN2a were studied only in the dorsal hippocampus (Figure 4.9) (n=3-6).

In the dorsal hippocampus, a significant increase (25%) in the expression of *bdnf* was found in the group exposed at 300 μT ($p<0.05$, one-way ANOVA). Small differences were also seen between exposure groups in three other genes at 300 μT but not at 100 μT (Figure 4.7A,B,E). These increases in the expression of *gria1* (29%), *gria2* (12%), and *ntrk2* (13%) were significant ($p<0.05$) when the sham and exposure groups were compared using a t-test. See Table 4.4 for details of the statistical analysis.

In the prefrontal cortex, significant changes in AMPA receptor gene expression were also found (Figure 4.7C,D,F). *gria1* expression was increased significantly by 19% in the 100 μT group compared to the sham exposed group ($p<0.01$, one-way ANOVA), while no significant change was seen in the 300 μT group (Figure 4.7C). In contrast, *gria2* expression significantly decreased

by 13%, in the 300 μT exposure group ($p<0.01$) but not the 100 μT group. Exposure did not affect the NMDA receptor subunits *grin1* or *grin2a* (Figure 4.7D). In contrast, *grin2b* expression was significantly decreased by approximately 20%, in both exposure groups compared to the sham exposed group ($p<0.05$, one-way ANOVA). There were no changes in *bdnf* or *ntrk2* expression after exposure at 100 μT or 300 μT (Figure 4.7F). See Table 4.4 for details of the statistical analyses.

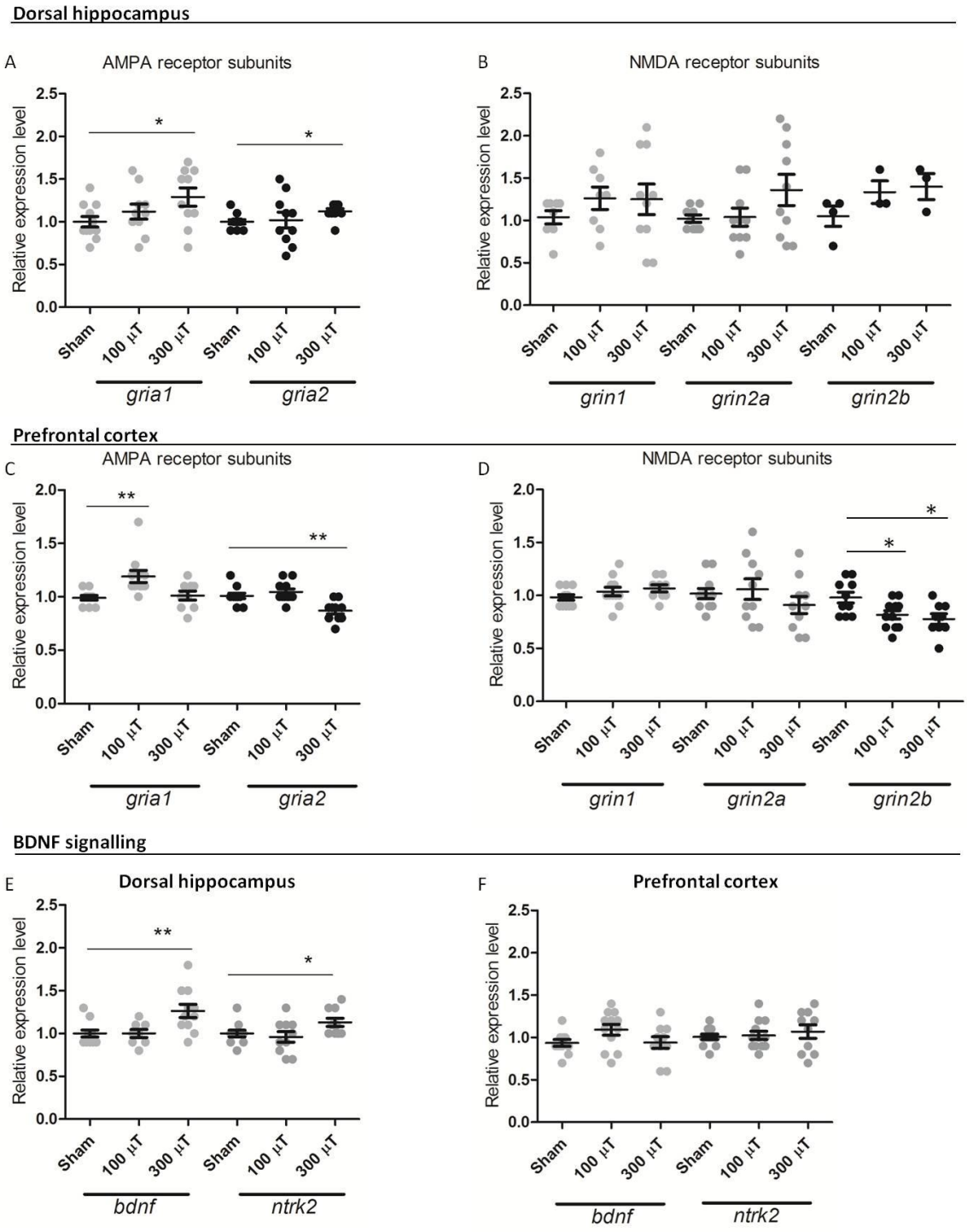


Figure 4.7 Subtle changes in synaptic plasticity genes were observed after 5 days repeated exposure to a 50 Hz MF. Gene expression of AMPA receptor subunits *gria1* and *gria2*, NMDA receptor subunits *grin1, grin2a* and *grin2b,* and *bdnf* signalling genes *bdnf* and *ntrk2* was investigated in the dorsal hippocampus (A,B,E), and the prefrontal cortex (C,D;F after 5 days repeated exposure for 4 hours per day) n=8-12, in mice exposed at magnetic flux densities of 100 µT, 300 µT or sham exposed). In the dorsal hippocampus significant increases in g*ria1, gria2, bdnf* and *ntrk2* were observed (A,B,E), In the prefrontal cortex a significant increase in *gria1* and significant decreases in *gria2* and *grin2b*

were observed (C,D). Data are presented as mean ± SEM. Statistical comparisons were carried out using a non-parametric one-way ANOVA with Dunn's *post hoc* test or between individual groups using an unpaired t-test, * p<0.05, ** p<0.01.

Table 4.4 Details of statistical analysis of effects of repeated MF exposure on expression of synaptic plasticity related genes

	Gene	**Difference between means of sham and 100 µT group**	**Difference between means of sham and 300 µT group**	**Group sizes (n)**	**Gaussian distribution?**	**One-way ANOVA or non-paramtric Kruskall-Wallis,**	***Post hoc* test, compared to sham group**	**Un-paired t-test, compared to sham group**
Dorsal hippocampus	*Grin1*	23%↑	22%↑	8-10	yes	p=0.50		100 µT: p=0.15 300 µT: p=0.39
	Grin2a	<10%↑	34%↑	9-10	yes	p=0.13		300 µT: p=0.88
	Grin2b	28%↑	35%↑	3-4	N too small	p=0.25		100 µT: p=0.17 300 µT: p=0.28
	Gria1	12%↑	29%↑	10-11	yes	p=0.07		100 µT: p=0.30 **300 µT: p=0.046***
	Gria2	<10%↑	12%↑	9-11	No	p=0.11		100 µT: p=1 **300 µT: p=0.01***
	bdnf	0%	25%↑	8-11	No	**p=0.01***	100 µT: n.s **300 µT: p<0.05***	100 µT: p=0.86 **300 µT: p=0.008****
	Ntrk2	<10%↓	13%↑	10-11	No	p=0.07		100 µT: p=0.72 **300 µT: p=0.03***
Prefrontal cortex	*Grin1*	<10%↑	<10%↑	9-11	yes	p=0.23		
	Grin2a	<10%↑	<10%↓	10-11	yes	p=0.39		
	Grin2b	16%↓	20%↓	9-11	yes	**p=0.01***	**100 µT: p<0.05*** **300 µT: p<0.05***	**100 µT: p=0.02*** **300 µT: p=0.01***

	Gria1	19%↑	<10%↑	9-11	no	**p=0.002****	**100 µT: p<0.01**** 300 µT: n.s.	
	Gria2	<10%↑	13%↓	10-11	yes	**p= 0.0003*****	100 µT: n.s **300 µT: p<0.01****	
	bdnf	15%↑	0%	10-12	yes	p=0.11		
	Ntrk2	0%	<10%↑	10-12	yes	p=0.72		

Again, to investigate the persistency of the changes in gene expression over time, as well as delayed effects of exposure, expression levels were assessed 7 days after the end of exposure (Protocol 2b, Figure 4.2, n=6). At this time point, only a near significant increase of 33% in *gria1* expression in the dorsal hippocampus was observed at 100 µT (p=0.057 t-test) (Figure 4,8A). There were no significant changes in gene expression in the prefrontal cortex (Figure 4.8C,D,F). See Table 4.5 for details of the statistical analyses.

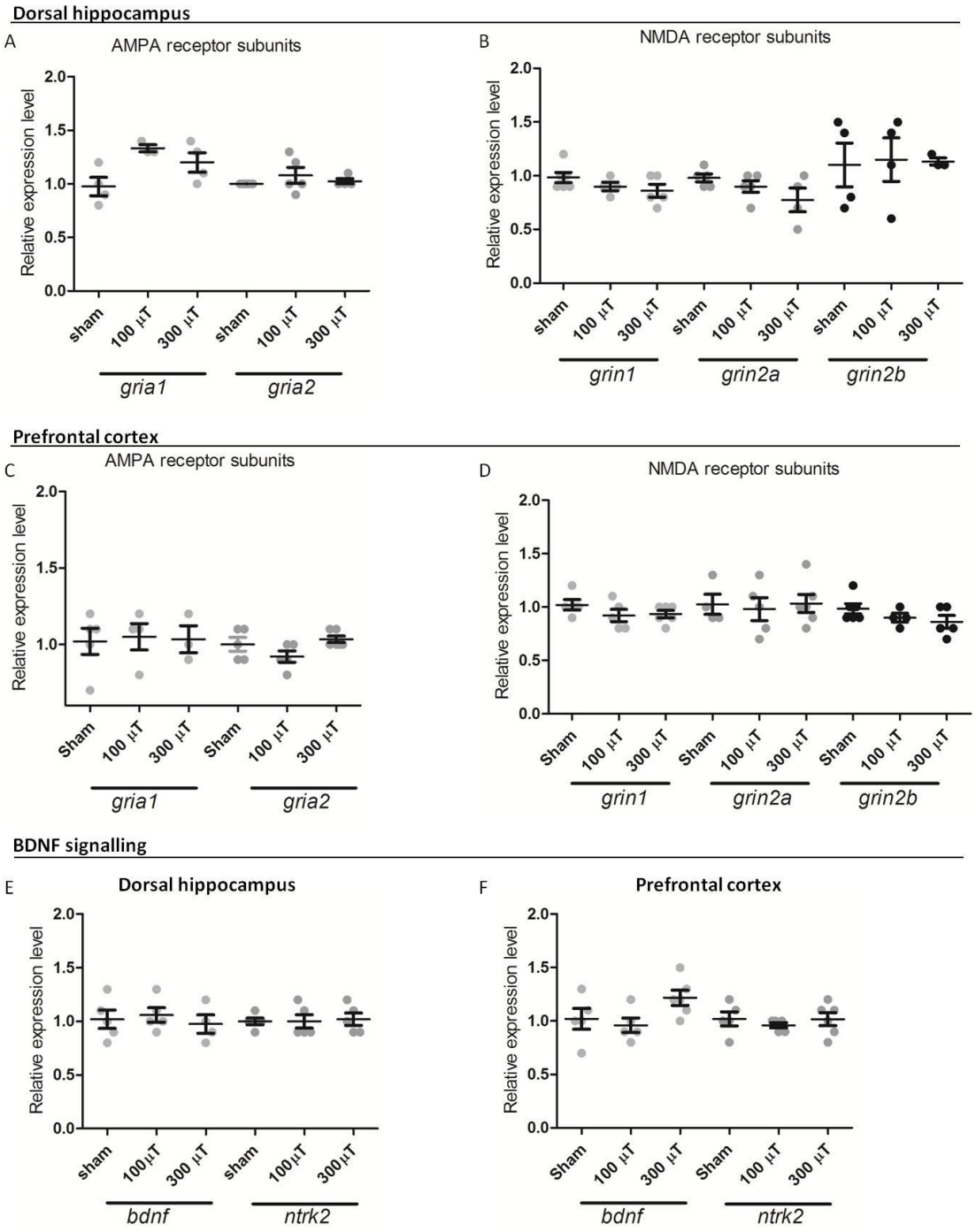


Figure 4.8 No changes in synaptic plasticity genes were observed 7 days after 5 days repeated exposure to a 50 Hz MF. Gene expression of AMPA receptor subunits *gria1* and *gria2*, NMDA receptor subunits *grin1, grin2a* and *grin2b,* and BDNF signalling genes *bdnf* and *ntrk2* was investigated in the dorsal hippocampus (A,B,E), and the prefrontal cortex (C,D;F)7 days after 5 days repeated exposure for 4 hours per day n=6, in mice exposed at magnetic flux densities of 100 µT, 300 µT or (sham exposed). No delayed or persistent effects were seen 7 days after exposure. Data are presented as mean ± SEM. Statistical comparisons were carried out using a non-parametric one-way ANOVA with Dunn's *post hoc* test or between individual groups using an unpaired t-test, no statistically significant differences were found.

Table 4.5 Details of the statistical analyses of persistent effects of repeated MF exposure 7 days after exposure on expression of synaptic plasticity related genes

	gene	Difference between means of sham and 100 µT group	Difference between means of sham and 300 µT group	Group sizes (n)	Gaussian distribution?	One-way ANOVA or non-paramtric Kruskall-Wallis,	*Post hoc* test, compared to sham group	Un-paired t-test, compared to sham group
Dorsal hippocampus	*Grin1*	<10%↓	12%↑	4-6	N too small	p=0.32		
	Grin2a	<10%↓	21%↓	4-5	N too small	p=0.24		300 µT: p=0.16
	Grin2b	<10%↑	<10%↑	3-4	N too small	p=0.97		
	Gria1	33%↑	23%↑	3-4	N too small	p=0.06		100 µT: p=0.057 300 µT: p=0.15
	Gria2	<10%↑	<10%↑	4-5	N too small	p=0.70		
	bdnf	<10%↑	<10%↓	4-5	N too small	p=0.69		
	Ntrk2	0%	<10%↑	5	N too small	p=0.93		
Prefrontal cortex	*Grin1*	<10%↓	12%↓	4-6	N too small	p=0.32		
	Grin2a	<10%↓	<10%↓	5-6	N too small	p=0.34		
	Grin2b	<10%↓	<10%↑	5-6	N too small	p=0.92		
	Gria1	<10%↑	<10%↑	3-5	N too small	p=0.94		
	Gria2	<10%↓	<10%↑	5-6	N too small	p=0.11		
	bdnf	<10%↓	19%↑	5-6	N too small	p=0.08		
	Ntrk2	<10%↓	0%	5-6	N too small	p=0.57		

Effects of repeated exposure on protein levels of glutamate receptor subunits

The effect of repeated exposure was investigated in terms of changes to protein levels of AMPA receptor subunits (GluA1 and GluA2) (Figure 4.9A) and NMDA receptor subunits (GluN1, GluN2a) (Figure 4.9B) (n=3-6). There were no significant effects on GluA1 or GluA2 levels in either exposure group when compared to the sham exposure group (Figure 4.9A) although there was greater variability in GluA1 protein levels in the group exposed at 300 µT. See Tabel 4.6 for results of the statistical analysis. For GluN1 the protein levels of both exposure groups was found to be lower (25-35%) than that of the sham exposed group. This was significant in the 100 µT group when using a t-test ($p<0.05$) (Figure 4.9B). For GluN2a, the average protein level was 60% higher in the 100 µT group, while it was slightly lower (18%) in the 300 µT group, this was found to be significant using a one-way ANOVA ($p<0.05$), but not by using Dunn's *post hoc* test.

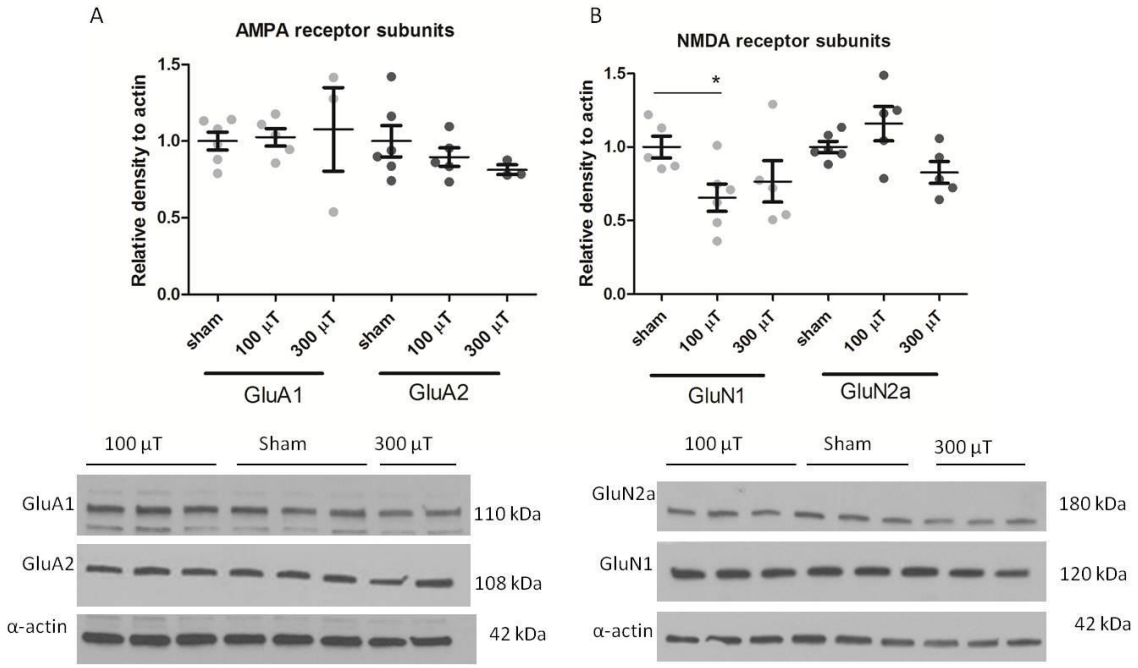


Figure 4.9 Repeated exposure to a 50 Hz MF affect glutamate receptor subunits levels. Protein expression of AMPA receptor subunits GluA1 and GluA2 (A) and NMDA receptor subunits GluN1 and GluN2a (B) was investigated in the dorsal hippocampus after 5 days repeated exposure for 4 hours per day n=3-6, in mice exposed at magnetic flux densities of 100 µT, 300 µT or sham. A significant decrease in GluN1 was observed after exposure at 100 µT, but not 300 µT. The GluN2a level was increased after exposure at 100 µT while it was decreased after exposure at 300 µT, this was statistically significant using a one-way ANOVA, but not by *post hoc* testing. Data are presented as mean ± SEM. Statistical comparisons were carried out using a non-parametric one-way ANOVA with Dunn's *post hoc* test or between individual groups using an unpaired t-test. *p<0.05.

Table 4.6 Details of the statistical analyses of effects of repeated MF exposure on glutamate receptor subunit levels

	gene	Difference between means of sham and 0.3W/kg group	Difference between means of sham and 3W/kg group	Group sizes (n)	Gaussian distribution?	One-way ANOVA or non-paramtric Kruskall-Wallis,	*Post hoc* test, compared to sham group	Un-paired t-test, compared to sham group
Dorsal hippocampus	*GluA1*	2% ↑	<10% ↑	3-6	N too small	p=0.69		100 µT: p=0.93 300 µT: p=0.55
	GluA2	11% ↓	19% ↓	3-6	N too small	p=0.39		100 µT: p=0.54 300 µT: p=0.26
	GluN1	35% ↓	24%↓	5-6	N too small	p=0.07		**100 µT: p=0.03*** 300 µT: n.s
	GluN2a	60% ↑	17% ↓	5-6	N too small	**p=0.03***	100 µT: n.s. 300 µT: n.s.	100 µT: p=0.13 300 µT: p=0.08

4.3.2.1.2 No changes in synaptic density after repeated exposures

Synaptic changes after MF exposure were investigated in terms of density of the pre-synaptic marker synaptophysin by immunohistochemistry, and also on changes in mRNA (*dlg4*) and protein levels of the post-synaptic density component PSD95.

Synaptophysin staining density was measured in the hippocampal subfields; *stratum. oriens* and *s.radiatum* in the CA1 and CA3, in the *s. lacunosum-moleculare,* and in the *s.moleculare* and hilus of the dentate gyrus (Figure 4.10A) (n=6). The effects of exposure on the regional synaptic density were compared using a two-way ANOVA. Significant effects of both exposure ($p<0.05$) and region ($p<0.0001$) were observed. However, when investigating this further using by Bonferroni's *post hoc* test, no significant differences between the sham and exposure groups were found as a result of exposure. See Table 4.7 for details of the statistical analyses.

No significant changes in *dlg4* gene expression levels were seen after exposure in the dorsal hippocampus, for either exposure group compared to the sham exposed group (Figure 4.10B). The average protein level in the hippocampus was higher (20-30%) in the two exposure groups than the sham group, but not significantly so (Figure 4.10E). In addition, no delayed effect was seen in *dlg4* gene expression levels seven days after the end of exposure (Figure 4.10F).

In the prefrontal cortex, a significant decrease of 13% was seen in the 300 μT group compared to the sham group ($p<0.05$), but seven days later, no differences were found between the exposure groups (Figure 4.10C,G). See Table 4.7 for results of the statistical analyses.

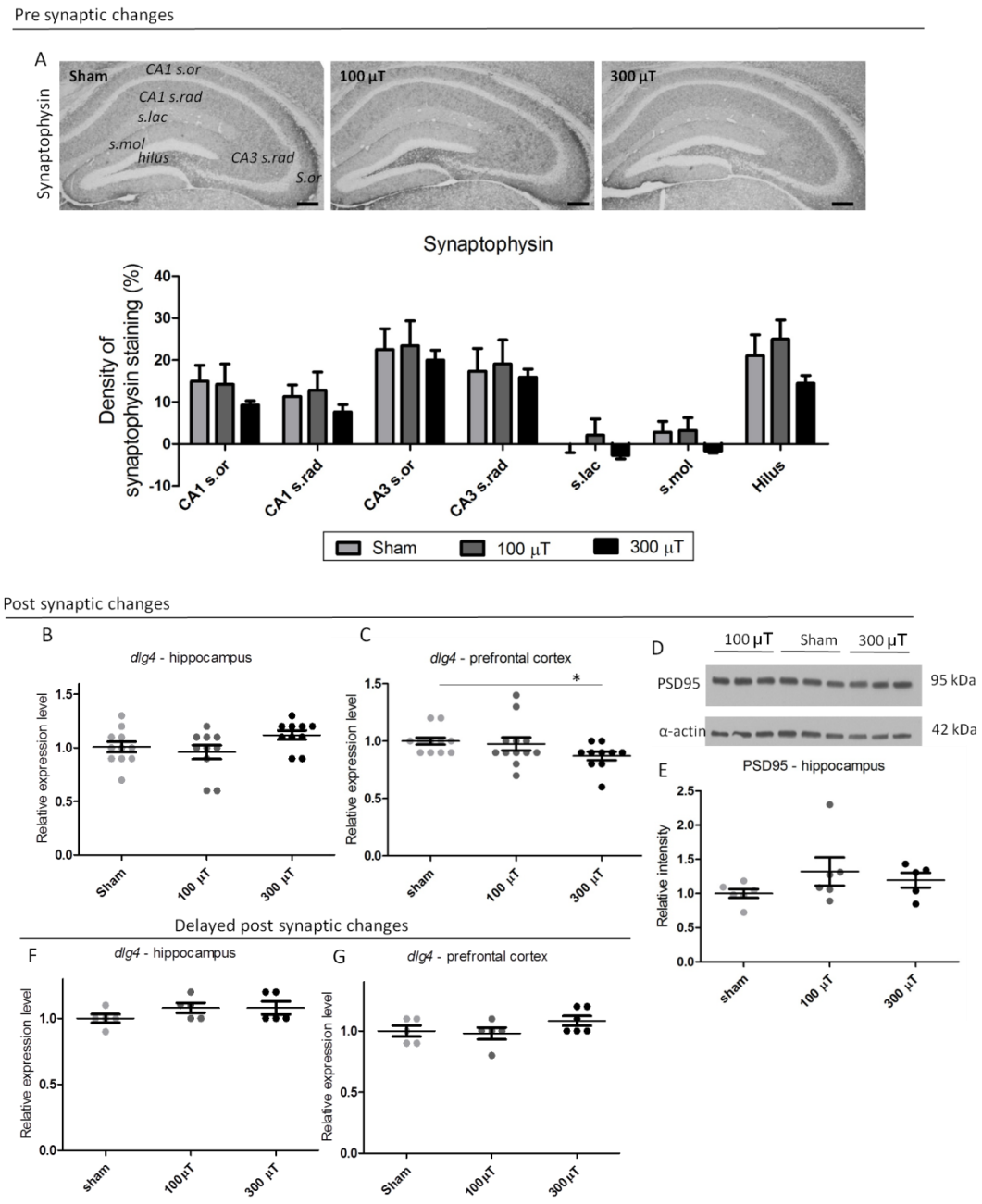


Figure 4.10 No synaptic changes after repeated MF exposure. Density of synaptophysin was used as a measure of presynaptic density. This was measured in the hippocampal regions *s.oriens* and *s.radiatum* of the CA1 and CA3, the *s.lacunosum-moleculare*, and in the *s.moleculare* and hilus of the dentate gyrus (A). No significant differences between exposure groups (sham, 100 µT and 300 µT were seen). Changes to the post-synaptic density was measured in terms of gene expression of *dlg4* in the dorsal hippocampus (B,F) and prefrontal cortex (C,G) immediately after exposure and seven days later. Protein levels of PSD95 were measured immediately after exposure in the dorsal hippocampus (D,E). A small significant decrease in *dlg4* expression was found in the prefrontal cortex immediately after exposure only. No significant differences were found between exposure groups. Scale bar: 200 µm. Statistical analysis was performed by non-parametric ANOVA and . *p<0.05

Table 4.7 Details of statistical analyses of effects of repeated MF exposure on regional synaptophysin staining density and PSD95 gene and protein levels

Two-way ANOVA,	**Interaction**	**Exposure effect**	**Region effect**	**Bonferroni's *post hoc* test, compared to sham**			
Synaptophysin density	F(12,105)= 0.14 p=0.99	F(2,105)=3.86 **p=0.02***	F(6,105)=16.97 **p<0.0001*** **	100 µT: n.s. in all regions 300 µT: n.s. in all regions			
One-way ANOVA	**Difference between means of sham and 100 µT group**	**Difference between means of sham and 300 µT group**	**Group sizes (n)**	**Gaussian distribution?**	**One-way ANOVA or non-parametric Kruskall-Wallis,**	***Post hoc* test**	**Mann-Whitney test compared to sham group,**
***Dlg*4 Hippocampus**	<10%↓	12%↑	10-11	yes	p=0.12		
***Dlg*4 Prefrontal cortex**	<10%↓	13% ↓	10-12	no	p=0.053		100 µT: p=0.3 **300 µT: p=0.013***
PSD95 **Hippocampus**	32% ↑	19% ↑	5-6	N too small	p=0.27		100 µT: p=0.18 300 µT: p=0.25
***Dlg*4 hippocampus** **Delayed**	<10%↑	<10%↑	5	N too small	p=0.30		
***Dlg*4 Prefrontal cortex** **Delayed**	<10%↓	<10%↑	5-6	N too small	p=0.30		

In summary, 5 days repeated MF exposure affected gene expression levels of glutamate receptor subunits and BDNF signalling molecules in the dorsal hippocampus and prefrontal cortex transiently, while it had no modulating effect on presynaptic density or gene and protein levels of PSD95, except for a small transient decrease in *dlg4* expression in the prefrontal cortex.

