

Sleep slow wave oscillation: effect of ageing and preceding sleep-wake history



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Abstract

Sleep is well-established to become more superficial and fragmented as we age, with deficits in cognitive processing also commonly observed. While effects have been identified in both humans and mice (used in this thesis), there are important species differences in these findings and importantly, very little is known about the neural dynamics underlying these changes. By integrating several state-of-the-art approaches from putative single unit electrophysiological recordings to behavioural and pharmacological assessments, this thesis aimed to provide novel insights into the neural mechanisms involved in the age-dependent changes in sleep and cognition in mice. Firstly, this thesis investigated the neural activity underpinning the known global sleep changes that occur with ageing. Surprisingly, the majority of neuronal measures quantified in this study were resilient to the effects of ageing. Therefore the global sleep disruptions identified with ageing are unlikely to arise from changes in local cortical activity. Secondly, diazepam injection was found to suppress neural activity, in addition to previously reported effects on electroencephalography (EEG). Subtle differences in the effects of diazepam were identified across age groups, which may account for the variability seen in the efficacy of benzodiazepines in older individuals. Thirdly, ageing and sleep deprivation were found to have only a few effects on performance in a spatial learning task, the Morris water maze (MWM). Suggesting that spatial learning may be fairly resilient to the effects of ageing and sleep deprivation. Finally, this thesis presents preliminary analyses that showed mice were able to perform two novel paradigms of the visual discrimination task, suggesting their suitability in studying the link between ageing, sleep and cognition. Together the studies presented in this thesis provide insights into the differences between global and local mechanisms affected by ageing. Only by understanding local mechanisms will we be able improve on current treatments aimed at helping with the unwanted effects of healthy ageing, such as cognitive decline and sleep disruptions.

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Declaration and Attributions

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Dr Chris Holton assisted with initial analyses of the water maze task, presented in Chapter 5.

Table of Contents

Glossary and Abbreviations	8
Chapter 1: An introduction to sleep, ageing and memory	9
1.1 An introduction to sleep	9
1.1.1 Electrophysiological correlates of sleep and waking	10
1.1.2 Homeostatic and circadian regulation of sleep	16
1.1.3 Local regulation of sleep	21
1.1.4 Function of sleep	24
1.2 An introduction to ageing	29
1.2.1 Anatomical and functional changes in the brain with ageing	29
1.2.2 Age-related changes in sleep	30
1.3 Pharmacological manipulation of sleep	35
1.4 Overall objectives of the project	38
Chapter 2: General Methods	41
2.1 Experimental animals	41
2.2 Surgical procedure for electrophysiological recordings	42
2.2.1 Electroencephalography and electromyography surgery	42
2.2.2 Microwire array implantation	44
2.3 Signal processing, vigilance state scoring and analysis	45
2.4 Spike sorting procedure for analysis of neuronal activity	46
2.5 Slow wave and OFF period detection	48
2.6 Sleep deprivation techniques	49
2.7 Behavioural testing	51
2.7.1 Histological verification of recording site	51
2.8 Statistical analysis	52
Chapter 3: The effect of ageing on cortical activity	53
3.1 Introduction	53
3.1.1 Experimental aims	55
3.2 Methods	56
3.2.1 Experimental animals	56
3.2.2 Surgical and recording procedures	56
3.2.3 Experimental protocol – baseline and sleep deprivation recordings	57
3.2.4 Relationship between LFP slow waves and cortical MUA	58
3.2.5 Neuronal phenotyping and vigilance-state dependency of cortical firing	59
3.2.6 Data and statistical analysis	59
3.3 Results	60
3.3.1 Characterisation of global sleep structure	62
3.3.2 Electrophysiological correlates of ageing: EEG and LFP	67
3.3.3 Electrophysiological correlates of ageing: Neuronal activity	69

3.3.4	Effect of preceding sleep-wake history	75
3.4	Discussion	83
3.4.1	Conclusions.....	89
Chapter 4:	<i>The effect of diazepam on cortical activity.....</i>	91
4.1	Introduction.....	91
4.1.1	Experimental aims	92
4.2	Methods	92
4.2.1	Experimental animals.....	92
4.2.2	Experimental protocol and specific analyses.....	92
4.2.3	Statistical analysis	95
4.3	Results	96
4.3.1	Effect of diazepam on sleep-wake architecture	96
4.3.2	Spectral power.....	101
4.3.3	Analyses of neural activity.....	108
4.4	Discussion	111
4.4.1	Neural characteristics.....	113
4.4.2	Conclusions.....	114
Chapter 5:	<i>The effect of ageing and sleep on spatial learning in the Morris water maze task 115</i>	
5.1	Introduction.....	115
5.1.1	The Morris Water Maze (MWM)	115
5.1.2	Sleep and spatial learning.....	118
5.1.3	Experimental aims	120
5.2	Methods	120
5.2.1	Experimental animals.....	120
5.2.2	Water maze design	121
5.2.3	Experimental protocol.....	122
5.2.4	Sleep deprivation procedure	125
5.2.5	Specific analyses	125
5.2.6	Statistical analysis	127
5.3	Results	127
5.3.1	Spatial learning in the MWM task.....	127
5.3.2	Spatial memory in the MWM task.....	132
5.3.3	Cued visual Trial.....	138
5.4	Discussion	140
5.4.1	Further Considerations.....	144
5.4.2	Conclusions.....	148
Chapter 6:	<i>The link between sleep, waking performance, cognitive function and ageing in two novel paradigms of the visual discrimination task</i>	149
6.1	Introduction.....	149
6.1.1	Experimental aims	150

6.2	Methods.....	151
6.2.1	Pre-experiment training in the VDT task and surgical preparation.....	151
6.2.2	Experiment 1: extended VDT	155
6.2.3	Experiment 2: the alphabet task	156
6.2.4	Statistical analyses	158
6.3	Results.....	158
6.3.1	Experiment 1 results: extended VDT.....	158
6.3.2	Experiment 2 results: the alphabet task.....	167
6.4	Discussion.....	176
6.4.1	Extended VDT.....	176
6.4.2	Limitations of the study.....	179
6.4.3	Overall conclusions	180
Chapter 7:	<i>Importance of findings and future directions</i>	<i>181</i>
7.1	Main findings.....	181
7.2	Novel observations.....	183
7.3	Is ageing different between humans and mice?	189
7.4	The role of extrinsic and intrinsic factors in the effects of ageing on sleep.....	191
7.5	Future directions	193
7.6	Overall conclusions.....	196
Bibliography		197

Glossary and Abbreviations

A comprehensive list of abbreviations and terminology used in this thesis are provided below.

- AMPA:** α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- Circadian rhythm:** a biological rhythm approximately 24 hours in length
- DZP:** diazepam
- EA mice:** early adulthood mice aged ~5 months old
- EEG:** electroencephalography
- EMG:** electromyography
- FFT:** Fast Fourier Transform
- GABA:** gamma-aminobutyric acid
- LA mice:** late adulthood mice aged ~12 months old
- LFP:** local field potential
- Local sleep:** occurrence of sleep-like activity occurring in localised, distinct regions of the cortex
- LTD:** long-term depression - strengthening of synapses
- LTP:** long-term potentiation - weakening of synapses
- MWM:** Morris water maze task
- MUA:** multiunit activity; summation of neuronal activity from a number of cells
- NMDA:** N-methyl-D-aspartate
- NREM sleep:** non-rapid eye movement sleep
- OA mice:** old aged mice aged ~24 months old
- OFF period:** absence of spiking (down-state) synchronised across neuronal populations
- ON period:** depolarised up-state caused by active neuronal firing across neuronal populations
- Preceding sleep-wake history:** vigilance states/behaviours occurred prior to the time of interest
- REM sleep:** rapid eye movement sleep
- SCN:** suprachiasmatic nucleus – the master circadian clock
- Sleep homeostasis:** regulation of sleep need
- Slow oscillation:** bi-stable slow (<1Hz) oscillatory alternation in membrane potential
- Slow wave:** regular waveform occurring at the SWA frequency range
- SWS:** slow wave sleep
- Spectral analysis:** how much specific frequencies contribute to a signal
- Spike:** a neuronal action potential
- SWA:** slow wave activity (0.5-4Hz)
- SWS:** slow wave sleep
- Synaptic homeostasis hypothesis:** during waking there is a progressive, net increase in the strength of cortico-cortical connections
- Synaptic plasticity:** change in the strength of synapses
- Two-process model:** propensity to sleep is determined by the interaction between both a circadian (process C) and a homeostatic component (process S)
- Vigilance state:** states can be classified as wake, NREM sleep or REM sleep
- VDT:** visual discrimination task
- Zeitgebers:** cues from the environment

Chapter 1: An introduction to sleep, ageing and memory

1.1 An introduction to sleep

Sleep is a highly dynamic process changing not only across the night but also the lifespan.

Behaviourally, sleep can be defined as a reversible, quiescent or inactive state that has an increased arousal threshold compared to quiet wake, involves specific sleeping sites and species-specific postures and usually occurs at a species-specific time of day (Campbell and Tobler, 1984; Deboer, 2015). Evidence that sleep is homeostatically regulated i.e. sleep loss is subsequently compensated, has later been added as a defining characteristic of sleep (Tobler, 1995). Sleep has been identified (in some form or other) in all species studied (Cirelli and Tononi, 2008; Tobler, 1995), most commonly in mammals and birds, but also simple organisms such as *Drosophila melanogaster* (fruit flies) (Donlea et al., 2018, 2014; Hendricks et al., 2000; Pimentel et al., 2016; Shaw et al., 2000), zebrafish (Prober et al., 2006; Yokogawa et al., 2007; Zhdanova et al., 2001) and *C.elegans* (Raizen et al., 2008). Therefore, there are a number of model systems available for studying sleep.

In humans and rodents recordings of brain activity have allowed sleep to be subdivided into non-rapid eye movement (NREM) and REM sleep. In humans, sleep can be further subdivided into three stages of NREM sleep (N1-N3), and REM sleep. Sleep progresses systematically through the different stages of NREM sleep with sleep becoming gradually deeper, before eventually entering a period of REM sleep. In humans, episodes of REM sleep occur every 90-120 mins, increasing in duration with each cycle (Olbrich et al., 2011). N2 sleep accounts for the majority of sleep (50%) with REM sleep comprising approximately 20-25% total sleep time (Olbrich et al., 2011). Stages 1 and 2 of NREM sleep are lighter stages consisting of a low voltage, mixed

frequency electroencephalogram (EEG) signal, with spindles and K complexes becoming apparent in stage 2 NREM sleep. Stage 3 is of particular interest in this thesis as it consists of slow wave sleep (SWS) where the EEG shows the highest spectral power in the slow wave activity range (SWA, traditionally defined as 0.5-4Hz). In contrast to humans, sleep in laboratory rodents is polyphasic, the NREM-REM cycle is considerably shorter (approximately 10-20 minutes in rats), and NREM sleep is not subdivided into sub-stages. However, importantly, the predominant types of EEG oscillatory activities occurring during sleep, as well as their dynamics, are similar between humans and animals, allowing for translation between species (Buzsáki et al., 2013).

Global sleep-wake states are controlled by arousal-promoting networks in the lateral hypothalamus, basal forebrain and upper brainstem (e.g. tuberomammillary nucleus (TMN), locus coeruleus (LC), lateral hypothalamus (LH), ventral periaqueductal gray (PAG)), and opposing sleep-promoting networks mostly located in the anterior hypothalamus (e.g. the ventral lateral preoptic nuclei (VLPO)) (Brown et al., 2012; Saper et al., 2010). These opposing systems are referred to as the putative 'sleep switch' (Brown et al., 2012; Saper et al., 2010), which is under the control of a number of neurotransmitters and neuromodulators. Wake-promoting systems include: cholinergic, adrenergic, histaminergic, dopaminergic, serotonergic and orexin/hypocretin nuclei (Fort et al., 2009; Franks, 2008; Nir and Tononi, 2010).

1.1.1 Electrophysiological correlates of sleep and waking

Sleep is a complex phenomenon that can be defined on numerous scales, from activity across 24 hours, down to the activity of individual neurons on the milliseconds scale. In order to learn more about sleep it is important to consider each of these scales (Vyazovskiy and Delogu, 2014). Recording brain activity is crucial for understanding what is happening during sleep, which may provide important insights into the overall function of sleep, as well as sleep regulation. Most

commonly brain activity is recorded by placing electrodes onto the cortical surface in rodents or on the scalp in humans and recording the electroencephalogram (EEG) (Buzsaki et al., 2012; Buzsáki, 2006). EEG signals are the result of the summation of electrical synaptic currents generated from populations of neurons in the surrounding region. In addition, electrodes can also be placed inside the brain to record similar activity but from more localised, smaller neuronal populations (Destexhe et al., 1999; Katzner et al., 2009). Depending on what filters are applied to the signals recorded from these electrodes, both local field potentials (LFPs – low pass filter applied to record slow frequency activity) and multiunit activity i.e. extracellular action potentials (high-pass filters used to record high frequency component) may be recorded. LFPs are mostly only recorded in animals as it is unethical to implant electrodes into the brains of healthy humans.

EEG and LFP recordings reflect the summation of electrical activity generated by neuronal populations. Briefly, the electrical membrane potential of neurons is determined by the gradient of ions across the membrane, which is mostly maintained by an exchange of positively charged potassium and sodium ions (resting membrane potential around -75mV). During depolarisation of the neurons there is an influx of positively charged sodium ions into the neuron which causes action potentials when the depolarisation threshold is exceeded. This current flow is then conducted/propagated to adjacent neurons which can be detected as fluctuations in electrical fields and visualised as waves in the surface EEG or at LFP electrodes (Tatum, 2014). The overall oscillations generated using EEG and LFP are similar, reflecting the slower postsynaptic potentials of neuronal activity, rather than the action potentials themselves (Buzsaki et al., 2012; Buzsáki, 2006). It should however be noted that EEG signals are often affected by factors such as diffusion and volume conduction associated with the numerous incoming inputs being integrated from distant sources and so do not have as good spatial resolution as LFP recordings.

Recording EEG and LFP allow specific brain oscillations to be used in defining vigilance states which have been shown to be conserved across species (Buzsáki et al., 2013). Frequency bands are often classified into infraslow oscillations (0.02-0.1Hz), slow oscillations (0.2-1Hz), delta (1-4Hz) spindle (7-15Hz), theta activity (4-10Hz), fast (20-60Hz) and ultra-fast (100-600Hz), and spectral analysis can be used to show how much specific frequencies are contributing to the signal (Buzsáki, 2006; Buzsáki et al., 2013).

Important new insights have been provided by chronic recordings of extracellular neuronal activity, which reveals characteristic associations between LFP or EEG signals and neuronal firing for each vigilance state (Figure 1-1). Specifically, in the wake state cortical neurons are continuously firing in a quasi-asynchronous manner, resulting in a low-voltage, high frequency EEG/LFP waveform, usually dominated by theta-activity. REM sleep is also predominated by theta frequency activity (7-12Hz in rodents), accompanied by a muscle atonia and ponto-geniculo occipital (PGO) waves associated with synchronised bursting activity originating in the pontine brain stem and propagating to the lateral geniculate nucleus and visual cortex. Parvalbumin GABAergic neurons in the medial septum act as pacemakers for the generation of theta oscillations, which in turn innervate inhibitory neurons in the hippocampus which itself also generates theta oscillations (Brown et al., 2012; Buzsaki, 2002). Finally, during NREM sleep the LFP/EEG is dominated by slow waves (slow wave activity: SWA 0.5-4Hz) which is underpinned by alternating synchronous periods of sustained neuronal firing dominated by intense synaptic activity and periods of neuronal silence (Amzica and Steriade, 1998; Steriade, 2001, 1978; Steriade et al., 1993a). As the network slow oscillation is a major focus of this thesis, more detail on this phenomenon is provided below (section 1.1.1.1).

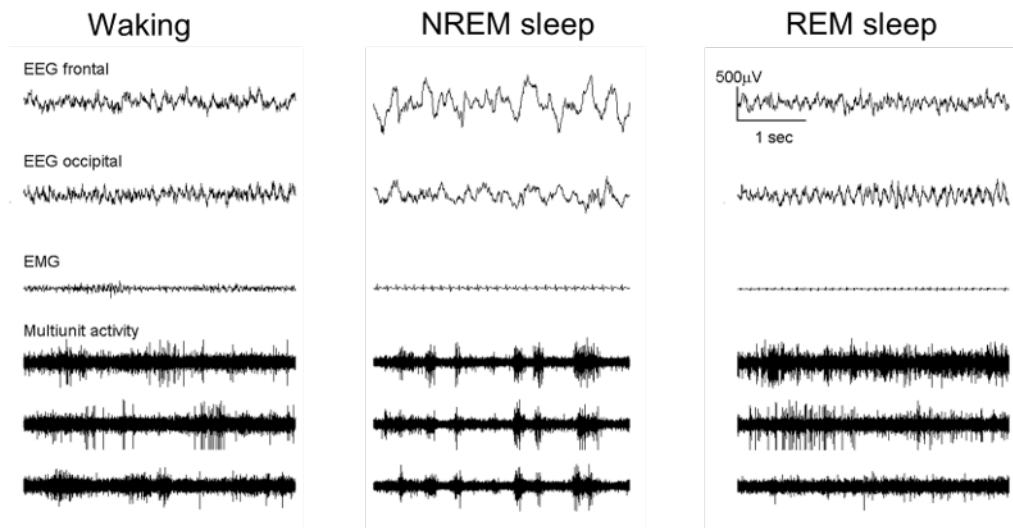


Figure 1-1: Representative examples of EEG from the frontal and the occipital derivations, the EMG, and multiunit activity traces during waking, NREM sleep and REM sleep from one individual EA mouse. Note stronger theta (6-9Hz) activity during waking and REM sleep, especially prominent in the occipital derivation, while during NREM sleep slow waves are present in both the frontal and the occipital derivation.

In humans, slow waves and delta oscillations may also be found during stage 2 sleep, associated with K complexes followed by sleep spindles. K complexes originate in layers I to II of the cerebral cortex and propagate to the thalamus where they are thought to synchronise the activity in the thalamus to result in spindles and slow waves (Cash et al., 2009). It is thought that K complexes are involved in information processing including memory consolidation while they are also involved in suppressing cortical arousal during sleep to therefore preserve sleep (Cash et al., 2009). In both humans and rodents sleep spindles occur during NREM sleep which are apparent as a burst of activity in the frequency range of 7-15 Hz (Dijk et al., 1993; Olbrich et al., 2011; Vyazovskiy et al., 2009b). The corticothalamic network is involved in the generation of delta and spindle oscillations (McCormick and Bal, 1997; Saper et al., 2001; Steriade et al., 1987). The dorsal thalamus receives inputs from ascending sensory pathways and brainstem regions and in turn sends projections to the cerebral cortex and reticular thalamic nucleus (RTN) via glutamatergic connections which drives the generation of spindles during the up state of the slow oscillation (Amzica and Steriade, 1998; Franks, 2008; Timofeev and Chauvette, 2013, 2011).

1.1.1.1 The slow oscillation

Of particular interest to this thesis is the neural network activity underpinning EEG/LFP slow waves, which are predominant during NREM sleep. Specifically, at the neuronal level, slow wave activity is underpinned by a bi-stable slow (<1Hz) oscillatory alternation in membrane potential (slow oscillation) within thalamocortical networks; from a depolarised up-state caused by active neuronal firing and synaptic activity (up-state or ON period), to a hyperpolarised down-state where both spiking and synaptic activity are absent due to a disfacilitation i.e. an absence of synaptic activity (down-state or OFF period) (Amzica and Steriade, 1998; Neske, 2015; Steriade, 2001, 1978; Steriade et al., 1993a). The summation of this synchronised activity gives rise to a regular waveform known as the EEG slow wave, occurring at a frequency of <4 Hz (slow wave activity; SWA). The down states of cortical neurons are associated with negative deflections in the scalp EEG signal, or positive potentials if recordings are performed from deeper cortical layers, and slow waves correlate with near-synchronous transitions between up and down states across cortical neurons (Steriade et al., 1993; Vyazovskiy et al., 2009). It is thought that the slow oscillation is able to achieve this long-range coherency through diffuse horizontal axon collaterals of cortical pyramidal cells and thalamocortical neurons from higher-order and intralaminar thalamic nuclei (Amzica and Steriade, 1998; Neske, 2015; Sheroziya and Timofeev, 2014). Figure 1-2 shows the relationship between LFP slow waves and the underlying neuronal activity (Vyazovskiy and Harris, 2013). It has been shown that the higher the spatial synchrony across neuronal populations and the longer the network down state, the larger the amplitude and steeper the slopes of the resultant EEG/LFP slow waves are, and fewer multipeak slow waves are detected (Esser et al., 2007; Panagiotou et al., 2017; Riedner et al., 2007; Steriade, 2006; Steriade et al., 1993a; Vyazovskiy et al., 2009b, 2007b). Importantly, recent intracerebral EEG recordings in humans, confirmed this association between slow waves and neural activity in humans (Nir et al., 2011), however it should be noted that this was performed in neurosurgical patients with epilepsy.

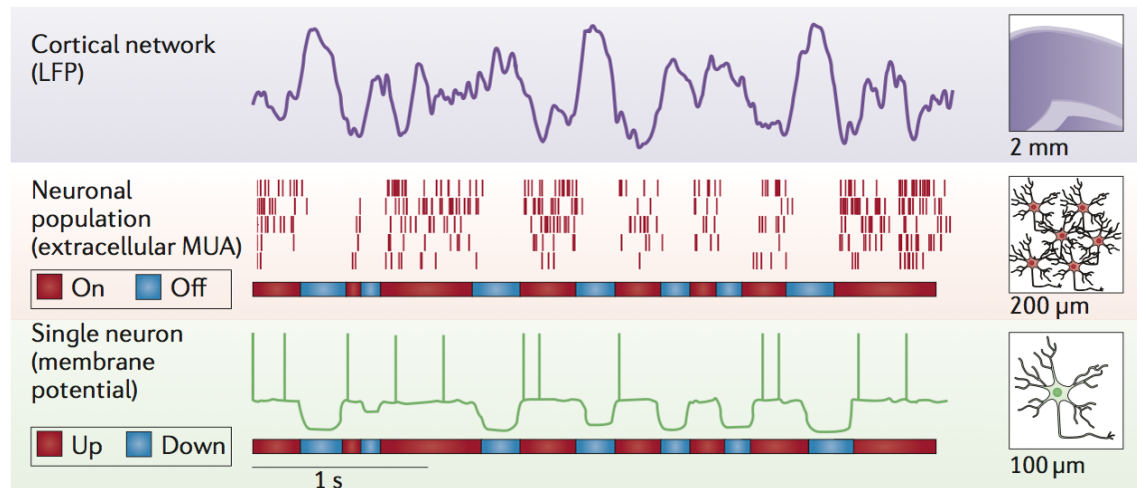


Figure 1-2: The association between LFP, neuronal population firing (extracellular multiunit activity (MUA)) and the membrane potential of single neurons, based on data recorded from deep cortical layers in a rat. Top: local field potential (LFP) recording showing slow waves (top). Middle: raster plot of neuronal population firing of 5 individual neurons show alternation between periods of firing (ON periods) and silence in firing (OFF periods). Bottom: schematic representation of the membrane potential expected in one individual neuron during slow wave sleep. Note the positive peak of slow waves recorded from deep cortical layers, are associated with population silence in firing (OFF periods) and a hyperpolarisation in individual neurons. (Figure from Vyazovskiy and Harris, 2013).

The slow oscillation was first identified in cortical and thalamic networks of cats, using intracellular and EEG techniques (Steriade et al., 1993a), but has also been shown to occur in cortical slabs (Lemieux et al., 2014; Timofeev et al., 2000) and in vitro slice preparations (Sanchez-Vives and McCormick, 2000) or in preparations where thalamic inputs are removed (Steriade et al., 1993b). Therefore, local cortical networks can generate slow oscillations in the absence of thalamic inputs. This has led to suggestions that the slow oscillation is the default state of networks. However, there is also evidence that thalamic oscillations may also play a role (Crunelli and Hughes, 2009). Layer 5 pyramidal cells are thought to be crucial in the initiation of up states underlying the slow oscillation as these show intrinsic rhythmicity and also have a subtype that show intrinsic bursting activity (Lőrincz et al., 2015; Neske, 2015). It is thought that up states may be initiated by the summation of spontaneous excitatory synaptic potentials that gradually increase depolarisation before eventually causing neurons to spike and drive the synchronous firing across the cortical network (an up-state) (Chauvette et al., 2010; Neske, 2015; Timofeev et al., 2000). In contrast, it has also been suggested that there is a subtype of

layer 5 pyramidal cells that persistently fires throughout a down state and these can cause the onset of an up-state once the refractory period of the previous up state has passed (Hasenstaub et al., 2007; Neske, 2015; Sanchez-Vives and McCormick, 2000).

1.1.2 Homeostatic and circadian regulation of sleep

1.1.2.1 *Homeostatic control*

Sleep is a strictly regulated process, under control at molecular, cellular and network levels.

Sleep homeostasis is based on the principle that sleep deficits are compensated for by an increase in the duration and intensity of subsequent sleep. As mentioned previously, all species studied to date are thought to show a homeostatic response to sleep loss, therefore this led to the homeostatic regulation of sleep being added to the main defining characteristics of sleep (Tobler, 1995). Studies have therefore used simplified animal models to investigate the molecular, cellular and network mechanisms underlying the regulation of sleep (Donlea et al., 2014; Huber et al., 2007; Leemburg et al., 2010; Pimentel et al., 2016; Vyazovskiy et al., 2009b, 2007b).