4.3.2.2 Cellular endpoints

4.3.2.2.1 Effects of repeated exposure on hippocampal neurogenesis

To investigate the effect of MF exposure on proliferation and differentiation, mice were injected with BrdU before exposure on days 1-4, (protocol 2d) (n=6). BrdU labelled cells were counted on every 6th section from -1.4 to -1.51 from bregma while the effects of exposure on differentiation was investigated in terms of changes to the fraction of BrdU+ cells that co-expressed DCX.

The average number of BrdU-labelled cells in the 100 µT group was lower than in the sham exposed group, while the average number in the 300 µT exposure group was higher (with two mice having higher numbers than the others) (Figure 4.11B). Using a non-parametric one-way ANOVA, a significant difference was found ($p<0.05$), when this was investigated further by Dunn's *post hoc* test no significant difference was found between the sham exposed group and either of the two exposed groups. When a t-test was used for pair wise comparison of the sham and 100 µT exposed groups a significantly lower number of BrdU-labelled cells was seen in the 100 µT group (17%) ($p<0.05$). See Table 4.8 for details of the statistical analyses.

Approximately 90% of the BrdU-labelled cells were DCX-positive in all three exposure groups, and no significant differences between the sham group and the MF exposed groups were found (Figure 4.11C).

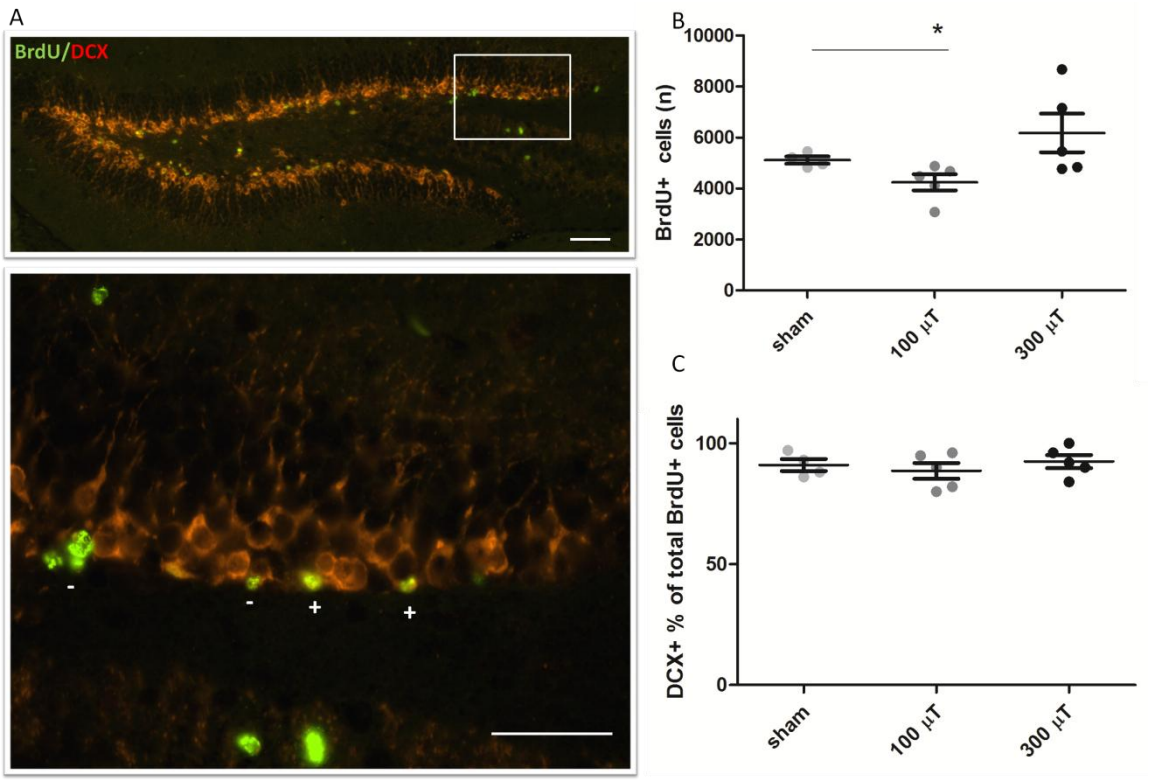


Figure 4.11 Effect of repeated exposure to a 50 Hz MF on hippocampal neurogenesis. Hippocampal neurogenesis was investigated after 5 days of exposure with daily BrdU injections. Serial sections were double labelled for BrdU and doublecortin (DCX) as markers of proliferation and neuronal differentiation. A large number of positive cells was observed in the subgranular zone and glanule cell layer (A). A significantly lower number of BrdU-labelled cells were observed in the 100 µT group compared to the sham group (B). Approximately 90 % of the BrdU+ cells were co-expressing DCX in all three exposure groups. Scale bar: 100 µm. Data are presented as mean ± SEM. Statistical comparisons were carried out using a non-parametric one-way ANOVA with Dunn's *post hoc* test or between individual groups using an unpaired t-test, * $p<0.05$.

Table 4.8 Details of statistical analyses of effects of repeated MF exposure on hippocampal neurogenesis

Parameter	**Difference between means of sham and 100 µT group**	**Difference between means of sham and 300 µT group**	**Group sizes (n)**	**Gaussian distribution?**	**One-way ANOVA or non-parametric Kruskall-Wallis,**	***Post hoc* test, compared to sham group**	**Un-paired t-test, compared to sham group, p=**
BrdU-labelled cells	17%↓	21%↑	4-5	N too small	**p=0.03***	100 µT: n.s. 300 µT: n.s.	**100 µT: 0.03*** 300 µT: 0.62
BrdU+/DCX+ cells	3% ↓	1.5%↑	4-5	N too small	p=0.65		

4.3.2.2.2 Effects of repeated MF exposure on glial cells.

Glial distribution in the hippocampus was investigated in the same brains used for studies of neurogenesis (n=6). Qualitatively, no differences in morphology of the microglia were observed between the MF exposed or sham exposed groups.

To investigate regional changes in microglial density after exposure, the total number of Iba-1+ cells was counted in the *s.moleculare*, granule cell layer (GCL) and the hilus of the DG and in the CA1 *s.radiatum* and *s.oriens* (Figure 4.12B). A near significant increase in microglial numbers was observed in the 300 μT group ($p=0.07$) when using a one-way ANOVA. This was found to be significant when using an unpaired t-test to compare these two groups ($p<0.05$). Differences between the exposure groups were also found in the regional distribution using a one-way ANOVA for comparison (Figure 4.12C). In all regions, except the hilus, the number of Iba-1+ stained cells was highest in the 300 μT group and lowest in the sham group. This was significant in the *s.moleculare* ($p<0.05$, ANOVA) and in the *s.radiatum* ($p=0.02$, t-test). See Table 4.9 for details of the statistical analyses.

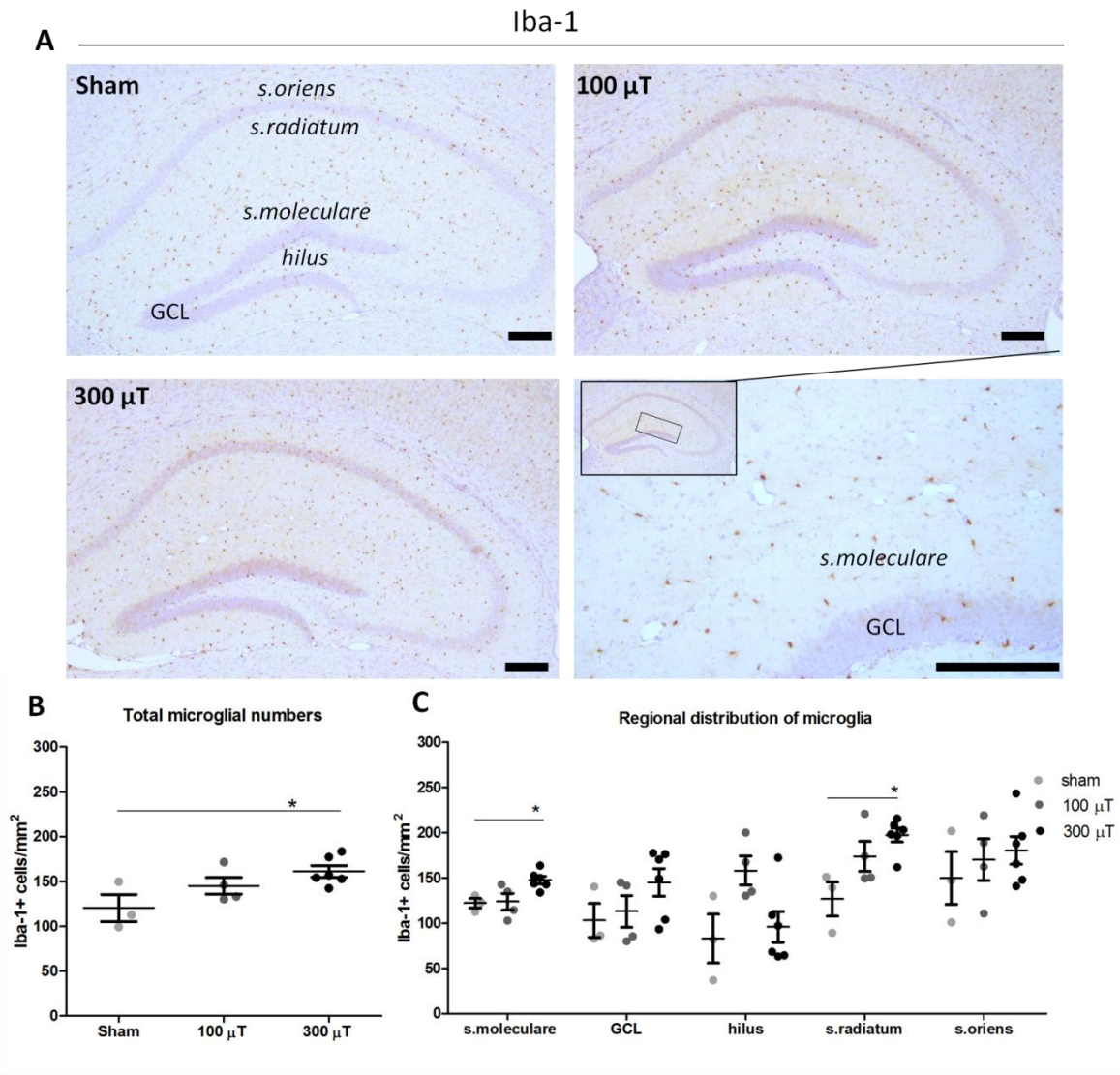


Figure 4.12 Increased number of microglia after repeated MF exposure. The effect of 50 Hz MF exposure (sham, 100 µT and 300 µT) on microglia was investigated in terms of changes in the number of Iba-1 positive cells. Positive cells were found in all regions of the hippocampus (A). The regional density of cells was counted in the the dentate gyrus (*s.moleculare*, granule cell layer (GCL) and the hilus) and in the CA1 (*s.radiatum* and *s.oriens*) (B,C). Significantly more microglia were found in the total area and in the *s.moleculare* and *s.radiatum* after exposure at 300 µT. Scalebar: 200 µm. Data are presented as mean cells per area (mm^2) ± SEM. Statistical comparisons were carried out using a non-parametric one-way ANOVA with Dunn's *post hoc* test or between individual groups using an unpaired t-test, * $p<0.05$.

To investigate regional changes in astroglia, the density of GFAP positive cells in the hippocampus were counted in the s*.moleculare* and the hilus, and in the *s.radiatum* and *s.oriens* in the CA1. A small significant increase in astroglial density was seen in the *s.moleculare* as a result of exposure when using the non-parametric one-way ANOVA, however, no significant differences were detected by *post hoc* testing. No significant differences were detected between the groups in the other regions. By one-way ANOVA the total number of astroglia was found to be significantly increased as a result of exposure, however this was not detected by *post hoc* testing (Figure 4.13). Qualitatively no morphological differences were seen between the exposure groups. See Table 4.9 for details of the statistical analyses.

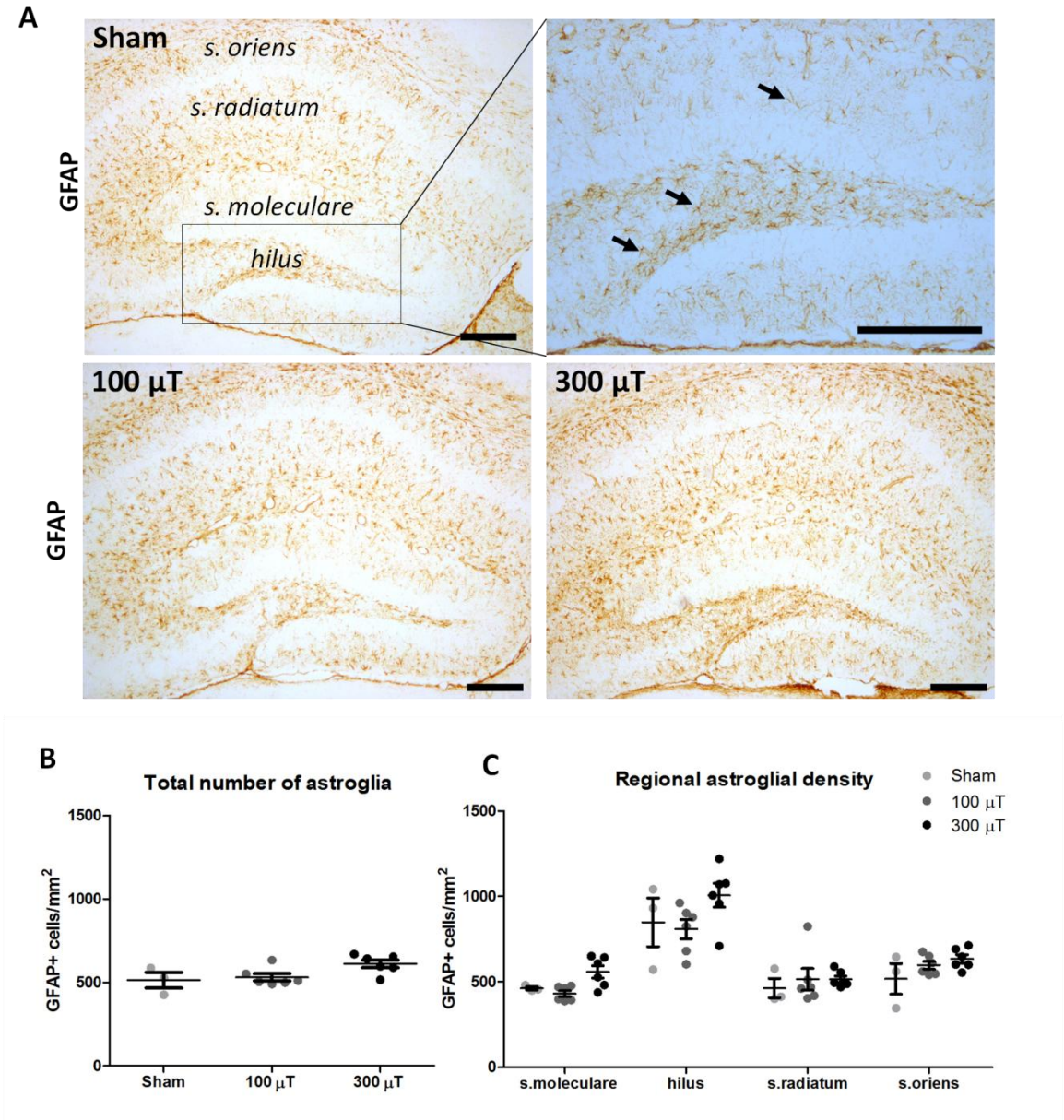


Figure 4.13 No effect of repeated MF exposure on astroglia. The effect of 50 Hz magnetic field exposure (sham, 100 µT and 300 µT) on astroglia was investigated in terms of changes in number of GFAP positive cells. Positive cells were found in all regions of the hippocampus except the pyramidal cell layer (A). The morphology of the GFAP+ astroglia can be seen in more detail in the picture in the upper right corner of (A), selected cells are indicated by black arrows. The density in the total area (B) and in the hippocampal regions was counted, in the dentate gyrus (*s.moleculare* and the hilus) and in the CA1 (*s.radiatum* and *s.oriens*) (C). Statistically significantly more astroglia were found in the *s.moleculare* and in the total area in the group exposed at 300 µT using a the Kruskall-Wallis analysis, but not with Dunn's *post hoc* test for comparison of the MF exposed groups to the sham group. Scalebar: 200 µm. Data are presented as mean cells per area (mm^2) ± SEM. Statistical comparisons were carried out using a non-parametric one-way ANOVA with Dunn's *post hoc* test.

Table 4.9 Details of the statistical analysis of effects of repeated MF exposure on microglia and astroglia

	Difference between means of sham and 100 µT group	**Difference between means of sham and 300 µT group**	**Group sizes (n)**	**Gaussian distribution?**	**One-way ANOVA or non-parametric Kruskall-Wallis,**	***Post hoc* test, compared to sham group**	**Un-paired t-test, compared to sham group,**
Microglia							
s.moleculare	0%	21%↑	3-6	N too small	p=**0.03***	100 µT: n.s. **300 µT: p<0.05***	**300 µT: p= 0.02***
GCL	10%↑	41%↑	3-6	N too small	p=0.10		300 µT: p=0.095
Hilus	91%↑	16%↑	3-6	N too small	p=0.064		100 µT: p=0.057 300 µT: p=0.90
s.radiatum	37%↑	56%↑	3-6	N too small	p=0.053		100 µT: p=0.23 **300 µT: p= 0.02***
s.oriens	13%↑	20%↑	3-6	N too small	p=0.69		
total	21%↑	34%↑	3-6	N too small	p=0.07		**300 µT: p=0.048***
Astroglia							
s.moleculare	3%↓	21%↑	3-6	N too small	**p=0.047***	100 µT: n.s. 300 µT: n.s.	100 µT: p=0.55. 300 µT: p=0.26
Hilus	5%↓	19%↑	3-6	N too small	p=0.09		100 µT: p=0.71 300 µT: p=0.26
s.radiatum	11%↑	11%↑	3-6	N too small	p=0.33		
s.oriens	15%↑	23%↑	3-6	N too small	p=0.34		300 µT: p=0.26
total	3%↑	19%↑	3-6	N too small	p=**0.047***	100 µT: n.s. 300 µT: n.s.	100 µT: p=0.095 300 µT: p=1.0

In summary, 5 days repeated MF exposure at 100 µT affected proliferation in the SGZ by decreasing the number of BrdU-labelled cells significantly, while, at 300 µT an indication of an increase in BrdU-labelled cells was seen. No changes to the proportion of BrdU-labelled cells that co-expressed doublecortin were found. On the other hand a significant increase in microglial density was found after exposure at 300 µT in the total area and in the *s.moleculare* and *s.radiatum* in the DG, while there were small indications of an increased astroglial density after MF exposure.

4.3.2.3 Functional endpoints

4.3.2.3.1 No effect of repeated exposure on memory retrieval

To evaluate the functional effect of repeated MF exposure on memory, mice were trained to find the submerged platform in the water maze (protocol 2c).

Mice were randomly assigned to the exposure groups (sham, 100 and 300 µT, n=6). No differences in motor function or behaviour were seen between mice in the three groups in the pre-trial (data not shown).

Mice in all three exposure groups learned equally well before exposure, evaluated in terms of distance swum and platform latency (Figure 4.14A-C). As shown in Figure 4.14C all mice swam at the same mean velocity of approximately 15 cm/s. See Table 4.10 for details of the statistical analyses.

Next, the mice were exposed to 50Hz MF for 5 days, 4 hours per day, at either 100 µT, 300 µT or sham exposure, and then tested for 1 minute in the probe trial, see Figure 4.14I for an example of a swim path. The mice swam approximately 1000 cm ± 100 cm during the trial (as shown in Figure 4.14E). Two mice in the sham group, and one from the 100 µT group did not find the platform during the trial, but overall, no significant differences were found between the

exposure groups. The number of platform zone crossings varied from 0-6, and was not significantly different between exposure groups (Figure 4.14G).

The mice in all exposure groups spent the majority of the time in the platform quadrant, 40% of the time for the sham and 100 µT groups and 50% for the 300 µT group. The remaining time was split equally between the three other quadrants (Figure 4.14H). No significant effect of exposure was found using a two-way ANOVA, but there was a significant effect of quadrant, indicating good retention of the task (See Table 4.10 for results of the statistical analyses).

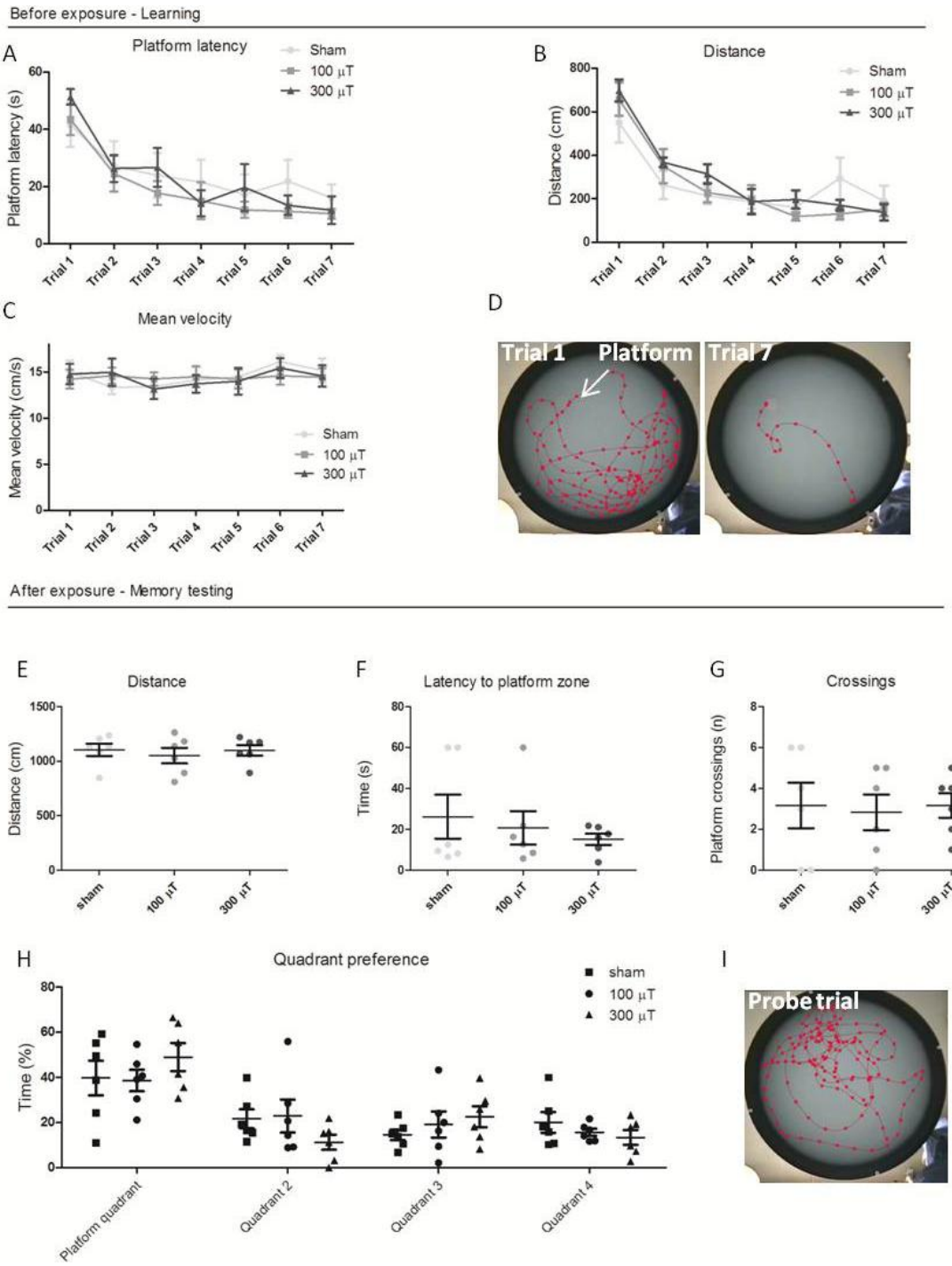


Figure 4.14 No effect of repeated MF exposure on memory in the water maze test. Mice were trained to find a submerged platform over 7 trials before exposure. (A) There were no differences in platform latency, (B) distance swum or (C) mean velocity between the three exposure groups. Examples of changes in the length of the swimming path in trials 1 and 7 are shown in (D). After training mice were exposed to a 50 Hz MF at sham, 100 µT or 300 µT 4 hours per day for 5 days. Mice were tested in the probe trial after exposure on the 5th day. No significant differences in (E) distance swum, (F) platform latency or (G) number of platform zone crossings were found. In all exposure groups (H) a preference for the platform quadrant was seen, and similar amounts of time were spent in the three

other quadrants. An example of a swimming path in the probe trial is shown in (I). Data are shown as mean ± SEM. Statistical analysis was performed using a repeated measures two-way ANOVA in the learning trials, and one-way ANOVA in the probe trial, except for quadrant preference where a two-way ANOVA was used. No statistically significant differences were found between the sham group and either of the two MF exposed groups.

Table 4.10 Details of the statistical analysis for water maze performance before and after repeated MF exposure

Two-way ANOVA, repeated measures			
Before exposure	**Interaction**	**Group effect**	**Trial effect**
Distance	F(12,105)=1.06 p=0.4	F(2,105)=0.81 p=0.45	F(6,105)=28.92 **p< 0.0001*****
Latency	F(12,105)=0.35 p=0.98	F(2,105)=1.43 p=0.24	F(6,105)=10.87 **p< 0.0001*****
Velocity	F(12,105)=0.30 p=0.99	F(2,105)=0.04 p=0.96	F(6,105)=0.81 p=0.57
2-way ANOVA **After exposure**	**Interaction**	**Exposure**	**Quadrant**
Quadrant preference	F(6,60)=1.36 p=0.25	F(2,60)=0.00 p=1.0	F(3,60)=18.42 **p< 0.0001*****
One-way ANOVA	**Percent difference between sham group and 100 µT group**	**Percent difference between sham group and 300 µT group**	**Kruskall-Wallis test, p=**
Distance	5%↓	0%	0.88
Latency	20%↓	42%↓	0.98
Platform crossings	11%↓	0%	0.97

In summary, 5 days of repeated MF exposure did not affect spatial memory in the water maze test.

4.4 Discussion

4.4.1 General introduction

This chapter investigated whether the young brain is sensitive to 50 Hz MF exposure at flux densities <1 mT, with particular focus on the plastic properties important for cognitive function. This work was prompted by studies reporting conflicting results with both enhancing and damaging effects of exposure on cognitive function.

No major effects of exposure were seen, while small changes were found after both acute and repeated exposure. See Table 4.11 for a summary. These changes did not affect memory as tested in the water maze.

Table 4.11 An overview of statistically significant effects of MF exposure

	Dorsal hippocampus	Prefrontal cortex
Acute exposure	*bdnf* 15% ↑ 100 µT *gria1* 33% ↑ 100 µT +7 days: bdnf 70% ↑300 µT	*gria1* 8% ↑ 300 µT
Repeated exposure	*bdnf* 26% ↑ 300 µT *ntrk2* 13% ↑ 300 µT *gria1* 29% ↑ 300 µT *gria2* 12% ↑ 300 µT GluN1 35% ↓100 µT BrdU-labelled cells 17% ↓100 µT Total Iba-1+ cells 34% ↑ 300 µT *s.moleculare* Iba-1+ cells 21%↑ 300 µT *s.radiatum* Iba-1+ cells 56%↑	*gria1* 19% ↑ 100 µT *gria2* 13% ↓ 300 µT *grin2b* 20% ↓ 100 µT, 300 µT *dlg4* 13% ↓ 300 µT

This work has investigated whether exposure to 50 Hz MF at 100 µT and 300 µT could have an effect on important processes in cognitive function with focus on learning and memory. Previous studies have reported conflicting results, and a majority of these used MF strengths which are not relevant for public or occupational exposures. Some previous studies have used exposure systems that may cause stress, so helmholtz coils with a very homogenous field was used where mice were allowed to move freely to minimize any stress not associated with MF exposure. Exposures were consistently performed at the same time of the day, and everything was done to minimise potential differences between experimental groups other than the magnetic flux density used for exposure.

The experiments presented in this chapter were carried out in 4 week old mice. This young age was chosen since the study of MF effects on the developing brain has been set as a priority (WHO, 2007a), and a number of studies have indicated that plastic processes are sensitive to modulation by MFs at this age (Cuccurazzu et al., 2010; Leone et al., 2014; Podda et al., 2014).

Effects of both acute and repeated exposure were investigated. It is possible that exposure induces acute changes in processes that affect cognitive function and where habituation is obtained over time, while it is likewise possible that exposure induces changes over time. Using both protocols provide information about these dynamics.

4.4.2 Experimental findings of effects of acute and repeated MF exposure

Little is known about the effects of MF exposure at flux densities below 1 mT on synapses. The findings presented in this chapter therefore provide important information for the understanding of sensitivity to exposure on synapses as an important component of cognitive function.

No loss of synapses or changes to gene expression or protein levels of PSD95 were observed after MF exposure in the dorsal hippocampus, while a small decrease in *dlg4* expression was found in the prefrontal cortex. Furthermore, synaptic changes at the receptor level were

detected in both brain regions. These changes were subtle and transient, where the response differed after acute and repeated exposure.

Glutamate is the major excitatory neurotransmitter in the brain, where it plays an important role in signalling during learning and memory formation. Changes to glutamate receptor density and composition affects this signalling (Paoletti et al., 2013) but only a few studies have looked at the sensitivity of this to 50 Hz MF exposure and none have investigated effects of acute exposure. One group found that in rats, exposure for 4 hours per day for 14 and 28 days at 0.5 mT affected NMDA receptor subunit protein levels, but not AMPA receptor subunit levels (Li et al., 2014a). In mice, exposure has been found to increase GluN2b expression after 28 days at 8 mT (Duan et al., 2014). Other findings relating to glutamate receptors have been reported: Salunke et al. (2014) found increased anxiety after exposure which could be ameliorated using an NMDA receptor antagonist and Manikonda et al. (2007) found decreased glutamate receptor binding after 90 days exposure, at 50 and 100 μT.

AMPA receptors mediate fast excitatory neurotransmission. Interestingly, *gria1* expression was increased after acute and repeated exposure in both the dorsal hippocampus and prefrontal cortex, although not consistently in both exposure groups. New AMPA receptor subunits are transcribed during learning for insertion into new synapses, and they are highly plastic and are continuously inserted into, and moved in and out of the synapse (Malinow and Malenka, 2002). The functional outcome of these changes in total hippocampal and prefrontal gene expression are difficult to predict. In the hippocampus, no effect of exposure at 100 μT was found on the GluA1 protein level, however, there were only three mice in the 300 μT group, where the measured levels varied greatly. Previously only one study has investigated effects of exposure (at 500 μT) and only on protein levels of the AMPA receptor subunits, finding no effect (Li et al., 2014a). For future studies, investigation of the GluA3 subunit would also be informative. The majority of AMPA receptors are composed of GluA1/2 or GluA1/3, with the majority of the

synaptic receptors being of the GluA1/2 type. Investigation of regional changes in receptor expression and composition after exposure would also be valuable because stress has been reported to have varying regional effects (Rosa et al., 2002).

NMDA receptors mediate slow excitatory neurotransmission. GluN1 is found in most NMDA receptors, and loss of GluN1 subunits does therefore indicate a loss of NMDA receptors. GluN1 forms heteromers with both GluN2a and GluN2b, alone or in combination. In this chapter no significant changes to NMDA receptor subunit levels were observed in the prefrontal cortex after acute exposure, while a decrease in *grin2b* expression was observed after repeated exposure. In the hippocampus, no changes in gene expression of the NMDA receptor subunits were seen, while the protein level of GluN1 was decreased after repeated exposure. This was not accompanied by changes in GluN2a; GluN2b levels were not measured due to time constraints. If no changes are found in GluN2a and GluN2b levels, investigations of the GluN2c and GluN2d subunits could be included, these are primarily expressed on interneurons (Monyer et al., 1994).

The more general investigation approach chosen in this chapter, looking at the overall mRNA and protein levels of the NMDA- and AMPA type ionotropic glutamate receptors should be seen as the initial study in a new line of studies. This could include investigation of regional expression levels and changes to phosphorylation of the various sites on the receptor subunits involved in regulation of receptor properties as mentioned above.

In summary, changes in gene expression of ionotropic glutamate receptors were found after both acute and repeated exposure, although the response differed. It must be considered that all changes were small in magnitude (12-20%), the effects were transient, and often a dose dependent sensitivity was seen. Statistical significance was in many cases only reached when using a less demanding t-test over a one-way ANOVA for multiple comparisons. Larger group sizes are needed for studies of effects on protein levels.

In addition to changes to glutamate receptor expression levels, *bdnf* and *ntrk2* expression was also found to be affected by MF exposure. One study has previously reported that exposure of healthy control mice lead to increased whole brain levels of BDNF after 3 weeks of exposure at 0.7 mT (Tasset et al., 2012). The MF induced changes to *bdnf* gene expression reported in this chapter were complex. In the dorsal hippocampus two hours acute exposure to 100 µT, but not 300 µT, was found to significant increase *bdnf* expression by 15%. After a delay of 7 days, *bdnf* expression was significantly increased by 70% in the dorsal hippocampus only after exposure to 300 µT. No change to *ntrk2* expression was found. After repeated exposure at 300 µT expression of both *bdnf* (26%) and *ntrk2* (13%) was significantly increased in the dorsal hippocampus. No differences in expression levels between exposure groups were seen 7 days later, or in the prefrontal cortex at either time point. Protein levels were not measured due to time constraints, but this would be very relevant to do.

In contrast to the indications of a trophic effect of exposure in terms of increased *bdnf* transcription, a decrease in proliferation in the SGZ was observed after exposure at 100 µT, but not 300 µT, for 5 days. A number of studies have investigated the effect of 50 Hz MF exposure at 1 mT on hippocampal neurogenesis, which have reported increases in proliferation, differentiation and survival of the new cells, and that these are functionally integrated into the tri-synaptic network which correlates with improved performance in the water maze and novel object recognition tests at the time of cell maturation in young mice (Cuccurazzu et al., 2010; Leone et al., 2014; Podda et al., 2014). Hence, the response to exposure at 100 and 300 µT is different than exposure at 1 mT. Although not significantly so, the number of BrdU-labelled cells in the 300 µT group was increased compared to the sham group.

Sensitivity of proliferation to both BDNF and NMDA receptor mediated signals have been reported (Seri and Alvarez-Buylla, 2002; Tabuchi, 2008). Repeated exposure induced increased *bdnf* gene expression in the 300 µT group, and decreased GluN1 protein expression in the 100

μT group. Whether the lower GluN1 protein level was associated with the lower number of immature neurons in the DG could be investigated by double-fluorescence for BrdU and GluN1. It is known that infusion of BDNF stimulates proliferation, while blocking of BDNF decreases the survival of cells. If changes in protein levels of BDNF are seen as a result of the increased *bdnf* gene expression observed after exposure at 300 μT then this could also impact these new neurons.

Regarding neuronal differentiation, Cuccurrazzu et al. (2010) have also shown (using a similar method to the one used in this chapter) that 95% of BrdU-labelled cells co-express DCX after 7 days exposure in 4-5 week old male C57Bl/6J mice. They also reported that MF exposure increased the number of immature DCX+ cells by 75% and the number of mature BrdU/NeuN double labeled cells 4 weeks later increased by 64%. Although this was not investigated here, future studies of <1 mT effects especially in light of the different response in proliferation to exposure at 100 μT would be informative.

Survival of the newly generated cells is dependent on stimulation (Zhao et al., 2008). If MFs affect synaptic plasticity and neuronal signalling through changes in glutamate receptor density and composition, this might impact the survival of these cells. The causal relationship between the two cannot be answered from the present experiments.

Microglia and astroglia are essential for synaptic plasticity, maintenance of synapses and for support of neurogenesis and they are highly sensitive to changes in the cellular environment. No studies appear to have investigated effects of power frequency MF exposure on glial cells in healthy mouse models *in vivo*.

In this chapter the regional density of microglia and astroglia was determined in the hippocampus. Slightly increased numbers of both microglia and astroglia were observed in the 300 μT group overall and in some regions compared to the sham exposed group. This was

statistically significant only for the total number of microglia and regionally in the *s.radiatum* and *s.moleculare.* For the total numbers this was only found using a non-parametric t-test for comparison of the sham and 300 µT groups. No statistically significant differences in astroglial numbers were found.

The microglial population can increase as a result of proliferation, or by migration to an injured site from an uninjured site of the brain, or by infiltration of circulating monocytes from the periphery (Wake et al., 2013). Investigation of microglial proliferation changes could be performed by double-immunohistochemistry of the BrdU-labelled brain sections for Iba-1 and BrdU. No evidence of injury to the hippocampus, which may attract microglia to the site, has been found in these experiments. Furthermore, visual inspection revealed no obvious differences in microglial morphology, that would reveal reactivity of the cells. However, proper morphological examination should be performed to be able to verify this. Measurements of alterations in Iba-1 density, cytokine secretion (such as IL1, IL6 and TNF) and surface protein expression (such as CD40, CD45 and CD68) would also be useful for investigation of reactivity of the microglia.

Microglia play important supportive roles in synaptic plasticity and hippocampal neurogenesis (Wake et al., 2009). The mice used in these experiments were exposed from the age of 4 to 5 weeks. Microglia play an important role in the formation and reorganisation of neuronal networks during development and adult life. The highest microglial density in the brain is observed in the second postnatal week, after which the density decreases rapidly, to reach a stable level from the age of 6 weeks that is maintained thoughout adult life (Nikodemova et al., 2015). It could be speculated that the increased microglial density may be a result of a MF induced decrease in the rate of apoptosis during this critical time. However, this should be investigated further before any conclusions to be drawn. Another explanation is that the increased microglial density is an indication of altered activity of the microglia, that in turn affect

synaptic plasticity and neurogenesis. Conversely, alterations in synaptic plasticity or neurogenesis could also affect microglial function. This could be investigated further by double-immunohistochemistry for microglial and synaptic markers, such as Iba-1 and PSD-95, to detect if the microglia contain more synaptic components due to alterations in their activity. The correlation between altered neurogenesis and microglial density could also be investigated more closely by inspecting the microglia in the SGZ and innermost part of the GCL. Since there appear to be no literature on effects of MF exposure on microglia *in vivo,* further studies are needed to understand the functional impact of the increased microglial density found after 5 days of repeated exposure.

To investigate the functional outcome of the cellular and molecular changes observed after exposure, mice were tested in the water maze for memory after exposure. No changes in performance were seen after acute or repeated exposure at 100 μT or 300 μT. The chosen protocol ensured a strong encoding of the platform position. Since no effect of exposure was detected using the current methodology, it would be relevant to investigate sensitivity of memory in different variations of the basic water maze test, for example by decreasing the number of learning trials and increasing the inter-trial interval to be able to detect more subtle impairments (van Praag et al., 1999b).

The experiments presented in this chapter fill out a gap in the knowledge of short-term MF induced effects on memory. Three other papers have reported a lack of effect on learning and memory after repeated exposure < 1mT but for periods of 14-90 days (Li et al 2014a and b and Cui 2012). In all three studies, learning in the water maze was tested, while only the two studies by Li et al (2014a,b) investigated effects on memory in the probe trial, but only one of the studies was in mice. Sienkiewicz et al. (1998) previously reported that exposure <1mT before daily testing in the RAM did not affect task performance.

Cuccurrazzu et al. (2010) showed that MF-induced neurogenesis affected learning and memory positively. On the contrary, ablation of neurogenesis using x-rays has been reported to affect performance in the water maze negatively (Snyder et al, 2005). In this chapter no differences in memory were found, after 5 days of exposure, at the same time-point when neurogenesis effects were investigated. Since a decrease in proliferation was found after exposure at 100 μT it would be very interesting to investigate this in the water maze 4-8 weeks later when these cells are matured and functionally integrated as in the studies by Leone et al. and Podda et al. (2014).

4.4.3 Summary

This chapter investigated whether the young brain is sensitive to 50 Hz MF exposure at flux densities < 1mT, with focus on cognitive function and plastic processes. An exposure system with a homogenous field, allowing mice to move freely was used for accurate exposure and minimized stress to the mice.

This is, to the best of my knowledge, the first *in vivo* studies in healthy rodents of the effects of exposure at 100 μT and 300 μT on hippocampal neurogenesis, glial cells, *bdnf* and synaptic density. Only a few studies have previously investigated effects on glutamate receptors and memory. These endpoints were chosen based on known high level of sensitivity of these to modulation and their central roles in many of the functions involved in learning and memory which would allow detection of modulating effects of exposure on many processes that would ultimately affect the cells and molecules of interest.

Some evidence of sensitivity of the young brain to MF exposure was found. Subtle complex changes in synaptic plasticity genes were observed after acute and repeated exposure. Proliferation was decreased after repeated exposure at 100 μT while the microglial density was increased after repeated exposure at 300 μT. Also a small decrease in *dlg4* gene expression was seen in the prefrontal cortex after repeated exposure. Exposure did not affect astroglia, or

memory. The latter is considered important because it suggests that the subtle changes do not cause a functional change.

While no major changes to the chosen endpoints were found, these experiments have identified a number of cellular and molecular endpoints for future studies of effects of MF < 1mT on cognitive function.

In this chapter the main focus has been on the hippocampus, although plasticity gene expression was also investigated in the prefrontal cortex. This region plays an important role in cognitive function. So working memory and changes to synaptic and cellular numbers in the PFC requires further investigation.

4.5 Conclusion

This chapter investigated if 50 Hz magnetic fields (MF) at magnetic flux densities <1 mT could stimulate molecular and cellular processes involved in cognitive function in young adult animals.

50 Hz MFs at 100 and 300 µT were found to affect plastic processes in the young mouse brain, but in a complex manner that differed from the reported findings at higher magnetic flux densities:

- Glutamate receptor subunits and BDNF signalling molecules were affected by exposures with regional and dose dependent differences.
- Proliferation was decreased after exposure at 100 µT for 5 days, but not after exposure at 300 µT.
- There was an overall increase in microglial density after exposure at 300 µT for 5 days.
- However, performance of a spatial memory task was not affected by acute or repeated exposure.

Chapter 5 Magnetic field effects on circadian rhythm control

5.1 Background

5.1.1 General introduction

Disturbances to circadian rhythms can have a great impact on both health and cognitive function (Lamont et al., 2007; Smarr et al., 2014). It has been proposed that MFs can disturb the circadian rhythm by affecting sleep quality (Akerstedt et al., 1999; Graham and Cook, 1999), melatonin levels (Stevens, 1987; Vanderstraeten et al., 2015) and via the cryptochrome protein (Vanderstraeten et al., 2015). Indications of 50 Hz MF effects on these parameters have been reported but no clear connections have been established. Additionally, a number of studies have reported findings which call for further investigation.

For example, a study of clock gene expression in human fibroblasts has reported that a magnetic flux density of 100 μT for 1 hour can induce rhythmic expression of the clock genes at a 24 hour rhythm in starved cells, and increase the gene expression levels in serum shocked cells (Manzella et al., 2015). Studies of whether this happens *in vivo* and in other cell types are needed.

CRY and PER form the negative loop of the transcriptional-translational feedback loop that is central in circadian rhythm control. It has been proposed that the CRY protein can be affected by MFs in mammals as in other organisms e.g. A.*thaliana* (Maeda et al., 2012), *E.coli* (Henbest et al., 2008) and *D.melanogaster* (Gegear et al., 2008, 2010). Such effects could potentially affect the transcriptional-translational feedback loop and therefore the circadian rhythm control. It is not possible to draw any conclusions on the basis of the published literature and this possibility is still being studied extensively.

Also, since light is the main zeitgeber of the circadian rhythm (Shigeyoshi et al., 1997), it seems logical that effects of MFs on the light response might also affect the circadian rhythm. No studies of this possibility appear to have been performed in mammals.

Two different approaches to study the effects of 50 Hz MFs on circadian rhythm control were chosen in this project. In the first, the effect of MF exposure on the response to light was investigated; and in the second, the effect of MF exposure on the rhythmic expression of the core clock genes (*cry1, cry2*, *per1, per2*, *bmal1* and *clock*) was investigated in the SCN, dorsal hippocampus and liver over a 24 hour period to investigate changes in rhythms and the differences between central and peripheral effects.

5.1.2 Sensitivity of the adrenal light response to power frequency MF exposure

If light reaches the intrinsically photoreceptive retinal ganglion cells (ipRGCs) in the dark period of the 12:12 light dark (LD) cycle, the cell membrane is depolarized leading to glutamate excretion in the SCN via the retinohypothalamic tract. This induces phosphorylation of CREB and its binding to CRE-containing promotor regions in light sensitive genes, e.g. *per1* and *per2,* in turn leading to a rapid increase in transcription of these genes (Albrecht et al., 1997; Naruse et al., 2004; Shearman et al., 1997).

A well-established protocol to study the light response consists of exposure of mice to a 30 minute light shock (LS) 2-4 hours into the dark period of the day, with assessment of the acute molecular response 30-60 minutes later, or the delayed behavioural response, days afterwards (Hughes et al., 2015). In addition to a strong response to the LS in the SCN the most rapid response is seen in the adrenal glands (Ishida et al., 2005). Significant increases in SCN and adrenal *per1* expression and in serum corticosterone levels have been reported 30-60 minutes after LS at ZT14-16 when the baseline expression of both is low but not at its lowest (Ishida et al., 2005; Kiessling et al., 2014, 2014; Loh et al., 2008; Pilorz et al., 2016; Shigeyoshi et al., 1997). The adrenal clock is regulated by both hormonal and autonomic mechanisms (Buijs et al., 2003;

Silver et al., 1996). Studies have shown that the increased corticosterone level in response to LS depends on splanchnic nerve stimulation and is independent of adrenocorticotropic hormone (ACTH) release from the pituitary gland (Ishida et al., 2005; Sollars et al., 2014). In addition to the acute changes in *per1* expression and corticosterone levels, nocturnal LS also induces sleep. This has been reported to be within 2-20 minutes after the onset of LS depending on the wavelength of the light and the balance between the responses in the SCN and the ventrolateral preoptic area (Pilorz et al., 2016). This protocol was chosen for investigations of effects of MF exposure on the nocturnal light response, allowing detection of modulating effects on both the light sensing in the eye, the propagation of the signal in the SCN and the signalling to the adrenal glands.

5.1.3 Sensitivity of clock gene expression to power frequency MF exposure

In the study by Manzella et al. (2015) presented above, MF exposure was reported to affect the rhythmic expression of clock genes in human fibroblasts. By combining this finding with the potential magnetosensitivity of mouse cryptochromes, the second part of the study presented in this chapter investigated effects of MF exposure on clock gene expression in the SCN, hippocampus and liver in terms of changes in expression levels, rhythmicity of clock gene expression and desynchronization between central and peripheral clocks. The SCN was chosen due to its orchestrating role as the master clock. Effects of exposure in the SCN would be propagated to the rest of the body's clocks which could then be detected in the hippocampus and liver. The hippocampus was included due to its central role in learning and memory formation and the known disruptive effect of circadian rhythm disturbances on many of its functions (Smarr, 2014). The liver clock has been extensively studied and a strong circadian rhythm is seen in its many physiological and metabolic functions. Both the hippocampal and the hepatic clocks have been reported to be more sensitive to chronic stress than the SCN clock (Christiansen et al., 2016). It is possible that a similar pattern of sensitivity would be seen in regards to MF exposure.

In the SCN, mRNA levels of *per1, per2* and *cry1* peak during the light phase of the day, while *bmal1* expression peaks during the dark phase (See Guilding and Piggins (2007) for further details). The peripheral clock gene expression is delayed compared to the SCN, with peak levels reported during the dark phase of the day in the hippocampus and liver (Chun et al., 2015; Jilg et al., 2010; Liu et al., 2007; Peirson et al., 2006). The rhythmic expression of the clock genes was evaluated in terms of changes in expression level and time of peak expression.

5.2 Experimental protocols

For investigating the effects of MF exposure on the response to light, mice were singly housed in a 12:12 hour LD rhythm and moved to the hatbox cages under DD conditions for 26 hours before LS and MF exposure. In the initial experiments using white light, LS and MF exposure was done at ZT16, in the later experiments at ZT14 (using blue light), for 30 minutes followed by a 30 minute period in the dark. There were 4 experimental groups: 1) LS-/MF- (no light shock, no magnetic field), 2) LS+/MF- (light shock, no magnetic field), 3) LS-/MF+ (no light shock, magnetic field), 4) LS+/MF+ (light shock, magnetic field) (Figure 5.1A). Mice were euthanized by CO_2 overdose in the hatboxes. The euthanized mice were moved to a procedure room and blood was collected by cardiac puncture prior to serum preparation. The adrenal glands, liver and dorsal hippocampus were collected for gene expression analysis and stored at -80^{o}C.

For investigations of the effects of MF exposure on core clock gene expression, mice were housed under standard 12:12 hours LD conditions in groups of 4 mice (Figure 5.1B). The mice were moved to the hatbox cages 12 hours before exposure and kept under DD conditions until the end of the experiment. Two groups of mice were included in this experiment, one group with exposure to the MF (+MF) and one group without MF exposure (-MF). The duration of MF exposure was 24 hours starting at the time of the subjective day (ZT0), this experiment was repeated 6 times, euthanizing mice at ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20 on the day after the end of exposure. The mice were kept in the dark until they were killed by CO_2 overdose and all dissections, except the brain, were carried out under red light conditions. The liver, SCN and dorsal hippocampus were dissected and frozen for gene expression analysis.