In order for the homeostatic regulation of sleep to take place, there must be sensors that detect how long an individual is awake and therefore how much sleep is required. This is determined by preceding sleep-wake history (e.g. the duration of prior wakefulness) as well as specific waking activities, whereby extending wakefulness beyond the normal level, through sleep restriction or sleep deprivation, increases SWA levels progressively further, while naps have been shown to decrease the level of SWA (Achermann and Borbély, 2003; Brown et al., 2012; Dijk et al., 1987; Huber et al., 2007; Werth et al., 1996b). Thus, the best indicator of 'sleep need' is the level of SWA, which is highest at the beginning of a sleep period, coinciding with a higher incidence of more prominent, higher amplitude EEG slow waves with steeper slopes which gradually dissipate and become more superficial across periods of sleep (Riedner et al., 2007; Tononi and

Cirelli, 2003; Vyazovskiy et al., 2011, 2009b, 2007b). In addition, human studies have shown a rise in EEG theta power as a function of the duration of wakefulness, which correlates with subjective reporting of alertness (Finelli et al., 2000). Additional markers of sleep homeostasis have also been identified at molecular, cellular and network levels. For example, adenosine is also thought to be involved in the regulation of the sleep-wake cycle, as its concentration builds with the duration of waking, leading to the inhibition of wake-promoting brain regions and so being sleep-promoting itself (Basheer et al., 2002; Porkka-Heiskanen et al., 2000).

1.1.2.2 Circadian control

Circadian regulation of the sleep-wake system relies on circadian pacemakers that are entrained by external zeitgebers (cues from the environment such as the levels of light) to set the timing of sleep to specific times of day (Daan et al., 1984; Duffy and Czeisler, 2009; Fuller et al., 2006). Entrainment to the light cycle may be determined by looking at the timing of sleep or activity levels across 24 hours, including the patterns of wheel running activity in mice. Factors such as light or food may then be manipulated in order to investigate how well organisms phase shift to entrain to new circadian cycles. Human studies often utilise constant routine or forced desynchrony protocols to manipulate the sleep-wake cycle, in order to investigate endogenous rhythmicity (Aeschbach et al., 1999).

Circadian oscillators have been identified in many different brain regions, throughout the body in peripheral organs such as the liver and skeletal muscle and even in isolated cell cultures (Balsalobre et al., 1998; Yamazaki et al., 2000; Zylka et al., 1998). These oscillators are coordinated by a master clock located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Ralph et al., 1990; Rusak and Zucker, 1979). Each of the approximately 20,000 neurons in the SCN are thought to be clock cells (Reppert and Weaver, 2002; Welsh et al., 1995). The SCN synchronises the activity of these additional so called 'slave oscillators' using both

electrical and chemical signals, which in turn control rhythms in overall physiology and behaviour (Preitner et al., 2002; Reppert and Weaver, 2002). The SCN has a period length of approximately 24 hours. Light signals are detected by the photopigment melanopsin which is located within a population of retinal ganglion cells in the eye. This sends signals to the SCN via the retinohypothalamic tract (RHT) to entrain circadian rhythms to the light cycle (Gooley et al., 2001; Hattar et al., 2002; Lucas et al., 2001; Provencio et al., 2000; Rusak and Zucker, 1979). The SCN itself may be subdivided into two regions: the core ventrolateral SCN where neurons express the clock genes that are responsive to light and are innervated by the RHT, raphe nuclei and intergeniculate leaflet; and the outer dorsomedial shell of the SCN where clock genes are instead internally rhythmic and receive modulatory inputs from the ventrolateral SCN and hypothalamic regions (Golombek and Rosenstein, 2010). In addition to light, the release of the hormone melatonin from the pineal gland, has also been shown to act as a zeitgeber for entrainment of the circadian system. The concentration of melatonin is increased during the night in both diurnal and nocturnal mammals with the highest concentration around dusk. This results in the hyperpolarisation of SCN neurons and an increased expression of two of the common clock genes *Per1* and *Per2* to reset the SCN clock at dusk (Gillette and McArthur, 1995; Kandalepas et al., 2016).

Molecular and genetic studies have been important for the identification of clock genes and their protein products. These have provided great insights into the molecular clock involved in circadian regulation, as well as the input and output mechanisms from the SCN that act to orchestrate circadian rhythms (Franken and Dijk, 2009; Reppert and Weaver, 2001, 2002). In particular it has been revealed that complex feedback loops (both positive and negative) maintain circadian regulation through transcription of RNA and protein levels of clock components. For example, CLOCK and BMAL1 are transcription factors that are essential drivers of circadian function. These act through a negative loop which drives the activation of three

period genes and two *cryptochrome* genes. These then negatively feedback on the system to inhibit transcription. In addition, these transcription factors also act through a positive feedback loop in which the activation of the orphan nuclear receptor gene *Rev-Erba* represses *Bmal1* transcription which is then de-repressed when CLOCK-BMAL1 inhibit *mPer* and *mCry* transcription (Preitner et al., 2002; Reppert and Weaver, 2002). The SCN also shows distinctive rhythmic patterns of neuronal activity across the circadian day, shown both in vitro (Jagota et al., 2000) and in vivo.

1.1.2.3 Models of sleep regulation

According to the two-process model of sleep regulation (Borbely, 1982; Daan et al., 1984) the propensity to sleep is determined by the interaction between a circadian (process C) and a homeostatic component (process S) (Figure 1-3A). According to the original model, sleep occurs when the homeostatic process S reaches the upper threshold (H) of process C, while conversely wake occurs when process S reaches the lower threshold (L) of process C (Figure 1-3A; Borbely, 1982; Daan et al., 1984). Therefore, the distance process S is from the threshold of process C determines the propensity to sleep (Borbely et al., 2016). In this original model, the homeostatic regulation of SWA (as discussed earlier) was used to model sleep pressure or process S which builds over periods of wakefulness and dissipates with sleep in proportion to its duration and intensity (Figure 1-3B). In addition, circadian oscillations, or process C, aligns sleep to specific times of day (Daan et al., 1984; Duffy and Czeisler, 2009; Fuller et al., 2006). This original two-process model has since been developed to account for intra-episodic dynamics in SWA (i.e. the dynamics within NREM sleep episodes), in the elaborated two-process model which utilised human data (Achermann et al., 1993; Achermann and Borbély, 1990).

Experimental evidence has provided further support that sleep is regulated by an interaction between homeostatic and circadian components. For example, lesioning the SCN causes animals

to become arrhythmic (Edgar et al., 1993; Mistlberger, 2005; Tobler et al., 1983), yet the homeostatic regulation of sleep is largely undisturbed (Mistlberger et al., 1983; Tobler et al., 1983). Neuronal activity of the SCN has been shown to be reflective of sleep-wake states (Deboer et al., 2003) and to be modulated by waking behaviours (Deboer et al., 2007; Ramkisoensing and Meijer, 2015; Yamazaki et al., 1998). Therefore, the activity of the SCN not only reflects circadian time but also ongoing state. Maintenance of a constant sleep pressure using a repeated short-sleep deprivation protocol in rats has shown that the spectral frequencies associated with the different vigilance states are regulated by both homeostatic and circadian components (Yasnikov and Deboer, 2011). Finally, clock genes have been shown to play a role in sleep homeostasis, as they are affected by sleep-wake history and disruptions to clock genes affect the homeostatic features of sleep (Franken, 2013).

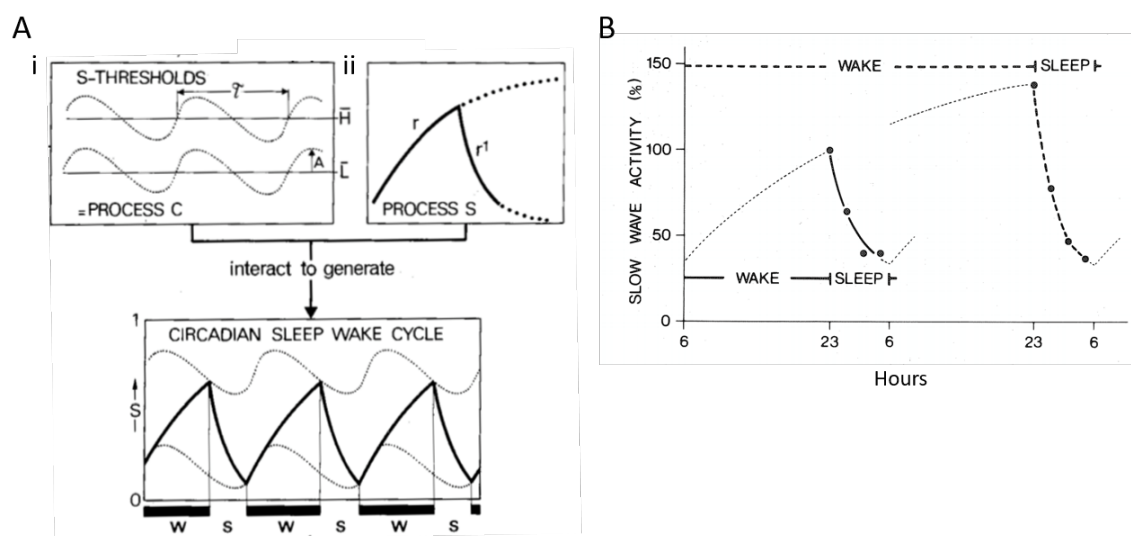


Figure 1-3: The two-process model of sleep regulation. (A) The model predicts that sleep is regulated by an interaction between two processes: Process C (circadian, panel Ai) and Process S (homeostatic, panel Aii). Circadian oscillations, or process C, aligns sleep to specific times of day (panel Ai). Process S reflects the homeostatic regulation of sleep and builds over periods of wakefulness (r) and dissipates with sleep (r_1) in proportion to its duration and intensity (panel Aii). This process is regulated by both an upper threshold (H) and a lower threshold (L), and when these thresholds are reached sleep (S) is initiated or terminated, respectively. (B) Process S is modelled based on SWA. Panel B shows the exponential decline of slow wave activity over four consecutive sleep cycles (value of first cycle = 100%) for a baseline night (continuous line) and after sleep deprivation (interrupted line). The exponential increase of slow wave sleep propensity during waking time is indicated by the dotted curve. [Figure modified from Borbely, 1982; Daan et al., 1984].

1.1.3 Local regulation of sleep

Until recently sleep was considered to be a global event, encompassing the entire cortex.

However, more recent hypotheses have suggested that sleep is a cortical circuit phenomenon initiated locally, and gradually encompassing more networks across sleep periods (Hinard et al., 2012; Krueger et al., 2016, 2008; Lemieux et al., 2014; Pigarev et al., 1997; Sanchez-Vives and McCormick, 2000; Vyazovskiy and Harris, 2013). Local sleep refers to the occurrence of sleep-like activity within localised, distinct regions of the cortex, regardless of ongoing behaviour, which has been shown both in rats and humans (Cajochen et al., 1999; Finelli et al., 2001; Naitoh et al., 1969). This suggests that sleep and wake are more of a continuum rather than distinct entities, where more wake-like activity may leak into sleep at the end of a sleep period and more sleep-like activity may leak into wake after prolonged wakefulness (Vyazovskiy and Harris, 2013), as depicted in Figure 1-4. This may also be observed in the highly variable activity occurring across the brain at the onset of sleep (Magnin et al., 2010). Therefore, sleep is thought to reflect fundamental properties of neuronal assemblies as well as the preceding activity of each network.

Human intracortical recordings, performed simultaneously from multiple brain regions, have shown that slow waves during sleep are mostly confined to local regions, with typically ~30% of the brain showing simultaneous slow waves (Nir et al., 2011). Specifically, slow waves originate in frontal regions of the cerebral cortex, and propagate posteriorly across the cortical surface along major anatomical pathways (Finelli et al., 2001; Huber et al., 2000a; Massimini et al., 2004; Murphy et al., 2009; Nir et al., 2011; Schwierin et al., 1999; Sirota and Buzsaki, 2005; Vyazovskiy et al., 2006b; Werth et al., 1996a). More anterior brain regions show a faster decline in SWA (Huber et al., 2000a; Zavada et al., 2009), while the increase in SWA after sleep deprivation is also higher in frontal cortical regions (Leemburg et al., 2010; Massimini et al., 2004; Schwierin et al., 1999). Sleep spindles have also been shown to mostly occur locally (Nir et al., 2011; Nobili et

al., 2012), with hippocampal spindles occurring a few minutes before the transition from wakefulness to NREM sleep (Nobili et al., 2012). It has been suggested that local cortical activity may reflect anatomical differences between cortical regions or it may be determined by fine synaptic modifications or changes in local neuronal connectivity as a result of preceding activity (Huber et al., 2004; Krueger and Tononi, 2011; Schwierin et al., 1999; Tononi and Cirelli, 2006; Vyazovskiy and Harris, 2013).

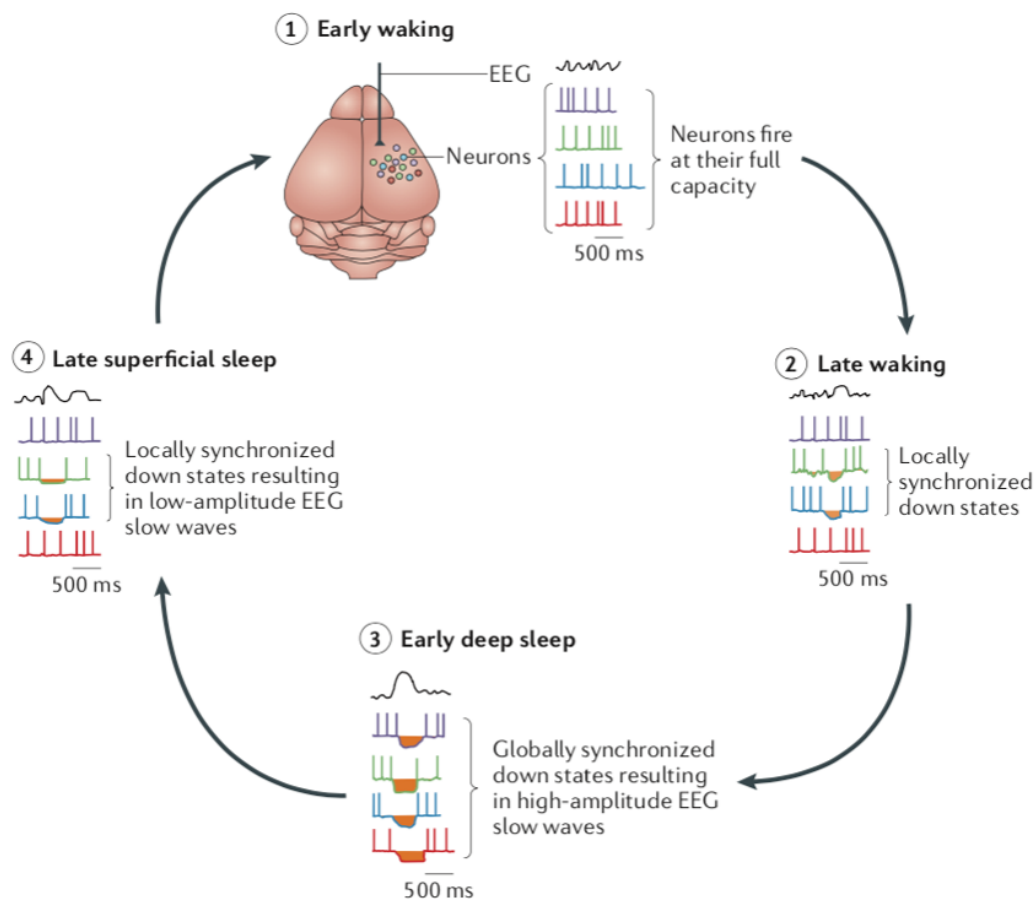


Figure 1-4: Local cortical activity during sleep and waking. (1) Early waking: EEG and neuronal activity show characteristic wake-like activity (low amplitude EEG and tonic neuronal firing). (2) Late waking: localised slow waves and down states may start to occur, perhaps due to the prolonged synaptic and spiking activity. They may reflect a cellular maintenance function. (3) Early deep sleep: the most globally synchronised down states occur during initial early deep sleep, resulting in the highest amplitude slow waves. (4) Late superficial sleep: as sleep progresses down states occur more locally, resulting in lower amplitude slow waves. This may indicate that individual neurons have obtained sufficient restoration and so long longer need to engage in synchronous down states. (Figure from Vyazovskiy and Harris, 2013).

The topographical differences in SWA across the cortex are influenced by preceding wakefulness (Esser et al., 2007; Huber et al., 2006, 2004; Vyazovskiy et al., 2011), while also being use dependent in that those brain regions more active during wakefulness show higher SWA during subsequent sleep (Huber et al., 2004; Kattler et al., 1994; Krueger et al., 2008; Krueger and Obál, 1993; Riedner et al., 2007; Vyazovskiy et al., 2011). For example, a localised increase in SWA has been observed in the motor cortex of rats after training in a pellet reaching task (Vyazovskiy et al., 2011). In contrast, human data has shown decreased levels of SWA in the sensorimotor cortex when an arm is immobilized during the day (Huber et al., 2006). Importantly, these tasks, both in rodents and humans, involve elements of synaptic potentiation which accompanies the increase in SWA, and these effects are often observed at a local level (Cirelli 2012). Local sleep during wake has been shown to be detrimental for performance in cognitive tasks (Cirelli and Tononi, 2008; Huber et al., 2004; Vyazovskiy et al., 2011). Importantly, this evidence this suggests that there is a local build-up of sleep need.

Interestingly, where there are great pressures not to sleep, species can adapt their sleep-wake architecture. For example, during migration birds undertake 'microsleep' in localised cortical regions (Rattenborg, 2006; Rattenborg et al., 2000). Some human sleep disorders such as sleep walking may also involve elements of local sleep in which there is a dissociation between the activity of the brain and behaviour (Mahowald et al., 2011; Mahowald and Schenck, 2005). In addition, aquatic species such as dolphins show sleep-like activity in one hemisphere at a time (Mukhametov, 1987; Mukhametov et al., 1977; Ridgway, 2002). As adaptations such as these are fairly uncommon, it may be that the benefits associated with sleeping one hemisphere at a time are only relevant under specific extreme circumstances (Rattenborg et al., 2000). It is possible therefore, that local sleep does not provide the same benefits as regular, more global sleep (Vyazovskiy and Harris, 2013).

1.1.4 Function of sleep

As sleep has been identified in all species studied to date (Cirelli and Tononi, 2008; Tobler, 1995) it must provide an evolutionary advantage to species, essential for survival. It has been suggested that specific behavioural and physiological measures of sleep may be universal across species, and differences may be related to metabolic rates, ecological factors such as diet and the risk of predation or such factors as body and/or brain size (Allison and Cicchetti, 1976; Capellini et al., 2008; Herculano-Houzel, 2015; Siegel, 2005; Tobler, 2005; Zepelin and Rechtschaffen, 1974). It is often argued that if sleep were not essential then its loss would not cause negative consequences. However, numerous studies suggest that sleep deprivation/staying awake can lead to a number of negative consequences including intrusions of sleep-like activity into wakefulness (Huber et al., 2004; Vyazovskiy et al., 2011); impairments in cognitive and emotional processing (Killgore, 2010; Vandekerckhove and Cluydts, 2010); temperature and metabolic dysregulation (Rechtschaffen and Bergmann, 1995); a general increased subjective feeling of sleepiness and tiredness or in extreme cases death (Montagna and Lugaresi, 2002; Shaw et al., 2002; Stephenson et al., 2007).

Although the exact function of sleep remains a topic for debate, there are many theories. It is thought that sleep may serve an energy saving role (Berger and Phillips, 1995); restore and replenish energy stores (Oswald, 1980); be involved in thermoregulation (Rechtschaffen and Bergmann, 1995); metabolic regulation (Knutson et al., 2007; Van Cauter et al., 2008), or have adaptive immune functions (Lange et al., 2010). However, evidence suggests that sleep may be more important for optimal brain functioning, as sleep may allow the brain to be detoxified from free radicals and other substrates which build during wakefulness (Inoué et al., 1995; Reimund, 1994), while also replenishing brain stores of substances such as glycogen (Oswald, 1980; Scharf et al., 2008). Sleep has also been shown to play a role in synaptic plasticity, maintaining a

balance of synaptic strength. Furthermore, sleep has been shown to play a role in learning and memory, which may involve its role in synaptic plasticity.

1.1.4.1 Sleep and synaptic plasticity: the role of sleep in memory

It is generally thought that memory formation involves a two-stage system: the initial rapid encoding of memories into a fast learning store, and the repeated activation of memories to strengthen and/or transfer them into long term stores (Rasch and Born, 2013). Extensive literature over the past decades suggest that sleep is an ideal state for new information acquired and encoded during waking to be consolidated into long term stores. Whether sleep plays a passive, permissive, active or no role at all in the consolidation of memory has been discussed greatly over the years (Diekelmann and Born, 2010; Ellenbogen et al., 2006; Feld and Born, 2017; Rasch and Born, 2013; Tononi and Cirelli, 2014). It is now more widely accepted that sleep plays an active role in the consolidation of memories into long-term stores. This can be observed in studies that showed that sleep after learning resulted in larger improvements in performance, compared to after periods of wakefulness, with sleep immediately after learning most beneficial (Benson and Feinberg, 1977; Gais et al., 2006; Payne et al., 2012; Talamini et al., 2008).

The neural basis of memory formation or encoding involves changes in synaptic efficacy. Much focus has been on the two main opposing forms of learning induced synaptic plasticity; long-term potentiation (LTP) or a strengthening of synapses, and long-term depression (LTD) or a weakening of synapses. LTP refers to an increase in the efficiency of synaptic transmission via the activation of NMDA (N-methyl-D-aspartate) receptors, that is sustained over time (Bliss and Collingridge, 1993; Morris et al., 1990, 1986). LTP involves an increase in synaptic response when repeated stimuli are applied. The first evidence of LTP came from a study in rabbits that showed an increase in the efficiency of synaptic transmission of hippocampal perforant path synapses and excitability of granule cells when high frequency stimulation was applied to the excitatory

perforant path fibres of the dentate area of the hippocampal formation (Bliss and Lomo, 1973). The hippocampus is well established to be involved in the formation of memories, and studying LTP in this region has been crucial for understanding the mechanisms underlying activity dependent synaptic plasticity (Bliss and Collingridge, 1993; Doyère Valérie and Laroche Serge, 2004; Morris et al., 1990, 1986). However, it is important to note that LTP has also been shown to occur in numerous other brain regions including: the frontal and prefrontal cortex (Maroun and Richter-Levin, 2003; Sutor and Hablitz, 1989; Xu and Yao, 2010); visual cortex (Sale et al., 2011) and anterior cingulate cortex (Zhuo, 2014).

The role of sleep on the efficacy of synapses and learning is yet unclear and two main hypotheses have been proposed: the active system consolidation hypothesis and the synaptic homeostasis hypothesis (Figure 1-5).

1.1.4.1.1 Active system consolidation hypothesis

The active system consolidation hypothesis suggests that memory traces are first encoded in the hippocampus and that repeated reactivation of these traces results in the successive strengthening of cortico-cortical connections to transform the memory traces into stronger long-term memories in other cortical and subcortical regions (Battaglia et al., 2004; Born and Wilhelm, 2012; Buzsaki, 1998; Eschenko et al., 2008) (Figure 1-5A). This occurs through slow oscillations during SWS driving the generation of hippocampal sharp wave ripples and thalamocortical spindles which become nested into the troughs of spindle-ripple events (Ellenbogen et al., 2006; Rasch and Born, 2013). During waking there is a tagging of cortical synapses through theta activity (theta coherence) in prefrontal-hippocampal circuitry. This ensures that the reactivation of neuronal assemblies during SWS occurs in the same neurons activated during wakefulness, though usually around 20-40 minutes after the original learning and at a faster rate (Battaglia et al., 2004; Fujisawa and Buzsaki, 2011; Hirase et al., 2001, 1999;

Ji and Wilson, 2007; Kudrimoti et al., 1999; Nadasdy et al., 1999; Qin et al., 1997; Rasch and Born, 2013; Ribeiro et al., 2007). This reactivation process is thought to destabilise memory traces to allow for memories to be integrated with new information, however periodic excursions into REM sleep may further strengthen these traces (Diekelmann and Born, 2010; Rasch and Born, 2013; Stickgold, 2005).

1.1.4.1.2 Synaptic homeostasis hypothesis

The synaptic homeostasis hypothesis suggests that sleep is crucial for maintaining the balance of synaptic strength that changes with waking experiences (Tononi and Cirelli, 2003) (Figure 1-5B). This is now considered one of the main functions of sleep. According to the synaptic homeostasis hypothesis, during waking there is a progressive, net increase in the strength of cortico-cortical connections. This is counteracted by a net decrease or downscaling of non-vital synapses during sleep, to renormalise synaptic strength and avoid hyperexcitability (Tononi and Cirelli, 2003a, 2006). These processes are thought to be similar to those observed in the homeostatic regulation of SWA and are therefore likely to be linked to the restorative function of sleep. Recent experimental evidence supports this hypothesis. In particular molecular markers such as synaptic proteins (e.g. presynaptic BRP) and postsynaptic glutamate AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors correlate with synaptic strength and are higher during wake (Diering et al., 2017; Gilestro et al., 2009; Vyazovskiy et al., 2008).