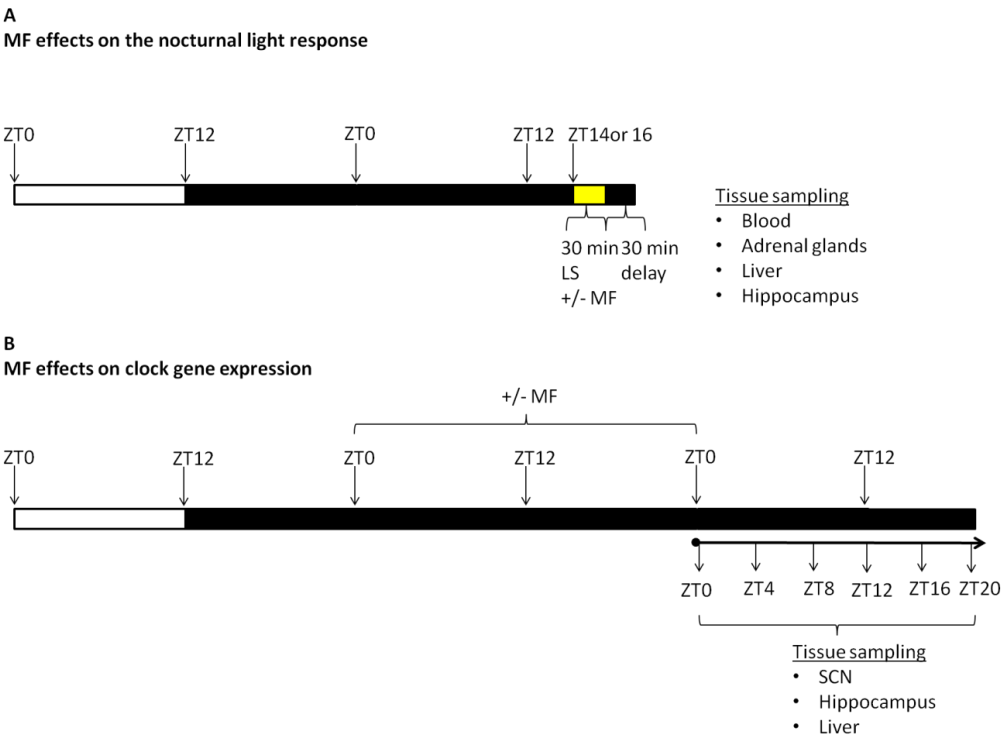


Figure 5.1 Two protocols were used. A 48 hour protocol was used for studies of MF effects on the nocturnal light response (A). Mice were housed under 12:12 LD conditions until 26-28 hours before exposure when they were moved to the hatboxes in constant darkness for habituation. At ZT14 or 16, single mice were exposed to a 30 minutes (white or blue) light shock (LS) ± MF exposure at 300 µT or 580 µT. Thirty minutes after the end of exposure the mice were euthanized by CO_2 overdose and blood, adrenals, liver and hippocampus were collected for further analysis. In the experiments using blue light, the behaviour of the mice was recorded during and after LS and MF exposure. For studies of MF effects on clock gene expression (B), mice were housed under 12:12 LD conditions until 12 hours before initiation of exposure where they were moved to the hatboxes in groups of 4 in constant dark. At ZT0 mice were exposed to a MF at 580 µT for 24 hours, a concurrent, unexposed group served as sham control. This was repeated 6 times when mice were euthanized at delays from 0-20 hours using CO_2.

5.2.1 Details of statistical analyses

Statistical comparison of experimental groups was carried out using two-way ANOVA for the effects of LS and MF exposure in the experiments using protocol A (Figure 5.1), or for MF exposure and time point in the experiment using protocol B. *Post hoc* testing was done by Bonferroni's *post hoc* test. For comparison of two groups an unpaired t-test or the Mann-Whitney test was used, depending on whether the data followed a Gaussian distribution, or not.

5.3 Results

5.3.1 Magnetosensitivity of the light response

8-12 week old male C57Bl/6J mice were used. These were housed in groups of four until 24 hours before exposure when they were placed individually in the compartments of the hatboxes.

5.3.1.1 Experiment to test sensitivity of method to measure the stress response

An swim stress experiment was performed to test the persistency of the elevated corticosterone concentration after stress, as well as the sensitivity of the corticosterone ELISA kit. Mice were housed in constant darkness for 26 hours before a 1 minute swim in a pool of water (20^{o}C). Groups of 3 mice were euthanized by CO_2 overdose 15, 30 or 60 minutes after the swim. At all three time points, an equally high increase in serum corticosterone was seen compared to the two control mice, increasing from 30 ng/ml to 150 ng/ml on average (Figure 5.2). The control mice were handled in the same way as the swim stress mice, but were not exposed to the swim stress.

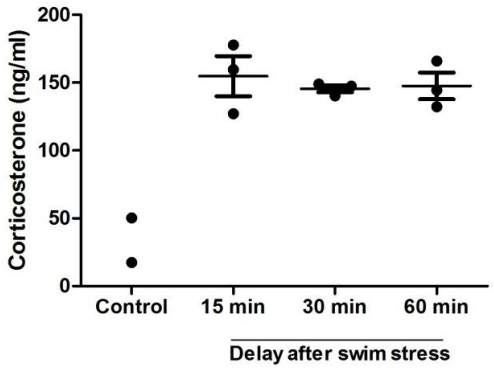


Figure 5.2 Experiment to test sensitivity of method to measure the stress response. An experiment was first performed to test the sensitivity of the ELISA kit for corticosterone measurements and the persistency of the stress response. 3 groups of 3 mice were subjected to swim stress in a pool filled with water for 1 minute. 15, 30 and 60 minutes after the stress, serum corticosterone levels were approximately 150 ng/ml in all three groups compared to levels < 50 ng/ml in the two control mice. Results are presented as mean ± SEM.

5.3.1.2 Exposure to 300 µT in combination with white light

To determine the effect of acute MF exposure at 300 µT with or without exposure to white light at 150 lux, serum corticosterone levels and adrenal *per1* expression were investigated 30 minutes after a 30 minute LS in groups of 8-9 mice (protocol A, as described in Figure 5.1). Measurements of serum corticosterone concentrations showed no significant differences between groups. The lowest corticosterone levels were observed in the LS-/MF- and LS-/MF+ groups at 6.92 ng/ml and 4.73 ng/ml respectively. Slightly higher corticosterone levels were measured in the two groups exposed to LS of 13.88 ng/ml in the LS+/MF- group and 27.97ng/ml in the LS+/MF+ group (Figure 5.3A). One mouse in the LS-/MF+ group showed an abnormally high corticosterone level of 223 ng/ml and was excluded from the analysis. A two-way ANOVA was conducted for the effects of MF with (+) and without (-)and LS with (+) and without (-). A significant effect of LS was observed as an increase in corticosterone ($p<0.05$), but no significant differences between the groups were found when using Bonferroni's *post hoc* test to compare LS+/MF+ to LS-/MF+ or LS+/MF- to LS-/MF-. No effect of MF exposure or an interaction between LS and MF were found. See Table 5.1 for details.

Adrenal *per1* expression was investigated in the same mice. No significant differences were seen between any of the groups using a two-way ANOVA for comparison as above (n=7-8). The results are presented relative to the mean expression in the LS-/MF- group, and the same level was observed in all four groups, indicating that exposure to a MF and/or a 30 minute LS to white light at 150 lux had no significant effect on adrenal *per1* expression (Figure 5.3B). See Table 5.1 for details of the statistical analysis.

Overall, a very small significant increase in corticosterone was found after LS at 150 lux. There were no other significant effects of LS and MF exposure at 300 µT for 30 minutes either together or on their own on either serum corticosterone levels or adrenal *per1* expression.

5.3.1.3 Exposure to 300 µT in combination with blue light

Since only a small effect of white light at 150 lux was found, the protocol was changed to use a blue light (blue LS) (457 nm) at 700 lux. The MF was kept at 300 µT. Low corticosterone levels (15-80 ng/ml) were observed in all groups (Figure 5.3C), although higher levels were found in the groups exposed to blue LS compared to those not exposed to blue LS (n=8). A two-way ANOVA for effects on MF and blue LS showed no interaction between blue LS and MF. However, there was a significant increase in corticosterone as a result of blue LS ($p<0.01$), Bonferroni's *post hoc* test showed that this was only significant in combination with MF exposure ($p<0.01$), while MF exposure alone did not affect the corticosterone level significantly. See Table 5.1 for details of the statistical analysis.

Differences in adrenal *per1* expression were also observed between groups, with a 30% increase after LS in the presence of a MF compared to LS-/MF+, while LS alone increased adrenal *per1* expression by 40% compared to LS-/MF-. The two-way ANOVA used to evaluate the effects of blue LS and MF exposure showed no interaction between blue LS and MF (Figure 5.3D). However, a significant effect of blue LS alone, and in combination with MF was found. See Table 5.1 for details of the statistical analyses.

Although not significantly different, a 30% decrease in *per1* expression was found after MF exposure in the blue LS-/MF+ group compared to the blue LS-/MF- group (n=8).

Overall, it was found that combined exposure to the MF at 300 µT and the blue LS at 700 lux for 30 minutes significantly increased both serum corticosterone levels and adrenal *per1* expression. Exposure to the blue LS alone significantly increased adrenal *per1* expression, while exposure to the MF alone resulted in a non-significant decrease in *per1* expression; corticosterone levels were not significantly changed by either treatment alone.

5.3.1.4 Exposure to 580 µT in combination with blue light

To determine the effect of acute MF exposure at a higher flux density of 580 µT with or without exposure to blue light at 700 lux, serum corticosterone levels and adrenal *per1* expression were investigated 30 minutes after a 30 minute LS in groups of 8 mice (protocol A, as described in Figure 5.1). In addition, the acute sleep response was also investigated.

5.3.1.4.1 Corticosterone and per1 responses

As seen previously, significant increases in serum corticosterone (Figure 5.3E) and adrenal *per1* levels were found after blue LS (Figure 5.3F). In both cases (MF- and MF+), significant effects of blue LS were found using a two-way ANOVA for LS and MF, while no interaction between the two factors was seen.

For corticosterone, blue LS caused significant increases alone and in combination with MF. The two-way ANOVA further showed that MF exposure alone had no effect on corticosterone. See Table 5.1 for details of the statistical analysis.

For adrenal *per1* expression, a two-way ANOVA for blue LS and MF effects showed no interaction between the two factors. However, significant effects of both MF and LS were found. The two-way ANOVA showed that MF exposure significantly decreased adrenal *per1* expression, but Bonferroni's *post hoc* test did not confirm this (see Table 5.1 for details). Blue LS was found to cause significant increases in adrenal *per1* both alone and in combination with MF exposure ($p<0.01$).

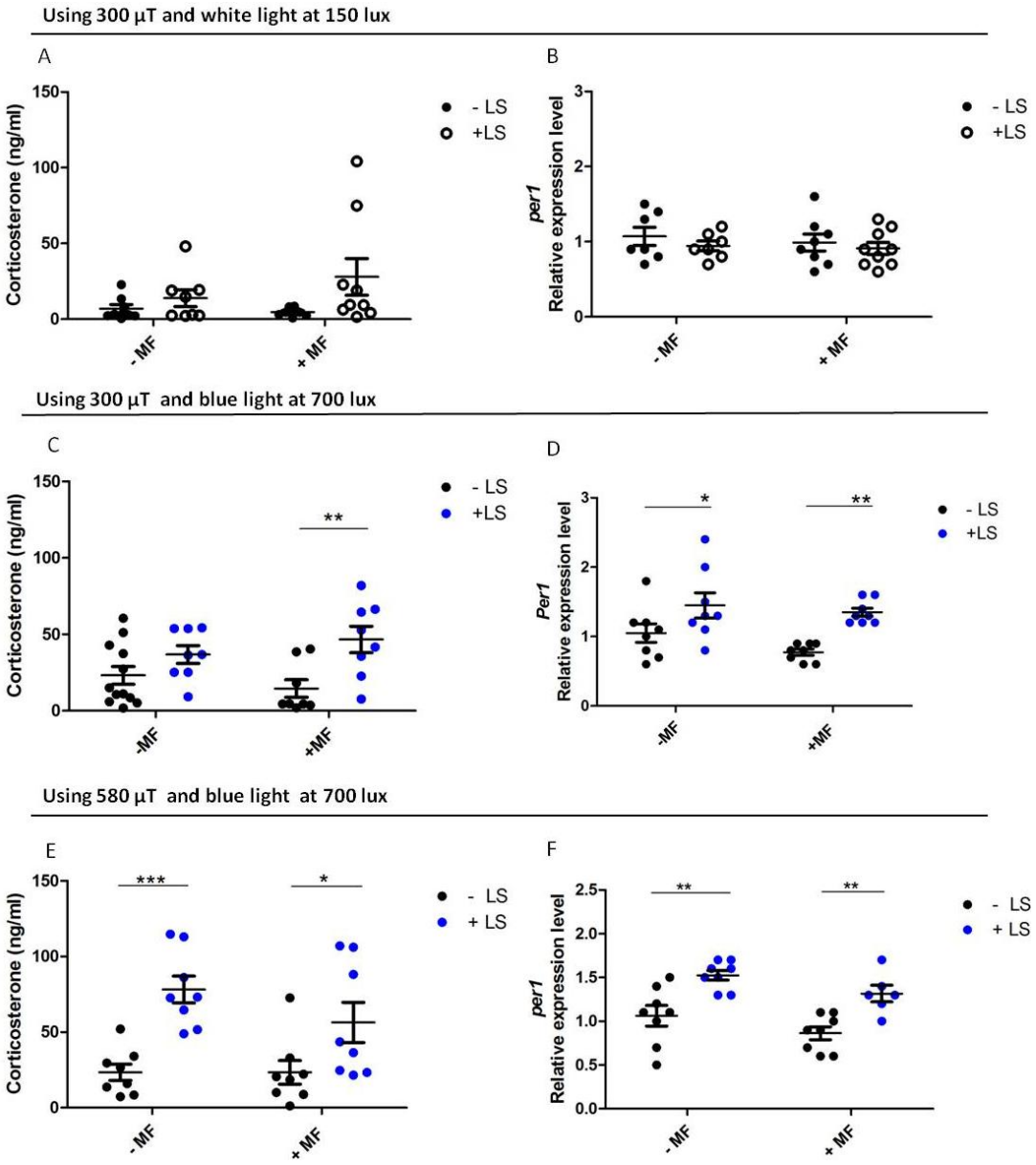


Figure 5.3 Magnetosensitivity of the light shock response. Mice were singly housed in a 12:12 hour LD cycle until the last 24 hours before the experiment when they were housed in constant dark for 26-28 hours. At ZT14 or ZT16 a 30 minute LS to either white or blue light ± MF was performed. 30 minutes later the mice were terminated using a CO_2 overdose and blood and adrenal glands were collected. In the first experiment white light at 150 lux ± exposure to a 300 µT 50 Hz MF was used (A,B). No significant differences in serum corticosterone (A) were found, while a small significant effect of LS was found on adrenal *per1* gene expression, but only by two-way ANOVA (B) (n=7-9). In the second experiment blue light (457nm) at 700 lux ± exposure to a 300 µT 50 Hz MF was used. Here a significant effect of blue LS+/MF+ was found on both corticosterone (C) and *per1* (D) and a significant effect of blue LS+/MF- was found on *per1* expression only (n=7-9). In the third experiment blue light at 700 lux was used ± exposure to a 580 µT 50 Hz MF. Significant increases in corticosterone (E) and adrenal *per1* gene expression (F) were seen in the blue LS+/MF-

and the blue LS+/MF+ groups compared to LS-/MF- and LS-/MF+ respectively (n=8). Exposure to MF significantly decreased *per1* expression, this was detected only by two-way ANOVA. Results are presented as mean ± SEM. Statistical comparison was carried out using a two-way ANOVA for white or blue LS and MF effects, *p<0.05, **p<0.01, ***p<0.001.

5.3.1.4.2 Behavioural responses

Like corticosterone levels, sleep is a sensitive indicator of circadian phase. Thus activity and sleep behaviour were monitored in the mice during the course of the experiment in the hatboxes (from onset of LS until 30 minutes after LS, when blood, adrenal glands, liver and dorsal hippocampus were dissected).

At the onset of exposure, 3 mice in the blue LS+/MF- group were sleeping, as was one mouse in the LS+/MF+ and one in the LS-/MF-. The mice exposed to LS all woke up at the onset of LS. Excluding the mice that slept at the onset of exposure did not affect the overall results (data not shown). In terms of sleep response to the blue LS, the variation within the groups was fairly large (Figure 5.4A). In the blue LS+/MF- group all mice fell asleep during the LS, with the latency to sleep varying from 8.5 minutes to 25.5 minutes. Half of the mice in the blue LS-/MF- group also slept during the 30 minute exposure period, while only 2 mice in the blue LS-/MF+ exposure group fell asleep. In the blue LS+/MF+ group 88% of the mice slept during the exposure period with latencies to sleep between 5.4 and 22 minutes. The average sleep latency was 17.17 ± 2.2 minutes in the LS+/MF- group and 16.91 ± 3.07 minutes in the LS+/MF+ group. Using a 2-way ANOVA to compare effects of MF and blue LS on sleep latency, no significant effects were found. See Table 5.1 for details. Since a number of mice were observed to go to sleep at the end of the blue LS, the persistency and delay of the sleep response was investigated in terms of sleep duration, during the exposure period and for the 30 minute period after the exposures (Figure 5.4B). A 2-way ANOVA was used to investigate effects of LS and MF on sleep duration, and no interaction between blue LS and MF was found. Blue LS significantly increased the sleep

duration both alone and in combination with MF exposure ($p<0.001$), while MF alone had no significant effect on sleep duration. See Table 5.1 for details of the statistical analysis.

The distances moved during exposure and 30 minutes after were measured as another means to detect potential differences in the response to exposure (Figure 5.4C). An immediate increase in movement was seen at the onset of LS in both groups, while no change in activity was detected in the LS-/MF- and LS-/MF+ groups. 12 minutes into the MF ± LS exposure, the activity of the LS exposed mice dropped to a lower level than in the other two groups. A repeated measures two-way ANOVA was used to compare the four experimental groups over time. Significant effects of both time and group, and an interaction, were found (See Table 5.1 for details). When comparing the four exposure groups at each of the 1 minute intervals by Bonferroni's *post hoc* test the significant differences were found to be transient. The distance moved at 20 and 25 minutes into the LS was significantly higher in the blue LS+/MF- and blue LS+/MF+ when compared to the blue LS-/MF- group, while this was not seen before, between or after LS.

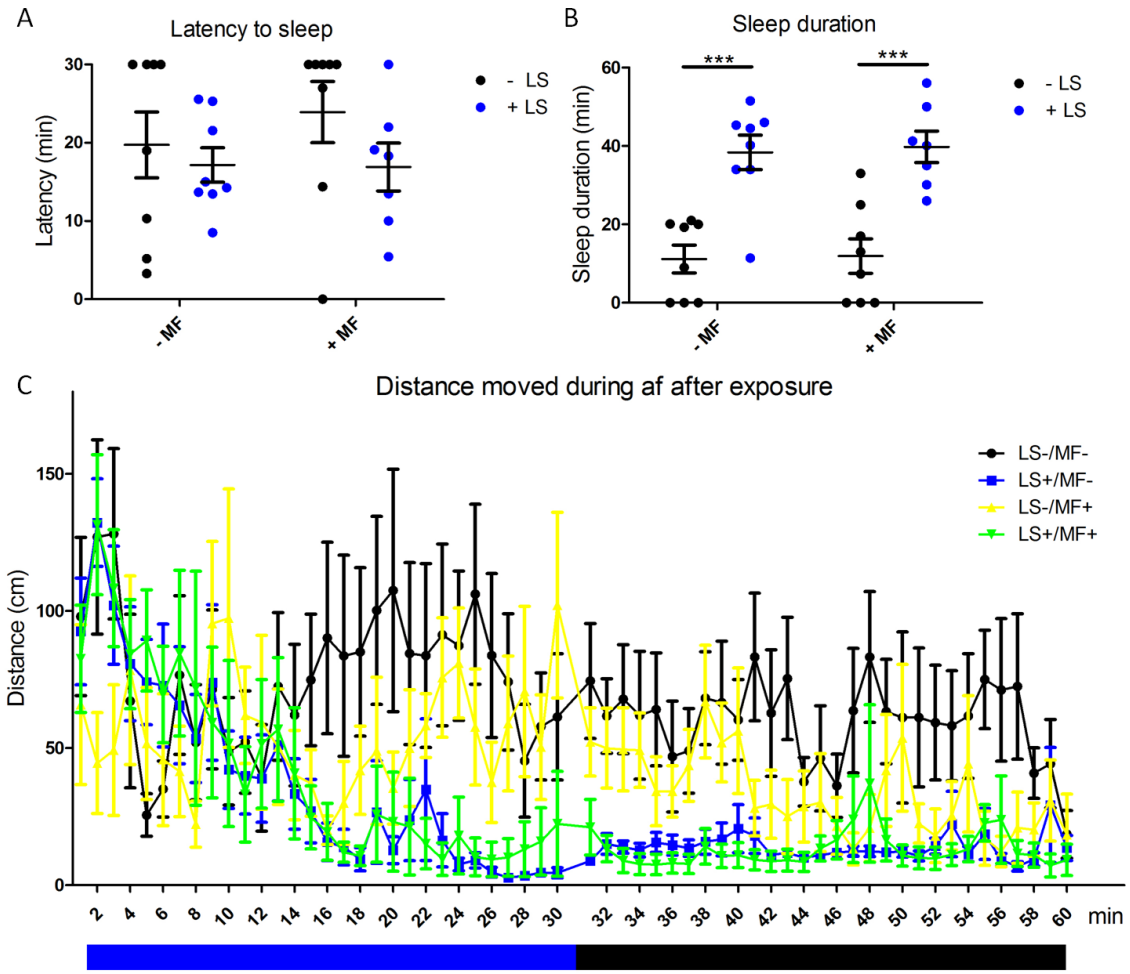


Figure 5.4 Behavioural response to blue LS and MF exposure at 580 µT. Latency to sleep, sleep duration and distance moved was recorded during the 30 minute blue LS ± MF exposure at ZT14 (blue bar) and for 30 minutes after exposure (black bar). Variation in latency to sleep after the start of exposure, and a greater tendency to sleep was observed in the groups exposed to blue LS, compared to their control groups (A). Significantly longer sleep duration was seen in the blue LS groups compared to their control groups (B). The movement of the mice was tracked during and after exposure (C), and the mice in the blue LS+/MF+ and blue LS+/MF- groups were found to move a greater distance after initiation of LS. This gradually reduced to the activity levels shown by the blue LS-/MF- and blue LS-/MF+ exposed groups at 12 minutes. Data are presented as mean ± SEM. Statistical comparison was carried out using a two-way ANOVA for blue LS and MF effects, or for exposure group and time in figure (C), ***p<0.001.

Table 5.1 Details of the statistical analyses of effects of MF ± LS on the adrenal glands

	Two-way ANOVA	**interaction**	**Light**	**MF**	**Bonferroni's *post hoc* test**
300 µT and white light	Adrenal *per1*	F(1,27)=0.07 p=0.79	F(1,27)=1.10 p=0.30	F(1,27)=0.35 p=0.56	
	Serum corticosterone	F(1,29)=1.24 p=0.27	F(1,29)=4.27 ***p=0.048**	F(1,29)=0.66 p=0.42	LS- vs LS+ MF-: p>0.05 MF+: p>0.05
300 µT and blue light	Adrenal *per1*	F(1,28)=0.54 p=0.47	F(1,28)=16.77 *****p=0.0003**	F(1,28)=2.48 p=0.13	LS- vs LS+ **MF-:* p<0.05** **MF+: **p<0.01**
	Serum corticosterone	F(1,32)=1.91 p=0.18	F(1,32)=11.79 ****p=0.002**	F(1,32)=0.01 p=0.93	LS- vs LS+ MF-: p>0.05 **MF+: **p<0.01**
580 µT and Blue light	Adrenal *per1*	F(1,26)=0.00 p=0.96	F(1,26)=26.52 *****p<0.0001**	F(1,26)=5.26 ***p=0.03**	LS- vs LS+ **MF-:** p<0.01** **MF+: **p<0.01** MF- vs MF+ LS-: p>0.05 LS+: p>0.05
	Serum corticosterone	F(1,28)=1.36 p=0.25	F(1,28)=22.21 *****p<0.0001**	F(1,28)=1.37 p=0.25	LS- vs LS+ **MF-:*** p<0.001** **MF+: *p<0.05**
	Latency to sleep	F(1,27)=0.41 p=0.53	F(1,27)=1.9 p=0.18	F(1,27)=0.32 p=0.58	
	Sleep duration	F(1,27)=0.01 p=0.94	F(1,27)=0.44.51 *****p<0.0001**	F(1,27)=0.07 p=0.80	LS- vs LS+ **MF-:*** p<0.001** **MF+: ***p<0.001**
	Repeated measures two-way ANOVA	**Interaction**	**Exposure group**	**Time**	**Bonferroni's *post hoc* test**
	Distance	F(177,1652)=1.41 *****p=0.0006**	F(3,1652)=5.77 ****p=0.0033**	F(3,1652)=4.82 *****p<0.0001**	MF-/LS- vs MF-/LS+ **20 min *p<0.05** **25 min*p<0.05** MF-/LS- vs MF+/LS- n.s. MF-/LS- vs MF+/LS+ **25 min *p<0.05**

A combined exposure to the MF at 580 µT and blue LS at 700 lux for 30 minutes significantly increased both serum corticosterone levels and adrenal *per1* expression. Exposure to the blue LS alone significantly increased both endpoints, and exposure to the MF alone resulted in a significant decrease in *per1* expression, but had no effect on corticosterone. The blue LS also induced a temporary period of increased movement in the mice, and this was followed by significantly longer sleep duration: MF alone did not engender any behavioural changes.

5.3.2 Magnetosensitivity of clock gene expression

In the second part of these experiments the effects of MF exposure on selected core clock genes were investigated after an acute, 30 minute exposure and after a 24 hour exposure. After acute MF exposure, *per1, cry1* and *cry2* gene expression was measured in the adrenal glands, dorsal hippocampus and liver. After 24 hours MF exposure, changes in the rhythmic expression of *per1, per2, cry1, cry2, clock* and *bmal1* were investigated in the SCN, dorsal hippocampus and liver over a 24 hour period. 4-5 months old male C57Bl/6J mice were used. These were housed and exposed in groups of four.

5.3.2.1 Clock gene expression in adrenals, liver and hippocampus after acute MF exposure and blue LS

The adrenal glands, liver and hippocampus were collected from the mice in the groups described in Section 5.3.1 (Protocol A in Figure 5.1). The expression of *per1*, *cry1* and *cry2* was investigated for acute effects of exposures to MF at 300 or 580 µT alone or in combination with blue LS (Figure 5.5 and 6).

Significant effects of MF and blue LS were found on *per1* expression in the adrenal glands as presented in Figure 5.3. In the liver and dorsal hippocampus, no effects of exposure to blue LS or MF, at 300 µT (Figure 5.5) and 580 µT (Figure 5.6), alone or in combination, were found. There were also no effects of LS or MF exposures on gene expression of *cry1* and *cry2* in the three tissues. However, there was a near significant interaction between the two factors on *cry1* expression in the liver after exposure at 580 µT ($p=0.052$), when this was investigated further by Bonferroni's *post hoc* test a significantly higher expression was found in the blue LS+/MF+ group compared to the blue LS+/MF- group ($p<0.05$). See Table 5.2 for details of the statistical analyses.

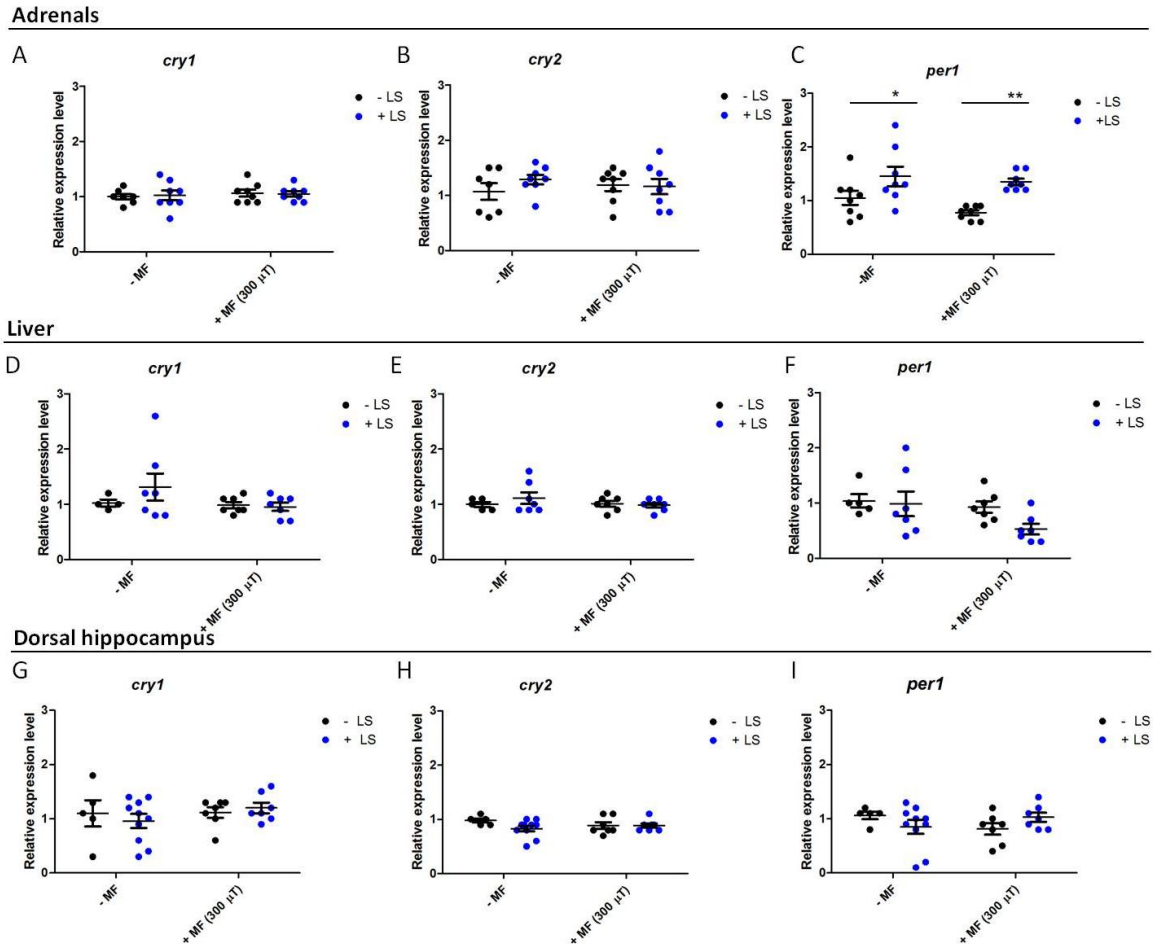


Figure 5.5 Clock gene expression in adrenal glands, liver and hippocampus after MF exposure at 300 µT ± blue LS. *per1, cry1* and *cry2* expression levels were measured in the adrenal glands, liver and hippocampus after exposure to a MF at 300 µT in the presence or absence of blue LS at 700 lux. No effect of blue LS on *per1* expression was observed in the liver or hippocampus, but significant changes were observed in the adrenal glands. No effect of MF and blue LS alone or in combination was found on the expression levels of *cry1* or *cry2* in either of the three tissues. Data are presented as means ± SEM. Statistical comparison was carried out using a two-way ANOVA of LS and MF effects, *p<0.05, **p<0.01. Note that data in (C) have also been presented in Figure 5.3.

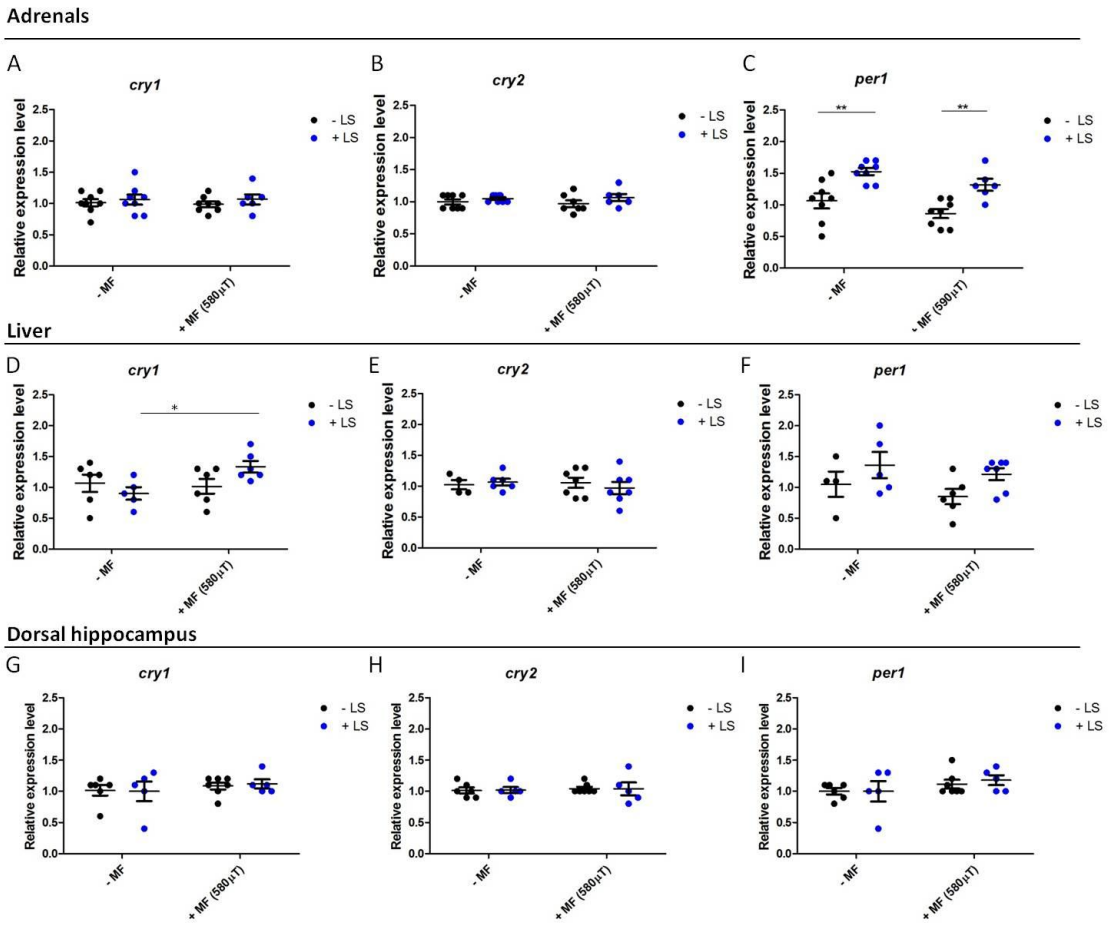


Figure 5.6 Clock gene expression in adrenal glands, liver and hippocampus after MF exposure at 580 µT ± blue LS. *per1, cry1* and *cry2* expression levels were measured in the liver, hippocampus and adrenal glands after exposure to 580 µT in the presence or absence of blue LS at 700 lux. No effects of blue LS on *per1* expression were observed in the liver or hippocampus, but significant increases were observed in the adrenals, while a significant decrease was observed after MF exposure alone. No effect of MF and blue LS alone or in combination was found on the expression levels of *cry1* or *cry2* in the adrenal glands and dorsal hippocampus. There was a near significant interaction between the two factors on *cry1* expression in the liver (p=0.052). Data are presented as means ± SEM. Statistical comparison was carried out using a two-way ANOVA of LS and MF effects, * p<0.05, **p<0.01. Note that data in (C) have also been presented in Figure 5.2.

Table 5.2 Details of statistical analysis of effects of MF +/- blue LS on clock gene expression

Tissue	MF exposure level	Gene	interaction	Blue LS	MF	Bonferroni *post hoc* test, comparing ± blue LS:
Adrenal gland	**300 µT**	*Cry1*	F(1,27)=0.08, p=0.78	F(1,27)= 0.01, p=0.92	F(1,27)= 0.45, p=0.51	
		Cry2	F(1,27)= 0.99, p=0.33	F(1,27)= 0.62, p =0.44	F(1,27)= 0.00, p=0.97	
		Per1	F(1,28)= 0.54, p =0.47	F(1,28)=16.77, p=**0.0003*****	F(1,28)= 2.48, p=0.13	**+MF p<0.01**** **-MF p<0.05***
	580 µT	*Cry1*	F(1,26)=0.05, p=0.83	F(1,26)= 0.94, p=0.34	F(1,26)=0.0, p=0.88	
		Cry2	F(1,26)=0.3, p=0.59	F(1,26)=3.06, p=0.09	F(1,26)= 0.02, p=0.89	
		Per1	F(1,26)=0, p=0.96	F(1,26)=26.52, **p<0.0001*****	F(1,26)=5.26, p**=0.03***	**-MF p<0.01**** **+MF p<0.01****
Liver	**300 µT**	*Cry1*	F(1,21)= 1.06, p=0.31	F(1,21)=0.72, p=0.41	F(1,21)=1.65, p=0.21	
		Cry2	F(1,22)= 1.04, p=0.32	F(1,22)= 0.37, p=0.55	F(1,22)= 0.67, p=0.43	
		Per1	F(1,22)= 1.3, p=0.27	F(1,22)=2.25 p= 0.15	F(1,22)=3.53, p=0.07	
	580 µT	*Cry1*	F(1,19)= 4.3, p=0.05	F(1,19)= 0.41, p=0.53	F(1,19)= 2.7, p=0.12	Bonferroni's *post hoc* test for -MF vs +MF: **+LS *p<0.05** -LS: n.s.
		Cry2	F(1,20)= 0.54, p=0.47	F(1,20)= 0.06, p=0.80	F(1,20)= 0.13, p=0.72	
		Per1	F(1,18)= 0.03, p=0.86	F(1,18)= 4.8, p**=0.04***	F(1,18)= 1.26, p=0.28	n.s
Dorsal hippocampus	**300 µT**	*Cry1*	F(1,25)= 0.63, p=0.43	F(1,25)= 0.04, p=0.85	F(1,25)= 0.8, p=0.38	
		Cry2	F(1,25)= 1.98, p=0.17	F(1,25)= 1.98, p=0.17	F(1,25)= 0.13, p=0.72	
		Per1	F(1,25)= 3.37, p=0.08	F(1,25)= 0.00, p=0.99	F(1,25)= 0.08, p=0.73	
	580 µT	*Cry1*	F(1,19)= 0.07, p=0.79	F(1,19)= 0.01, p=0.93	F(1,19)= 0.99, p=0.33	
		Cry2	F(1,19)= 0.00, p=0.96	F(1,19)= 0.00, p=0.99	F(1,19)= 0.16, p=0.70	
		Per1	F(1,19)=0.12, p=0.73	F(1,19)= 0.12, p=0.73	F(1,19)= 2.42, p=0.14	

In summary, there were no significant effects of acute exposure to blue LS or MF alone or in combination on *per1* expression in the liver or dorsal hippocampus, or on *cry1* and *cry2* expression in the adrenal glands or dorsal hippocampus. However, in the liver a near significant interaction between MF exposure at 580 µT and blue LS was found on *cry1* expression, Bonferroni's *post hoc* test revealed that the increased *cry1* expression in the blue LS+/MF+ compared to blue LS+/MF- was statistically significant.

5.3.2.2 Clock gene expression in the SCN, liver and hippocampus after 24 hours MF exposure

Gene expression of the core clock genes *per1, per2, cry1, cry2, clock* and *bmal1* was measured in the SCN, liver and dorsal hippocampus in mice exposed for 24 hours to a 50 Hz MF at 580 µT (Protocol B in Figure 5.1). Tissues were sampled at 6 different time points 4 hours apart after the end of exposure to detect differences in the expression of the core clock genes between control mice that were not exposed to the MF (-MF) and mice exposed to the MF (+MF) over the course of a day. The mice were housed in constant darkness in the hatboxes to avoid the strong zeitgeber effect of light on clock gene expression.

5.3.2.2.1 The expression of the core clock genes in the SCN was not affected by MF exposure

In the SCN, no strong rhythm in gene expression was observed in any of the genes (n=3-8), and a two-way ANOVA for MF and time revealed only subtle and transient effects (Figure 5.7).

A significant effect of time point was found on *per1* and *per2* expression, with peak expression levels at ZT12 (See Table 5.3 for details of the statistical analyses). The rhythm of *per1* expression appeared to be advanced in the MF exposed group compared to the unexposed group, however when the expression within the group was inspected, one outlier was found to account for the apparent peak at ZT8 in the +MF group. A lower *per1* expression (45%) at ZT12 was seen in the +MF group compared to the -MF group (Mann-Whitney test at ZT12, -MF vs. +MF $p<0.05$). At ZT16, no differences were observed between the exposure groups.

cry2 was expressed at the same level at all six time points in both exposure groups, except at ZT8 when a much higher expression level was detected in the MF exposed group. A two-way ANOVA revealed an interaction between MF and time point ($p<0.05$). However, the high average expression level at ZT8 was caused by two samples with much higher expression levels than in the remaining six samples in the group (unpaired t-test at ZT8, -MF vs. +MF $p=0.10$).

In both exposure groups, very little variation in expression of *cry1, bmal1* and *clock* was seen over the course of the day. However, *bmal1* expression varied greatly at ZT20 in the -MF group, but this was caused by two mice with high expression levels and three mice with low expression levels.

Although no overall effect of MF exposure was detected on gene expression in the SCN, there appeared to be time specific differences between the two exposure groups in *per1, per2* and *clock* at ZT12, *cry2* at ZT8 and in *bmal1* at ZT20, this was investigated further by comparison of the groups using a Mann-Whitney t-test. No significant differences between the groups were found other than on *cry2* at ZT8.

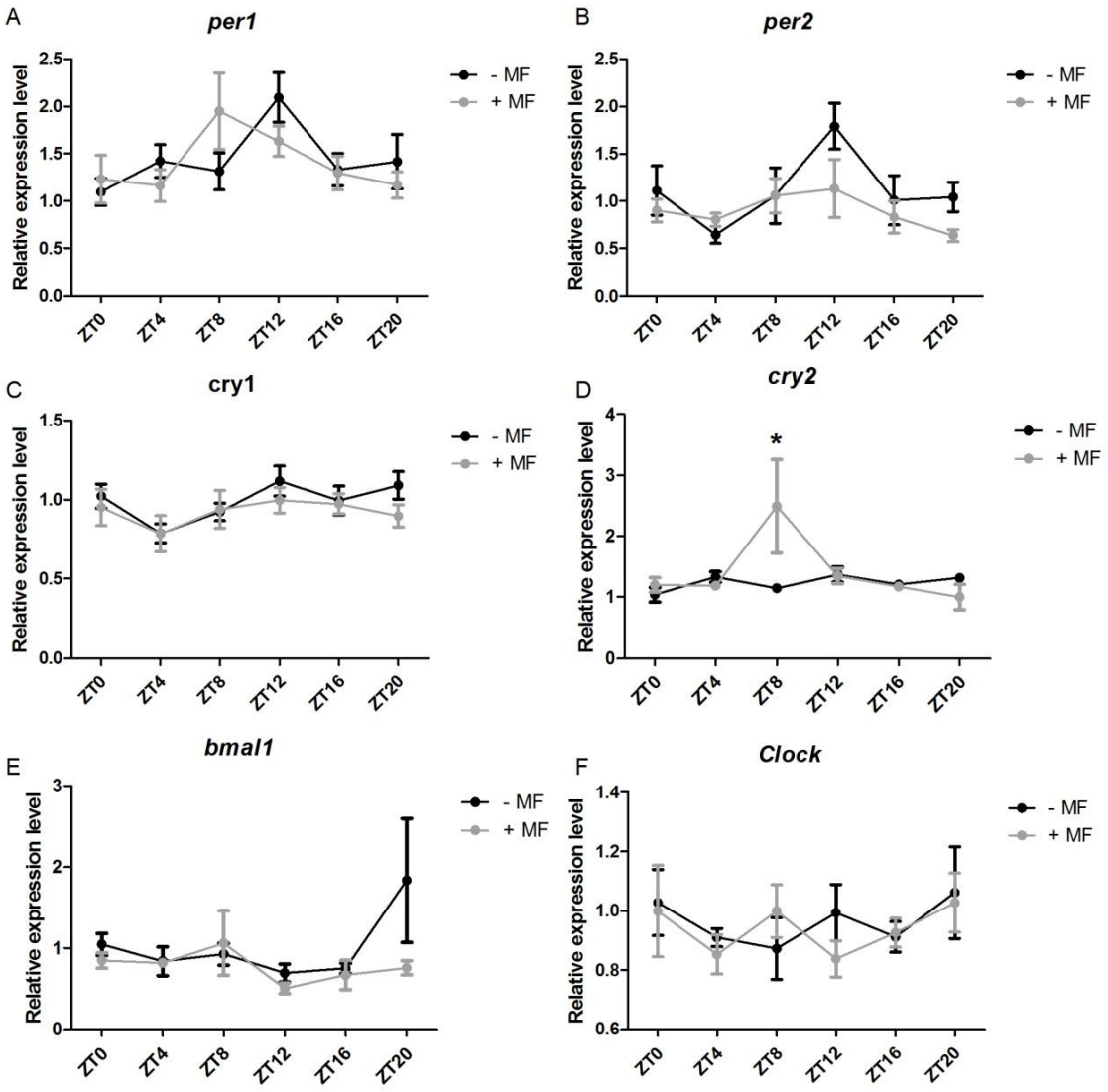


Figure 5.7 Clock gene expression in the SCN after MF exposure at 580 µT for 24 hours. *per1, per2, cry1, cry2, bmal1* and *clock* gene expression levels were measured at 4 hour intervals on the day following 24 hours of MF exposure at 580 µT in the dark. Minor changes in gene expression levels were detected over the course of the day. Indications of differences between the MF exposed and the unexposed controls were observed, however, statistical analysis revealed no significant differences except a transient increase in *cry2* expression at ZT8. Data are presented as mean ± SEM. Statistical comparison was carried out using a two-way ANOVA for investigation of effects of MF exposure and time point on expression levels, and using Mann-Whitney t-test for comparison of groups at single time points. *p<0.05.

5.3.2.2.2 MF exposure induced transient changes in core clock gene expression in the liver

In the liver, changes in expression level of the clock genes were observed over the course of the day in both exposure groups (n=3-7) (Figure 5.8). The expression of *per1* and *bmal1* increased 10-15 fold from the lowest to the highest with opposite phases, *per1* expression peaked at ZT12, whereas *bmal1* peaked at ZT0. The changes in *per2* expression were smaller than in *per1*, but a peak in *per2* gene expression levels was observed at ZT16, while less marked changes in expression levels of *cry1, cry2* and *clock* were seen over the course of the day.

Two-way ANOVA was used to investigate the effects of MF exposure and timepoint on gene expression. A significant interactions between the two factors was found on expression of *bmal1* ($(F5,51)=4.29$ $p<0.01$, Bonferroni's *post hoc* test ZT16 -MF vs +MF $p<0.01$) and *clock* $(5,52)=2.47$ $p<0.05$, Bonferroni's *post hoc* test MF- vs MF+ n.s. at all time points). There was no main effect of MF exposure, while there was a significant effect of time point on expression of *per1, per2, clock* and *bmal1.* (See Table 5.3 for details).

Since there was no overall effect of MF exposure on gene expression, but time specific differences between the two exposure groups were apparent, these were investigated further using the Mann-Whitney test for comparison. This revealed a significant difference in expression level of *bmal1* at ZT16 (MF+ vs MF- $p=0.01$) with a 78% higher level in the MF exposed group. Near significant decreases in expression of *per1* at ZT12 (MF+ vs MF- $p=0.08$) and in *clock* at ZT4 (MF+ vs MF- $p=0.07$) were also found after MF exposure.

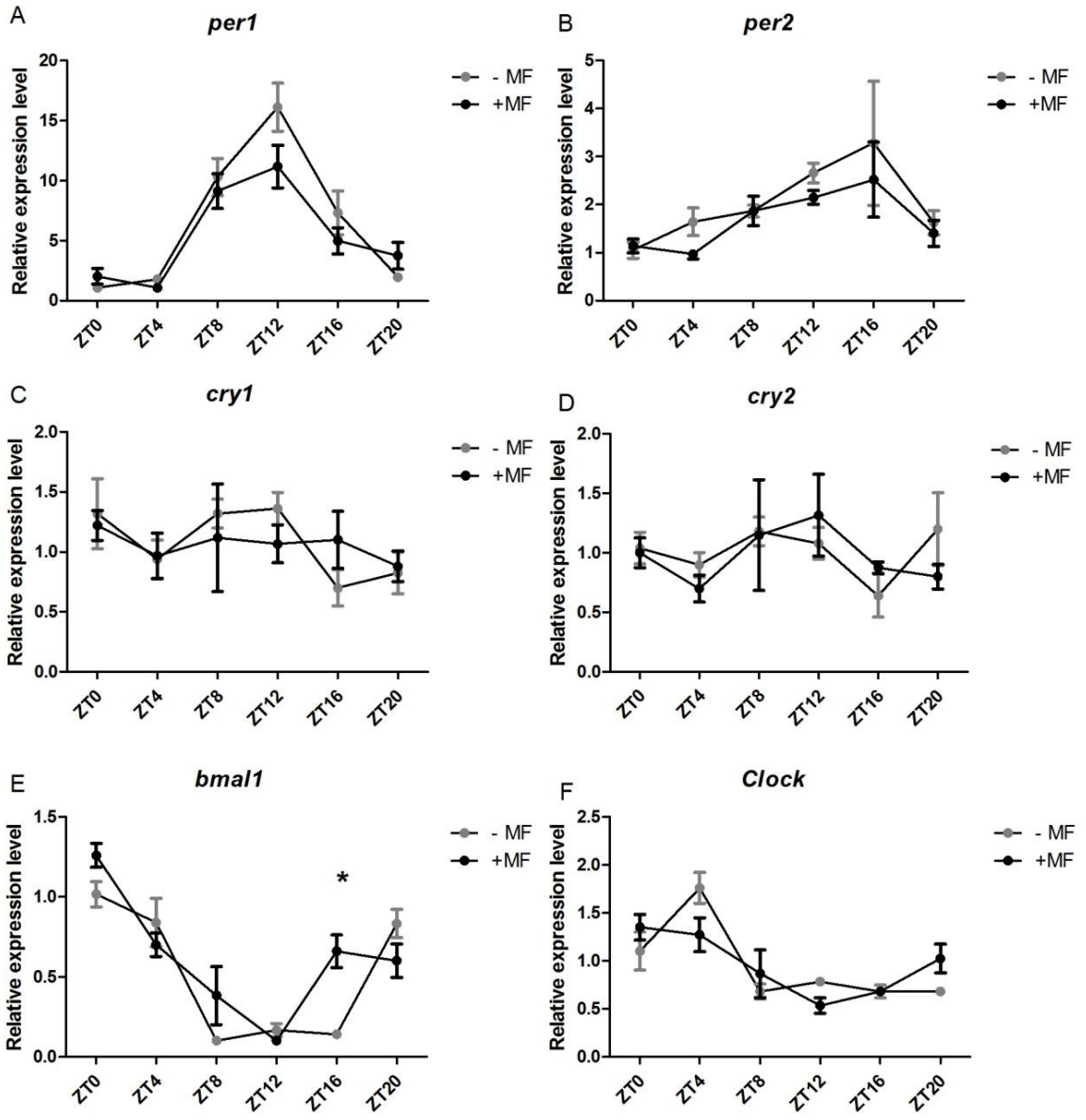


Figure 5.8 Clock gene expression in the liver after MF exposure at 580 µT for 24 hours. *per1, per2, cry1, cry2, bmal1* and *clock* expression levels were measured at 4 hour intervals on the day following 24 hours of MF exposure at 580 µT in the dark. Changes in expression levels of *per1* and *bmal1* in particular were observed over the course of the day. MF exposure was found to significantly increase *bmal1* expression at ZT16, while near significant decreases in both *per1* and *clock* were found at ZT12 and ZT4 respectively when compared to unexposed controls. Data are presented as means ± SEM. Statistical comparison was carried out using a two-way ANOVA for the investigation of the effects of MF exposure and time point on expression levels, and using Mann-Whitney's t-test for comparison of groups at single time points, *p<0.05.