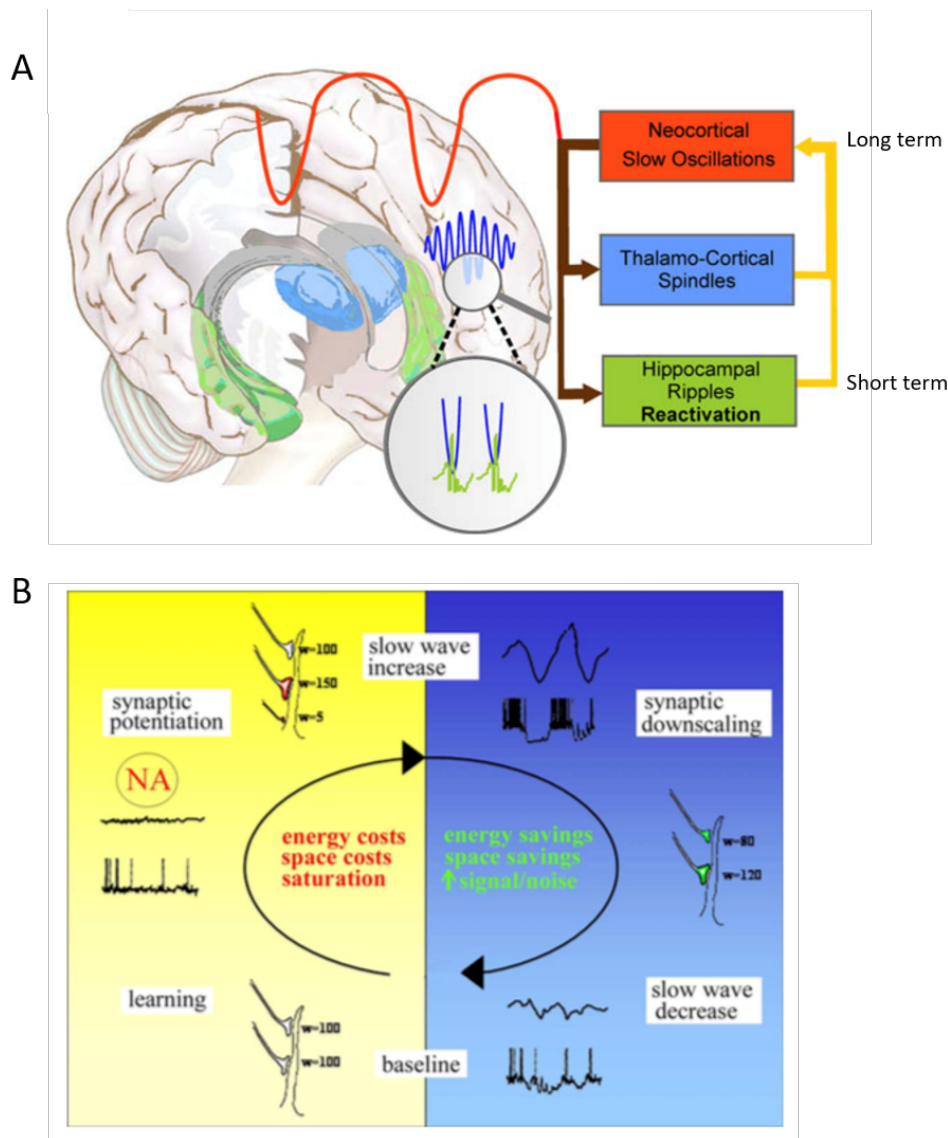


Figure 1-5: The two main hypotheses that suggest the main function of sleep is in memory processing. (A) the active system consolidation hypothesis suggests that new memory traces are encoded in the hippocampus and reactivated during slow wave sleep to strengthen the memory traces and transfer them to long term stores in the neocortex. This process occurs via hippocampal sharp wave ripples (green) and thalamocortical spindles (blue). (B) The synaptic homeostasis hypothesis suggests that synaptic downscaling occurs during slow wave sleep to renormalise the synaptic potentiation that occurs with waking. This process is thought to reflect the homeostatic regulation of sleep, as is often observed as a decrease in slow wave activity across the time asleep. Figure modified from Born and Wilhelm, 2012 (A) and Tononi and Cirelli, 2006 (B).

These theories highlight a role for sleep in learning and memory, which is often investigated using behavioural techniques. For example, studies in rodents often utilise odours, sight or sound to investigate discrimination-based learning. Importantly, it is also well established that ageing leads to a cognitive decline, and with it, behavioural deficits in tasks that involve information processing. These include deficits in spatial memory, episodic memory, spatial

navigation and working memory (Burke and Barnes, 2006; Lester et al., 2017; Lindner, 1997), with motor deficits including locomotor activity and spatial memory among the most common (Barreto et al., 2010; Ingram et al., 1981). It is possible that the well-established disruptions of sleep with ageing (see section 1.2.2) may contribute to the cognitive decline observed with ageing. However, the mechanisms underlying this link are largely unknown. This thesis has utilised two experimental tasks in order to study the role sleep plays in the memory deficits occurring in ageing. These are: the visual discrimination task (and an extension of this: the alphabet task) and the Morris water maze. Details of these tasks, including a background and results can be found in their relevant results chapters (chapters 5 and 6, respectively).

1.2 An introduction to ageing

The ageing population is rapidly increasing, with the National Institute of Aging estimating that the worldwide population of humans over the age of 65 is set to increase from 8% to 16% of the global population between 2010 and 2050 (National Institute on Aging, 2011). Ageing is a strictly regulated biological process, which can be highly advantageous to a species, increasing its evolutionary success. Ageing involves both expected 'programmed ageing' elements which reflect normal physiological changes across the lifespan, but also unwanted negative processes that are the result of exposure to stressors (Enzinger et al., 2005; Meyer et al., 1999). In order to understand ageing at a fundamental level, it is important to consider ageing in the absence of other pathological conditions, so called healthy ageing.

1.2.1 Anatomical and functional changes in the brain with ageing

Ageing involves a number of anatomical and functional modifications at molecular and cellular levels which have important consequences on overall behaviour and cognitive function (Bishop et al., 2010; Burke and Barnes, 2006; Kirkwood, 2010; Kourtis and Tavernarakis, 2011; Morrison

and Baxter, 2012; Yeoman et al., 2012; Zoncu et al., 2010). At a structural level, healthy ageing is often associated with a loss of synaptic connectivity rather than a loss of neurons per se, as is often observed in neurodegenerative diseases. For example, animal studies have shown that ageing is often associated with changes in spine density and morphology as well as decreases in the number of synaptic connections at neuromuscular junctions and in the hippocampus (Burke and Barnes, 2010, 2006; Dumitriu et al., 2010; Morrison and Baxter, 2012; Peters et al., 2008; Petralia et al., 2014). Human studies have also shown a decrease in grey and white matter with ageing (Coffley et al., 1992; Enzinger et al., 2005; Ge et al., 2002; Marner et al., 2003; Ziegler et al., 2012). In addition, both human and rodent data suggest that the function of brain networks is also often altered in ageing, which may be observed as alterations in synaptic transmission (Porkka-Heiskanen et al., 2004; Wang et al., 2011; Wigren et al., 2009), calcium homeostasis (Toescu and Vreugdenhil, 2010) or an increased susceptibility to cellular stress (Kourtis and Tavernarakis, 2011; Naidoo, 2009; Scharf et al., 2008). Studies have shown that reduced synaptic connectivity may be compensated for by increasing the electrical responsiveness of neurons, leading to the generation of larger synaptic field potentials (Barnes and McNaughton, 1980). As sleep is associated with a number of restorative functions (section 1.1.4), the effects of ageing may also be compensated for by changes in sleep.

1.2.2 Age-related changes in sleep

Age-dependent changes in sleep may reflect compensatory responses, in which sleep is important in maintaining cellular homeostasis and optimal waking functions as an organism ages. As the ageing population is ever expanding, it is becoming increasingly important to establish the biological/neural mechanisms underpinning the effects of ageing on sleep, which are currently unclear. Therefore, one of the main aims of this thesis was to characterise the neural signatures of ageing during sleep.

1.2.2.1 Global sleep-wake architecture

It is well established that in humans, sleep is deepest up until adolescence, after which it is progressively harder to maintain, becoming fragmented and superficial (Agnew et al., 1967; Dijk et al., 1989; Feinberg et al., 1984; Ohayon et al., 2004a; Roffwarg et al., 1966). A meta-analysis of 2391 adults aged 19-102, showed the main effects of ageing to include: a reduction in total sleep time, decreased sleep efficiency, decreased amount of SWS and increased number of awakenings after sleep onset (Ohayon et al., 2004a). Human sleep is characterised by a decrease in the percentage time asleep when in bed, from approximately 90-95% in adolescence to 80% in individuals over the age of 70 years (Ohayon et al. 2004b). Older human subjects have an alteration in their sleep composition, resulting in a larger proportion of superficial sleep stages, at the expense of deeper sleep stages predominated by slow wave sleep.

Although only a few studies have investigated the effects of ageing on sleep in mice, the majority are able to identify some changes parallel to humans in the distribution and timing of sleep. In rodents, the age-dependent effects on total sleep amount are less consistent than in humans. However these studies generally report an increase in total sleep with age (Banks et al., 2015; Colas et al., 2005; Hasan et al., 2012; Panagiotou et al., 2017; Welsh et al., 1986; Wimmer et al., 2013), which conflicts with human studies that instead report a reduction (Ohayon et al., 2004a; Roffwarg et al., 1966). In addition, there is evidence that ageing may not monotonically affect sleep across the lifespan, instead sleep in mice was found to increase up until 1 year of age and then decrease by 2 years of age (Hasan et al., 2012). The differences in sleep duration across species may be related to differences in longevity (Grandner, 2017; Itani et al., 2017; Zawisza et al., 2015). However, the link between sleep duration and longevity are often complicated to interpret due to limitations in the techniques used to quantify sleep duration, which often rely on self-reporting measures, and the common occurrence of comorbidities (Cirelli, 2012; Youngstedt and Kripke, 2004).

More consistent effects are observed in the effects of ageing on sleep-wake architecture, particularly the fragmentation of sleep. Ageing in rodents is well established to increase the fragmentation of the sleep-wake cycle, leading to more bouts of shorter duration wake and sleep (Colas et al., 2005; Hasan et al., 2012; Panagiotou et al., 2017; Welsh et al., 1986; Wimmer et al., 2013). This increased fragmentation predominantly occurs during the dark phase which leads to a reduced sleep-wake amplitude (distribution of sleep-wake over 24 hours) (Banks et al., 2015; Colas et al., 2005; Eleftheriou et al., 1975; Hasan et al., 2012; Naidoo et al., 2011; Nakamura et al., 2011; Valentinuzzi et al., 1997; Wimmer et al., 2013). In a more recent study, an innovative spike-slab statistical approach was used to further investigate the structure of the sleep-wake cycle, which is unevenly distributed between long and short bouts in rodent species (Wimmer et al., 2013). In contrast, REM sleep has been shown to be relatively unaffected by age, suggesting a weaker effect of ageing on REM sleep distribution (Hasan et al., 2012).

1.2.2.2 Effect of ageing on the circadian system

Early studies consistently found that aged populations of mice, rats and hamsters, all showed a loss of circadian rhythmicity. These studies often utilised running wheel behaviour to assess both entrainment as well as circadian components such as free running circadian periods, and re-entrainment to phase changes. For example, some studies have shown ageing to lengthen the free-running circadian period of wheel running activity (Possidente et al., 1995; Valentinuzzi et al., 1997; Welsh et al., 1986), whereas others have shown a shortening of period length or it to remain stable (Banks et al., 2015). Access to a running wheel is also well established to aid the entrainment of the circadian system (Edgar et al., 1991; Gu et al., 2015; Yasumoto et al., 2015). However, it should be noted that ageing is associated with a reduction in locomotor activity and running in a wheel (Banks et al., 2015; Kopp et al., 2006; Possidente et al., 1995; Valentinuzzi et

al., 1997; Welsh et al., 1986), and so data using wheel running to assess the effects of ageing must be interpreted with caution.

As mentioned previously ageing is associated with a reduction in circadian rhythm amplitudes leading to a less distinct separation between light and dark phase activity (Banks et al., 2015; Nakamura et al., 2011; Possidente et al., 1995; Valentinuzzi et al., 1997; Welsh et al., 1986). Individual neurons in the SCN of older animals also show reduced synchronisation with ageing, leading to suggestions that reduced circadian rhythmicity with ageing may arise from weakened coupling between oscillators in the SCN (Farajnia et al., 2014, 2012; Ramkisoensing and Meijer, 2015). Additional factors such as the regulation of body temperature, adrenal corticosterone, pineal and plasma melatonin are also affected by ageing, as reviewed in (Ingram et al., 1982; Webb, 1978). Numerous studies have shown that the circadian system has a major role in influencing ageing and longevity (Kondratov 2007, Kondratova and Kondratov 2012, Hood and Amir 2017). Although the mechanisms by which this occurs are unclear, possibilities are discussed in a recent review by Hood and Amir (2017).

1.2.2.3 Characteristics of cortical activity

One of the most notable effects of ageing is to decrease SWA spectral power, which has been identified in humans (Agnew Jr. et al., 1967; Dijk et al., 1989; Feinberg et al., 1984) and replicated in some mouse studies (Banks et al., 2015; Colas et al., 2005; Hasan et al., 2012; Wimmer et al., 2013). In one study, this reduction was only apparent in the oldest age groups, rather than a gradual decline in delta power across age (Hasan et al., 2012). Studies have also shown that aged mice have reduced theta frequency power during wake and a slower theta peak frequency during both REM sleep and wake, whereas the EEG spectra during NREM sleep were unaffected (Wimmer et al., 2013). Importantly, in contrast to these previous studies, recent evidence suggests that ageing may in fact be associated with an increase in SWA power

(Panagiotou et al., 2017). A possible explanation for these discrepancies is that these studies often report normalised values, which vary greatly among studies. Therefore, it is unclear whether the reduction in SWA reported in earlier studies is a true reflection of the effects of ageing in mice, and instead it may be associated with an increase in SWA or a redistribution of spectral power between frequencies. This would therefore conflict with the well-established reduction in SWA consistently reported in human studies.

Elevated sleep pressure, as a result of sleep deprivation, has been shown to enhance age differences identified in sleep characteristics such as delta power and time asleep (Hasan et al., 2012). As well as being associated with a reduction in SWA in human and some mouse studies, ageing is also thought to attenuate the response to sleep deprivation, leading to suggestions that ageing may reduce the homeostatic sleep need or may instead diminish the capacity to generate and sustain NREM sleep (Cirelli, 2012; Klerman and Dijk, 2008; Mander et al., 2017), (Figure 1-6). Importantly, the build-up of SWA (modelled as process S in the two-process model) has been shown to be shallower in older adults, with smaller rebounds in SWA after prolonged periods of wake also observed (Landolt et al., 1996; Landolt and Borbely, 2001) (Figure 1-6). However, this is conflicted by recent evidence in mice suggesting that ageing may instead be associated with an increased SWA, accompanying increased amplitude and steeper slope of individual EEG slow waves, in which fewer were multipeak waves (Panagiotou et al., 2017). As these properties of slow waves are indicative of an increased synchronisation of cortical neurons, this led the authors to suggest that older mice may rather have an increase sleep pressure (Panagiotou et al., 2017). This highlights the necessity to investigate the cortical neural underpinnings of slow waves in order to fully establish the effects of ageing.

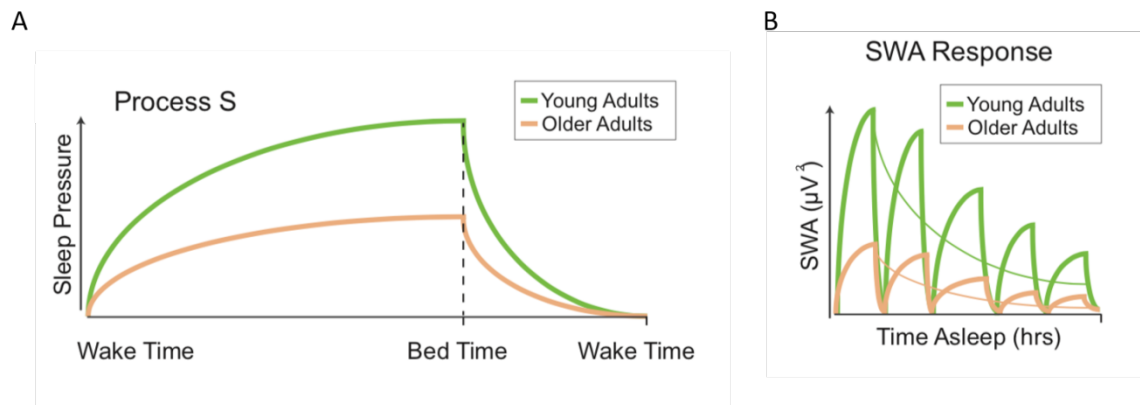


Figure 1-6: The homeostatic regulation of sleep may be affected by ageing. (A) Process S reflects the homeostatic need to sleep (see two-process model section for details). It is thought that in older adults (orange) the buildup of sleep pressure across wake periods and the dissipation across sleep periods is slower and weaker compared to younger adults. (B) The reduction in the homeostatic need to sleep is associated with a decreased amount of slow-wave activity (SWA) during each sleep cycle, compared to young adults. Figure modified from Mander et al., 2017.

Despite numerous studies characterising the overall sleep-wake architecture and EEG characteristics with ageing, very little is known about the underlying neural dynamics. As previously discussed, sleep regulation is thought to reflect fundamental properties of neuronal assemblies (Hinard et al., 2012; Krueger et al., 2016, 2008; Lemieux et al., 2014; Pigarev et al., 1997; Sanchez-Vives and McCormick, 2000; Vyazovskiy and Harris, 2013), and so the effects of ageing cannot be fully understood without considering local cortical and neural activity in addition to the whole-brain view, which currently have not been investigated in the context of ageing. It is possible that the global changes in sleep previously observed may be distinct from those occurring at the level of local cortical circuits.

1.3 Pharmacological manipulation of sleep

There are a number of ways to improve sleep ranging from behavioural techniques using cognitive behavioural therapy (CBT) for insomnia (Espie et al., 2007; Freeman et al., 2015; Morin et al., 2006) or physiological techniques either targeting the circadian system through chronotherapeutics (Wirz-Justice, 2009) or sleep through pharmacological interventions (Amanti, 2018; Olfson et al., 2015). Despite this range of treatments available, and the

disadvantages associated with the use of pharmacological interventions such as dependency and tolerance, hypnotics remain a prevalent treatment for improving sleep (Amanti, 2018; Kripke, 2000; Olfson et al., 2015; Schutte-Rodin et al., 2008).

Hypnotic drugs such as benzodiazepines are commonly used to improve sleep in the elderly. However, in many older individuals these treatments lack efficacy, have a slow elimination which results in unwanted prolonged drowsiness side effects or can have an increased sensitivity to the drugs. For these reasons they are now often not recommended for use in elderly populations (Amanti, 2018; Borbély et al., 1983; Greenblatt et al., 1983; Nicholson et al., 1982). In particular diazepam has been shown to have impaired clearance in elderly populations due to its action involving oxidative transformation (Nicholson et al., 1982). However despite these issues, in the US the use of benzodiazepines increased from 4.1% to 5.6% between 1996-2013 (Bachhuber et al., 2016), with the percentage of individuals using benzodiazepines increasing with age from 2.6% (aged 18-35) to 8.7% (over 65 years) (Olfson et al., 2015).

Benzodiazepines, such as diazepam, potentiate inhibitory GABAergic neurotransmission by increasing the affinity for the inhibitory neurotransmitter GABA to bind to GABA type A receptors (GABA_A). This leads to an enhanced GABA-induced chloride influx, enhancing inhibitory GABAergic neurotransmission, which results in downstream effects on brain oscillations. In particular, benzodiazepines result in a reduction of EEG power in the slow-wave activity range (0.5-4 Hz) and an enhancement of activity in the spindle frequency range (9-14Hz) during NREM sleep, which has been noted in both humans and rodent studies (Aeschbach et al., 1994; Borbély et al., 1983; Kopp et al., 2003; Kopp et al., 2004; Lancel et al., 1996; Lancel and Steiger, 1999; Tobler et al., 2001).

GABA_A -receptors are highly complex structures, formed from the various combinations of their numerous subunits which in turn determine the receptor function, such as the affinity of different benzodiazepines for the GABA_A receptor, with the $\gamma 2$ -subunit containing the specific binding site for benzodiazepines (Fritschy and Mohler, 2004). GABA_A receptors have been shown to be widespread throughout the brain, with their distribution highly heterogeneous and representing putative receptor subtypes in distinct neuronal populations (Fritschy and Mohler, 2004). It was originally proposed that $\alpha 1$ and $\alpha 3$ GABA_A subtypes may be responsible for the effect of benzodiazepines, as these have been shown to be present in the corticothalamic network (Fritschy and Mohler, 2004), which is involved in the generation of delta and spindle oscillations (McCormick and Bal, 1997; Saper et al., 2001; Steriade et al., 1987). However, mouse models with diazepam-insensitive $\alpha 1$ or $\alpha 3$ GABA_A receptors still showed the typical reduction in delta power post injection of diazepam (Kopp et al., 2003; Tobler et al., 2001). $\alpha 2$ GABA_A receptors may instead be involved in efficacy of benzodiazepines as they are present or project from the basal forebrain or preoptic regions of the ascending system (Fritschy and Mohler, 2004). These are thought to cause a hyperpolarisation of ascending projections to the corticothalamic system, leading to the generation of slow oscillations during NREM sleep (McCormick and Bal, 1997; Saper et al., 2001; Steriade et al., 1987). $\alpha 2$ GABA_A receptors also present in hippocampal formation responsible for the generation of theta activity during waking and REM sleep (Fritschy and Mohler, 2004; Kopp et al., 2004), which have been shown to be enhanced in mice injected with diazepam (Kopp et al., 2003; Tobler et al., 2001). In another study mice in which the $\alpha 2$ GABA_A receptors were mutated to be insensitive to diazepam, there was a marked attenuation of the reduction in delta power, an increase in theta activity and an absence of the increase in high frequencies (above 16-18Hz) after diazepam injection (Kopp et al., 2004). This data supports a role of $\alpha 2$ GABA_A receptors in the action of diazepam. Interestingly benzodiazepines do not affect the homeostatic regulation of sleep, as evidence

suggests they do not greatly affect the time course of SWA, despite reducing absolute power (Aeschbach et al., 1994).

While the molecular mechanisms of action of benzodiazepines have been elucidated in great detail, the neural mechanisms underlying the actions of benzodiazepines on brain activity during sleep are currently unclear. It is therefore crucial to further understand the physiological mechanisms underlying the efficacy of these drugs. Therefore, in this thesis I have focussed on characterising the effects of a commonly used benzodiazepine, diazepam on LFPs and neural activity.

1.4 Overall objectives of the thesis

There is considerable evidence that sleep is important for optimal brain functioning, with factors such as the total amount, and brain oscillations well-established to be involved. Numerous studies in both rodents and humans have shown sleep and cognitive functions to be profoundly affected by ageing, however the neurophysiological mechanisms underlying these effects are not well known. In this thesis I performed chronic recordings of sleep and neural activity from the neocortex of young and older mice. I performed electrophysiological recordings, sleep deprivation, pharmacological manipulations and behavioural tasks to study the mechanisms linking sleep, ageing and brain function. Therefore, the main objectives of this project were:

- **Objective 1:** To characterise the spatio-temporal properties of cortical neural activity in freely moving young and older mice and to elucidate neurophysiological markers of ageing and preceding sleep-wake history.
- **Objective 2:** To investigate the effects of a commonly used hypnotic, diazepam, on cortical neural activity and to determine whether there are age-dependent differences in the neural responses to diazepam.

- **Objective 3:** To determine the effect of ageing and sleep on behavioural performance in a hippocampal dependent task: the Morris water maze.
- **Objective 4:** To develop a novel behavioural paradigm to study sleep, sleep deprivation and cognitive function, which may be applicable to ageing studies in the future.

Altogether the overall aim was to utilise several methodologies, from electrophysiological recordings to pharmacological interventions and cognitive tests, to provide insights into the cortical and neural mechanisms underpinning the link between sleep, ageing and cognition.

Chapter 2: General Methods

2.1 Experimental animals

Experiments were carried out in male C57BL/6J mice (see individual results sections for details regarding numbers and ages of the mice). Throughout this thesis mice are subdivided into Early adulthood (~5 months old), Late adulthood (~12 months) and Old age (~24 months old) age groups where applicable. These ages were selected based on previous studies that investigated the effects of ageing on sleep characteristics in mice (Eleftheriou et al., 1975; Hasan et al., 2012; Valentinuzzi et al., 1997), so that results would be translatable between studies. It is thought that the OA mice in this thesis correspond to an age of approximately 70 years in humans (Dutta and Sengupta, 2016).

During training for the visual discrimination task (Chapter 6) animals were individually housed in individually ventilated cages, to allow them to be food restricted for the purpose of the tasks (see chapter 6 for details). For electrophysiological recordings, mice were individually housed in custom-made clear plexiglass cages (20.3 x 32 x 35 cm) with free access to a running wheel (Campden Instruments, Loughborough, UK, wheel diameter 14 cm, bars spaced 1.11 cm apart inclusive of bars) and food available ad libitum. Cages were housed in ventilated, sound-attenuated Faraday chambers (Campden Instruments, Loughborough, UK, two cages per chamber). To ensure that circadian period or phasing differences did not influence the results, mice were housed under entrained conditions (standard 12:12 h light–dark cycle (lights on 0900, ZT0) light levels 120–180 lux). While in the main ageing chapter (Chapter 3) running wheel patterns and the onset of activity at dark onset were calculated to ensure all animals were entrained to the light-dark cycle. Room temperature and relative humidity were maintained at $22\pm1^{\circ}\text{C}$ and $50\pm20\%$, respectively. All procedures conformed to the Animal (Scientific

Procedures) Act 1986 and were performed under a UK Home Office Project Licence in accordance with institutional guidelines.

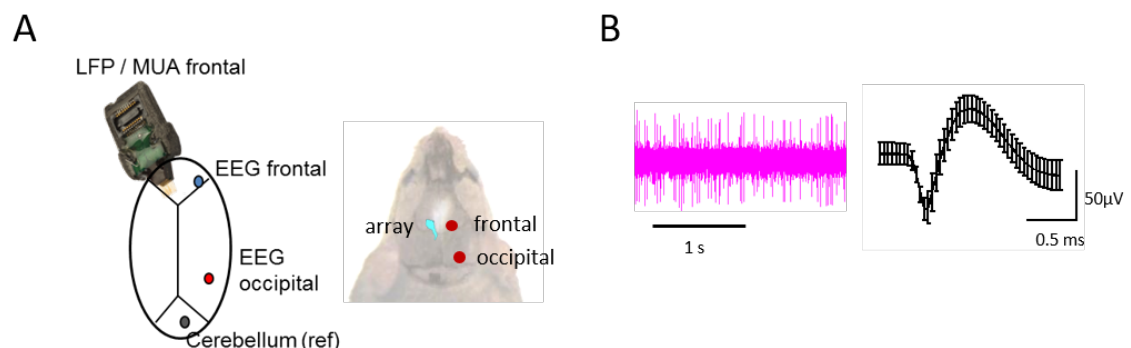
2.2 Surgical procedure for electrophysiological recordings

2.2.1 Electroencephalography and electromyography surgery

Surgical procedures were carried out as previously described (Cui et al., 2014; Fisher et al., 2016; McKillop et al., 2018), under aseptic conditions. Isoflurane anaesthesia at 3–5% was used for induction and 1–2% for maintenance. Mice were first prepped by shaving the head and neck and cleaning the area with three rounds of alternating washes of ethanol and povidone iodine. Mice were then fixed in a stereotaxic frame using ear bars. Metacam (1–2 mg/kg, subcutaneous (s.c.)), dexamethasone (0.2 mg/kg, s.c.) and vetergesic (0.08 mg/kg, s.c.) were administered preoperatively. Artificial tears were applied to the eyes to prevent them drying out, and internal temperature was monitored throughout the surgery using a heating pad feedback system and anal probe. An incision was made down the midline of the skull from the eyes to the edge of the neck muscle (taking care not to cut the muscle). Bulldog clips were used to retract the skin and expose the skull. The skull was then thoroughly cleaned, using both saline and ethanol. Etching gel was applied for a couple of minutes and the surface of the skull was scratched gently with the edge of a scalpel to ensure all tissue is removed from the skull surface so that the head mount would firmly attach to the skull bone.

Once the skull surface was thoroughly cleaned, coordinates were marked on the skull surface to identify the location where recording electrodes were to be implanted. Three EEG screw electrodes were implanted above the following cortical regions as follows (co-ordinates are in reference to bregma): frontal cortex EEG (motor area, anteroposterior (AP) 2 mm, mediolateral (ML) 2 mm); occipital cortex EEG (visual area, V1, AP -3.5–4 mm, ML 2.5 mm) and cerebellum

(see Figure 2-1 for a schematic of the electrode locations). The cerebellum screw was a reference electrode for both the frontal and occipital EEG signals. Reference and ground screw electrodes were implanted above the cerebellum and contralaterally to the occipital screw, respectively. These screw electrodes were attached to wires and soldered to custom-made headmount connectors (Pinnacle Technology Inc. Lawrence, USA) prior to surgery. The wires were approximately 1cm long to raise the headmount above the skull so that pressure to the head could be reduced during cable attachment. Holes were drilled using a high-speed drill and screws were implanted using pliers and a small screwdriver. Screws were implanted so that they should just be touching the surface of the dura, while not damaging the brain. An additional free-standing screw was implanted above the contralateral (left) occipital cortex, to act as an anchor screw to stabilise the implant. Finally, two single stranded, twisted, stainless steel wires were inserted either side of the nuchal muscle to record electromyography (EMG). All screws and wires were secured to the skull using dental acrylic.



*Figure 2-1: **General experimental setup.** (A) Schematic representation of the position of the frontal and occipital EEG electrodes as well as the microwire that was used to record local field potentials (LFPs) and multiunit activity (drawn in reference to (Paxinos and Franklin, 2001)) shown on the dorsal surface of the mouse head. (B) Example multiunit activity, and associated spike waveform during waking.*

The recording configuration was selected for a number of reasons. Recording from anterior (frontal) and more posterior (occipital) cortices is a standard procedure for studies investigating sleep (Cui et al., 2014; Huber et al., 2000b). This configuration is particularly useful as SWA predominates in the frontal derivation, and theta activity predominates around the occipital

cortex (due to the proximity of this area to the hippocampus). Recording from these two cortical regions therefore allows for characterisation of two of the main frequency bands important in sleep-wake electrophysiology and facilitates the scoring of state transitions, such as from NREM to REM sleep.

At the end of the surgery mice were injected with saline to replace fluids that may have been lost during the surgery, and animals were continuously monitored until they became conscious and had palpebral and toe pinch responses, and respiration patterns had returned to near normal. All mice were monitored closely post-operatively and scored daily for altered behaviour (natural and provoked), their appearance, changes in respiratory rate and signs of grimace (Langford et al., 2010), until they were scored as normal for a minimum of two days. Mice were provided with dexamethasone 0.2 mg kg for 2 days and metacam 5 mg kg for a minimum of 3 day, as necessary.

2.2.2 Microwire array implantation

For chapters 3 (ageing) and 4 (diazepam), a 16-channel polyimide–insulated tungsten microwire array (Tucker-Davis Technologies Inc (TDT), Alachua, FL, USA) was implanted into deep layers of the primary motor cortex, in addition to the previously described EEG screw electrodes. The array consisted of 16-channels (2 rows each of 8 wires), with a wire diameter of 33 μ m, electrode spacing 250 μ m, row separation L-R: 375 μ m and tip angle of 45 degrees. As the skull surface has a natural curvature, arrays were customised to have one row of electrodes 250 μ m longer than the other (Fisher et al., 2016; McKillop et al., 2018).

One day before surgery animals received dexamethasone (0.2 mg kg, orally) to suppress the local immunological response. Surgery was then carried out as previously described for EEG with additional steps, specific for the implantation of the microwire array. A 1x2 mm craniotomy was

made using a high-speed drill with midpoint coordinates relative to bregma as follows: AP +1.5–2 mm, ML 2 mm. Dorsal ventral coordinates were taken when the longer row of electrodes were touching the surface of the brain and were lowered 0.7–0.8 mm below the pial surface into layer 5 of the motor cortex. A two-component silicone gel (KwikSil; World Precision Instruments, FL, USA) was used to seal the craniotomy and protect the surface of the brain from the dental acrylic used to fix the array to the skull.

2.3 Signal processing, vigilance state scoring and analysis

Data were acquired using a Tucker Davis Technology (TDT, Alachua FL, USA) Multichannel Neurophysiology Recording System. Cortical EEG was recorded from frontal and occipital derivations. EEG/EMG/LFP data were filtered between 0.1–100 Hz, amplified (PZ5 NeuroDigitizer pre-amplifier, TDT Alachua FL, USA) and stored on a local computer at a sampling rate of 256.9 Hz. Extracellular neuronal spike data were recorded from the microwire array at a sampling rate of 25 kHz (filtered between 300 Hz - 5kHz). During recordings, an amplitude threshold was applied to the signal using an online spike sorting procedure (using OpenEx software (TDT)) ($>2 \times$ noise level, at least $-25 \mu\text{V}$) so that only those spikes that exceeded the threshold were detected and stored for further analysis (voltage and time stamps of the 0.48 ms before, 1.36 ms after the threshold crossing were stored as 46 samples). Custom-written Matlab scripts (The MathWorks Inc, Natick, Massachusetts, USA) were then used to resample the LFP, EEG and EMG data at a sampling rate of 256 Hz and then open source Neurotraces software was used to transform data into European Data Format (EDF).

LFP, EEG, EMG and MUA data were then visualised offline using the software SleepSign (Kissei Comtec Co, Nagano, Japan) and each 4-second epoch was scored into its respective vigilance state. Waking was defined as a low voltage, high frequency EEG with a high level or phasic EMG

activity. NREM sleep was defined as a signal of a high amplitude and low frequency, as well as the occurrence of EEG slow waves. REM sleep was defined as a low voltage, high frequency EEG signal, which was distinguished from waking by a low level or absence of EMG activity. The same software was used to calculate power spectra for each 4-second epoch of the scored EEG and LFP data, using a Fast Fourier Transform (FFT) routine for 4-s epochs (Hanning window, 0.25Hz resolution).

2.4 Spike sorting procedure for analysis of neuronal activity

Offline spike sorting was performed on the recorded multiunit activity, in order to identify putative single units, as previously described (Fisher et al., 2016; McKillop et al., 2018). For the main ageing chapter (Chapter 3) spike sorting was performed by either concatenating the 12-hour baseline light period and the 12-hour baseline dark period, or by concatenating the baseline 12-hour light period and the sleep deprivation 12-hour period. For the diazepam study (Chapter 4) the first 2 hours and last two hours of each 12-hour light and dark period were concatenated for the three recording days (baseline, injection and recovery), separately for vehicle and diazepam conditions.

As a first step, artefactual waveforms were removed using custom written Matlab scripts. For each remaining spike, the segment occurring between the 5th and 35th time stamps were then subjected to a principal component analysis (PCA) and was then described by 31 variables, each being a linear combination of the original sampling values. This segment was chosen as this is where the initial negative peaks after threshold crossing and subsequent afterhyperpolarisations are located and so this segment is more informative about the overall spike waveform. All spikes were then clustered based on a k-means algorithm (Jolliffe, 2002), implemented according to Lloyd's algorithm (Lloyd 1982), using the k-means function in Matlab. This is a partitioning

method which aims to divide n observations into k clusters in which each observation belongs to the cluster with the nearest mean, serving as a prototype of the cluster. Clustering was performed for $k=2-5$ clusters ($k=2, 3, 4$ or 5) and the number of clusters for each channel was then manually determined based on visual inspection of the signal to noise ratio, waveshape of the resulting action potentials, stability of the amplitude across time and ISI distribution histogram. As a final step, the quality and stability of each cluster was then manually assessed and rated, based on the average spike waveform with standard deviation, interspike interval distribution, the time course of peak-to-peak amplitude across the recording period, the corresponding time course of average firing rates and the autocorrelogram of the spike trains (an example of which is shown in Figure 2-2). Clusters that had low quality or stability were excluded from analyses.

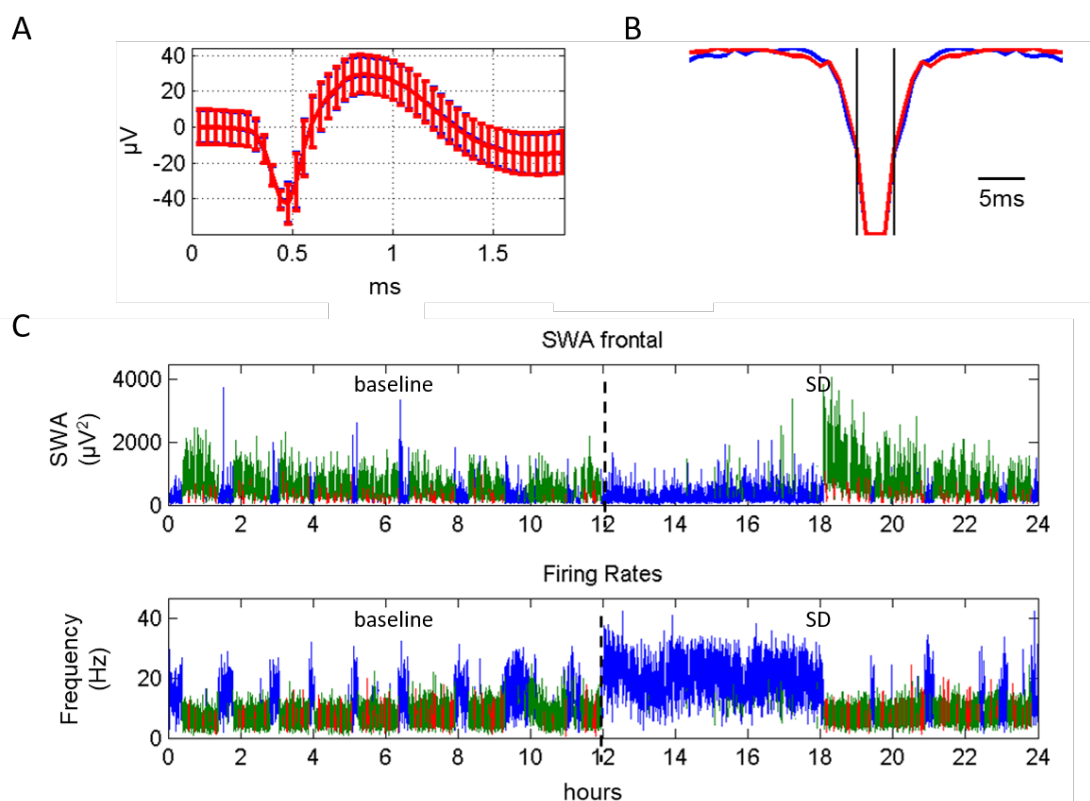


Figure 2-2: Spike sorting procedure: Once clustering analysis had been performed, the quality and stability of each putative neuron was assessed using specific features: (A) the spike waveform and (B) autocorrelogram of one individual putative single unit, (C) the time course of EEG SWA (frontal derivation, top panel) and the average firing rates for a same neuron (bottom panel). Note 12-hour baseline and sleep deprivation (SD) light period recordings were combined and used for spike sorting. (A) and (B): red and blue represent baseline and sleep deprivation days, respectively. (C): blue, green and red represent Wake, NREM sleep and REM sleep, respectively. Data are shown for one putative single unit only.

2.5 Slow wave and OFF period detection

In order to detect slow waves in the LFP, firstly the signal was band pass filtered between 0.5-4 Hz (stopband edge frequencies 0.3-8 Hz) with MATLAB `filtfilt` function exploiting a Chebyshev Type II filter design (The MathWorks Inc, Natick, Massachusetts, USA) (Achermann and Borbely, 1997; Fisher et al., 2016; McKillop et al., 2018; Vyazovskiy et al., 2009b). Slow waves were then defined as positive deflections of the filtered LFP signal between two consecutive negative deflections below the zero-crossing, in which the peak amplitude of the wave was larger than the median amplitude detected across all waves.

OFF periods were classified as described previously (McKillop et al., 2018). OFF periods were detected by first concatenating all individual spikes together with all periods in which neuronal firing stopped for a minimum of 20 ms (not exceeding 1000ms). Next, OFF periods were subdivided into one hundred 1% percentiles and the LFP slow waves triggered by the onset of an OFF period were averaged across all recording channels. There was a strong association between the amplitude of LFP slow waves and the duration of OFF periods, in which slow wave amplitude increased with increasing OFF period duration (Figure 2-3). In this thesis, slow waves at least 50% the amplitude of the largest slow waves (100th percentile) were selected and the periods of neuronal silence associated with these slow waves were defined as OFF periods. Therefore, this study investigates the largest slow waves and the corresponding longest OFF periods only. These were selected as large slow waves are well established to reflect network activity and to be sensitive to the effects of sleep deprivation, while smaller slow waves are more difficult to distinguish from noise. In order to assess this association in further detail, the OFF periods detected using the above methodology were then aligned and the resulting slow waves associated instead with the neuronal silence were calculated.

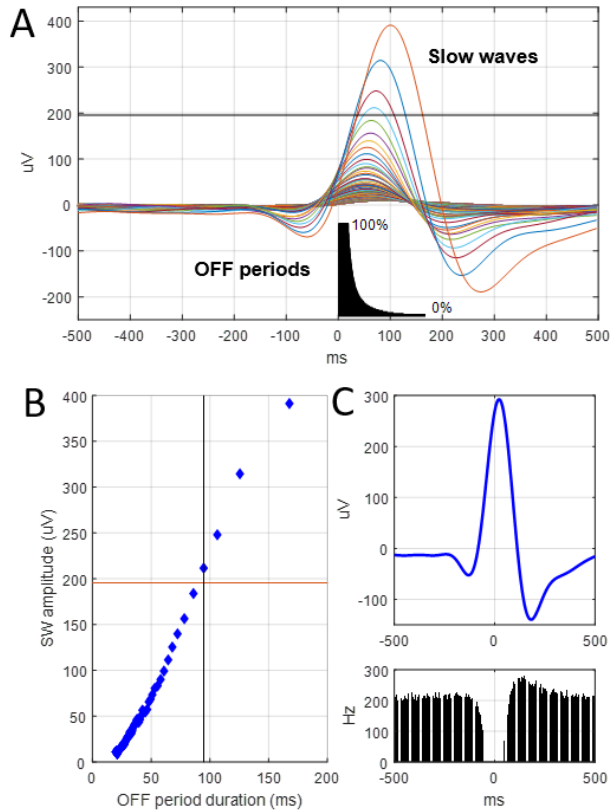


Figure 2-3: Algorithm for OFF-periods detection. (A) Average LFP slow waves calculated as a function of OFF-period durations. All OFF periods were subdivided into 100 1% percentiles, and the corresponding average LFP signal aligned to the OFF period onset was calculated. Note that short OFF periods are not associated with a noticeable change in the amplitude of the LFP slow waves, which increases progressively with longer OFF period duration. (B) The relationship between the duration of OFF periods and the amplitude of corresponding average LFP slow wave. OFF periods were defined as periods of generalised network silence, which were associated with a slow wave at least 50% the amplitude of the largest slow waves. The corresponding thresholds are depicted as horizontal (SW amplitude) and vertical (minimal OFF period duration) lines. (C) Top: average LFP slow wave. Bottom: corresponding profile of MUA centered on the mid-point of the OFF-periods defined based on the above criteria.

2.6 Sleep deprivation techniques

In Chapters 3 (ageing) and 6 (visual discrimination task) of this thesis, sleep deprivation was performed using a well-established gentle handling technique, as described previously (Cui et al., 2014; Fisher et al., 2016). This technique utilises the well-established tendency for mice to exhibit exploratory behaviour when exposed to novel objects such as cardboard, colourful plastic and tissue paper. In this study novel objects were provided when the behaviour or polysomnographic recordings of an individual mouse showed sleep-like activity. This technique is therefore more ethologically relevant to natural periods of wakefulness, and is less stressful for

the animals compared to other sleep deprivation methods, such as the disk-over-water method (Rechtschaffen and Bergmann, 1995).

In chapter 5 (water maze task), sleep deprivation was instead performed using a forced locomotion protocol. For the duration of the sleep deprivation protocol animals were singly housed in a cylinder (31.5 cm diameter by 10 cm length) constructed of plexiglass rods, allowing waste to be collected below the chamber. The cylinder was positioned horizontally inside a Plexiglas frame in a sound-attenuated and light and temperature-controlled room, to regulate sensory modalities known to affect sleep. Sleep deprivation was performed using a non-invasive enforced activity protocol whereby the cylinder intermittently turned throughout the deprivation period. A motor was activated to turn the cylinder around its axis for 8 seconds (265° of rotation at 11.5cm.s^{-1}), in a pseudo-random rotation direction (left or right). This is sufficient to initiate the righting reflex and thus awaken the animals. Motor activation was determined using parameters from a Weibull distribution fitted to the intervals between sleep attempts in historical mouse data. The algorithm used in this study was modified from one which was previously used with rats (McCarthy et al., 2017), however it should be noted that this remains to be validated against EEG data. This algorithm was chosen based on previous suggestions that it may be the best non-invasive technique for producing functional deficits and increasing the need for recovery sleep (observed as an increase in recovery sleep, delta power and sleep continuity post sleep restriction), while it does not lead to significant increases in stress (as determined by urinary corticosterone levels) (McCarthy et al., 2017). This method has also been shown to achieve similar functional deficits to those observed using EEG-driven biofeedback protocols (McCarthy et al., 2017).

2.7 Behavioural testing

In order to assess the link between sleep, ageing and cognition a number of cognitive tasks were utilised in this project. Firstly, the Morris Water Maze was used in Chapter 5 to assess whether spatial memory is disrupted by ageing and whether sleep deprivation has an influence on this. Secondly, I also designed novel adaptations of the visual discrimination task, details of which are provided in Chapter 6.

2.7.1 Histological verification of recording site

In this project, microwire arrays were implanted into deeper layers of the primary motor cortex. In order to verify the location of the electrode recording sites, histological verification was performed. To do this, at the end of the experiment, mice were anaesthetised and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde solution, and brains removed for histology. The brains were photographed to aid the localisation of the exact electrode positions. Brains were then sectioned into 50µm coronal slices using a freezing Microtome electrode spacing 250µm (Leica, Germany) and the region of interest was mounted onto microscope slides and stained using cresyl-violet (Nissl) staining, a marker of cell bodies. This method relies on the damage caused by the individual tracts of the array being visible. However, as the removal of the electrodes often causes damage to the cortex, these tracts were often not visible in the slices. Therefore, in later recordings, electrodes were first coated with the fluorescent dye Dil (DiI18(3), Invitrogen), before their implantation, so that fluorescence microscopy may aid the visualisation of the electrode tracts. Figure 2-4 shows example images from both the Nissl stain and Dil fluorescence techniques, in which electrode tracts can be identified.

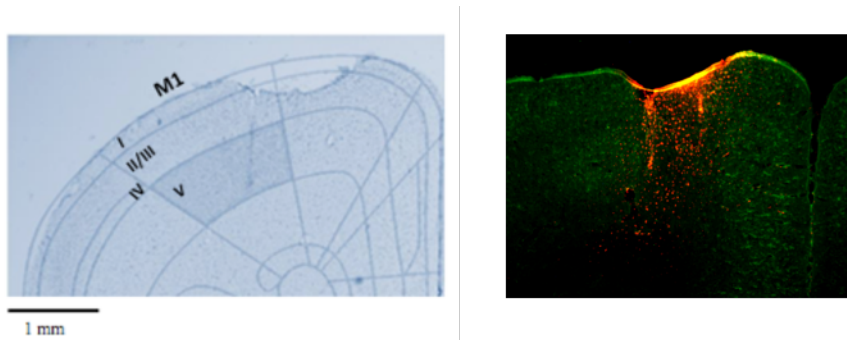


Figure 2-4: Histological verification of recording site. Left panel: an example slice in which Nissl staining was used to verify the electrode location. The image is overlaid onto a schematic of the mouse brain obtained from the Allen Mouse Brain Atlas. One electrode tract is clearly identifiable as being in layer V of the motor cortex (M1). Right panel: an example slice in which electrode tracts were visualised using fluorescence microscopy. In more recent recordings electrodes were first coated with the fluorescent dye Dil (DiI18(3), Invitrogen), before their implantation, so that fluorescence microscopy may aid the visualisation of the electrode tracts. The left panel was modified from Fisher et al., 2017, which used some of the same animals as in this study.

Due to the size of the microwire arrays, which were approximately 1mm by 2mm, with an electrode spacing of 250µm, it is likely that the recording sites covered areas that represented numerous body parts (Harrison et al., 2012). However, the lack of precision in these histological techniques, make it difficult to map exactly which areas the recording sites are recording from.

2.8 Statistical analysis

Data were analysed using Matlab (The Math Works, Inc., Natick, MA, USA) and SigmaPlot 14.0, SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp) and Matlab were used for statistical testing. All values reported are mean $\pm$ s.e.m, unless explicitly stated. Details of the statistical tests and the numbers of animals used in each of the analyses are stated in the specific figure legends.

Chapter 3: The effect of ageing on cortical activity

3.1 Introduction

Ageing is associated with numerous changes in global sleep characteristics including the overall structure of the sleep-wake cycle and associated electroencephalogram (EEG), with sleep becoming more fragmented and superficial with ageing (Altena et al., 2010; Banks et al., 2015; Bano et al., 2012; Gu et al., 2015; Hasan et al., 2012; Klerman et al., 2013; Klerman and Dijk, 2008; Ohayon, 2004a; Panagiotou et al., 2017; Shiromani et al., 2000; Vyazovskiy et al., 2006a; Wimmer et al., 2013). A thorough overview of the effects of ageing on sleep is provided in the general introduction chapter of this thesis (section 1.2.2). Previous studies often relied on EEG recordings, which reflect the summation of activity from many local and distant sources. However, in order to get a better idea of the network mechanisms underpinning the age-dependent changes in sleep it is important to study activity at a more local level, by studying neuronal activity in distinct cortical regions.

No study to date has investigated the cortical network mechanisms underpinning the observed effects of ageing at the surface EEG level. This is an important omission, as numerous studies suggest that sleep may be initiated at the level of local cortical networks, such that single neurons and local neuronal populations have important contributions to global sleep dynamics (Fisher et al., 2016; Grosmark et al., 2012; Krueger et al., 2016; Rodriguez et al., 2016; Siclari and Tononi, 2017; Vyazovskiy et al., 2009b, 2009b, 2011; Watson et al., 2016). This is supported by evidence that slow waves and OFF periods can occur locally during wake, especially after sleep deprivation when sleep pressure is high (Vyazovskiy et al., 2009b, 2011). Details as the local regulation of sleep are provided in the general introduction chapter (section 1.1.3).

Although the neural activity underpinning slow waves was outlined in detail in the general introduction chapter of this thesis (section 1.1.1.1), I have provided a brief overview below. Slow waves during NREM sleep reflect the synchronous occurrence of population neuronal silence (OFF periods) within thalamocortical networks, corresponding to the hyperpolarisation of single neurons, while also being influenced by distant sources (Crunelli et al., 2015; Destexhe et al., 1999; Neske, 2015; Timofeev, 2013; Vyazovskiy and Harris, 2013). These periods of population silence (OFF periods) alternate with periods of neuronal firing (ON periods). Slow wave activity (SWA; the frequency range at which slow waves occur 0.5-4 Hz) is highest at the beginning of a sleep period, especially after periods of prolonged wakefulness such as after sleep deprivation, and decreases progressively across the sleep period (Tobler and Borbély, 1986; Vyazovskiy et al., 2009b, 2011). This is associated with an increased incidence of slow waves and OFF periods, as well as longer duration OFF periods at the beginning of a sleep period (Rodriguez et al., 2016; Vyazovskiy et al., 2007b, 2009b). These measures are therefore considered markers of increased physiological sleep pressure at the network level. Under increased sleep pressure, e.g. when sleep deprived, not only is SWA high, but the slow waves are more frequent, steeper, have a higher amplitude and there are fewer multipeak waves (Esser et al., 2007; Riedner et al., 2007; Vyazovskiy et al., 2007b), all of which reflect a high synchronicity of ON-OFF periods across neuronal populations (Steriade et al., 1993a; Steriade, 2006; Vyazovskiy et al., 2007b, 2009b).

Although the local dynamics of the network neural mechanisms underlying slow waves have been well characterised during sleep and after sleep deprivation, this has not been investigated in the context of ageing. A recent study suggests that SWA is increased in old aged mice which was linked to the occurrence of more frequent, larger amplitude, steeper sloped slow waves (Panagiotou et al., 2017). Therefore, it would be reasonable to hypothesise that old age mice may have an increased synchronicity across neuronal populations, which may also result in longer OFF periods. Together these findings led the authors to suggest that ageing may be

associated with an increased sleep pressure or in other words a higher homeostatic sleep need (Panagiotou et al., 2017). It should however be noted that previous evidence has also suggested ageing may instead be associated with a decrease in SWA in mice (Banks et al., 2015; Colas et al., 2005; Hasan et al., 2012; Wimmer et al., 2013). This, in combination with the lack of a robust increase in SWA during recovery sleep after sleep deprivation (Hasan et al., 2012; Lafortune et al., 2012; Munch et al., 2004; Wimmer et al., 2013), has led to suggestions ageing is instead associated with a reduced homeostatic sleep need (Cirelli, 2012; Hasan et al., 2012; Klerman and Dijk, 2008; Mander et al., 2017). Only by studying the neuronal activity underpinning this network phenomenon will it be possible further investigate whether this is true, or whether in fact ageing rather involves a diminished capacity to generate and sustain deep NREM sleep, both of which have been hypothesised (Cirelli, 2012; Klerman and Dijk, 2008; Mander et al., 2017).