5.3.2.2.3 MF exposure induce subtle and transient changes in expression of the core clock gene in the dorsal hippocampus

In the dorsal hippocampus, fairly stable expression levels of *per1, per2, cry1* and *cry2* were observed over the course of the day following MF exposure, with very similar patterns of gene expression in both exposure groups (Figure 5.9). However, more variation over the course of the day was detected in *clock* and *bmal1* expression.

Two-way ANOVA was used to investigate the effects of MF exposure and time point on expression of the six genes. A significant interaction between the two factors were seen on *clock* expression ($F(5,43)=4.63$ $p<0.01$). This was due to changes in gene expression in opposite directions at ZT8 compared to ZT4, with a decrease in expression in the +MF group, and an increase in the -MF group. This difference was found to be significant only at ZT8 when the two exposure groups (n=3-4) were compared by Bonferroni's *post hoc* test ($p<0.0001$). In addition, near significant effects of time were also found on *bmal1* ($F(5,50)=2.36$ $p=0.053$) and *clock* ($F(5,43)=2.29$ $p=0.06$) expression. Additional sporadic differences between groups were also seen, as given in Table 5.3.

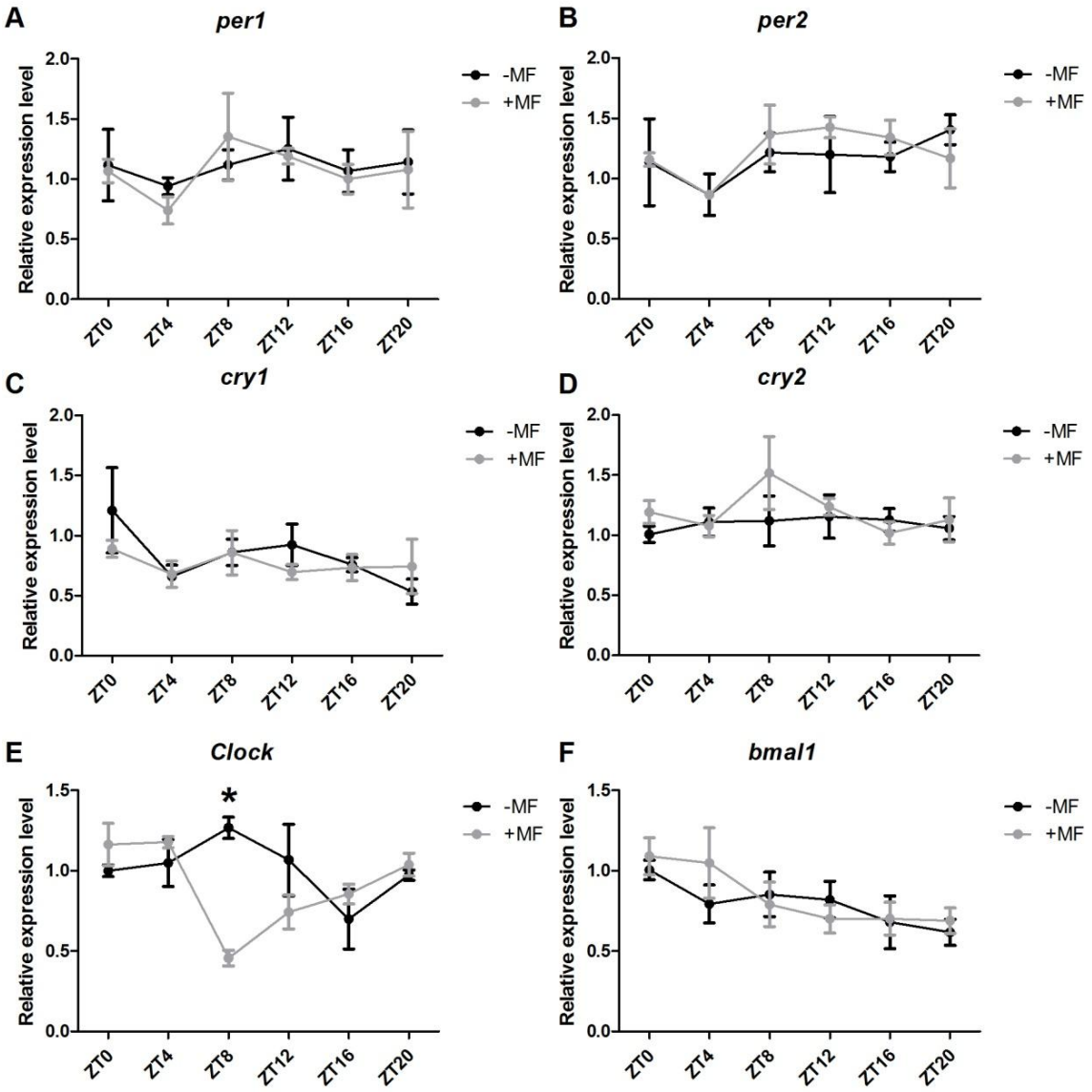


Figure 5.9 Clock gene expression in the dorsal hippocampus after MF exposure at 580 µT for 24 hours. *per1, per2, cry1, cry2, bmal1 and clock* expression levels in the dorsal hippocampus were measured at 4 hour intervals on the day following 24 hours of MF exposure at 580 µT in the dark. Small changes in gene expression were observed in all genes over the course of the day, with little effect of MF exposure. Only a significant decrease in clock expression was detected at ZT8 after MF exposure compared to unexposed control. Data are presented as mean ± SEM. Statistical comparison was carried out using a two-way ANOVA for investigation of effects of MF exposure and time point on expression levels, and using Mann-Whitney t-test for comparison of groups at single time points. No significant differences between the two exposure groups were detected.

Table 5.3 Details of statistical analyses of effects of MF exposure on core clock gene expression in the SCN, liver and dorsal hippocampus.

		Two-way ANOVA					
		Interaction	MF	ZT	Time: Bonferroni's *post hoc* test, compared to ZT0	Exposure: Bonferroni's *post hoc* test, -MF vs +MF	Mann-Whitney test, -MF vs +MF
Dorsal hippocampus	*Per1*	F(5,48)=0.25 p=0.95	F(1,48)=0.09 p=0.77	F(5,48)=1.02 p=0.41			
	Per2	F(5,47)=0.38 p=0.86	F(1,47)=0.25 p=0.61	F(5,47)=1.94 p=0.11			
	Cry1	F(5,47)=0.77 p=0.57	F(1,47)=0.46 p=0.50	F(5,47)=2.03 p=0.92			
	Cry2	F(5,49)=0.63 p=0.68	F(1,49)=1.17 p=0.29	F(5,49)=0.71 p=0.62			
	Clock	F(5,43)=4.63 ****p=0.002**	F(1,43)=2.18 p=0.15	F(5,43)=2.29 p=0.06		**ZT8***p<0.0001**	ZT8 p=0.057
	Bmal1	F(5,50)=0.53 p=0.75	F(1,50)=0.29 p=0.59	F(5,50)=2.36 p=0.053			
liver	*Per1*	F(5,55)=1.75 p=0.14	F(1,55)=2.16 p=0.15	F(5,55)=30.90 *****p<0.0001**	**ZT8 ***p<0.001 ± MF ZT12***p<0.001 ±MF ZT16 ** p<0.01 - MF**		ZT12 p=0.08
	Per2	F(5,51)=0.31 p=0.90	F(1,51)=1.71 p=0.2	F(5,51)=4.57 ****p=0.002**	**ZT12 * p<0.05 - MF ZT16 ** p<0.01 - MF**		
	Cry1	F(5,50)=0.53 p=0.75	F(1,50)=0.02 p=0.89	F(5,50)=41.26 p=0.30			
	Cry2	F(5,48)=0.49 p=0.78	F(1,48)=0.06 p=0.81	F(5,48)=1.21 p=0.32			
	Bmal1	F(5,51)=4.29 ****p=0.0025**	F(1,51)=3.22 p=0.08	F(5,51)=32.52 *****p<0.0001**	**ZT4 *** p<0.001 + MF ZT8 *** p<0.001 ± MF ZT12 ***p<0.001 ± MF ZT16 ***p<0.001 ±MF ZT20 ***p<0.001 +MF**		**ZT16 *p=0.011**

		Interaction	**MF**	**ZT**	**Time:** **Bonferroni's *post hoc* test,** **compared to ZT0**	**Exposure:** **Bonferroni's *post hoc* test,** **-MF vs +MF**	**Mann-Whitney test,** **-MF vs +MF**
	Clock	F(5,52)=2.47 ***p=0.04**	F(1,52)=0.01 p=0.93	F(5,52)=11.83 *****p<0.0001**	**ZT4 ** p<0.01 +MF** **ZT12 ***p<0.001 + MF** **ZT16 ** p<0.01 + MF**		ZT4 p=0.07
SCN	*Per1*	F(5,59)=1.55 p=0.19	F(1,59)=0.08 p=0.77	F(5,59)=2.75 ***p=0.03**	**ZT12 * p<0.05 +MF**		
	Per2	F(5,59)=0.81 p=0.55	F(1,59)=2.67 p=0.11	F(5,59)=2.58 ***p=0.04**	ns		
	Cry1	F(5,72)=0.42 p=0.83	F(1,72)=1.63 p=0.21	F(5,72)=1.94 p=0.098			
	Cry2	F(5,71)=2.59 ***p=0.03**	F(1,71)=1.04 p=0.31	F(5,71)=1.9 p=0.10		**ZT8** ****p<0.01**	ZT8 p=0.10
	Bmal1	F(5,60)=1.27 p=0.29	F(1,60)=2.42 p=0.13	F(5,60)=1.67 p=0.16			
	Clock	F(5,59)=0.49 p=0.78	F(1,59)=0.16 p=0.69	F(5,59)=0.81 p=0.55			

In summary, MF exposure at 580 µT for 24 hours did not significantly affect the overall expression level or the rhythm of core clock gene expression in the SCN, liver or dorsal hippocampus. The only significant effects of MF exposure was an increase in *bmal1* expression in the liver at ZT16, an increase in *cry2* expression in the SCN at ZT8 and a decrease in *clock* expression at ZT8 in the dorsal hippocampus. Near significant changes in expression levels were also detected on *per1* and *clock* expression in the liver at ZT12 and ZT4 respectively.

5.4 Discussion

The aim of this chapter was to investigate whether 50 Hz MFs can disturb circadian rhythm control. It is important to have knowledge about such possibilities because they could have detrimental effect on health and severely impair cognitive function (Lamont et al., 2007; Smarr et al., 2014).

5.4.1 Magnetosensitivity of the light response

To date, no papers appear to have been published on the magnetosensitivity of the adrenal response to nocturnal LS in rodents.

However, a number of studies have investigated light-dependent magnetosensitivity in mammals indirectly, focusing on the cryptochromes. For example, transgenic *Drosophila* modified to express mammalian (human) CRY have been used as a model system (Fedele et al., 2014b; Foley et al., 2011). Wild type (WT) *Drosophila* express CRYI which is a photoreceptor that is known also to be sensitive to MFs under blue light, and various assays have been developed to study this (Yoshii et al., 2009). Mammalian CRY1 and CRY2 are both of the CRYII type which is involved in circadian rhythm control and not photoreception (Vanderstraeten et al., 2012). Foley et al. (2011) showed that *Drosophila* deficient in its native CRY, but expressing human CRY2, were sensitive to MF exposure (at 10-500 μT), and this was detected as physical movement of the flies away from the MF exposure source. This response was dependent on light. Prompted by this finding, Fedele et al (2014) studied light-dependent magnetosensitivity of *Drosophila* expressing human CRY2 or CRY1 in another behavioural assay, and found that only flies expressing CRY2 were sensitive to MF exposure at 300 μT and blue light, measured as hyperactivity of the flies. In contrast to WT flies, no behavioural response to blue light alone was observed in the mutant flies. Based on the magnetosensitivity of human CRY, but with the limitations for studying this *in vivo*, magnetosensitivity of rodent CRY has been investigated in SCN slice cultures from mice expressing luminescent PER2. No change in *per2* expression level or rhythm was observed after 50 Hz MF exposure at 50-500 μT under blue light. It was therefore

concluded that the cellular environment plays an important role in both the light and MF response. It is however possible that MFs can affect mammalian CRY in its native environment in a manner that could not be detected in these assays. Since these studies were published no evidence for MF sensitivity of mammalian CRY has been found, and the precise mechanisms underlying the reported behavioural responses remain unknown.

In addition to the proposed CRY mediated magnetosensitivity, it is possible that MFs can affect the light response through other mechanisms, such as via signalling from the retina to the SCN, the SCN response, or the propagation of the signal to the adrenal glands and onwards.

5.4.1.1 Adrenal response to MF exposure alone or in combination with LS

The protocol for this work was initially based upon studies published by Ishida et al. (2005), Loh et al. (2007) and Kiessling et al. (2014). The same strain, sex and age of mice were used, with the same duration of the LS, and corticosterone was measured at the same time point after LS.

Ishida et al. (2005) found that at CT16 a 30 minute LS at 400 lux increased *per1* transcription in the adrenal glands, but not the liver or kidneys, peaking 1-2 hours after initiation of LS. They also showed that LS using dim and intense light at 4, 40, 400 and 2000 lux caused the serum corticosterone concentration to increase significantly compared to the serum from mice kept in the dark. The light response was found to be dependent on an intact SCN and innervation of the adrenal glands. Loh et al. (2008) similarly showed that at ZT16 a 30 minute LS (50 lux) increased both adrenal *per1* transcription (5x) and the serum corticosterone levels (7x). They further showed that vasoactive intestinal peptide (VIP) in the SCN played an important role in the light response. Kiessling et al. (2014) then investigated the effect of LS (350 lux) during the dark and light phases of the day and found little effect on *per1* and corticosterone during the light phase, but large increases in both, in the early dark phase (ZT16).

The spectral characteristics of the light sources used previously in the literature have not been described. The white light source used here has a broad spectrum LED with a peak at 445 nm.

However, only a very small adrenal response to white LS could be detected using this light, although a few mice did exhibit large increases in corticosterone. Because it was not possible to increase the light intensity with the existing equipment, the experiment was repeated using blue light to increase the involvement of Melanopsin. Melanopsin plays a role in non-visual photoreception in the retina with maximum absorption at 480 nm (Hughes et al., 2012). An adrenal response to LS using blue light has been reported in mice, in addition to a delayed sleep response compared to light at other wavelengths (Pilorz et al., 2016). Pilorz et al. used 470 nm blue LED at an intensity of 14.9 log quanta (approximately 200 lux) to induce adrenal *per1* transcription and increase serum corticosterone levels. In the blue light experiments presented in this chapter an LED lamp with a peak emission at 457 nm was used at 700 lux. Using the same protocol as for white light, increases in both serum corticosterone and adrenal *per1* expression could be detected following LS.

Significant increases in both corticosterone levels and *per1* expression were seen following combined exposure to MF at either 300 or 580 µT with blue LS, but these changes were largely attributable to an effect of the LS since comparable increases were seen using the LS alone.

However, exposure at 580 µT significantly reduced adrenal *per1* expression in both groups that were exposed to MF compared to the groups that were not exposed to this field. The size of this decrease was small and differences between treatment groups were not confirmed using a *post hoc* test. The possibility that MFs might have an inhibitory effect on *per1* expression was also suggested by the results using 300 µT, when a 30% reduction in *per1* expression was found between the group exposed to the MF without LS and the group that was not exposed to either the field or the LS.

No significant effects on corticosterone levels were seen at either flux density, and additionally it was not possible to see any potential trends either due to the relatively low levels of corticosterone exhibited in the animals without LS and the variability at the data.

5.4.1.2 Behavioural response to blue light and magnetic field exposure

As another measure of light response, the activity of the mice was tracked during and after 30 minutes exposure to MF ± blue LS. LS during the early dark phase of the day has been found to induce sleep in mice (Pilorz et al., 2016). The blue LS-induced latency to sleep, alone and in combination with MF exposure, was comparable to that reported by Pilorz et al. (2016) of 17.5 ± 1.64 minutes for blue LS at 200 lux. MFs alone did not affect sleep latency or the cumulative sleep duration.

There were no behavioural indications that the mice sensed the field. Mice that were asleep at the onset of exposure were woken up by the light, but this was not seen as a result of MF exposure. Furthermore, initiation of the LS caused an immediate increase in activity, while there was no change in activity after MF exposure alone.

5.4.1.3 Summary of acute effects of MF exposure in the presence and absence of light

A literature review suggests that this is the first *in vivo* study of the modulating effects of 50 Hz MF on the molecular mechanisms of the light response. No effect of MF exposure at 300 or 580 µT on the adrenal response to nocturnal LS was observed. However, a small decrease in adrenal *per1* gene expression was found after MF exposure in the absence of light. No changes in activity of the mice were detected after MF exposure.

The two proposed interaction mechanisms between MFs and biological tissues that have gained most interest, are the radical pair mechanism and effects on magnetite (WHO, 2007). These mechanisms were not investigated directly in this experiment.

To explain the mechanism underlying the decreased *per1* expression after MF exposure further experiments are needed, especially studies of *per1* expression in the SCN to identify the level of interaction of the MF, and then to identify which cells or molecules are affected. It would also be relevant to investigate if acute MF exposure can decrease *per1* expression at other times of the

day, e.g. during peak expression time in the light phase of the day or in the late night where decreases causes phase shifts, as has been achieved by other non-photic modulators such as increased activity and treatment with morphine, serotonin-agonists and NPY (Biello and Mrosovsky, 1995; Hamada et al., 2004; Maywood et al., 1999; Mistlberger and Holmes, 1999; Rea et al., 1994). Studies of the phase shifting after exposure at ZT14 in addition to *per1* and corticosterone levels are also important to better understand the physiological impact of the MF induced decrease in *per1* expression. Sequential MF exposure and LS would be interesting to assess whether the decreased *per1* expression after MF exposure would impair the LS response.

5.4.2 Magnetosensitivity of clock gene expression

Possible disruptive effects of MF exposure on circadian clock gene expression was investigated after 30 minutes of exposure and after 24 hours of continuous exposure. The expression of *per1, cry1* and *cry2* expression was investigated in the adrenal glands, liver and dorsal hippocampus after acute MF exposure with or without blue light, and the expression of *per1, per2, cry1, cry2, bmal1* and *clock* was investigated in the SCN, liver and dorsal hippocampus after MF exposure for 24 hours.

5.4.2.1 Hepatic and hippocampal response to acute MF and blue LS exposure

In addition to evaluation of the adrenal response to MF with or without blue LS changes in *per1* expression in the liver and dorsal hippocampus were evaluated after exposure at flux densities of 300 μT or 580 μT. This allowed investigation of the effects of exposure on the signalling from the SCN and adrenal glands to the liver and hippocampus. Disturbances to the circadian clocks in these tissues are known to affect cognitive and metabolic functions and thereby the general health (Hastings et al., 2008; Lamont et al., 2007; Zwighaft et al., 2016). In this chapter, it was found that a 30 minute MF exposure at magnetic flux densities of either 300 μT or 580 μT did not affect gene expression of *per1* in the liver or dorsal hippocampus 30 minutes after exposure, alone or in combination with blue LS. This is in contrast to the observed response in the adrenal glands, therefore the MF effect causes different responses in different organs. It is possible that the sensitivity of *per1* is specific to the adrenal cells, or like the light response, the response is first propagated from the SCN to the adrenal glands where the most sensitive cells or molecules may be located.

Cryptochrome gene expression was also measured in the adrenal glands, hippocampus and liver after MF exposure in the presence or absence of blue LS to investigate the acute effects of MFs on *cry* transcription. No acute sensitivity to light was observed, which is in accordance with its lack of photoreceptor properties in mammals (Owens et al., 2012). Also no effect of MF exposure was detected. However, a near significant interaction between MF and blue LS was

detected on *cry1*, but only in the liver. This was due to an increase in *cry1* expression in the blue LS+/MF+ group compared to the three other exposure groups, indicating a possible additive effect of the two exposures. It seems unlikely that light in combination with MF has an acute effect on *cry1* expression in the liver but not in the adrenal glands where the light response is first detected.

5.4.2.2 Changes in rhythmic expression of core clock genes after 24 hours MF exposure

Strong rhythmic expression of *per1, per2 and bmal1* was observed in the liver of all mice, corresponding with that reported in the literature (Liu et al., 2007; Loh et al., 2011; Peirson et al., 2006). Smaller changes in expression were seen over the course of the day in the SCN and dorsal hippocampus. The increase in gene expression after MF exposure demonstrated by Manzella et al. (2015) in fibroblasts could not be replicated in any of the three tissues *in vivo*. The only significant effects of MF exposure was an increase in *bmal1* expression in the liver at ZT16, an increase in *cry2* expression in the SCN at ZT8 and a concomitant decrease in *clock* expression at ZT8 in the dorsal hippocampus. Fairly large variations within experimental groups were observed, for example a t-test did not detect a significant difference in *clock* expression between the two exposure groups in the hippocampus at ZT8 even though a 64% decrease was seen after MF exposure.

Transient near significant differences in gene expression between the two exposure groups were detected, but there appeared to be no pattern in sensitivity of the genes or a time-window of sensitivity. There were indications of both increases and decreases in gene expression after MF exposure, which further complicates the understanding of any possible effect of exposure on clock gene expression. It should be noted that there were no differences between the exposure groups at ZT0 (where the MF was just turned off) on the expression of any of the genes, indicating that a possible sensitivity to MFs would be either time-dependent and/or transient.

The SCN is known to show great rhythmic expression of the core clock genes, but this could not be detected in these experiments. Isolation of the SCN is technically difficult due to its size and position. SCN punches were made on frozen coronal sections using a flat tip needle. Even though this was done accurately, it is likely that surrounding tissue has been included which could then mask the high SCN expression levels. Conclusions on MF effects in this region based on these experiments have to be made with caution, and perhaps a better assessment could be done using *in situ* hybridization on brain sections which would allow accurate investigation of gene expression changes in the SCN. As discussed previously, a significant decrease in *per1* expression was detected in the adrenal glands after acute MF exposure, but not in the hippocampus or liver. In these experiments MF exposure also had no effect on *per1* expression in the liver and dorsal hippocampus. Possible changes in the SCN could not be detected.

The 24 hour exposure protocol was chosen to ensure exposure during all processes that occur at a 24 hour cycle. No changes in the rhythmic expression were detected in the tightly regulated SCN or in the peripheral clocks. Thus, there appear to be no effect of exposure on any physiological processes that affect clock gene expression or synchronization of clocks, to an extend that exceeds what the body can regulate.

The lack of effect of 24 hours of exposure on *per1* expression in the SCN, liver or dorsal hippocampus suggests that the mechanism for the acute effect of exposure in the adrenal glands is either adrenal specific or transient. The adrenal response using the 24 hours exposure protocol should be investigated.

5.5 Conclusion

In this chapter, possible effects of 50 Hz MFs at 300 and 580 µT on circadian rhythm control have been investigated.

From the experiments, it can be concluded that:

1) MFs do not affect the acute light response in terms of the light detection in the retina, the signalling in the retinohypothalamic tract to the SCN, the detection and propagation of the signal in the SCN, and the further signalling to the adrenal glands, to a degree that can be detected in the adrenal response.

2) Acute MF exposure at 580 µT decreases *per1* expression in the adrenal gland, but not in the liver or dorsal hippocampus.

3) Acute MF exposure does not affect transcription of *cry1* and cry*2* in the dorsal hippocampus or adrenal glands, while indications of an increase in *cry1* expression were detected in the liver.

4) MF exposure for 24 hours does not affect the overall expression levels or rhythm of expression of the core clock genes (*per1, per2, cry1, cry2, bmal1* or *clock*) in the liver or dorsal hippocampus. However, in the liver *bmal1* expression was transiently increased at ZT16.

Chapter 6 Summary of Effect of Exposure to Electromagnetic Fields on Brain Function and Behaviour in Mice

This thesis investigated the effects of low level electromagnetic fields at power or mobile phone frequencies on cognitive function. A broad variety of molecular and cellular endpoints known to be sensitive to external modulators were chosen for investigation, and these were correlated with functional effects in spatial memory tests.

Conflicting effects of RF exposure on spatial memory have been reported in the literature. Some studies have reported large effects on performance in a water maze and other tasks, but others have found exposure was without any significant effect, and it is difficult to reconcile these differences. However, performance is known to be affected by many factors including stressors that may have occurred either during exposure or testing, so eliminating all potential confounding is crucial. Here a reverberation chamber exposure system was used where external stressors were controlled as much as possible, and the animals were free to move during exposure, which has distinct advantages over some previous studies where the animals were restrained in tubes for dosimetric purposes. In addition, the reverberation chambers produced a controlled and homogeneous field, which would not be the case with studies using a mobile phone as exposure source. All animals were also tested in the water maze under controlled and consistent conditions to eliminate spurious influences as much as possible. No effects of exposures at up to 3 W/kg were found on spatial learning and memory in young adult C57Bl6/J mice, adding weight to the viewpoint that the processing and use of spatial information in rodents is not susceptible to disruption by signals used by mobile phones. In the literature very little has been reported on the effects of exposure on synapses and neuronal signalling, therefore a broad approach was chosen for the studies presented in Chapter 3. Exposure at an SAR of 0.3 W/kg for 4 weeks had no effect on any of the chosen endpoints. Exposures at 3 W/kg induced small changes including decreased *grin2a* expression in the dorsal hippocampus and

increased *ntrk2* expression in the prefrontal cortex. Previously, only protein levels of glutamate receptor subunits have been investigated and at much higher levels. Synaptic density, PSD95 levels, hippocampal neurogenesis, glial density and c-fos levels were not affected by exposure. Previous studies have likewise reported no effects of exposure on microglia, proliferation and on c-fos levels, while differentiation of newly generated cells in the hippocampus has not previously been studied. Conflicting effects on astroglia have been reported with the majority of reports finding increased GFAP density in astroglia.