3.1.1 Experimental aims

This chapter aimed to address objective 1 of my thesis: ***‘To characterise the spatio-temporal properties of cortical neural activity in freely moving young and older mice, and to elucidate neurophysiological markers of ageing and preceding sleep-wake history’***. The primary aim of this chapter was to investigate whether ageing is associated with changes in cortical neural activity, including the characteristics of neuronal OFF periods and corresponding LFP slow waves during baseline sleep and after sleep deprivation. This chapter is descriptive and exploratory in nature, because no study to date has investigated how ageing affects local neural activity in the neocortex. In order to investigate the effect of ageing on sleep and wakefulness this chapter will perform a full characterisation of the global sleep-wake structure, including electrophysiological correlates of sleep before addressing the most novel aspect of this project, the neuronal properties underlying sleep. It may be expected that if the global alterations associated with ageing are at least partially the result of disruptions in local cortical networks, this would be

reflected in local network properties, such as reduced spiking activity or changes related to localised slow oscillations.

3.2 Methods

3.2.1 Experimental animals

Recordings were carried out in male C57BL/6J mice subdivided into three age groups; early adulthood (EA, 4.6 ± 0.3 months, $n=10$), late adulthood (LA, 12.1 ± 0.3 months, $n=11$) and older age (OA, 24.6 ± 0.4 months, $n=10$). Although it is unclear what these age ranges equate to in humans, it has been suggested that the OA group in this study is likely to correspond to an age of approximately 70 years in humans (Dutta and Sengupta, 2016). Although the aim of this study was to study healthy ageing, it is not certain that the animals used in this study did not have any underlying age-related pathologies.

As this was a novel study with a number of uncertainties, power calculations were not performed prior commencing the study, and instead group sizes were based on the number of animals used in previous studies relevant to this, including ageing studies (Colas et al., 2005; Eleftheriou et al., 1975; Farajnia et al., 2014; Hasan et al., 2012; Nakamura et al., 2011; Panagiotou et al., 2017; Possidente et al., 1995; Valentinuzzi et al., 1997; Welsh et al., 1986; Wimmer et al., 2013). The average group size of these studies was on average 6.9 mice per age group, therefore the group sizes of this study (EA: $n=10$, LA: $n=11$ and OA: $n=10$, or at least $n=5$ for some of the comparisons) were deemed appropriate.

3.2.2 Surgical and recording procedures

Mice were group housed until they underwent surgery. All mice contributing to this chapter underwent surgery to implant both EEG screw electrodes and a microwire array into layer 5 of

the motor cortex (as described in the general methods section (Chapter 2). Animals were closely monitored during their recovery after surgery and their appearance, behaviour and signs of grimace were scored daily out of 5. Once they reached a score of zero for two consecutive days they were deemed to have sufficiently recovered from surgery. On average this took 6.1 ± 0.4 , 6.7 ± 0.6 and 9.7 ± 0.9 days, for EA, LA and OA mice, respectively. Therefore, OA animals took approximately 3 days longer to recover from surgery compared to both EA and LA mice (Kruskal-Wallis with Mann-Whitney post hoc: $X^2(2)=13.684$, $p<0.0001$, EA vs OA $p<0.0001$, LA vs OA $p=0.004$). The implantation of microwire arrays can cause an immune response which can deteriorate the electrophysiological signal and also destabilise the implant; therefore longitudinal recordings were not performed in this study. For this reason mice were implanted shortly before they were required for surgery.

Electrophysiological data were acquired as outlined in the general methods chapter (Chapter 2). Both a 24-hour long baseline and sleep deprivation day were scored to identify vigilance states. Artefactual epochs were also scored so that they may be removed from appropriate analyses (percentage of total recording time: EA $8.7 \pm 9.8\%$, LA $8.8 \pm 7.5\%$, OA $7.9 \pm 11.0\%$).

3.2.3 Experimental protocol – baseline and sleep deprivation recordings

The primary aim of this study was to investigate the effects of physiological healthy ageing on spontaneous waking and sleep, rather than the effects of specific manipulations beyond the conventional sleep deprivation. For this reason mice were kept in a 12:12 light-dark cycle, under standard laboratory conditions. Mice were transferred to the recording chambers and habituated to both the cage and recording cables for a minimum of 3 days before recording (Cui et al., 2014; Fisher et al., 2016). Patterns of running wheel activity were monitored to ensure that the mice were well entrained to the light dark cycle and that the recordings to be used in baseline analysis were representative for that animal. Furthermore, to confirm that differences

in circadian phase were not present in this study, the onset of activity after lights off was calculated for all animals based on EEG/EMG defined wakefulness. Most animals were awake within minutes from dark onset, irrespective of age (EA: 0.8 ± 0.3 , OA: 1.1 ± 0.5 ; LA: 1.1 ± 0.3 mins, ns. Wilcoxon test). Furthermore, plotting the time course of EEG/EMG defined waking around dark onset revealed that the three age groups did not show marked differences in the amount of wakefulness prior to dark onset (data not shown), suggesting that there were no major changes in circadian period or phase, which could account for the differences.

As neuronal activity during sleep has not previously been investigated in freely moving mice in the context of ageing, most analyses concentrated on characterising the general effects of ageing during undisturbed baseline periods. In order to determine the effect of ageing on sleep need and the response to sleep deprivation, an additional day, in which sleep deprivation was performed, was also analysed. Previous evidence suggests that the capacity to generate a homeostatic rebound in sleep in response to prolonged wakefulness is disrupted in ageing (Hasan et al., 2012). Sleep deprivation was performed for 6-hours starting at light onset using the well-established gentle handling technique (Cui et al., 2014; Fisher et al., 2016; Vyazovskiy et al., 2002), see general methods section for details (section 2.6). Although the aim of sleep deprivation was to abolish sleep completely, it is common for some sleep to occur, which was on average 5.9 ± 2.3 , 7.4 ± 7.15 and 12.3 ± 6.6 minutes (mean $\pm$ SD), for EA, LA and OA mice, respectively, during the 6-hour period.

3.2.4 Relationship between LFP slow waves and cortical MUA

As previously mentioned, slow waves are underpinned by periods of neuronal silence, in which firing activity ceases. These periods of neuronal silence are synchronous across neuronal populations and are referred to as OFF periods. Despite being investigated in a number of studies in young mice (Amzica and Steriade, 1998; Nir et al., 2011; Riedner et al., 2007; Steriade

et al., 1993a; Steriade, 2001; Vyazovskiy et al., 2007b, 2011; Vyazovskiy and Harris, 2013), there is no straightforward way for defining OFF periods, which often varies greatly across studies. Throughout this thesis spike sorting was first performed in order to detect putative single units across recording channels of the microwire array and then OFF periods were defined based on their association with slow waves, see general methods section (Chapter 2) for details. To investigate the effect of ageing on the fundamental underpinnings of the slow oscillation, the incidence and duration of OFF periods was quantified, along with the incidence of slow waves. The association between slow waves and OFF periods has also been investigated.

3.2.5 Neuronal phenotyping and vigilance-state dependency of cortical firing

Although mostly qualitative in nature, the neuronal firing associated with specific vigilance states and transitions between states was also investigated. The average firing rate (i.e. the number of spikes per 1 second) during each 4-sec epoch was calculated and separated into the three vigilance states: waking, NREM sleep and REM sleep. Distribution histograms were calculated for each putative neuron (examples of which are shown in Figure 3-9B). The peak firing frequencies during each state were calculated using these histograms, sorted according to peak firing rates and spectrograms were plotted to visualise the variability in firing rate properties according to vigilance state (Figure 3-9,) and transitions between states (Figure 3-12 and Figure 3-13).

3.2.6 Data and statistical analysis

Data were analysed using Matlab (The Math Works, Inc., Natick, MA, USA), Microsoft excel and SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp). In most cases ANOVAs were used to identify differences between the three age groups.

Welch F tests with Games-Howell post hoc tests were used in cases where data failed homogeneity testing, including for the weight and sleep data in Figure 3-5. In cases where data were not normally distributed, non-parametric Kruskal-Wallis tests (Mann-Whitney post hoc tests) were instead used. Where applicable critical p-values were adjusted for multiple testing ($p \times \text{number of tests}$). For time-course data such as in Figure 3-3B, repeated measures ANOVAs were used to identify differences. It should be noted that in Figure 3-14C-E two animals had a single missing value and so data for these time points were estimated using a multiple imputation technique within SPSS (5 imputations used). Details on the statistical tests, as well as the numbers of animals used in each of the analyses are provided in the relevant figure legends. In some cases mice were excluded from analyses due to technical reasons, such as poor signal quality. All values are mean $\pm$ s.e.m, unless stated otherwise. For fragmentation analysis a minimum episode criteria was applied, which was 16 seconds for all vigilance states. For all other analyses involving NREM sleep episodes, a minimum duration of 2 minutes was instead used. Brief awakenings of up to 4 seconds were allowed for and did not count as a change of state in all analyses.

3.3 Results

In this study I was successful in obtaining stable, good quality electrophysiological recordings (EEG, LFP and MUA) from 5, 12 and 24 months old mice. Upon visual inspection of the raw signals the three age groups were indistinguishable (Figure 3-1). Specific characteristics are discussed later in this chapter, however noteworthy is that in all three age groups, slow waves at the level of EEG and LFP recordings were associated with neuronal silence in MUA, as is classically seen during NREM sleep.

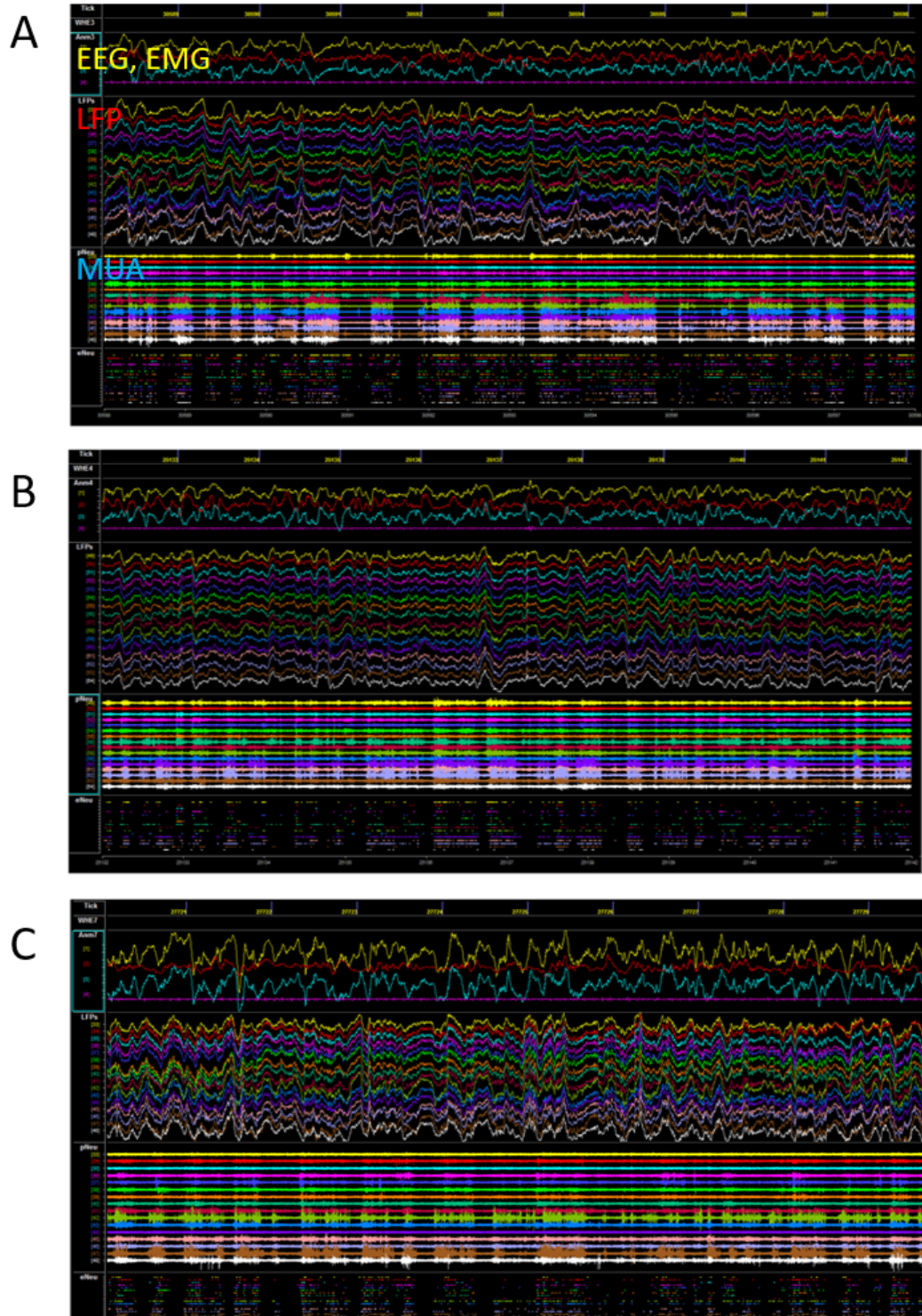


Figure 3-1: Representative examples of electrophysiological recordings during NREM sleep for (A) early adulthood (EA), (B) late adulthood (LA) and (C) old age (OA) mice. Signals displayed from top to bottom: EEG (electroencephalography frontal, occipital, differential, electromyography (EMG), LFPs (local field potentials, 16 channels), MUA (multiunit activity, 16 channels). Note: the occurrence of synchronous silent (OFF) periods in the multiunit activity in all examples.

3.3.1 Characterisation of global sleep structure

To investigate the effect of ageing on cortical activity, it was first important to establish the more global effects on sleep-wake structure. As mice are nocturnal animals, they are predominantly awake during the dark period (active phase) and asleep during the light period (sleep phase). It is well established that ageing leads to a redistribution of vigilance states across 24-hours, so that they are less distinctly associated with the light-dark cycle. This is referred to as a reduction in the daily sleep-wake amplitude. The 24-hour baseline recordings were scored into the respective vigilance states and plotted to show the distribution of sleep-wake activity over 24 hours. Notably, OA mice had an increased fragmentation of the sleep-wake cycle while ageing also increased the amount of NREM sleep, as can be seen in the representative examples in Figure 3-2.

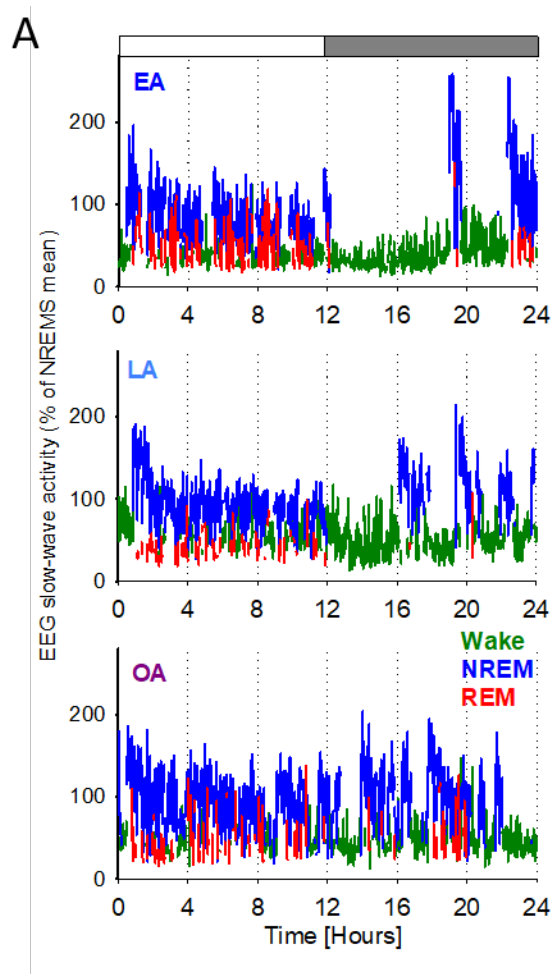


Figure 3-2: Hypnograms of individual representative animals from each age group (EA=early adulthood, LA=late adulthood, OA=older age). 24-h profile of EEG slow-wave activity (SWA, EEG power between 0.5-4.0 Hz, represented as % of 24-h mean) recorded from the frontal cortex. Wake, NREM sleep and REM sleep are represented by green, blue and red colour-coding, respectively. The bar at the top of the panel depicts the 12-h light and 12-h dark periods.

Quantification of the total amount of each vigilance state for the 12-hour light and dark periods (expressed as a % of total recording time) identified significant age-dependent effects, predominantly during the dark period (Figure 3-3A). Specifically this consisted of a decrease in the amount of wake (EA: $77.01 \pm 2.24\%$; LA: $66.88 \pm 4.24\%$; OA: $52.70 \pm 1.66\%$, Welch test (Games Howell): Factor Age=[$F(2,17.462)=37.436$, $p<0.0001$]), and increases in the amount of NREM sleep (EA: $19.87 \pm 1.99\%$; LA: 28.69 ± 3.50 ; OA: $40.05 \pm 1.41\%$; Welch test (Games Howell): Factor Age=[$F(2,17.390)=33.754$, $p<0.0001$]) and REM sleep (EA: $2.08 \pm 0.25\%$; LA: $2.10 \pm 0.46\%$; OA: $3.75 \pm 0.45\%$; One-way ANOVA (Tukey): Factor Age=[$F(2,30)=5.573$, $p=0.009$]), during the dark period (Figure 3-3A). In contrast, the total amount of REM sleep was significantly increased during the light period (EA: 12.23 ± 0.94 ; LA: 10.71 ± 0.68 ; OA: 9.46 ± 0.33 , Welch test (Games Howell): Factor Age=[$F(2,17.390)=33.754$, $p<0.0001$]). Plotting the amount of each vigilance state (in 2 hour intervals) across the 24-hour baseline day, revealed that age-dependent differences in the amounts of vigilance states were most prominent at the beginning of the dark phase, where the amount of wakefulness was decreased (repeated measures ANOVA: factor 'age': $F(2,28)=20.6$, $p<0.0001$, 'age x time interval': $F(11,157)=4.8$, $p<0.0001$), and NREM sleep was increased (repeated measures ANOVA: factor 'age': $F(2,28)=21.0$, $p<0.0001$, 'age x time interval': $F(11,153)=4.4$, $p<0.0001$) (Figure 3-3B). In contrast the time course of REM sleep was not significantly different between age groups (repeated measures ANOVA: factor 'age': ns), however there was a significant 'age x time interval' interaction: ($F(12,169)=3.8$, $p<0.0001$). Therefore, although the total amount of REM sleep showed age-dependent differences, no significant age-dependent differences were detected for the time course of REM sleep. Together this suggests that the decrease in wakefulness is mostly accounted for by an increase in NREM sleep. Importantly, all animals awoke only minutes after dark onset, which was not significantly different between age groups (EA: 0.8 ± 0.3 , OA: 1.1 ± 0.5 ; LA: 1.1 ± 0.3 min, n.s., Wilcoxon test), suggesting that all ages were well entrained to the light-dark cycle and so this is unlikely to explain the differences in sleep-wake architecture.

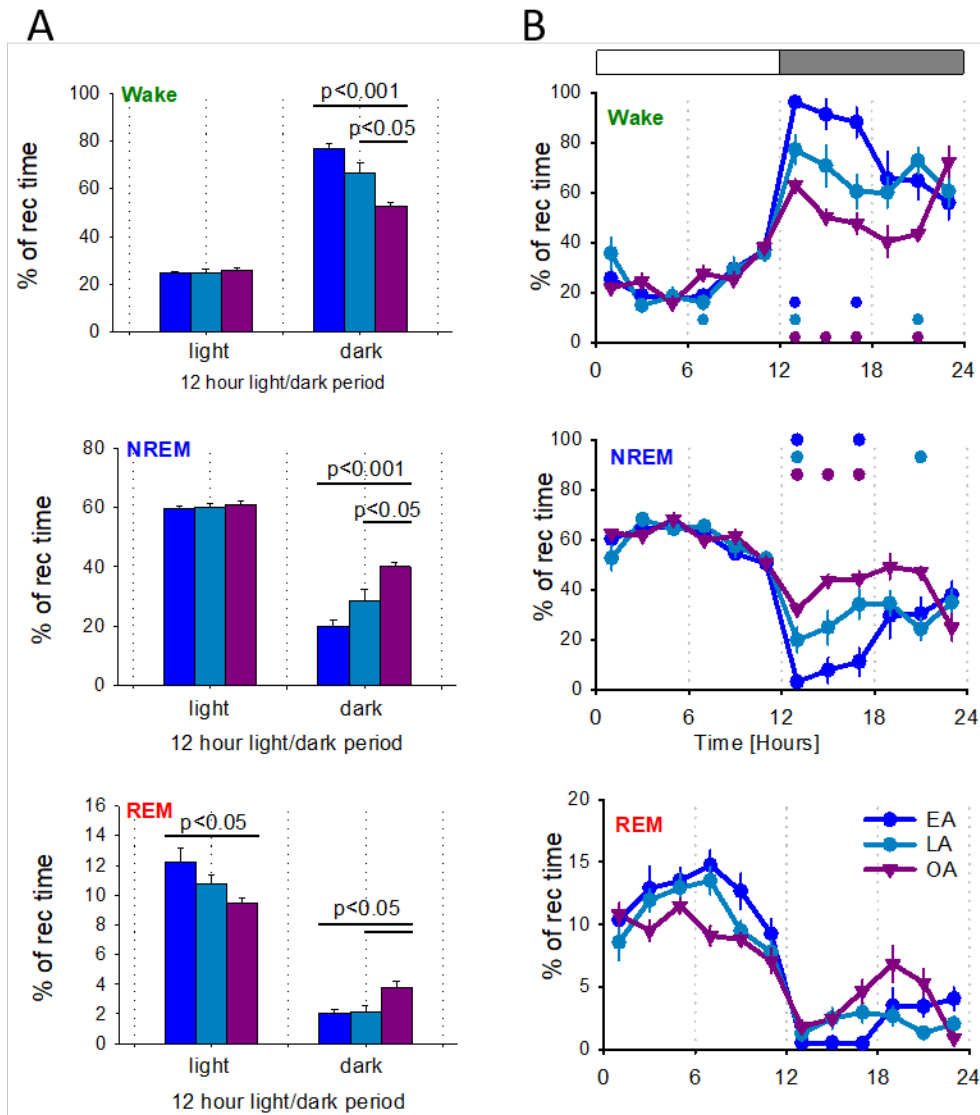


Figure 3-3: The global alterations of sleep-wake architecture with ageing in mice. (A) total amount of waking, NREM and REM sleep during 12-hour baseline light and dark periods, represented as a % total recording time. For Wake and NREM sleep a Welch F test (Games-Howell post hoc) was used to compare age groups. For REM sleep a one-way ANOVA was instead used (due to meeting homogeneity requirements). **(B)** Time course of waking, NREM and REM sleep during 24-h baseline day, shown in 2-h intervals. The amount of each vigilance state is represented as % of the total recording time. Significant differences between ages shown in blue, cyan and pink for EA vs LA, LA vs OA and EA vs OA, respectively. Both (A) and (B) show mean values, SEM, n=10 (EA), n=11 (LA) and n=10 (OA).

To determine whether ageing was associated with an increased fragmentation of the sleep-wake cycle, the average number and duration of NREM sleep, wake, and REM sleep episodes were calculated for the 24-hour baseline recording. Note, only episodes that were at least 16 seconds in duration (4 epochs) were included in this analysis. Brief awakenings of up to four 4-second epochs were not considered as changes of state and so did not break continuity of episodes. On average, the number of NREM sleep and waking episodes were significantly higher in older

animals (NREM sleep EA: 125.20 ± 6.08 , LA: 143.27 ± 8.86 , OA: 185.00 ± 6.63 episodes, one-way ANOVA $F(2,28)=16.60$, $p<0.0001$; wake EA: 53.50 ± 6.92 , LA: 74.09 ± 5.55 , OA: 107.60 ± 8.00 episodes, one-way ANOVA $F(2,28)=15.53$, $p<0.0001$; Figure 3-4). In contrast, no significant age-differences were identified in the number of REM sleep episodes (EA: 85.60 ± 6.12 , LA: 76.73 ± 5.95 , OA: 85.80 ± 4.87 , one-way ANOVA $F(2,28)=0.85$, $p=0.44$; Figure 3-4). Despite differences in their number, the average duration of NREM sleep episodes did not differ between age groups (EA: 4.86 ± 0.29 , LA: 4.77 ± 0.21 , OA: 4.19 ± 0.15 minutes, one-way ANOVA $F(2,28)=2.52$, $p=0.10$). However, older animals were found to have significantly shorter episodes of both wake and REM sleep (wake EA: 15.90 ± 2.60 , LA: 9.10 ± 0.71 , OA: 5.33 ± 0.33 minutes, one-way ANOVA $F(2,28)=11.99$, $p<0.0001$; REM sleep EA: 1.18 ± 0.02 , LA: 1.17 ± 0.04 , OA: 1.08 ± 0.02 minutes, one-way ANOVA $F(2,28)=3.47$, $p=0.05$; Figure 3-4).

An important factor, and potential confound of this study, is the well-established fact that in mice body weight increases with age. In this study weight was not only found to increase with ageing (EA, 26.4 ± 0.9 ; LA, 31.5 ± 0.6 ; OA: 34.1 ± 1.3 ; Welch F test: $F(2,17)=15.7$, $p<0.0001$; Figure 3-5A), but this also significantly correlated with the total amount of sleep (NREM and REM sleep, $r=0.60$, $p<0.001$) (Figure 3-5B). Total amount of sleep also significantly increased with age (% of recording time: EA, 47.0 ± 1.0 ; LA, 50.6 ± 1.5 ; OA: 57.1 ± 0.8 ; Welch F test: $F(2,18)=31.3$, $p<0.0001$), resulting in a strong positive correlation between total sleep amount and age ($r=0.76$, $p<0.001$), (Figure 3-5B). In this study, all animals had free access to running wheels for the duration of the experiment (but not prior to). As expected, younger mice had an increased amount of running-wheel activity compared to older mice (RW-revolutions per hour of waking: EA: 485.8 ± 87.0 ; LA: 280.8 ± 84.7 ; OA: 38.1 ± 20.6 , Welch F test: $F(2,15)=15.0$, $p<0.0001$, Figure 3-5C). However, all age groups showed a significant positive correlation between the amount of waking and running wheel activity ($r=0.84$, $p<0.001$). Importantly, running speed was also found to decrease with

ageing Figure 3-5C. Therefore, the combination of running amount and speed may be important contributors to the effects of ageing on sleep-wake architecture.

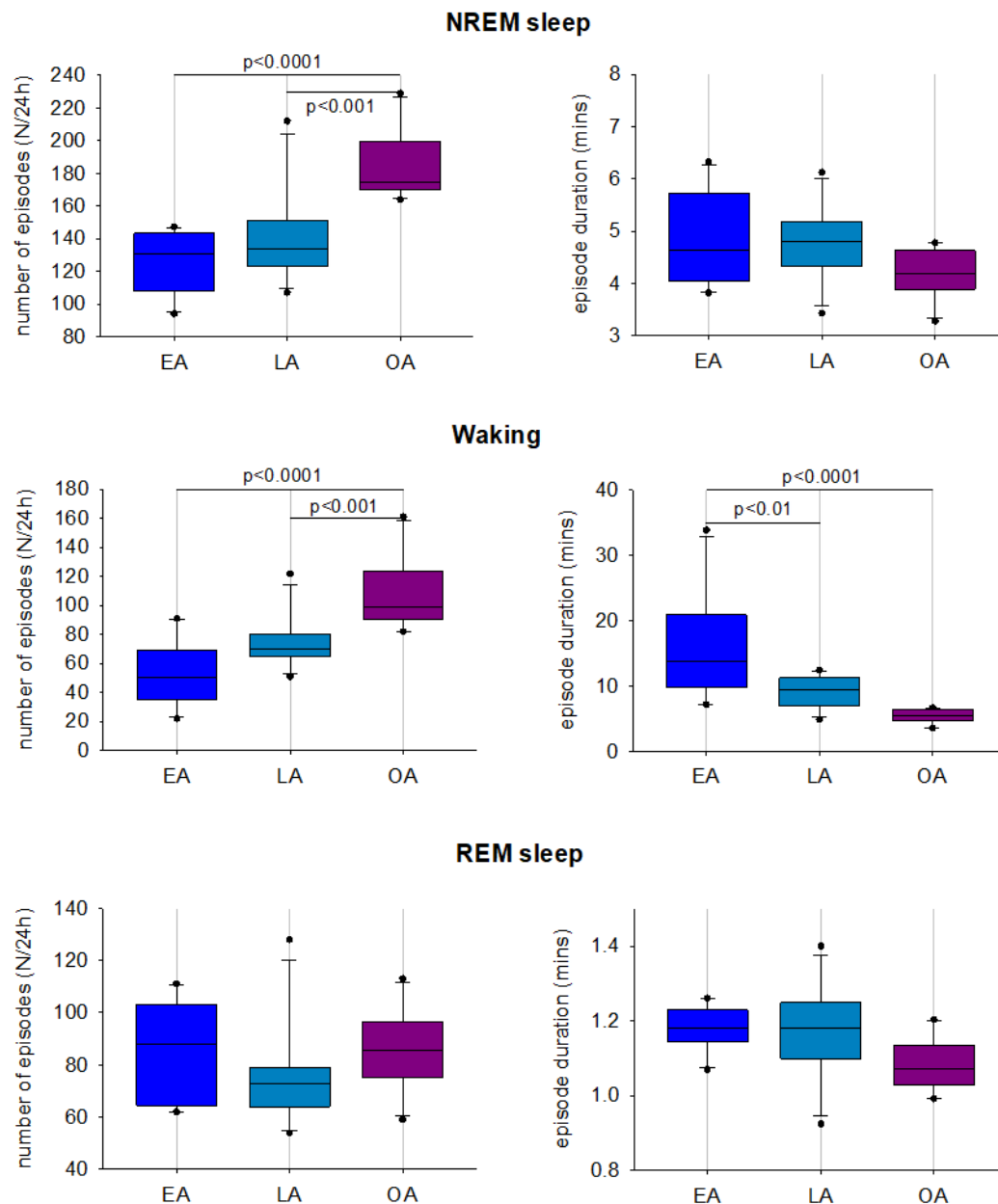


Figure 3-4: **Fragmentation of the sleep-wake cycle with ageing.** Both the average number (left panel) and duration (right panel) of episodes were calculated for each vigilance state, for the entire 24 hour baseline recording. Mean values, SEM. EA n=10, LA n=11, OA n=10. One-way ANOVAs (Tukey post-hoc) were used to compare age groups. Significant age comparisons are reported on the figure.

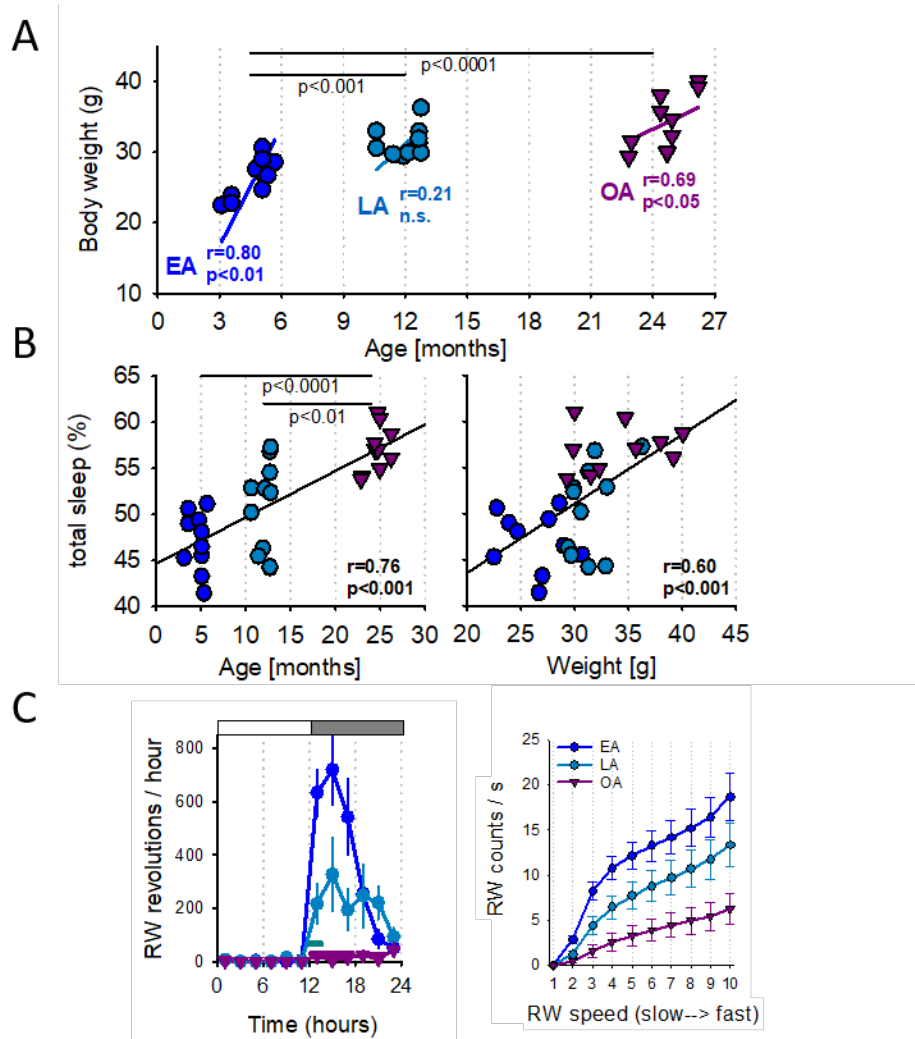


Figure 3-5: Association between age, weight, total sleep and running wheel activity (RW-activity). (A) The relationship between age and body weight is shown for individual mice (filled symbols). The straight lines depict linear regression lines which were calculated separately for each age group. (B) The relationship of age (left) and body weight (right) with the amount of total sleep shown as % of recording time over 24-h. (A and B): R and p-values correspond to Pearson's product moment correlation. Welch F test (Games-Howell post hoc) used to compare age groups. (C) Left panel: The amount of running decreases with ageing. RW activity was quantified as the number of counts per hour and shown for the three age groups. Right panel: all 4-s epochs were subdivided into ten 10% percentiles as a function of running speed (the first percentile includes all epochs without wheel running), and the corresponding average running speed was calculated. All panels are mean values, SEM, $n=10$ (EA), $n=11$ (LA) and $n=10$ (OA).

3.3.2 Electrophysiological correlates of ageing: EEG and LFP

Consistent with a recent report (Panagiotou et al., 2017), LA and OA mice were found to have higher spectral power density in slow frequencies as compared to young controls (Figure 3-6). However, significant age effects were only identified for frontal EEG recordings and not for the occipital EEG or the average LFP signal, for any vigilance state. Specifically the frontal EEG

derivation showed significant differences for frequency bin*age interactions but no direct effect of age during NREM sleep (freq*age: $F(5,63)=2.60$, $p=0.04$; age: $F(2,26)=2.74$, $p=0.08$; repeated measures ANOVA) and REM sleep (freq*age: $F(7,90)=2.15$, $p=0.05$; age: $F(2,26)=2.14$, $p=0.14$; repeated measures ANOVA). The specific frequencies at which age differences were identified are indicated on the relevant graphs in Figure 3-6. During wake there was a significant effect of age (EA vs LA mice, $p=0.03$), but not the frequency bin*age interaction for frontal EEG recordings (freq*age: $F(3,42)=1.48$, $p=0.23$; age: $F(2,26)=4.38$, $p=0.02$; repeated measures ANOVA).

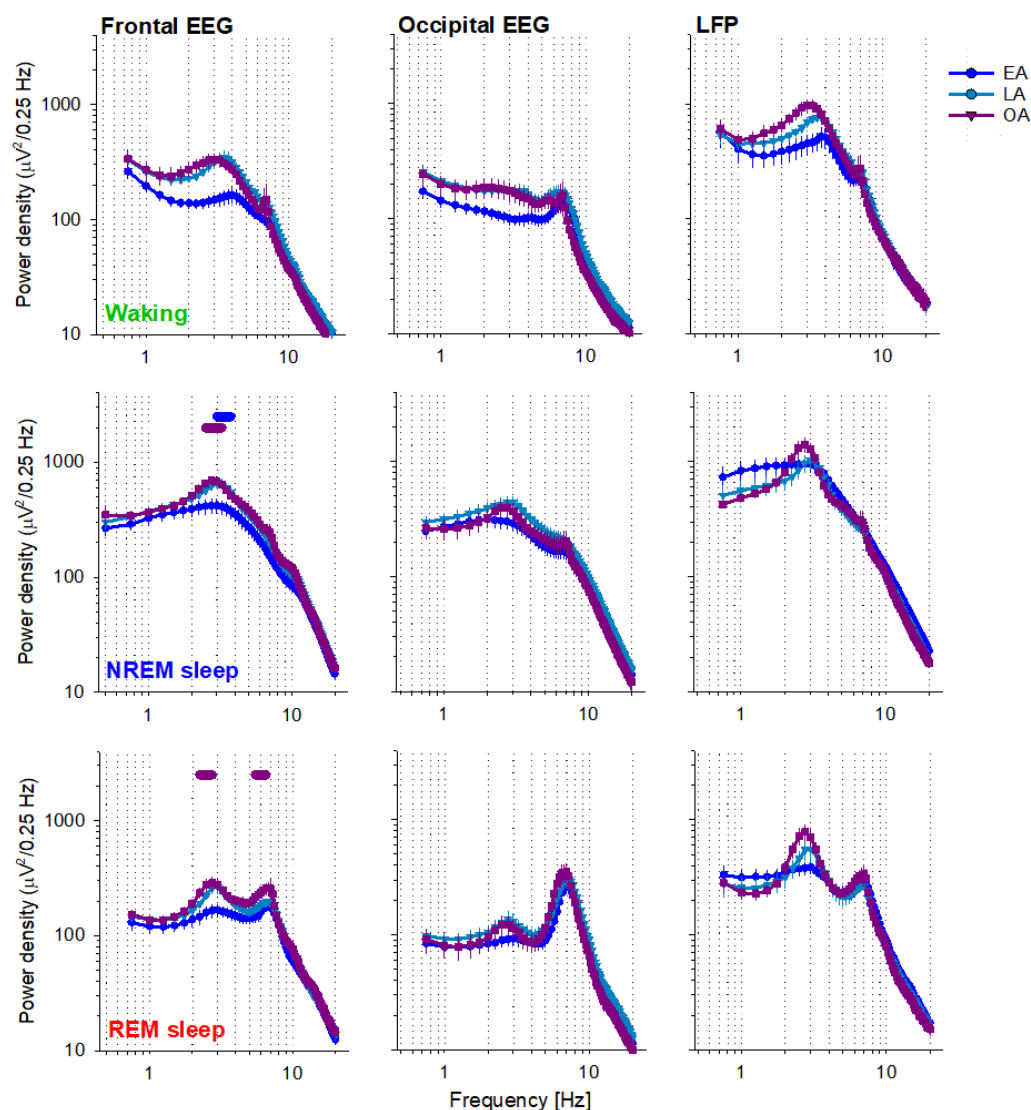


Figure 3-6: Power spectra during Waking (A), NREM sleep (B) and REM sleep (C), calculated for the frontal EEG (left), occipital EEG (middle) and a single LFP channel (right). Power densities are shown on a logarithmic scale for frequencies between 0-20, binned in a 0.25 frequency resolution. Mean values, SEM. Where significant frequency bin*age differences were identified using repeated measures ANOVAs, the frequency bins at which significant differences were identified are shown on the graph in blue and pink for EA vs LA and EA vs OA, respectively. Frontal EEG: EA $n=10$, LA $n=10$, OA $n=9$. Occipital EEG: EA $n=10$, LA $n=10$, OA $n=9$. LFP average: EA $n=8$, LA $n=7$, OA $n=10$.

As slow wave activity (SWA, 0.5-4Hz) is a well-established marker of sleep homeostasis and has been shown to be greatly affected by ageing, the dynamics of SWA across the 12-hour baseline light period were next calculated for frontal EEG recordings. Absolute SWA showed a gradual decline across the 12-hour light period (repeated measures ANOVA factor time= $[F(2,57)=55.69, p<0.0001]$). Older mice had a higher absolute SWA compared to EA mice, though this was not significant (repeated measures ANOVA factor age= $[F(2,26)=3.12, p=0.06]$, Figure 3-7A). There was however, a significant interaction between time and age (repeated measures ANOVA factor time*age= $[F(4,57)=4.232, p=0.003]$). When SWA was normalised to the 12-hour mean, no age differences remained, such that all age groups showed similar dynamics across the 12-hour light period (repeated measures ANOVA; time= $[F(2.755,71.628)=73.731, p<0.0001]$; time*age= $[F(6,72)=4.231, p=0.001]$; age ns; Figure 3-7B).

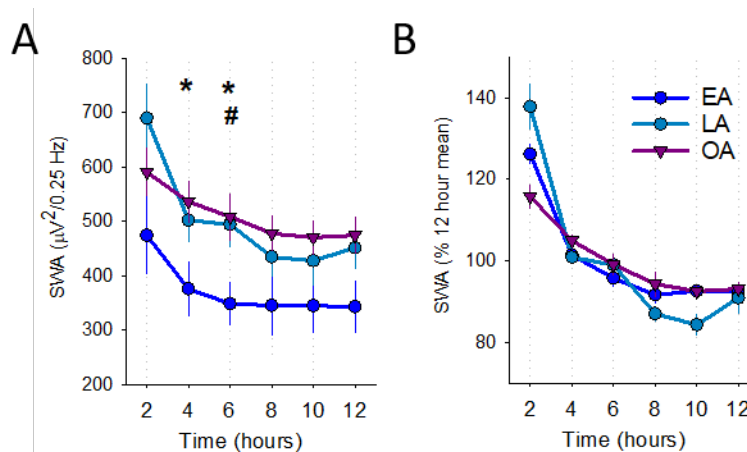


Figure 3-7: **Dynamics of frontal EEG slow wave activity (SWA) across the 12-hour baseline light period.** Frontal EEG SWA is shown in 2-h intervals for both absolute SWA (A) and SWA normalised to the 12-hour mean of the light period (B). All data are mean $\pm$ SEM. $n=10, n=10, n=9$ for EA, LA and OA mice, respectively. Repeated measures ANOVA with pairwise comparisons used for statistical testing. * $p<0.05$ EA vs OA, # $p<0.05$ EA vs LA.

3.3.3 Electrophysiological correlates of ageing: Neuronal activity

3.3.3.1 Vigilance state specificity in neuronal firing

The next aim of this chapter was to investigate the vigilance state specificity of neuronal firing.

Figure 3-8A shows a representative plot of the average firing rates of multiunit activity across the 12 hours light, plotted for each recording channel.

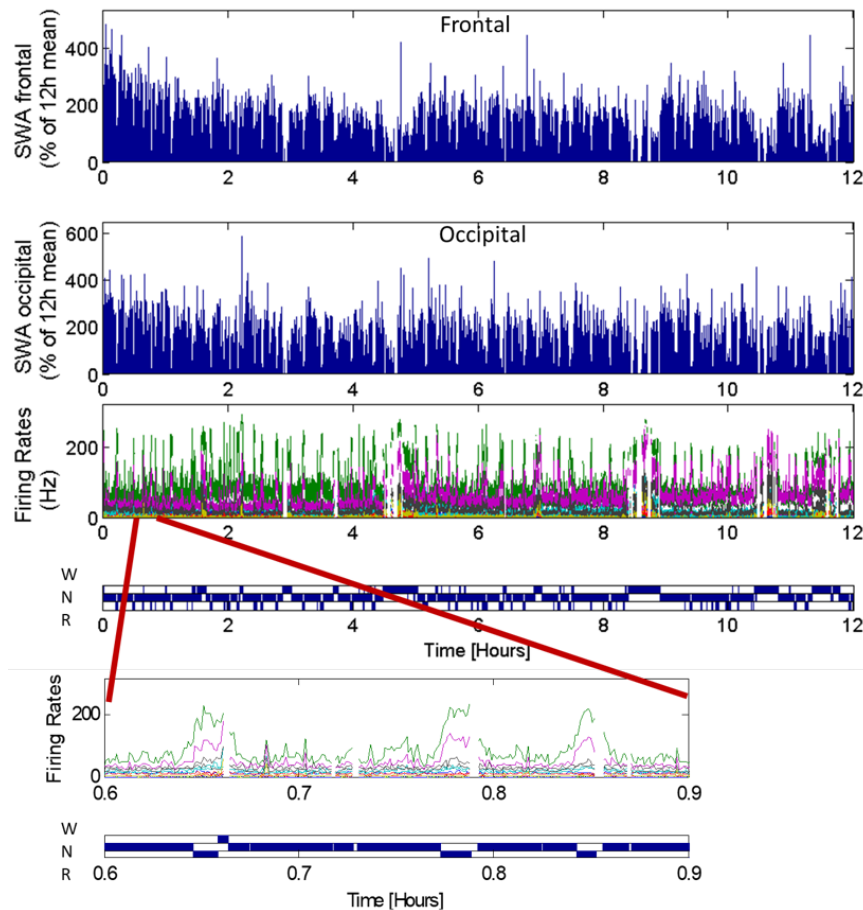


Figure 3-8: **Time course of firing rates across a 12 hour baseline light period, for a representative mouse.** Top two panels show slow wave activity (SWA) for frontal and occipital recording derivations. Third panel shows the average firing rates of each individual recording channel (depicted in different colours), plotted for consecutive 4-s epochs. Fourth panel shows the hypnogram of vigilance states (wake: W, NREM: N and REM: R). Bottom panel (inset) zooms into a 30 minute period, to illustrate the state specific changes in firing rates across time.

As each recording electrode records the activity from all neurons in its vicinity (multiunit activity), spike sorting was next performed to identify putative neurons. On average, out of 16 microwire channels, 9.2 ± 1.0 , 9.3 ± 1.0 and 9.2 ± 1.0 channels showed robust MUA in EA, LA and OA animals, respectively, and subsequent spike sorting resulted in 17.4 ± 2.9 , 15.6 ± 1.7 and 16.7 ± 2.5 putative single units detected in each animal, which was not significantly different between ages.

In order to investigate the vigilance state dependency of neuronal firing in more detail, the distribution of the firing rates of all putative neurons across all vigilance states were plotted as a proportion of the total number of neurons (Figure 3-9A). This revealed that there was a higher

proportion of slow spiking neurons in older mice (Figure 3-9A). However, this may be related to the higher amount and presumably intensity of waking in EA animals, as compared to LA and OA mice (Figure 3-5C). Not only is activity variable between different neurons, but also within individual neurons, so next I performed neuronal ‘phenotyping’ for each putative neuron. Histograms of the distribution of firing rates across all 4-second epochs for each putative neuron were calculated separately for NREM sleep, REM sleep and waking. Example histograms are shown in Figure 3-9B. The peak frequency for each putative single unit was then determined using these histograms (Figure 3-9B) and then plotted separately for each vigilance state (Figure 3-9C). Interestingly, this data indicates that neurons do not have specific firing characteristics according to their vigilance state. Instead a full repertoire of firing rates was observed in all vigilance states, which was similar across the three age groups. These data highlight that the distribution of neuronal firing often deviates from normality, and may instead have a lognormal distribution (Watson et al., 2016). Therefore, calculations of the average firing rates during specific vigilance states may not give a true reflection of neural activity.

On average 13.7 ± 3.3 , 15.9 ± 6.6 and $14.5 \pm 4.5\%$ of all putative single units, in EA, LA and OA mice, respectively, discharged at a higher average firing rate during sleep (including both NREM and REM sleep) as compared to during waking, and the proportion of such ‘sleep-active’ cortical neurons was similar between ages. However, plotting the distribution of individual neurons as a function of their spiking activity revealed that during wake faster spiking neurons were more common in EA mice, while no significant differences were identified for NREM sleep or REM sleep (Figure 3-9D). Finally, no statistical differences were observed in the distribution widths of the firing rates between age groups, for any vigilance state (Figure 3-9E). Therefore, neurons largely retain their vigilance state-dependent firing profile across the lifespan.

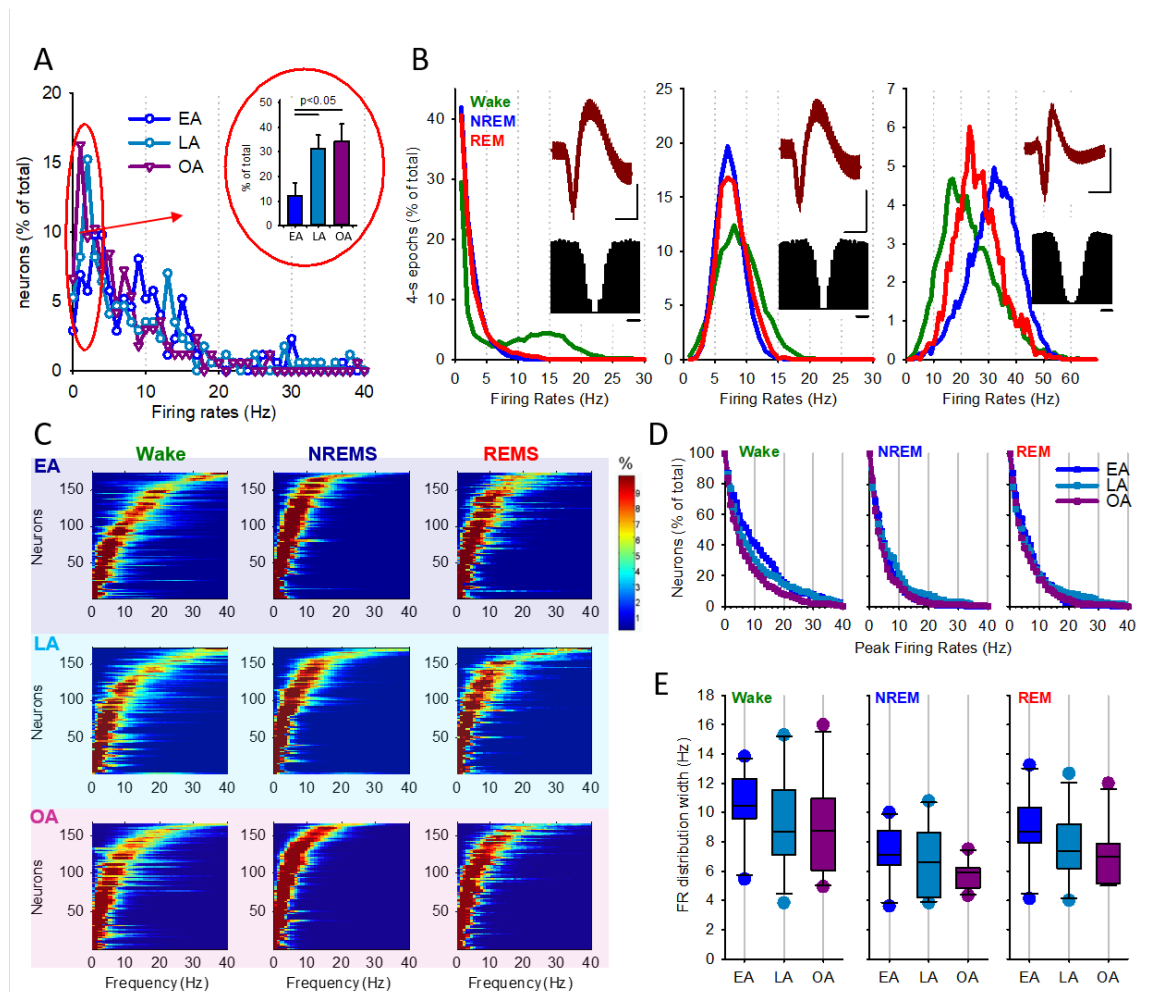


Figure 3-9: Ageing and the vigilance state dependence of cortical neuronal firing. **(A)** The distribution of firing rates of all putative neurons across all vigilance states, plotted as a proportion of the total number of neurons. Inset shows the proportion of neurons that fire at slow firing rates (0-3 Hz), which was significantly different between age groups (one-way ANOVA (Tukey post hoc)). **(B)** The distribution of firing rates across 4-s epochs is expressed as a percentage of the total number of epochs, for three representative individual putative single units. Wake shown in green, NREM sleep in blue and REM sleep in red. Subplots show the corresponding average spike waveform ($\pm$ std. dev) and autocorrelogram. **(C)** The peak firing rates for all putative neurons was determined from the distribution histograms (representative examples shown in panel B), sorted by their peak firing rate, and plotted in ascending order for each vigilance state separately. **(D)** The proportion of neurons discharging at a specific frequency is shown separately for Wake, NREM and REM sleep. **(E)** Mean firing rate distribution widths for Wake, NREM and REM sleep are shown for the three age groups. Mean values, SEM. EA n=10; LA n=11; OA n=10. No statistical differences between age groups were identified (One-way ANOVA).