The second part of Chapter 3 considered the sensitivity of the senescent brain to RF exposure at 3 W/kg. Only few studies have investigated differences in sensitivity of the adult and aged brain. SAMP8 and SAMR1 mice at 7 months of age were used, and only small significant differences were found between the two strains when sham exposed. However indications of differences in sensitivity to RF exposure were seen between the two strains on a number of the investigated endpoints, this included *bdnf* expression levels, GluA2 protein levels, presynaptic density and astroglial density. Microglial density was decreased in both strains after exposure and *grin1* expression in the hippocampus was increased after exposure in the SAMP8 mice only. Adult hippocampal neurogenesis was not affected by exposure. Despite the indications of a greater sensitivity of the SAMP8 mice compared to SAMR1 mice and young adult C57Bl/6 mice, all changes were still small although significant.

In summary, the results presented in Chapter 3 have shown that RF exposure at low levels where external stress is minimised has very little effect on the plastic processes that are involved in cognitive function and that there might be a greater sensitivity of the same processes in older mice.

Long-term exposure to power frequency MF exposure below 1 mT has been reported to have no significant effects on spatial learning and memory, while acute exposure prior to behavioural testing has been found to impair memory, and exposure during behavioural testing has been

reported to improve memory. The studies presented in this thesis found no effect of exposure on spatial memory 4 hours after acute exposure. In line with the literature, no effect of repeated exposures was found. Only very few studies have previously investigated effects of power frequency MFs on the plastic properties involved in cognitive function. These have reported reduced dendritic spine density and glutamate binding to receptors, and increased glutamate receptor protein levels after high level exposures. The studies presented in this thesis are, to the best of my knowledge, the first investigating gene expression of glutamate receptor subunits, presynaptic density, effects on PSD95 and on prefrontal cortex and hippocampal BDNF signalling. No changes to presynaptic density were found. The studies revealed small effects of both acute and repeated exposures with complex and transient changes in glutamate receptor expression levels and BDNF signalling molecules. In particular, *gria1* expression was increased after both acute and repeated exposure and in both the dorsal hippocampus and prefrontal cortex. However, this effect was not seen 7 days after the end of exposure in either region. *bdnf* gene expression was increased after both acute and repeated exposure but only in the dorsal hippocampus. Seven days after the end of an acute, but not repeated, exposure the expression level was still increased. Previously, studies have also reported persistent effects of exposures at 1 mT for up to 1 month after the end of exposure, this was assessed by the number of newly generated cells and a number of differentiation factors. The experiments presented in this thesis found that exposures up to 300 μT did not enhance proliferation and differentiation of hippocampal neurons. On the contrary, a small decrease in proliferation was seen, however, this was only after exposure at 100 μT and not 300 μT. Glia cells are known to play an important supportive role for synapses, the results presented here appear to be the first investigating the effects of MF exposure on these. No effects of exposure on astroglial density were found, while an increase in microglial density in the hippocampus was found, with no apparent changes to the cellular morphology. This could be due to possible alterations to the rate of apoptosis seen from the third to sixth postnatal week, or to changes in synaptic plasticity or neurogenesis that

trigger microglial reacitivity and/or proliferation. Additional work would be required to decide which of these possibilities might be correct.

In summary, the studies of the effects of power frequency MF exposure at 100 μT or 300 μT presented in Chapter 4 provide novel information about the sensitivity of plastic processes that are involved in cognitive function, showing subtle, though transient, effects.

In Chapter 5, the effects of MFs at magnetic flux densities up to 580 μT on circadian rhythm control were investigated. Effects on the adrenal response to light shock have not been investigated previously, while the rhythmic core clock gene expression has been reported to be sensitive to exposure in human fibroblasts. The adrenal light response was not found to be sensitive to modulation by exposure, while acute MF exposure alone decreased *per1* expression in the adrenal glands but not the liver or hippocampus. The physiological impact of this change is not clear from these experiments. There appeared to be a dose-response relationship with the greatest decrease after exposure at 580 μT. Only transient and subtle effects of exposure were seen for clock gene expression in the liver and hippocampus, in contrast to what had been reported in the human fibroblasts. The method for dissecting the SCN could be further optimised before any conclusions can be drawn on possible effects in this region.

In summary, the experiments presented in Chapter 5, have shown that MF exposures up to 580 μT might have subtle effects on circadian physiology.

The overall aim of this thesis was to improve the understanding of effects of low level EMFs on cognitive function. The experiments have shown that exposure to 50 Hz or 1800 MHz fields can cause subtle changes to molecules and cells without affecting spatial memory, and that 50 Hz magnetic fields can induce transient changes in expression of genes involved in circadian rhythm control.

Appendix 1 Reported effects of power frequency MFs on cognitive function in rodents

Table A1.1 Studies of effects of power frequency MFs on performance in learning and memory tests by rodents

Reference	Exposure level and duration	Model	Learning and memory tests	Other endpoints	Results
(Kavaliers et al., 1996)	60 Hz Helmholtz coils 0.1 mT 5 min exposure during testing	Deer mice	Water maze pool inside coils	Opioid system, enklephalin	Improved female performance to level corresponding to males. Find involvement of opiod system in this improvement.
(Sienkiewicz et al., 1998a)	50 Hz, yoked magnet 0.0075 mT, 0.075 mT, 7.5mT daily exposures for 45 min before training in RAM	C57Bl/6J	RAM		Transient decrease in performance at 7.5mT, increased number of errors and reduced rate of acquisition without affecting accuracy of the test. Delay of 45 min between exp and training resulted in no effect of exposure. Continued exp for 4 days after training no amnesic effect
(Sienkiewicz et al., 1998b)	50 Hz, yoked magnet 0.75mT daily exposures for 45 min before training in	C57Bl/6J	RAM		4 independent experiments reduced rate of acquisition without affecting accuracy of the test.

	RAM				
(Lai et al., 1998)	60 Hz Helmholtz coils, 1mT 1 hour per training session	Sprague-Dawley rats	Water maze, exposure before training		No effect in learning, but impaired performance in probe trial
(Sienkiewicz et al., 2001)	50 Hz, coils 0.0075, 0.075 or 0.75mT 45 min, exposed after familiarization trial	C57Bl/6J	NOR test, 1 hour retention interval		No effect
(Mostafa et al., 2002)	50 Hz Cylindrical copper cage 0.2mT 1 or 2 weeks continuous exposure	Wistar rats	NOR test, 15 min retention interval	Increased plasma corticosterone	Impaired performance after 2 weeks of exposure but not 1 week.
(Jadidi et al., 2007)	50 Hz coils, 2 mT, 8 mT 20 minutes before or after training	Wistar rats	Water maze: 2 trials one day and 48h later probe test		Spend less time in target quadrant in WM when exp to 8 mT immediately after training but not when given before
(Fu et al., 2008)	50 Hz, coil 1.1mT for 7d exp and 2mT for 28d exp	ICR mice	2-trial Y-maze		Impaired memory of novel arm after 28d exposure

	1h/d for 7 days or 28 days				
(Liu et al., 2008)	50 Hz, Helmholtz coils, 2mT 1h/d or 4h/d for 4 weeks	Sprague-Dawley rats	Water maze (5 sessions of 4 trials)		reduced latency and improved performance in the probe trial
(He et al., 2011)	50 Hz, Helmholtz coils 2 mT 1 h or 4 h per day, 7d/w for 4 weeks	Sprague-Dawley rats	Water maze	OF and EPM after 3 weeks, 1h/d:no significant effect, 4 h/d: increased levels of anxiety	Exposure for 4 h/d improved platform latency and memory in probe test
(Cui et al., 2012)	50 Hz, coils 0.1 mT , 1 mT 4 h/day for 12 weeks	C57Bl/6	Water maze over 4 days, and cued version. No probe trial	increased oxidative stress in hippocampus and striatum	Significant impairment of learning at 1 mT but not 0.1 mT
(Duan et al., 2013)	50 Hz, Helmholtz coils 8 mT for 4 h/day for 28 days	ICR mice	Water maze	Do also see increased oxidative stress, and neuronal loss	Longer escape latency in learning trials and less time spend in platform quadrant and distance moved
(Wang et al., 2013) *this has not	50 Hz, coils 2 mT 60 min/day from	Kunming mice	Water maze	Y-maze, no effect	Improved learning and memory However, the sham-exposed animals showed abnormally poor memory in the

been included in the text.	postnatal day 23 to 35.				probe trial
(Deng et al., 2013)	50 Hz, Helmholtz coils, 2 mT 4h/d 6d/w 8weeks	Kunming mice	Water maze 4 daily trials for 5 days of training with changing start positions, same order each day,	Correlate with cell loss and increased oxidative stress	Impaired learning, and decreased quadrant preference in probe trial
(Li et al., 2014b)	50 Hz, Helmholtz coils 0.1mT contin 90 days	Sprague-Dawley rats	Water maze		No effect of exposure on learning or memory
(Li et al., 2014a)	50 Hz, solenoid coils 0.5mT 14 or 28 days 4h/d	Sprague-Dawley rats	Water maze	WB for AMPA and NMDA receptor subunits. Find effects	No effect of exposure
(Leone et al., 2014)	50 Hz, Solenoid coil 1mT 3.5h/d 12 days	C57Bl/6	Water maze 30d after end of exposure NOR test 36d after exposure	Increased hippocampal neurogenesis.	Improved performance decreased latency on all days, and higher quadrant preference in probe test, Improved performance in NOR test
(Podda et al., 2014)	50 Hz, solenoid, 1mT 3.5 hours, 6 days	C57BL/6J	Water maze and NOR	Increased survival of new cells	24 days old cells: improved water maze performance
(Zhao et al., 2015)	50 Hz, Helmholtz coils 0.4, 0.6, 1 mT 12h/d for 7 days or 21	ICR mice	NOR	no change in OF Lower dendritic spine density at 7 and 10 days in 1mT groups, but not	impaired performance in NOR after 1 mT for 7 days

	days			after 14 days. In hippocampus	
(Lai et al., 2016)	50 Hz 0.1mT 20h/d 24 weeks	Sprague- Dawley rats	Water maze	No effects of exposure in EPM, tail suspension test, forced swim test, fear conditioning Cell loss, HE and NeuN.	No effects of exposure

Abbreviations: d:day, EPM:elevated plus maze, h:hour, NOR:novel object recognition, OF:open field, RAM:radial arm maze, w:week

Table A1.2 Studies of effects of power frequency MFs on synaptic plasticity in rodents

Reference	Exposure level and duration	Model	Region and method	Other endpoints	Results
(Harry et al., 2000)	60 Hz Coils 2 uT, 200uT, 1000 uT 18.5h/d, 7d/w p0-p20	Male rat pups unclear which type	Forebrain levels of *gap43* an axonal growth marker and the growth factors *cntf, bdnf, ngfb, ntf3* and *ntf4.*		No significant effect
(Manikonda et al., 2007)	50 Hz Helmholtz coils 0.05 and 0.1 mT 90d	Wistar rats 3 week	Hippocampus	Increased activity Increased intracellular calciumlevels Increased calcium dependent kinaseactivity Decreased CaMK activity	Decreased NMDA binding in ^{3}H glutamate binding assay in 0.1 mT group, indicating decreased receptor activation
(Varró et al., 2009)	50 Hz, Helmholtz coils 0.5 mT 1 or 4 days , 15h/d	Wistar rats	Neoocortical and hippocampal slices, EPSP analysis, LTP, seizure sensitivity		Enhanced short and long-term synaptic facilitation was found in hippocampal slices.
(Tasset et al., 2012)	60Hz Coils head exposure 0.7mT 2x2h/d 21d	Wistar rats 3M	Whole brain		EIA: increased >100 % BDNF and GDNF levels

(Balassa et al., 2013)	50 Hz, Helmholtz coils 0.5 or 3 mT 7 days of continuous exposure in the second prenatal week, or from postnatal day 3,	Wistar rats 2 months old at sacrifice	Neocortical and hippocampal slices EPSP analysis and LTD and LTP		increased the electrophysiological response Greatest effect in brains exposed postnatally
(Xiong et al., 2013)	50Hz, coils 0.5mT 4h/d 14 and 28d	Sprague dawley rats	Entorhinal cortex Dendritic spine density, dendrite shape		Reduced spine density and morphology
(Duan et al., 2014)	50 Hz Helmholtz coils 8mT 4h/d, 28d	ICR mice 3 Week old n=10	Hippocampus HPLC		Increased glutamate and GABA concentration, and destroyed balance between these two. Also increased protein level of GluN2B
(Komaki et al., 2014)	50 Hz, Solenoid coils 0.1 mT 3 months, 2h/d	Wistar rats	Electrode inserted into the hippocampus, LTP, and paired-pulse ratio		Significantly greater response to stimulation was observed in exposed brains
(Li et al., 2014a)	50Hz solenoid coils 0.5 mT 4h/d, 14 or 28d	Sprague dawley rats, male 220-250g	Hippocampus, entorhinal cortex, prefrontal cortex	no functional effect in water maze	No effect on AMPA receptor protein levels (WB) – increased GluN2A and B in hippocampus after both 14 and 28d. In entorhinal cortex increased Grin1 and 2a after 14d, decreased 2a and increased 2b after 28d. In

					the Prefrontal cortex increased grin1 and 2a after 14d and after 28d only grin1
(Salunke et al., 2014)	50 Hz Helmholtz coils 1mT 120d, 8h/d	Swiss albino mouse 22-26g n=6-8	Brain homogenate of striatum, cortex, hippocampus and hypothalamus. HPLC	Find increased anxiety in exposed mice with an involvement of glutamate.	HPLC - Find significantly higher glutamate and GABA levels in hippocampus and hypothalamus but not in striatum and cortex.
(Chung et al., 2015b)	60 Hz Helmholtz coils, 1.8-2 mT. 2 or 5 days	Sprague dawley rats	cerebellum, cortex, hippocampus, thalamus and striatum	Changes in noradrenaline, vanillylmandelic, serotonin, 5-hydroxyindoleacetic acid, dopamine, aspartic acid glutamine, taurine, tyrosine and NO	glutamate ↑thalamus, GABA ↓cortex, cerebellum and hippocampus ↑striatum and thalamus.
(Sun et al., 2016)	50 Hz, Hemholtz coils 1mT 8-10d,	C57Bl/6 from p0	In calyx of Held synapses	Increased protein levels of voltage gated calcium channels	Increased frequency of EPSP, and higher amplitude of post-tetanic potentiaiton, increased calcium influx and endocytosis

Abbreviations: d:day, EIA: EPSP: excitatory postsynaptic potential, enzyme immunoassay, GABA: gamma aminobutyric acid, GDNF: glia derived neurotrophic factor, h: hour, HPLC: high performance liquid chromatography, LTP: long-term potentiation, NO: nitric oxide, w:week, WB: western blot

Table A1.3 Studies of effects of power frequency MFs on hippocampal neurogenesis in rodents

Reference	Exposure level and duration	Model	Endpoints	Other endpoints	Results
(Piacentini et al., 2008)	50 Hz, solenoid 1mT up to 12 days	Cells from cortex of p0 CD1 mice	Proliferation and differentiation	Calcium channel activity and expression	Increased proliferation dependent on voltage gated calcium channels
(Cuccurazzu et al., 2010)	50Hz, solenoid 1mT, 4d 7h/d	C57BL/6J	Proliferation and differentiation	Differentiation factors *Hes1, Mash1, Ngn1, Ngn2, NeuroD1, NeuroD2.* All genes were increased in expression, although only Hes1, *Mash1* and *NeuroD1* was significant. By western blotting a 40% increase in both NeuroD1 and 2 was found. Electrophysiology: after 7d/7h exposure and 30-37days maturation. Functional integration was found.	63% more BrdU+ cells 24h after 7h/d 7days. There are few GFAP and nestin+ cells. Majority are BrdU/DCX+ (95%). There are 75% more in the MF group. There is also a significant increase after 1h and 2h exposure for 7days (46% and 65% increase respectively. 4 weeks after end of exposure 97-99% af the BrdU+ cells are found to be NeuN+. The level is 64% higher in the exposed group ($p<0.05$). when comparing the number of BrdU/DCX and BrdU/NeuN+ cells at the two time points only 50% of the BrdU+ cells are found to survive, this is similar in both groups.

(Leone et al., 2014)	50Hz, solenoid 1mT, 3.5h/d for 12 days	C57BL/6J n=3-4	Proliferation and differentiation and functional impact	1 month after end of exposure. Water maze. 4d acquisition training, 6 trials/d. 40s trials. Decreased latency on all days, in the probe trial the exposure mice spend significantly more time in the platform quadrant. NOR: 24h delay. Higher discrimination ability of exposed mice compared with controls. qPCR: *Hes1, Mash1, Ngn1, NeuroD1.* CREB activation after exposure as *in vitro* on the neuronal differentiation gene promotors and acetylation of H3K9 on these regulatory regions	Significantly more BrdU-labelled cells and BrdU/DCX+ cells 24h after exposure, and 171% increase in BrdU/NeuN+ cells 40 days after end of exposure
(Podda et al., 2014)	50 Hz, solenoid, 1mT 3.5 hours, 6 days	C57BL/6J	Survival Day 18 and day 24	Improved performance in NOR and water maze tests	Significantly higher number of BrdU+ cells, BrdU/DCX+ cells, BDNF/NeuN+ cells at 18 days, and BrdU/NeuN at 24 days after labelling.

Abbreviations: BDNF: brain derived neurotrophic factor, BrdU: 5'-bromodeoxy-2'uridine, d: day, DCX: doublecortin, GFAP: glial fibrillary acidic protein, h: hour, NeuN: neuronal nuclei, w:week.

Table A1.4 Studies of effects of power frequency MFs on neurodegeneration in rodents

Reference	Exposure level	Model	Cellular and molecular endpoints	Functional endpoints	Results
(Poulletier de Gannes et al., 2009)	50 Hz, Merritt coils 0.1 and 1mT2h/d,5d/week for 7w	SOD-1 mice		Rotarod and weight	No effect of exposure during disease development
(Tasset et al., 2012)	60 Hz, Helmholtz coils 0.7mT 2x2h/d, 3 weeks	Huntingtons model, 3-nitroproprionic acid injection in rats	Striatum. Growth factor expression, oxidative stress, LDH assay, Nissl staining,	OF, forced swim test	Reduced brain injury
(Deng et al., 2013)	50 Hz Helmholtz coils 2mT 4h/d, 6d/week, 8 weeks	Kunming mice with aluminium overload	Hematoxylin-eosin staining, p-tau, oxidative stress	Water maze	exposure impaired performance in water maze and pathology, no co-effect between aluminium and MF
(Zhang et al., 2014)	50 Hz Helmholtz coils 0.1mT 12 weeks conti	Sprague-Dawley rats	No effect on amyloid levels in cortex, hippocampus and plasma, cell morphonlogy using HE	Water maze	No effect of exposure
(Liebl et al., 2015)	50 Hz, Merritt coils 1mT Continuous 16mo	Mouse /APP23, $SOD1^{G85R}$, $SOD1^{G93A}$, $SOD1^{WT}$	Gene and protein levels SOD-1, APP, GFAP, HSP25, Iba-1, Abeta, SOD activity, Iba-1 and GFAP staining,	Water maze in AD mice	No effects in any tests in any models

(Liu et al., 2015)	50 Hz, coils, 0.38-0.42mT Continuous for 60 days	Rats with D-galactose ip and Abeta25-35 intrahippocampal	Cell morphology by nissl staining, and hippocampal electron microscopy, proteomic analysis	Water maze	Improved performance in water maze, cell damage after exposure, recovery after 5 days. The proteomics showed changes to a number of proteins involved in AD pathology and synaptic signalling
(Hu et al., 2016)	50 Hz, bronxe wire 0.5mT 20h/d 3 months	3xTg AD model and C57Bl/6	Tau, p-Tau, GluA1/2, GluN1,2a,2b, PSD93 and 95, Aβ, enzymes involved in p-tau, oxidative stress markers and apoptosis markers, NeuN, ROS assay	Water maze and fear conditioning	Improved cognitive function, increased GluA2, GluN1, PSD95, Decreased apoptosis and ox stress, decreased tau and p-tau, and in activity in enzymes involved in this. No effect in WT controls.
GLIA					
(Teimori et al., 2016)	50 Hz, Helmholtz coils 3mT 4h/d 10 weeks	Wistar rat	Ultrastructural analysis of hippocampus and TUNEL staining		Ultrastructural damage in glia and neurons, and increased apoptosis

Abbreviations: AD: alzheimers disease, APP: amyloid precursor protein, d: day, GFAP: glial fibrillary acidic protein, HE: hematoxylin eosin, h: hour, HSP: heat -shock protein, OF: open field, SOD: superoxide dismutase, w: week.

Appendix 2 Reported effects of RF exposure on cognitive function in rodents

Table A2.1 Studies of effects of RF exposure on performance in learning and memory tests by rodents

Reference	Exposure level and duration	Model	Learning and memory tests	Other endpoints	Results
(Sienkiewicz et al., 2000)	900 MHz, GTEM cell 45 min/d, 10 days 0.05 W/kg Whole body	C57Bl/6	RAM immediately after or 15 or 30 min after end of exposure		No effect of exposure
(Dubreuil et al., 2002)	loop antennae to the head. 900MHz, restrained in tubes 45min before daily training 1 or 3.5W/kg to brain	Sprague-dawley rats	8-arm RAM or dry land water maze for 10-14 days of training		No effect of exposure in either test
(Dubreuil et al., 2003)	loop antennae to the head. 900MHz, 45 min daily 7,9 or 12 days or 45 min before or between NOR testing restrained in tubes 1 or 3.5W/kg to brain	Sprague-dawley rats	RAM NOR (15 or 60 min delay).		No effect of exposure on memory. In NOR (60 min delay), significantly less exploration in exposed and sham exposed compared to controls.
(Yamaguchi et al., 2003)	1.439 GHz, monopole antenna 1 h/day for 4 days 7.5 or 25W/kg to brain 1.7 or 5.7 W/kg whole body	Sprague-dawley rats	T-maze		No effect of 7.5 W/kg 2˚C heating

(Ammari et al., 2008a)	900 MHz, Signal generator connected to loop antennae held just above the head of the restricted rat 45 or 15 min, 5d/w 8 or 24 weeks. 1.5 or 6 W/kg to brain	Sprague-dawley rats	RAM		no difference between groups
(Megha et al., 2012)	900 and 1800 MHz, GTEM cell, 2h/d, 30 days SAR 0.5953 and 0.5853 mW/kg respectively, whole body	Fischer rats	Water maze	Increase in pro-inflammatory cytokines and oxidative stress Increased transfer latency in elevated plus maze	Impairment in learning and memory
(Deshmukh et al., 2013)	900 MHz GTEM cell, sham exposure 2h/d, 5d/w 30d 84.738 µW/kg	Adult Fisher 344 rats	Water maze memory test, after end of exposure on days of exposure	Increased lipid and protein oxidation, no effect on glutathione Elevated plus maze, impaired performance	Impairments in water maze performance
(Klose et al., 2014)	900 MHz wavegioe, restraine rats 5-120 min/d, 5d/w, for 7 days, 14 days-19 months 0.7, 2.5, 10 W/kg to brain	Juvenile, adult and presenile rats	RAM and water maze	No effect in rotarod orOF	No effect
(Deshmukh et al., 2015)	900/1800/2450 MHz GTEM cell	FIscher 344 rats	EPM and water maze	Increased HSP70 and DNA damage	Indications of impairment

	2h/d, 5d/w 180d 0.5953, 0.5853 or 0.6672 mW/kg respectively.				
(Tang et al., 2015a)	900 MHz, central antenna, immobilised rats 3h/d 14 or 28d 2 W/kg to head	Sprague-dawley rats	Water maze	Altered neuronal ultra structure, BBB leakage, increased MAPK-phosphatase-1, reduced p-ERK.	Impaired performance after 28 days
(Wang et al., 2017)	1800 MHz, horn antenna 30 min 1.9-3.3W/kg in brain	3-4weeks old C57Bl/6 females	NOR	Changes to spine density, altered electrophysiology in prefrontal cortex and hippocampus	Increased exploration of novel object day 3-8 post exposure

Abbreviations: BBB: blood brain barrier, d:day, EPM:elevated plus maze, h:hour, NOR:novel object recognition, OF:open field, RAM:radial arm maze, w:week

Table A2.2 Studies of effects of RF exposure on synaptic plasticity in rodents

Reference	Exposure level and duration	Model	Endpoint	Other endpoints	Results
(Crouzier et al., 2007)	1800 MHz Horn antenna 24h 0.048 W/kg whole body, 0.078 W/kg head Or 0.36 W/kg whole body, 0.53 W/kg head	Sprague-dawley rats	Sleep EEG	No effect on acetylcholine in hippocampus or on lipid metabolism	No general effect On day 3, less REM sleep in low exposure group
(Vorobyov et al., 2010)	915 MHz Circular antennae over cage 3x10 min/day, 3 days over 5 day period 0.007W/kg	Wistar rats	Wake EEG in cortex and hypothalamus during exposure on 5 days		Increased β-wave activity and cumulative effect of exposure
(Prochnow et al., 2011)	2 GHz head waveguide 2h	Wistar rats	EPSP in hippocampus	Increased corticosterone and ACTH in sham and exposed groups	10 W/kg decreases both LTP and LTD non-thermal