3.3.3.2 Relationship between LFP slow waves and cortical MUA

As previously discussed, there is a well-established association between slow waves at the level of the EEG or LFP and network silence at the neuronal level (known as OFF periods when referring to synchronous network silence). In this study, the association between LFP slow waves and neuronal silence was surprisingly indistinguishable between age groups, as can be seen in

the example traces shown in Figure 3-10A. Plotting the neuronal activity underlying the average LFP slow waves revealed that there was a clear-cut suppression of neuronal firing rates associated with the positive LFP wave in all three ages (Figure 3-10B).

Next, all OFF periods (periods of neuronal silence) were detected according to previous methodologies (see section 2.5 for details). These were aligned to their onset and the resultant LFP slow waves associated with these silence periods averaged. Interestingly, the average amplitude of the slow-wave triggered by OFF-periods was reduced in both LA and OA mice as compared to EA mice (ANOVA: $F(2,28)=4.9$, $p=0.015$, Figure 3-10C), despite similar average OFF period durations which were 133.9ms, 141.9ms and 140.0ms for EA, LA and OA mice, respectively (n.s.). This suggests that the silence of individual cortical neurons may, to some extent, become uncoupled from the slow network LFP oscillation with ageing.

To investigate this possibility further, the average incidence of LFP slow waves and population OFF periods was calculated for an undisturbed baseline 12-h light period. The incidence of slow waves and OFF periods increased significantly with age (slow wave incidence: Welch F test: $F(2,18)=24.9$, $p<0.0001$, OFF period incidence: one-way ANOVA: $F(2,28)=3.9$, $p=0.031$, Figure 3-10D).

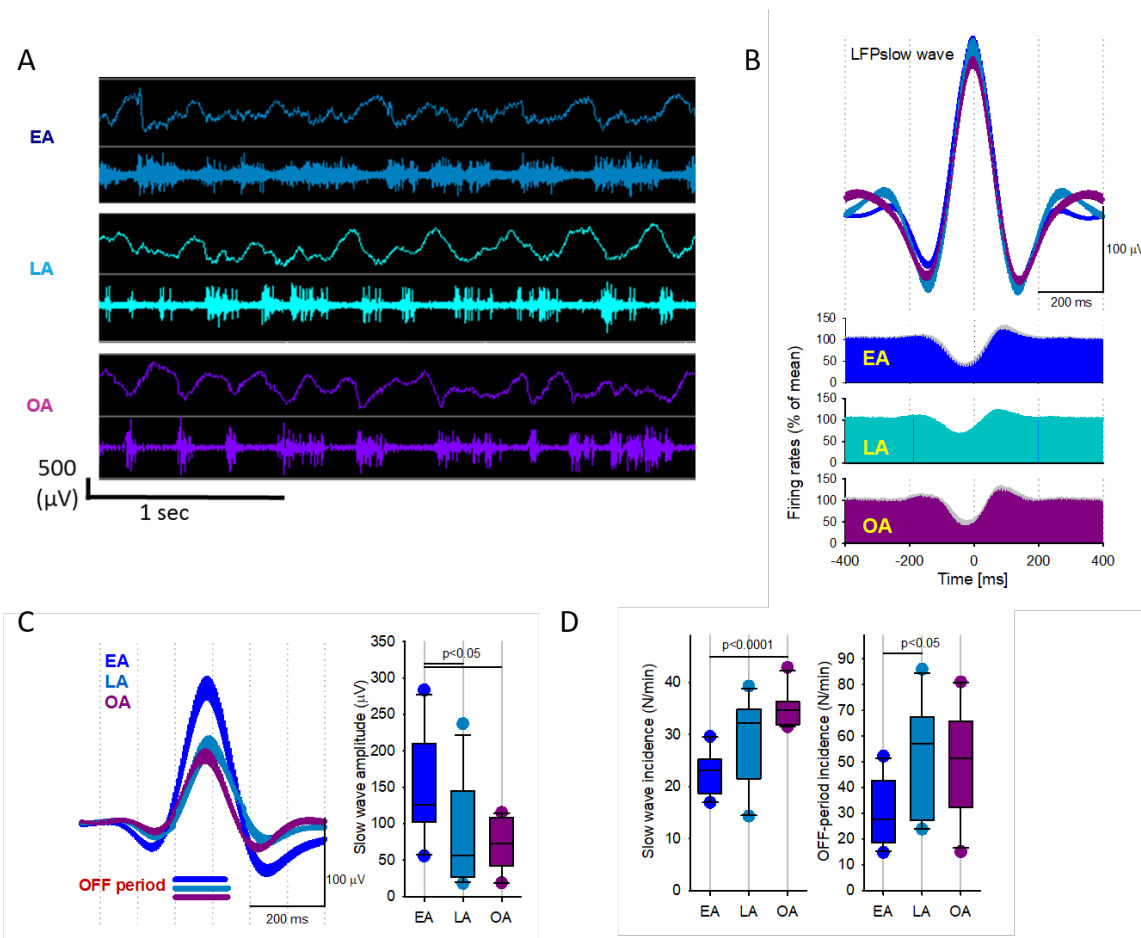


Figure 3-10: The relationship between LFP slow waves and cortical MUA in young and older mice (A) LFP and MUA traces from representative animals from each age group. (B) Average LFP slow wave (top) and corresponding slow wave – triggered average MUA (plots below) in the three age groups. (C) Average LFP slow wave triggered by the onset of generalised neuronal silence (an OFF period) across all recorded neurons (left panel). This is quantified in the right panel. A one-way ANOVA (Tukey post-hoc) was used to compare age groups. (D) The effect of ageing on the incidence of slow waves and OFF periods during baseline NREM sleep. For slow wave incidence a Welch F test (Games-Howell post hoc) was used to compare age groups. For OFF period incidence a one-way ANOVA (Tukey post-hoc) was used to compare age groups. For all panels, data is mean, SEM. EA: $n=10$; LA: $n=11$; OA: $n=10$.

3.3.3.3 Influence of running wheel behaviour on neuronal activity

As previously mentioned, the general amount of running activity, as well as running speed, decreases with ageing (Figure 3-5). Our group has previously shown that large subpopulations of cortical neurons show a reduction in firing rates during stereotypic wheel running, especially at high speeds (Fisher et al., 2016). In this study running was also associated with a reduction in firing rates, which was particularly apparent at higher running speeds (Figure 3-11). Figure 3-11A shows a representative example of the activity recorded from an individual neuron in an EA mouse. In this example, the neuron produces fewer spikes as running speed increases. Average

data confirms that this reduction in firing rates is true for all three age groups, especially at higher running speeds (Figure 3-11B).

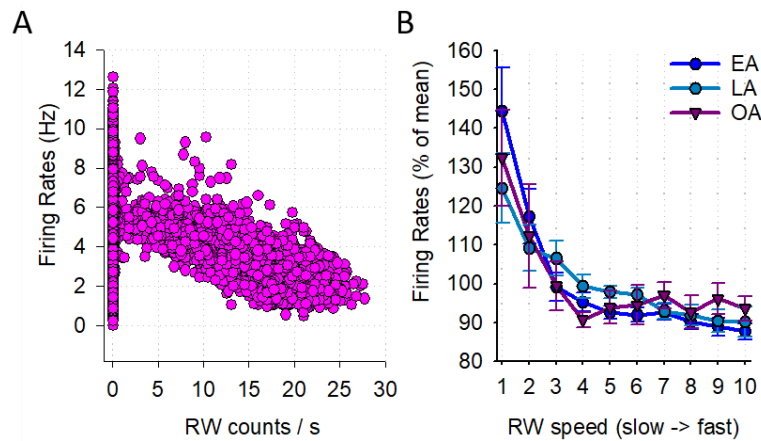


Figure 3-11: Association between running activity and neuronal firing rates. (A) The relationship between wheel running speed and the firing rates of one individual representative cortical neuron in one EA animal. **(B)** The effect of running speed on the average neuronal spiking activity. All 4-s epochs were subdivided into 10% groups as a function of running speed and corresponding firing rates were calculated for each neuron and averaged within each animal prior to calculating the mean between animals within each age group. Note that irrespective of age, firing rates decrease during running, especially at high speeds.

3.3.4 Effect of preceding sleep-wake history

The effects of preceding sleep-wake history can be studied at various temporal scales. In this thesis I have subdivided this into two components: a small-scale component consisting of the transitions between vigilance states; and a large-scale component in which the effect of sleep deprivation is considered.

3.3.4.1 Neuronal dynamics at vigilance state transitions (small-scale)

Firstly, the dynamics of neuronal firing in the first 2 minutes after the onset of NREM sleep were considered (Figure 3-12A). The relative incidence of slow waves was found to increase across the first 2 minutes of a NREM sleep episode, which was larger and more rapid in EA mice as compared to LA and OA mice (Figure 3-12B). Calculation of the incidence of slow wave during the 2nd minute of NREM sleep as a percentage of the corresponding value during the first 12-seconds after the onset of a NREM sleep episode confirmed that OA mice had a reduced relative

increase in the incidence of slow waves as sleep progressed into the second minute (Kruskal-Wallis H test $\chi^2(2) = 9.979$, $p=0.007$, with a mean rank age score of 23.22 for EA, 12.73 for LA and 11.6 for OA mice, Figure 3-12E). Interestingly, although the incidence of OFF periods also gradually increased during the first 2-minutes of NREM sleep episodes, no significant differences were identified between the age groups (Figure 3-12C).

In order to investigate the dynamics of neuronal firing rates across the initial 2 minutes after the onset of NREM sleep, the activity of individual putative single units across all animals was plotted and sorted as a function of their relative firing rates attained during the second minute after the episode onset. Similar to the findings presented in Figure 3-9C, neurons once again showed an entire spectrum of possible changes: some increasing, others decreasing and some not changing their spiking activity (Figure 3-12D). Visual inspection of these dynamics revealed that OA mice had a larger proportion of neurons that increased their spiking activity in the initial 2 minutes of NREM sleep episodes (darker red apparent in the spectrogram, Figure 3-12D), which was confirmed by quantification of the distribution of all putative neurons as a function of the change in their firing frequency within NREM sleep episodes (Figure 3-12F). Finally, I calculated the proportion of neurons that showed at least a 30% increase in their rate of discharge during the second minute of NREM sleep episodes relative to the first 12-s after the initiation of corresponding NREM sleep episodes. This revealed that OA mice had a more intense relative neuronal spiking in the middle of NREM sleep episodes (mean rank age scores 12.9 for EA, 13.86 for LA and 21.45 for OA mice, Kruskal-Wallis H test $\chi^2(2) = 6.4$, $p=0.04$, Figure 3-12G). Together, the reduced relative increase in the incidence of slow waves and the increased proportion of neurons that increased or sustained firing during the initial 2 minutes of NREM sleep, suggests that the progression from superficial to deeper sleep intensities may be attenuated in older mice.

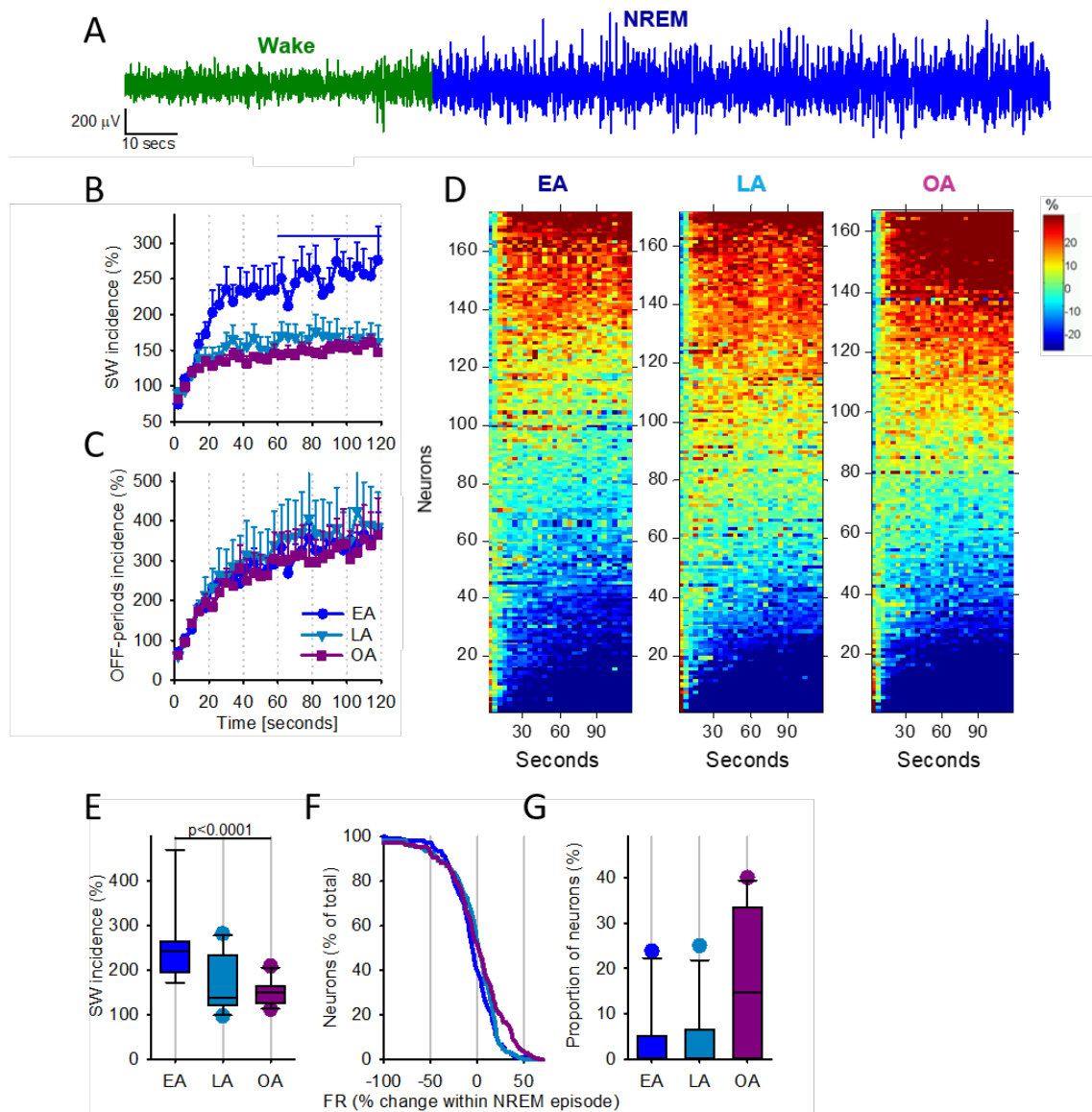


Figure 3-12: Intraepisodic dynamics of cortical firing at the onset of NREM sleep. (A) A representative example of a cortical LFP recording at the transition from waking to NREM sleep. (B) The time course of relative LFP slow wave incidence during the first 2 minutes after the onset of NREM sleep episodes. The values are represented as % of the first 12-sec. Mean values, SEM. (C) The same for the incidence of OFF periods. (D) The dynamics of firing rates during the first 2 min after the onset of a NREM sleep episode is shown for all individual putative single units across all animals. Neurons are sorted as a function of their relative firing rates attained during the 2nd minute after the episode onset. (E) Mean values of slow wave incidence during the 2nd minute of NREM sleep episode shown as percentage of the corresponding value during the first 12-sec after the onset of NREM sleep episode. Mean values SEM. EA $n=9$, LA $n=11$, OA $n=10$. A non-parametric Kruskal-Wallis test with Mann-Whitney post hoc test (exact, two tailed) was used to test for significant differences between age groups. Note: post hoc tests for EA VS OA and LA vs OA gave p values of 0.033 and 0.037, respectively, this was not significant after correcting for multiple testing (critical value $p=0.0167$). (F) Distribution of all putative neurons as a function of the change in their firing frequency within NREM sleep episodes. (G) The proportion of neurons, that show at least a 30% increase in their rate of discharge during the second minute after NREM sleep onset relative to the first 12-s. Mean values, SEM. A non-parametric Kruskal-Wallis test with Mann-Whitney post hoc test (exact, two tailed) was used to test for significant differences between age groups. EA vs OA: $U=4$, $z=-3.348$, $p<0.0001$. Note: post hoc testing for EA VS LA gave a p value of 0.031, however this was not significant after correcting for multiple testing (critical value $p=0.0167$).

Transitions from NREM sleep to REM sleep were next investigated (Figure 3-13A). On average, 51.9 ± 3.7 , 45.8 ± 2.0 and 45.3 ± 2.5 NREM-REM sleep transitions contributed to the analysis below for EA, LA and OA animals respectively. The distribution of firing rates for example individual putative single units during NREM sleep (blue) and REM sleep (red) are shown in Figure 3-13B. Together with spectrograms of the dynamics of firing rates during the last minute of NREM sleep and first minute of REM sleep for all individual putative neurons (Figure 3-13C), these figures highlight that neurons show a great diversity in their state dependent firing during sleep. Calculation of the proportion of putative single neurons that discharged, on average, at a higher rate during REM sleep as a percentage of NREM sleep revealed that the number of 'REM-sleep active' cortical neurons was not significantly different between the three age groups (Figure 3-13D). Interestingly, some neurons exhibited similar patterns of activity during both NREM and REM sleep.

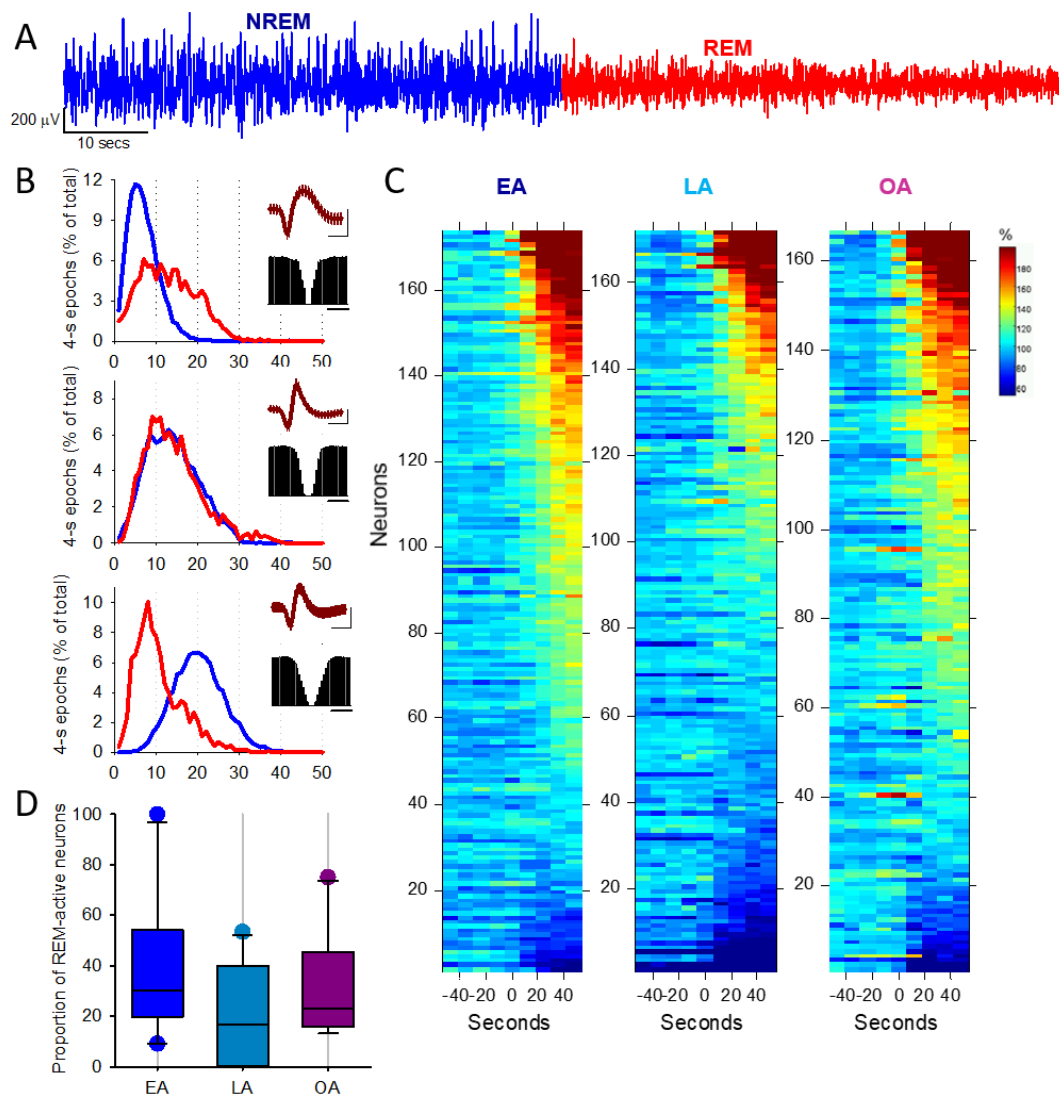


Figure 3-13: The effects of aging on the neuronal dynamics at the transition from NREM to REM sleep. (A) Representative example of a cortical LFP recording at the transition from NREM sleep to REM sleep. **(B)** Distribution of firing rates across 4-s epochs in NREM sleep (blue) and REM sleep (red) shown for three representative individual putative single units. Each subplot also shows the average spike waveform ($\pm$ std. dev) and autocorrelogram. **(C)** The dynamics of firing rates during the last minute of NREM sleep and the first minute of subsequent REM sleep shown for all individual putative single units across all animals. The neurons are sorted as a function of their relative firing rates attained during REM sleep. 100% represents the average over the last 1 minute of NREM sleep prior to the transition, for each individual neuron. **(D)** The proportion of putative single neurons, discharging on average at a higher rate during REM sleep as percentage of NREM sleep in the three age groups. Mean values, SEM. EA $n=10$, LA $n=11$, OA $n=10$. One-way ANOVA did not identify any significant differences between age groups.

3.3.4.2 Effect of sleep deprivation on local neural activity (large-scale)

It has previously been suggested that the capacity to generate a rebound in SWA after sleep deprivation is diminished in older humans and rodents (Lafortune et al., 2012; Munch et al.,

2004; Wimmer et al., 2013b). However, in this study all age groups generated similar increases in SWA after 6-hours of sleep deprivation, as can be seen in the representative plots for each age group (Figure 3-14A). All three age groups showed similar reductions in SWA across the 6 hour recovery period after sleep deprivation, for both the frontal EEG and the LFP (Figure 3-14B). However, SWA during the first hour recovery sleep after sleep deprivation was significantly attenuated in OA mice for the frontal EEG recording only (EA 218.1 ± 8.5 ; LA 197.8 ± 7.3 ; OA 181.1 ± 8.3 , ANOVA factor 'age' $F(2,18)=5.4$, $p=0.015$, Figure 3-14B). The time course of slow wave and OFF period incidence, as well as the duration of OFF periods was quantified over the 12 hour baseline light period as well as for the 6 hour recovery sleep period after sleep deprivation. All measures decreased significantly across the baseline light period (repeated measures ANOVAs: slow wave incidence factor 'time interval' $F(5,96)=19.6$, $p<0.0001$, Figure 3-14C; OFF period incidence factor 'time interval' $F(4,74)=12$, $p<0.0001$, Figure 3-14D; OFF period duration factor 'time interval' $F(6,116)=13.6$, $p<0.0001$, Figure 3-14E), however only the time course of OFF period incidence was significantly different between age groups (repeated measures ANOVA: factor 'age' $F(2,21)=7$, $p=0.005$, EA vs LA $p=0.009$, LA vs OA $p=0.01$). All measures increased significantly after sleep deprivation, as compared to the same 6 hours during the baseline recording day (repeated measures ANOVA factor 'day': slow wave incidence $F(1,42)=108.6$, $p<0.0001$, Figure 3-14C; OFF period incidence $F(1,42)=29.9$, $p<0.0001$, Figure 3-14D; OFF period duration: $F(1,42)=84.5$, $p<0.0001$, Figure 3-14E). This increase after SD was not significantly different between age groups (ANOVA, factor 'age': slow wave incidence: $F(2,42)=0.185$, $p=0.832$, Figure 3-14C; OFF periods incidence: $F(2,42)=0.99$, $p=0.38$, Figure 3-14D; OFF periods duration: $F(2,42)=2.776$, $p=0.074$, Figure 3-14E). Once again, all three age groups showed a gradual decrease in slow wave incidence (factor 'time interval': $F(3,53)=61.3$, $p<0.0001$; factor 'time interval'*'age': $F(5,53)=1.6$, $p=0.179$, Figure 3-14C), OFF period incidence (factor 'time interval': $F(2,35)=50.0$, $p<0.0001$; factor 'time interval'*'age': $F(3,35)=1.7$, $p=0.142$, Figure 3-14D) and OFF period duration (factor 'time interval': $F(3,56)=54.1$, $p<0.0001$; factor

‘time interval’*‘age’: $F(5,56)=0.654$, $p=0.67$, Figure 3-14E) over the 6 hours recovery after SD.

Therefore, in this study the response of local cortical network activity to sleep deprivation was not markedly affected by ageing, except in the initial level of EEG SWA in the frontal derivation.