	2 or 10 W/kg to brain				
(Hidisoglu et al., 2016)	2100MHz Antenna placed in centre of carousel 2h/day, 1 or 10 weeks 0.57-0.95 W/kg to head	Wistar rats	Visual evoked potentials	Decreased oxidative stress after 1 week, increased after 10 weeks.	7 days exposure decreased latency of the potentials, while 10 weeks exposure increased the latency
(Wang et al., 2017)	1800 MHz Horn antenna over cage 30 min 3.3 W/kg in brain	C57Bl/6	EPSP in hippocampus CA1 and mPFC	Changes to spine density in prefrontal cortex and hippocampus Increased exploration of novel object day 3-8 post exposure	Exposure significantly increased the action potential firing frequency by 49.83%. exposure significantly reduced the delay to elicit an action potential. reduced the action potential half-width by 16.48%. and significantly depolarized the resting potential mPFC: exposure significantly reduced the delay to elicit an action potential. reduced the threshold. the action potential half-width was also significantly reduced by 15.01% and decreased resting membrane potential

c-fos					
(Morrissey et al., 1999)	Dipole antenna 1.6GHz 1h 0.35-11.21 W/kg brain 0.19-6.42 W/kg whole body	Balb/c mice	c-fos mRNA through brain		At high thermal exposure levels, see increase
(Stagg et al., 2001)	Dipole antenna 1.6GHz 2h 0.6, 1.6, 5 W/kg to brain	Fischer rats	c-fos cerebrum and cerebellum	Ornithine decarboxylase enzyme, *jun,* ACTH, corticosterone up to 5 W/kg no effect of exposure	No effect, but high stress in restrained shams as well
(Finnie, 2005)	Dipole antenna 900 MHz 1h 4W/kg	C57Bl/6	c-fos IHC		No effect, but high stress in restrained shams as well
(Finnie et al., 2007)	Dipole antenna 900 MHz 1h/d, 5d/week, 104 weeks 4W/kg	C57Bl/6	c-fos IHC		No effect of exposure

Neurotransmitters					
(Mausset-Bonnefont et al., 2004)	900 MHz Loop antennae over head 6 W/kg to head 15 min, head	Wistar rats	Cortex hippocampus striatum		Autoradiografi – receptor binding. NMDA-R: ↓Striatum and cortex Hipp n.s. WB – NMDAR: Crude: 10-20%↓ in all. GluN1 in cortex, GluN2a cortex and hippocampus, GluN2b in striatum. In PSD: in Striatum all subunits 90 %↓, cortex: 30-80% decrease in all. In hippocampus GluN2b decreased. P of NR1 showed decrease in cortex only. IHC: GFAP. 3d after exposure find great increase in GFAP reactivity in all three areas.
(Khadrawy et al., 2009) No acces, details from EMF-portal.org	900 MHz 2 central antennaes in circular cage for 16 rats SAR 1.165 W/kg head 1h/d, 1M, 2M, 4M 4M +1 M washout	Rattus norvegicus 1M or 4M old	cortex		HPLC: glutamate, aspartate, glutamine, GABA, glycine, taurine Adult: 1M ↓glutamate, aspartate 2M ↑glutamate, aspartate, glycine and taurine 4M ↑Aspartate, GABA, glycine, taurine Young: 4M ↓glutamine, ↑GABA and taurine

					+1M ↓glutamine, ↑GABA and glycine
(Noor et al., 2011)	900 MHz, not specified source SAR 1.165 W/kg 1h/d, 1M, 2M, 4M 4M +1 M washout	Rattus norvegicus 1M or 4M old	Midbrain, cerebellum, medulla		HPLC: Cerebellum: increase in both excitatory and inhibitory aa in young and adult Midbrain: young, ↓ excitory amino acids Adult, ↑glycine 1M, ↑GABA 1 + 4M Medulla:Young: 1,2M, ↓glutamate and aspartate 4M ↑glutamine Adult, ↑inhibitory amino acids.
Trophic factors					
(Maskey and Kim, 2014)	835 MHz, horn antennae in cage 1.6 W/kg whole body 8h/d 3 months	ICR Mouse,	auditory system: lateral superior olive, superior paraolivary nucleus, medial superior olive and medial nucleus of the trapezoid body		BDNF, GDNF. Significant decrease in all regions

Abbreviations: BDNF: brain derived neurotrophic protein, d: day, EEG: electroencephalography, EPSP: excitatory postsynaptic potential, GABA: gamma aminobutyric acid, GDNF: glial derived neurotrophic factor, h: hour, HPLC: high performance liquid chromatography, IHC: immunohistochemistry, LTD: long-term depression, LTP: long-term potentiation, w: week

Table A2.3 Studies of effects of RF exposure on hippocampal neurogenesis in rodents

Reference	Exposure level and duration	Model	Endpoint	Other endpoints	Results
(Kim et al., 2008)	849MHz or 172.5 MHz 6 and 12 months 7.8 W/kg brain, Or 5.28 or 7.8 W/kg brain and 1.55 W/kg whole body	C57Bl/6J	PCNA SGZ and SVZ	No effect on apoptosis, DCX, NeuN, GFAP in hippocampus and cerebellum	No effect of exposure on proliferation
(Odaci et al., 2008)	900 MHz, antenna in tube 1h/day through grstation Peak at 2 W/kg	Wistar rats	CV DG		Fewer cells in DG at 4 weeks of age
(Bas et al., 2009)	900 MHz, antenna in tube Continuous through gestation Whole body peak at 2 W/kg	Wistar rats	CV CA	No skeletal effects	Fewer cells and more dark cells at 4 weeks of age
(Kim et al., 2015)	848.5 MHz reverberation chamber 1h/d or 8h/day for 2 weeks 2 W/kg whole body	Sprague dawley rats	BrdU injected daily last 5 days of exposure SGZ and SVZ		No effect of exposure on proliferation
(Son et al., 2015)	1950 MHz reverberation chamber 60 days	C57Bl/6J	PCNA DG and CA1	No effect on NeuN, GFAP, Iba-1, OF, passive avoidance test	No effect of exposure on proliferation

	5W/kg				
(Xu et al., 2017)	1800MHz 8h/day, 3 days 1.16 W/kg brain	kunming mice P7 and p21	BrdU injection daily before exposure and phospho histone H3 and brain-lipid binidng protein	Increased caspase3 and fluoroJade	P7; increased BrdU but decreased phosphohistone and BLBP. P21: no effect

Abbreviations: BrdU: bromo-deoxy uridine, BLBP: brain lipid binding protein, CA: cornu ammonis, CV: cresyl violet, d: day, DCX: doublecortin, DG: dentate gyrus, GFAP: glial fibrillary acidic protein, h: hour, NeuN: neuronal nuclei,OF: open field, PCNA: proliferating cell nuclear antigen, SGZ: subgranular zone, SVZ: subventricular zone, w: week

Table A2.4 Studies of effects of RF exposure on glia and neurodegeneration in rodents

Reference	Exposure level and duration	Model	Endpoint	Other endpoints	Results
(Kumlin et al., 2007) No access, info from EMF-portal.org	Radial waveguide 900 MHz 2h/d. 5d/week, 5 weeks 0.3W/kg or 3 W/kg whole body	Wistar rats	BBB leakage,cell death	OF, plus maze, startle response, water maze Improved learning in water maze, no effect in other tests	No effect on BBB leakage, cell death
(Kim et al., 2008)	Carousel around dipole antenna 849 MHz or 1762.5MHz, 1h/day, 5 days/week, 6 or 12 months 5.28-7.8 W/kg in brain	C57Bl/6 mice	Hippocampus and cerebellum TUNEL, HE staining,	GFAP, NeuN, PCNA	No effec of exposure
(Dasdag et al., 2009)	Carousel with central antenna 900 MHz 2h/d, 10 months 0.17-0.58W/kg brain	Wistar rats	Parietal lobe, Caspase-3, p53, total antioxidant capacity, total oxidant status, catalase activity.		Reduced apoptosis, increased total antioxidant capacity and catalase activity.

(de Gannes et al., 2009)	TEM cell 900 MHz 2 hours 0.14W/kg and 2 W/kg brain	Fischer 344 rats	Cresyl violet, dark neurons, BBB leakage, fluoroJade B, TUNEL, 14 and 50 days after exposure		No effect of exposure
(Bouji et al., 2012)	900 MHz, loop antennae by head 15 min 6 W/kg head	6W or 12M Sprague dawley rats	Increased IL1beta olfactory bulb in 12M olds. No effect on IL6.	IHC, OD, soma diameter, processes number and distance between astrocytes in hippocampus, cortex and striatum. Increased corticosterone in 6 w olds, increased memory in fear conditioning in 12 months old.	No effect of exposure on any parameters
(Maskey et al., 2012)	835 MHz, waveguide 8h/day 3 months 1.6 W/kg	ICR mice	hippocampus Propidium iodide TUNEL	Decreased calbindin D28k and increased GFAP	find apoptotic cells
(Megha et al., 2012)	900 and 1800MHz. GTEM cell app. 0.6 mW/kg 30d 2h/d	Fischer rats	oxidative stress (lipid perocidation, protein oxidation, glutathione reduction), inflammation (IL-6, TNFα).	EPM, water maze, impaired performance in both tests	increased oxidative stress, IL6 and TNF-alpha
(Hidisoglu et al., 2016)	2100 MHz, antenna in centre of carousel, 0.57-0.95 W/kg	Wistar rats	iNOS, nNOS in lateral geniculate nucleus, oxidative stress (lipid peroxidation, NO, glutathione,	EEG, see different effects of 1 and 10 weeks exposure. Shorter and longer latencies of visual evoked potentials respectively	Lipid peroxidation: decreased after 1 week, increased after 10 weeks. Increased activity og catalase

	head 2h/d, 1 week or 10 weeks		SOD, glutathione peroxidase and catalase acticity. CV staining		and glutathione peroxidase after 1 week, increased NO level after 1 week. Decreased SOD activity After 10 weeks: decreased glutatione peroxidase and catalase activity, and decreased NO level.
(Kim et al., 2017b)	835MHz Waveguide with central antenna 5h/d 12 weeks 4 W/kg	C57Bl/6	Cerebral cortex autophagy markers	Rotarod no effect Increased activity OF Decreased Bax and Bcl-2	qPCR autophagy genes: *AMPK1α , Ulk1, Beclin1/2, Atg9A, Atg4A/B, Atg5, and LC3A/B),* Elevated autophagy genes (25-250%) Protein: Beclin and LS3B increased Observed autophagosomes, damages axon myelin by transmission electron microscopy
(Kim et al., 2017a)	835MHz Waveguide with central antenna 5h/d 4 weeks	C57Bl/6	Brainstem and cerebral cortex Autophagy markers		qPCR: Beclin1,2, Atg9, 4A,B, 5, LC3A/B, Most increases 1.5-3x in cortex,

	4 W/kg whole doby				but not in brain stem, protein levels of LX3BII and Beclin1 were increased in cortex, but not in brain stem Bcl2, Bax decreased in cortex. In brainstem bax increased, Bcl-2 decreased
Astroglia					
(Mausset-Bonnefont et al., 2004)	900 MHz loop antenna over head 15 min 6 W/kg in brain	Male wistar rats	IHC immunoreactive area in cortex, hipp and striatum 3d delay		Significant increases- 20-25% in cortex and hipp and 140% in striatum
(Brillaud et al., 2007)	900MHz 6W/kg brain Antenna by head 15min	Rat	GFAP 3,6 or 10d delay		increase after 2 d in frontal cortex and putamen and after 3d in the cerebellum as well.
(Ammari et al., 2008b)	900 MHz Loop antenna by head 1.5 or 6 W/kg brain 45min/d or 15min/d,	Male SD rats	IHC area in prefrontal cortex, caudate putamen, lateral globus pallidus of striatum, DG and cerebellum cortex 10 d delay		Significant increased area in all brain regions after exposure at 6 W/kg but not 1.5W/kg. 50% in hipp

	5d/w, 6M				
(Kim et al., 2008)	849 and 1763 MHz central dipole antenna 5.28-7.8 W/kg brain 1.55 W/kg whole body 1h/d, 5d/w, 6 or 12M	Male and female C57bl/6 mice	IHC, hippocampus and cerebellum	No effect on TUNEL,GFAP, PCNA, NeuN	No difference
(Ammari et al., 2010)	900 MHz, loop antenna by head 1.5 and 6 W/kg brain 45 or 15 min, 5d/w, 2M	Male SD rats	IHC GFAP, Prefrontal cortex, cerebellar cortex, dentate gyrus of the hippocampus, lateral globus pallidus of the striatum, and the caudate putamen. 3 and 10d delay		Significantly increased area stained in all brain regions after 3 days at both doses. 40% in DG 1.5 W/kg. 15% in 6 W/kg group. At 10d delay a significant difference only in 6 W/kg in the pfc and DG of 140%.
(Maskey et al., 2010)	835 MHZ Waveguide with central antenna SAR of 1.6 W/kg 8h/d , 3M	ICR mice	IHC GFAP in the hippocampus	Decreased calbindin, increase TUNEL and Propidium iodide	5-15% increases in OD in CA3, CA1 , GCL and hilus but not ML. Also more hypertrophic astroglia in exposed mice.
(Bouji et al., 2012)	900 MHz, loop antenna by head	6W or 12M Sprague dawley	IHC, OD, soma diameter, processes number and distance	Increased IL1beta olfactory bulb in 12M olds, increased corticosterone in 6 w	No effect of exposure on any parameters

	15 min 6 W/kg head	rats	between astrocytes in hippocampus, cortex and striatum 2 days delay	olds, increased memory in fear conditioning in 12 months old. No effect on IL6	
(Maskey et al., 2012)	835MHZ horn antenna over cage SAR of 1.6 or 4W/kg 8h/d, 1 month	Male ICR mice	IHC GFAP hippocampus Morphology and OD	Calbindin and calretinin decrease in both exposure groups	increased OD in CA1 in the 1.5 W/kg group, and in the GCL, ML and hilus in both groups
(Court-Kowalski et al., 2015)	900 MHz central dipole antenna 4 W/kg whole body 1h/d, 5d/w, 102 W	Female C57bl/6	GFAP IHC Phenotype and antigen amount in cerebellum, cortex or corpus callosum		No difference
(Son et al., 2015)	1950 MHz reverberation chamber 5W/kg 2h/d, 5/w. 2M	Female C57bl/6	IHC in CA1 and DG	No effect on NeuN, PCNA, Iba-1 or Passive avoidance test	No difference
(Barthélémy et al., 2016)	900 MHz loop antenna by head, 15 min or 45 min 1.5 or 6 W/kg whole body	Sprague dawley rats	GFAP (ELISA).	Myelin basic protein, Abeta1-40 protein, no effect. fear conditioning, reduce memory in 6 W/kg group. No effect in elevated plus maze or OF	119% increase in cytosolic fraction in hipp at 6 W/kg. In striatum 114% in striatum in 1.5W/kg and in olfactory bulb 46% 6 W/kg

Microglia					
(Finnie et al., 2010)	900 MHz central dipole antenna 60 min or 5d/w 104 weeks 4W/kg whole body	mice	IHC Iba-1 cortex and hippocampus		No difference
(Son et al., 2015)	1950 MHz Reverberation chamber 2h/d, 5/w. 2M 5W/kg	Female C57bl/6	IHC in CA1 and DG	No effect on NeuN, PCNA, GFAP or Passive avoidance test	No difference
BBB					
(Salford et al., 2003)	915 MHz, mobile phone coupled to TEM cell 2h 0.002W/kg whole body	Fischer rats	BBB integrity, albumin leakage	CV staining for darkly stained neurons in cortex, hippocampus, basal ganglia	Found albumin leakage and darkly staned neurons in exposed rats.
(Masuda et al., 2009)	915 MHz, TEM cell 2h 0.02, 0.2. 2 W/kg whole body	Fischer rats	BBB integrity, albumin leakage	HE staining and CV staining for darkly stained neurons	No effect of exposure

(McQuade JM et al., 2009)	915 MHz, TEM cell 30 min App. 0.002, 0.02, 0.2,2,20 W/kg	Fischer rats	BBB integrity, albumin leakage		No effect of exposure
(Sirav and Seyhan, 2011)	900 and 1800 MHz, Horn antenna 20 minutes 0.00426 or 0.00146 W/kg respectively	Wistar rats	BBB integrity, evans blue leakage and albumin		Increased permeability in males but not in females
(Tang et al., 2015b)	900 MHz, monopole antenna, immobilised 3h/d, 14 or 28 days 2 W/kg in head	Sprague-Dawley rats	Albumin and evans blue leakage	Water maze, improved performance afer 28 days in probe trial TEM to study ultrastructure of neurons in hippocampus and parietal cortex. Edema and unclear neuronal boundary HO-1 and MAPK-phosphatase increased and d p-ERK decrease after 28days	Increased albumin in both exposure groups in cortex, only in hippocampus after 28 days. Evans blue leakage after both periods.
(Sırav and Seyhan, 2016)	900 and 1800 MHz, Horn antenna 20 minutes 0.02 W/kg	Wistar rats	BBB integrity, evans blue leakage		Increased permeability in males. In females increased leakage at 900 MHz.

Alzheimers disease models					
Reference	**Exposure level and duration**	**Model**	**Memory**	**Other endpoints**	
(Arendash et al., 2010)	918 MHz, antenna in middle of faraday cage. 2x1h/d, 8.5-9.5 months 0.25W/kg	APPsw, and WT Young adults (2-2.5months old) and aged adults (4 months)	RA-water maze, cognitive interference test, y-maze, OF, EPM	Cortex and hippocampus Aβ levels and aggregates, SOD and glutathione levels and activity, DNA repair enzyme activity, protein oxidation	Improved performance in cognitive test in both young and aged Reduced amyloid aggregation, aged, No effect on oxidative stress Microarray showed different effect of exposure in WT and Tg model
(Dragicevic et al., 2011)	918 MHz, antenna, cages around in a faraday cage. 2x 1-h /day1month 918 MHz SAR=0,25-1,05W/kg whole body	APPswe+PS1 and aged normal mice APPsw PS1, APPsw, PS1, and NT 15-17M		Aβ-40 level and mitochondrial function. Cerebral cortex, hippocampus, striatum, and amygdala	5-10x increased soluble Aβ-40 Enhanced mitochondrial function by 50 –150% . 216% higher reactive oxygen species levels, 56% lower membrane potential level, 71% lower ATP levels, and 71% lower Complex IV activity.

(Arendash et al., 2012)	918MHz, central antenna, 0.25W/kg Whole body 2-2.5 months old: 2x1h/day, 5d/week, 6-7months. 5 months old: 2x1h/day, 5d/week, 8.5 months.	AβPPsw Tg mouse	Young: Radial arm water maze, cognitive interference test, y-maze, sensorimotor function, anxiety. - No effect on RAWM. - Cognitive interference test: exposure ameliorated the cognitive decline. - No effect on anxiety or sensorymotor function Aged - RA-WM, (before, 2,5,8months) - cognitive test, - WT and Tg mice showed cognitive improvement compared to non-radiated Tg mice after 8months.	No effect oxidative stress in young AD model. Young: More soluble Abeta in hipp and frontal cortex (20-50%) but not significant. Aged: less Abeta in hipp and enthorinal cortex (32-35%) + increased levels of soluble Abeta. (but great variations, not that convincing)	
(Dasdag et al., 2012)	900 MHz, antenna in centre of carousel, restrained. 2h/day, 7d/week,	Wistar rats (not Alzheimer model)		b-amyloid (40) (ELISA) in brain increase in Abeta40. Carbonyl and malondialdehyde (marker of stress and lipidperoxidation)	

	10 months 0.17-0.58 W/kg			Significantly increased carbonyl.	
(Jeong et al., 2015)	1950 MHz, reverberation chamber 2h/d, 5d/w, 8 months 5 W/kg	5xFAD and WT 1.5 months	OF, passive avoidance, Y-maze	Hipp and enthorinal cortex: Soluble and aggregated Aβ and APP level BACE level Astroglia and microglia Microarray	Decrease in soluble and aggregated Aβ and APP Decreased BACE in hippocampus Decreased GFAP and Iba-1 protein levels in Tg model, but not WT. Normalised behaviour in OF and passive avoidance Improved spatial memory in Y-maze
(Son et al., 2016)	1950 MHz, reverberation chamber 2h/d, 5d/w 3 months 5 W/kg whole body	5xFAD 1.5 months old	OF, NOR, Y-maze spontaneous alternations, water maze	Soluble and aggregated amyloid , APP,	No effect of exposure

Multiple sclerosis					
(Anane et al., 2003)	900 MHz, loop antenna by head. Restrained rats 2h/d, 21 days 1.5 or 6 W/kg	Lewis rats with EAE	Clinical disease scoring 0-4 for 35 days		No effect of exposure on onset, duration or termination of EAE
Epilepsy					
(Lopez-Martin et al., 2006)	900 MHz, antenna 2h 0.15-0.24 W/kg whole body, 0.27-0.42 W/kg brain	Sprague-Dawley rats treated with picrotoxin	EEG c-fos in cortex, hippocampus, thalamus		Increased number of sezures and more c-fos+ cells No effect of exposure in control rats
(Carballo-Quintas et al., 2011)	900 MHz, antenna 2h 1.38-1.44 W/kg brain 0.78-0.81 W/kg whole body	Sprague-Dawley rats treated with picrotoxin	c-fos in cortex, hippocampus GFAP after 3 days seizures		Increased c-fos up to three days after exposure. In some regions a decrease was seen after three days but not after 90 min or 24 hours. increased GFAP
Aged rodent					
(Finnie et al., 2007)	Dipole antenna 900 MHz 1h/d, 5d/week, 104 weeks 4W/kg	C57Bl/6 2 years of exposure	c-fos IHC		No effect of exposure

(Kim et al., 2008)	849 and 1763 MHz central dipole antenna 5.28-7.8 W/kg brain 1.55 W/kg whole body 1h/d, 5d/w, 6 or 12M	Male and female C57bl/6 mice **1 year exposure**	IHC, hippocampus and cerebellum	No effect on TUNEL,GFAP, PCNA, NeuN	No difference
(Arendash et al., 2010)	918 MHz, antenna in middle of faraday cage. 2x1h/d, 8.5-9.5 months 0.25W/kg	APPsw, and **WT** Young adults (2-2.5months old) and aged adults (4 months)	RA-water maze Cognitive interference test Y-maze	PARP, SOD, GSH, protein oxidation	Young: Increased spontaneous alternations in WT mice Decreased PARP and mitochondrial SOD Aged: Improved performance of WT mice in cognitive interference test
(Arendash et al., 2012)	918MHz, central antenna, 0.25W/kg Whole body 2-2.5 months old: 2x1h/day, 5d/week, 6-7months.	AβPPsw and **WT** mouse	OF, Y-maze, and some motor function tests RA-water maze		No effect of exposure in WT mice

	5 months old: 2x1h/day, 5d/week, 8.5 months.				
(Bouji et al., 2012)	900 MHz, loop antenna, restrained rats 15 min 6 W/kg	Sprague-Dawley rats 6 weeks or 12 months	GFAP IHC, IL1beta, IL6, corticosterone levels	Fear conditioning, effect of exposure in 12 months olds	Increased IL-1beta in olfactory bulb in 12 months olds, not in cortex, subortical nuclei, cerebellum Increased corticosterone in 6 weeks old, not 12 months
(Klose et al., 2014)	900 MHz wavegioe, restraine rats 5-120 min/d, 5d/w, for 7 days, 14 days-19 months 0.7, 2.5, 10 W/kg to brain	Juvenile, adult and presenile rats **19 months exposure**	RAM and water maze No effect in rotarod, OF		No effect No effect in rotarod, OF
(Court-Kowalski et al., 2015)	900 MHz central dipole antenna 4 W/kg whole body 1h/d, 5d/w, **102 W**	C57bl/6 2 years of exposure	GFAP IHC Phenotype and antigen amount in cerebellum, cortex or corpus callosum		No difference
(Bouji et al., 2016)	900 MHz, loop antenna, restrained,	Wistar rats 4-6 months	GFAP IHC, IL1beta, IL6, corticosterone	OF, EPM, fear conditioning, RAM	Pooled young and old rats, see decreased anxiety after

	45 min on some days for 26 days 6 W/kg brain	or 22-24 months			exposure. No other effects.

Abbreviations: APP: amyloid precursor protein, BBB: Blood brain barrier, CV: cresyl violet, d:day, EEG: electroencephalography, EPM: elevated plus maze, FAD: familial alzheimer's disease, GFAP: glial fibrillary acidic protein, GSH: glutathione, HE: hematoxylin eosin, h: hour, IHC: immunohistochemistry, NeuN: neuronal nuclei, NO: nitric oxide, OD: optical density, OF: open field, PCNA:proliferating cell nuclear antigen, RAM: radial arm maze, SOD: superoxide dismutase, TUNEL: terminal deoxynucleotidyl transferase, w: week, WT: wildtype

Appendix 3 Experimental protocols

Reagents for protein extraction

2X protein extraction buffer

2% SDS

100 mM Tris base pH 8.0

Made up in H_2O

Add proteinase inhibitors and phosphatase inhibitors immediately before use.

Proteinase inhibitors

cOmplete protease inhibitor cocktail tablets (Sigma, 000000011697498001).

Phosphatase inhibitors - Okadaic acid and sodium orthovanadate

Okadaic acid (Abcam, ab120375. 100 µM stock in DMSO. Final concentration of 100 nM in protein lysis buffer

Sodium orthovanadate (Sigma, S6508) 100 mM stock is made in water adjusted to pH 10. A final concentration of 1mM in the protein lysis buffer

Western blotting

TBST

Tris base pH 7.4	30 g
NaCl	80 g
KCl	2 g
Tween 20	5 ml

In 10 litres of water

SDS running buffer

Tris Base	30.2 g
Glycine	188 g
20 % SDS	50 ml

In 10 litres of water. pH 8.3

5x protein sample loading buffer

Tris HCl pH 6.8	250mM
DTT	500 mM
SDS	10 %
Bromophenol blue	0.5 %
Glycerol	50 %

Store at -20˚C.

Reagents for BrdU-double fluorescence staining

0.1 M borate buffer

0.3814 g Na-tetraborat 10 H2O

100 ml H_2O

pH= 8.5, using HCl

2M HCl in TBS

17 ml concentrated HCl

83 ml TBS

Reagents for Hematoxylin staining

Scott's tap water

3.5 g $NaHCO_3$

20 g $MgSO_4$

In 1 litre of tap water

Reagents for 0.5 % Cresyl Violet solution

2.5 g cresyl violet acetate

250 ml distilled water

1.5 ml acetic acid

Stir for 20 minutes

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