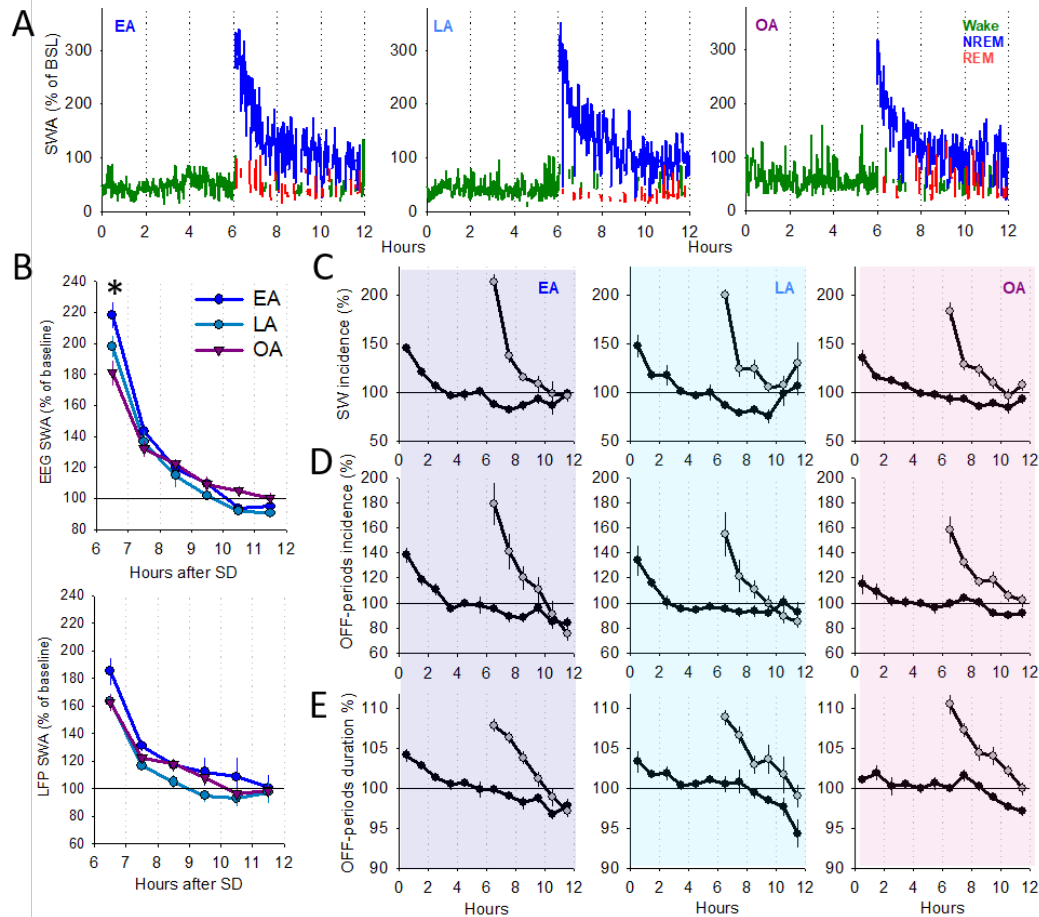


Figure 3-14: Effects of sleep deprivation on cortical slow waves and OFF periods in older mice. (A) Representative hypnograms of individual animals from each age group (EA=early adulthood, LA=late adulthood, OA=older age). 12-h profile of EEG slow-wave activity (SWA, EEG power between 0.5-4.0 Hz, represented as % of baseline 24-h mean) recorded in the frontal cortex (wake = green, NREM sleep = blue, REM sleep = red). Sleep deprivation (SD) was performed for 6 hours from light onset. (B) Time course of EEG (top) and LFP (bottom) slow-wave activity (SWA, 0.5-4 Hz) for the 6 hours period after 6-h SD. Mean values, SEM (EA $n=7$; LA $n=5-6$; OA $n=9$). Asterisk indicates a significant difference between EA and OA mice in the first hour after SD, $p=0.01$ (One-way ANOVA with Tukey post-hoc test). (C) The effect of SD on the incidence of LFP slow waves for a 12-h baseline period and the 6-h period following SD. Values are shown in 1-h intervals. Mean values, SEM. EA $n=7$; LA $n=7$; OA $n=10$. Repeated measure ANOVAs were used to identify age differences during baseline and recovery after SD. One-way ANOVAs used to identify age-differences in the initial rebound after sleep deprivation (see text). (D,E) The same analyses were performed as in (C) but for OFF period incidence and duration.

In order to investigate the relationship between the amount of NREM sleep during the baseline 12-hour dark period prior to sleep deprivation and the recovery sleep after sleep deprivation pearson correlation analyses were performed. The incidence of both slow waves and OFF

periods were significantly negatively correlated with the amount of NREM sleep during the 12-hour baseline dark period before sleep deprivation ($r = -0.51$, $p < 0.05$ for both, Figure 3-15B and C). However, the amount of NREM sleep during the 12 hour baseline dark period before sleep deprivation did not correlate with the amount of NREM sleep after sleep deprivation (Figure 3-15A). This suggests that sleep-wake history prior to SD has an influence on the measures of local cortical dynamics during recovery sleep across ages.

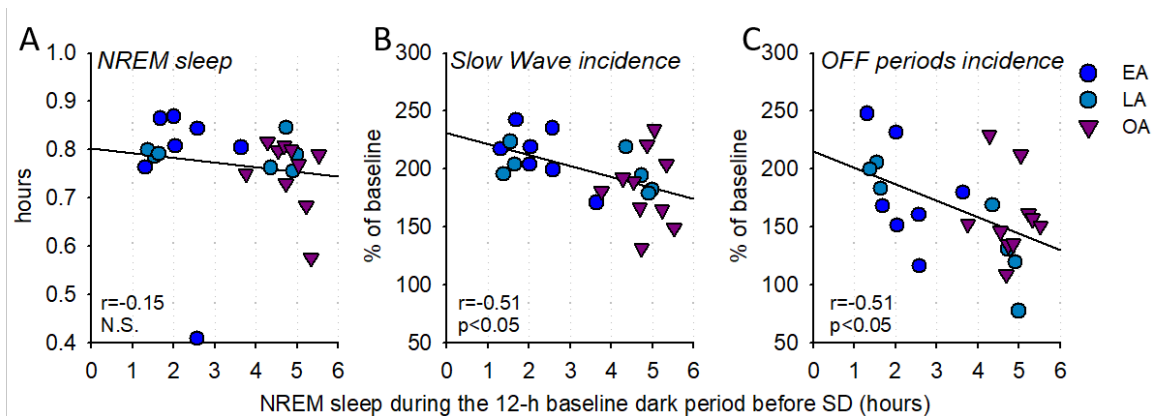


Figure 3-15: The relationship between the amount of NREM sleep during the baseline 12-h dark period before sleep deprivation (SD) and the initial amount of NREM sleep (panel A), slow wave incidence (panel B) and OFF periods incidence (panel C) during the first 1-h interval after SD. The effect of SD on the incidence of LFP slow waves and OFF periods is represented as percentage from corresponding mean baseline values. Filled symbols correspond to individual animals. The straight line depicts linear regression across all three age groups. Pearson's correlation values reported.

To determine whether there is a faster build-up of SWA within NREM sleep episodes, which may reflect a higher sleep pressure, next the incidence of LFP slow waves and OFF periods were quantified during the first 2 minutes after the onset of NREM sleep, both during baseline and during the recovery sleep after sleep deprivation (Figure 3-16). EA mice showed an increase in both parameters after sleep deprivation, however this was attenuated or even absent in LA and OA mice (percentage change relative to baseline; slow wave incidence: EA $+20.3 \pm 5.5\%$; LA $+2.0 \pm 5.5\%$; OA $-3.9 \pm 4.7\%$, one-way ANOVA factor 'age' $F(2,21) = 5.8$, $p = 0.01$; OFF period incidence: EA $+50.1 \pm 14.1\%$; LA $-7.6 \pm 7.6\%$; OA $-2.3 \pm 18.3\%$; Kruskal-Wallis test $\chi^2(2) = 8.214$, $p = 0.014$, with a mean rank score of 18.86 for EA, 10.86 for LA and 9.2 for OA mice, Figure 3-16).

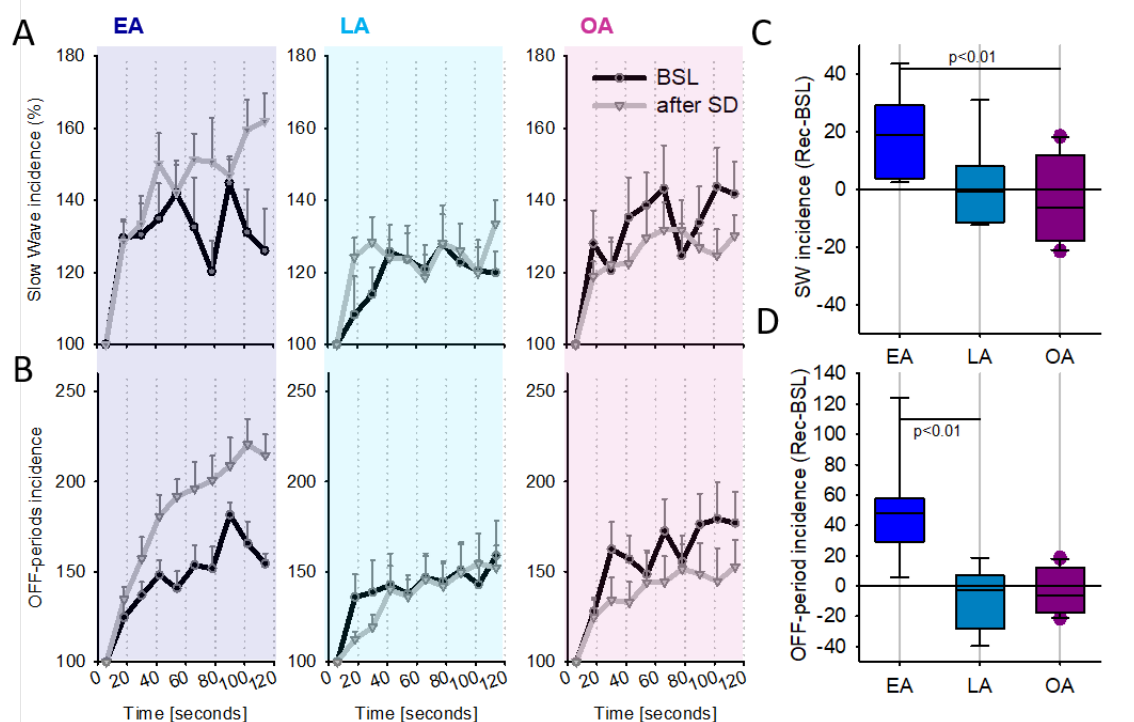


Figure 3-16: Intraepisodic dynamics of slow waves and OFF periods during NREM sleep episodes after sleep deprivation. (A) The time course of slow wave incidence during the first 2-min after the onset of individual NREM sleep episodes. The values are expressed as a percentage of the first 12-sec after NREM sleep onset. Mean values correspond to all episodes during the first 2 hours after sleep deprivation and the corresponding baseline interval (SEM). EA n=7, LA n=7, OA n=10. (B) Same as (A) but for the incidence of population OFF periods. (C) The difference between the incidence of slow waves after SD and during the baseline prior to SD, during the second minute after NREM sleep onset. Mean values, SEM. EA n=7; LA n=7; OA n=10. ANOVA with Tukey post hoc test used to test for significance between age groups. (D) The same analyses as in (C) but for the incidence of OFF periods. A non-parametric Kruskal-Wallis test with Mann-Whitney post hoc test was used to test for significant differences between age groups. EA Vs LA: $U = 2$, $z = -2.88$, $p = 0.002$. Note: although EA vs OA post hoc testing gave a p value of 0.033 this was no longer significant after correcting for multiple testing (critical value $p = 0.0167$).

3.4 Discussion

This project had two main aims; to investigate the neural activity underpinning the known global changes that occur with ageing; and to determine the link between preceding sleep wake history and these cortical mechanisms. As this was the first study to record neuronal activity in freely moving mice to address the link between sleep and ageing, initial analysis focussed on the general characterisation of cortical EEG, LFP and neuronal activity across the different age groups of mice. Consistent with previous reports (Colas et al., 2005; Hasan et al., 2012; Panagiotou et al., 2017; Welsh et al., 1986; Wimmer et al., 2013b), in this study ageing resulted

in an overall increase in the total daily amount of sleep and fragmentation of the sleep-wake cycle. While there was also an increase in SWA with ageing, in support of recent evidence (Panagiotou et al., 2017). However, it should be noted that the increase in SWA contrasts with human studies (Agnew Jr. et al., 1967; Dijk et al., 1989; Feinberg et al., 1984), as well as other studies in mice (Banks et al., 2015; Colas et al., 2005; Hasan et al., 2012; Wimmer et al., 2013b) that instead showed a decrease in SWA with ageing.

The most novel aspect of this project, was the characterisation of the neural activity underpinning the effects of ageing of sleep, which currently has not been investigated in freely moving mice. Before initiating these experiments I hypothesised that ageing may disrupt local cortical population activity, leading to reduced neuronal spiking, a decreased incidence of network OFF periods during sleep and deficits in the homeostatic response to sleep deprivation. This hypothesis was based on the notion that sleep is initiated at the level of local cortical networks (Hinard et al., 2012; Krueger et al., 2008; Lemieux et al., 2014; Pigarev et al., 1997; Sanchez-Vives and Mattia, 2014), with slow waves during NREM sleep being the result of the summation of localised neuronal population activity under the influence of synaptic modifications or changes in neuronal connectivity arising from preceding sleep-wake history (Krueger and Tononi, 2011; Vyazovskiy and Harris, 2013). Therefore, by studying properties of the slow oscillation, such as the incidence of slow waves and their underpinning firing (OFF periods), these local regulatory mechanisms can be investigated. Surprisingly, the majority of neuronal measures quantified in this study were resilient to the effects of ageing, including the generation of consolidated OFF periods and local slow waves, the general vigilance-state specific firing profiles of individual neurons, and the homeostatic response to sleep deprivation.

In this study LFP slow waves were associated with a suppression of MUA in all three age groups, which when quantified only had modest age-dependent differences. Therefore this study

suggests that the basic properties of neurons are mostly intact with ageing. In this study the most notable effects of ageing at the level of neuronal activity was an increase in the incidence of LFP slow waves and OFF periods, as well as there being a higher proportion of neurons that increase their firing rates after the onset of sleep. Previous evidence in humans showed that SWA and properties of slow waves, gradually reduce with ageing (Dijk and Beersma, 1989; Landolt and Borbely, 2001; Mander et al., 2013). However, in contrast to these studies, in this study, and one other investigating EEG (Panagiotou et al., 2017), SWA was found to instead increase with ageing. The increased SWA, as well as evidence for leakage of slow-frequency oscillations into wakefulness (Leemburg et al., 2010; Panagiotou et al., 2017), have led to suggestions that the older mice may have an increased sleep pressure. This conflicts with previous suggestions that ageing may reduce the homeostatic sleep need (Mander et al., 2017; Wimmer et al., 2013b). Interestingly, the dynamics of SWA during the recovery period after SD have also been shown to have a slower decay rate in older mice (Panagiotou et al., 2017). Therefore, although older mice seem to have an increased sleep pressure, they are less able to dissipate this sleep pressure (Panagiotou et al., 2017).

In order to investigate the possible effects of ageing on the homeostatic regulation of sleep, recovery sleep after sleep deprivation was next investigated. Higher sleep propensity may be identified as a faster build-up of SWA within NREM sleep episodes. In this study the robust increase in the incidence of slow waves and OFF periods in the first 2 minutes of NREM sleep after sleep deprivation (compared to baseline values), was greatly attenuated in LA and OA mice. One interpretation is that older mice are less able to progress into deeper stages of NREM sleep, which is consistent with the notion that ageing may primarily diminish the capacity to generate and sustain NREM sleep (Cirelli, 2012a; Klerman and Dijk, 2008; Mander et al., 2017). It is possible that this may be due to a ceiling effect taking place, whereby the higher absolute number of slow waves and OFF periods during baseline recordings (Figure 3-10) may limit

further increases after sleep deprivation. Alternatively, this reduced capacity, may be due to increased neuronal activity or excitability during NREM sleep in older animals (Klerman and Dijk, 2008). It is also possible that the baseline differences in the amount of NREM sleep between age groups may influence the effects of sleep deprivation. One could argue that as the older animals slept more during the dark period before sleep deprivation, they were relatively less sleep deprived at the beginning of sleep deprivation. If they had a similar amount of wake and sleep, then one could, at least in theory, expect that they may have an even larger homeostatic response. This is supported by correlations that showed that the amount of sleep during the first 1-h interval after SD was only weakly associated with the amount of sleep before SD (Figure 3-15), whereas the magnitude of the increase in the incidence of LFP slow waves and OFF periods after SD showed a significant negative relationship with the amount of NREM sleep during the preceding dark period. This is consistent with the notion that sleep loss is mostly compensated for by increased sleep intensity, rather than sleep amount, and confirms that these measures are sensitive to sleep-wake history and reflect sleep homeostasis. Furthermore, this finding indicates that the excess sleep present in older animals is, in fact, restorative, as it appears to contribute to the dissipation of sleep pressure. This is supported by evidence that sleep-wake history even before sleep deprivation has a substantial influence on subsequent response to SD (Vyazovskiy et al., 2007a).

The next aim of this study was to determine whether ageing may be reflected at either or both the single cell level or larger scale processes such as global neuromodulation. Previous studies have shown that the activity of individual neurons changes according to global behavioural state (Fisher et al., 2016; Hobson and McCarley, 1971; Niethard et al., 2016; Vyazovskiy et al., 2009b), typically observed as a decrease in firing rates during NREM sleep (Vyazovskiy et al., 2009b). Importantly, despite this, great variation has been observed across individual neurons, which has also been shown to be influenced by the cortical region recorded from as well as the recording

technique itself (Hobson and McCarley, 1971; Niethard et al., 2016). This has led to suggestions that the electrophysiological characteristics, connectivity pattern, ongoing behaviour or preceding sleep-wake history may all together determine the firing phenotype of specific cortical neurons (Ascoli et al., 2008; Fisher et al., 2016; Kropff et al., 2015; McGinley et al., 2015; O'Keefe and Dostrovsky, 1971; Poulet and Petersen, 2008). In this study, an entire spectrum of changes was identified during each of the vigilance states. Therefore, neurons could not be subdivided into distinct categories based only on their firing characteristics. Importantly, despite large variations in activity across the neuronal populations, the firing profiles of individual putative neurons during waking and sleep was largely stable across the life span. OA mice were found to have a higher proportion of 'sleep active' neurons (i.e. neurons that discharged at a higher rate during NREM sleep and REM sleep compared to waking), however, this effect may be explained by the higher amount of waking and therefore arousal in EA mice, compared to older age groups. This is confirmed by comparisons of the firing rate distributions, which were not significantly different between age-groups. Therefore, the vigilance state-dependent firing profiles of individual neurons may be resistant to the effects of ageing. As neuronal activity is stable, despite drastic changes in the global sleep-wake distribution, this suggests that a different mechanism may be regulating the control of localised cortical activity and states.

As both the amount of NREM sleep and the number of transitions into NREM sleep were increased in older animals in this study, it is possible that markers of ageing may be observed at the transition between states. Previous studies have shown that neuronal firing rates often reduce at the transition to NREM sleep (possibly due to increased neuronal silence during slow waves) (Vyazovskiy et al., 2009b), while there is a corresponding increase in SWA and slow wave amplitude in the initial 1-2 minutes of NREM sleep episodes (Cui et al., 2014; Vyazovskiy et al., 2009a). In this study, although all three age groups had an increased incidence of slow waves during the first 2 minutes of NREM sleep, this was attenuated and slower in older mice. This, in

combination with evidence that older mice had increased relative firing in the middle or NREM sleep episodes, suggests that ageing may reduce the capacity to progress from more superficial NREM sleep into deeper stages. This would therefore result in sleep which is more superficial and easier to disrupt. These effects are well established to occur with ageing, apparent as an increased fragmentation of the sleep-wake cycle. Though it should be noted that, in mice, this is often more predominantly the result of wake becoming more fragmented rather than sleep. It is also possible that ageing leads to a disruption in this excitation/inhibition balance in firing rates, which must be maintained stable in order to avoid increased energy requirements and neurotoxicity (Haider et al., 2006; Laughlin et al., 1998). This may lead to increased firing rates and with it a faster build-up of sleep pressure in older age, ultimately leading to a more fragmented wake as a result of the inability to sustain longer periods of wakefulness (Colas et al., 2005; Hasan et al., 2012; Welsh et al., 1986; Wimmer et al., 2013b). These effects may also reflect an instability of local states (Doran et al., 2001; Parrino et al., 2012). Both of these suggest a disruption in sleep intensity, which may interfere with the restorative functions of sleep. Alternatively, this may be the result of a ceiling effect taking place whereby the higher initial incidence of slow waves and OFF periods may limit possible further increases across the NREM sleep episode. However, as the number of OFF periods was also initially higher, yet no age-differences were identified in the incidence of OFF periods across the initial 2 minutes of NREM sleep episodes, this suggests the capacity to increase was still possible.

In this study individual putative neurons were highly variable at the transitions between NREM-REM sleep, and there were no differences identified between age groups. Therefore once again, ageing does not seem to greatly influence the dynamics of cortical neuronal activity.

Noteworthy, is that a large proportion of neurons did not change their activity between NREM and REM sleep, consistent with previous suggestions that these two sleep states share important features at the local cortical level (Funk et al., 2016).

Finally, healthy ageing is thought to involve a number of structural changes, such as a loss of synaptic connections, and a reduction in the stability of synaptic connection, while alterations in synaptic transmission have also been observed (Dumitriu et al., 2010; Grillo et al., 2013; Morrison and Baxter, 2012; Peters et al., 2008; Petralia et al., 2014). It is also possible that the proposed role for slow waves in the homeostatic rebalancing or remodelling of synaptic networks (Chauvette et al., 2012; Krueger et al., 2013; Tononi and Cirelli, 2014; Vyazovskiy and Harris, 2013; Watson et al., 2016), may be affected by ageing and be observed as an increase in the incidence of local slow waves in older mice.

3.4.1 Conclusions

This study provides evidence that healthy ageing in mice does not greatly affect vigilance state related local neural activity, despite pronounced global changes in the daily amount and distribution of waking and sleep. This suggests that the global sleep disruptions identified with ageing are unlikely to arise from changes in local cortical activity. It is possible that the mechanisms underlying the previously observed global changes in sleep are distinct from those responsible for local sleep regulation. One hypothesis is that local neural activity may be maintained over ageing, due to compensatory or protective mechanisms. This has major implications for future studies, as the data presented here suggest that in order to fully understand the effects of ageing, different levels of organisation (i.e. local and global activity) must be considered together.

Chapter 4: The effect of diazepam on cortical activity

4.1 Introduction

Hypnotic drugs such as benzodiazepines are commonly used to improve sleep in people of all ages. However, in many elderly individuals these treatments lack efficacy or individuals can have an increased sensitivity or a slow elimination of the drugs leading to unwanted effects such as prolonged drowsiness (Borbély et al., 1983; Greenblatt et al., 1983; Nicholson et al., 1982). In particular diazepam has been shown to have impaired clearance in elderly populations due to its oxidative transformation (Nicholson et al., 1982). It is therefore crucial to further understand the physiological mechanisms underlying the efficacy of these drugs, which are not currently well understood. While the molecular mechanisms underlying the efficacy of benzodiazepines have been elucidated in great detail (see section 1.3, for details), the effects of benzodiazepines on neural activity are currently unclear. Therefore, in this study I have focussed on characterising the effects of a commonly used benzodiazepine, diazepam, on LFPs and neural activity.

In an earlier chapter I reported that although there are disruptions in the overall sleep architecture and characteristics of the EEG with ageing, local neural mechanisms during sleep are mostly unaffected by ageing (Chapter 3:). Therefore it is possible, that as with ageing, global and local cortical mechanisms may be differentially affected by benzodiazepines. Furthermore, the mechanisms underlying the efficacy of diazepam may vary across ageing, accounting for differences in the efficacy of diazepam.

4.1.1 Experimental aims

The aim of this chapter was to address objective 2 of the thesis: ***‘To investigate the effects of a commonly used hypnotic, diazepam, on cortical neural activity and to determine whether there are age-dependent differences in the neural responses to diazepam’***. The initial aim of this study was to address whether reduced ‘global’ EEG slow-wave activity after diazepam was associated with changes at the local network level. As the evidence presented in Chapter 3: (McKillop et al., 2018) suggests that the local neural mechanisms underlying the effects of ageing, were distinct from the global mechanisms, it may be hypothesised that two are also distinct in the effect of diazepam.

4.2 Methods

4.2.1 Experimental animals

This study was carried out in male C57Bl/6J mice subdivided into two age groups, early adulthood (EA, 5.23 months old, n=3-4) and late adulthood (LA, 12.83 months, n=3-4). Note that the numbers of animals used in this study were fairly low, and so the results presented in this chapter are considered preliminary. These animals were a sub-group of those used in the main ageing study of this project (Chapter 3:), and therefore had previously undergone surgery to implant EEG screws and microwire arrays (section 2.2). At the time of this study, animals were 26.63 ± 0.78 days post-surgery.

4.2.2 Experimental protocol and specific analyses

This study was a cross-over design with all animals receiving both an injection of diazepam (3mg/kg, Hameln Pharmaceuticals Ltd, UK) and vehicle (saline with 0.3% Tween), with 96 hours between each injection. Diazepam was dissolved in the vehicle and injected at a volume of

10mg/ml. Injections were performed at light onset. Animals were randomised so that both conditions were represented by both age groups per day. Animals were undisturbed throughout the duration of the study and recorded continuously. Electrophysiological data were recorded and processed as previously described in the main methods chapter (section 2.3). The 24-hours prior to each injection was used as a baseline for the injections. The 48 hours after injections was used to assess the effect of, and recovery after, injections. Therefore, overall a baseline day, injection day and recovery day were scored per injection (to determine vigilance states, see section 2.3), and used in further analysis (vehicle injection: Veh BL, Veh and Veh Rec; diazepam injection: dzp BL, dzp, dzp Rec). Details of the protocol are provided in Figure 4-1.

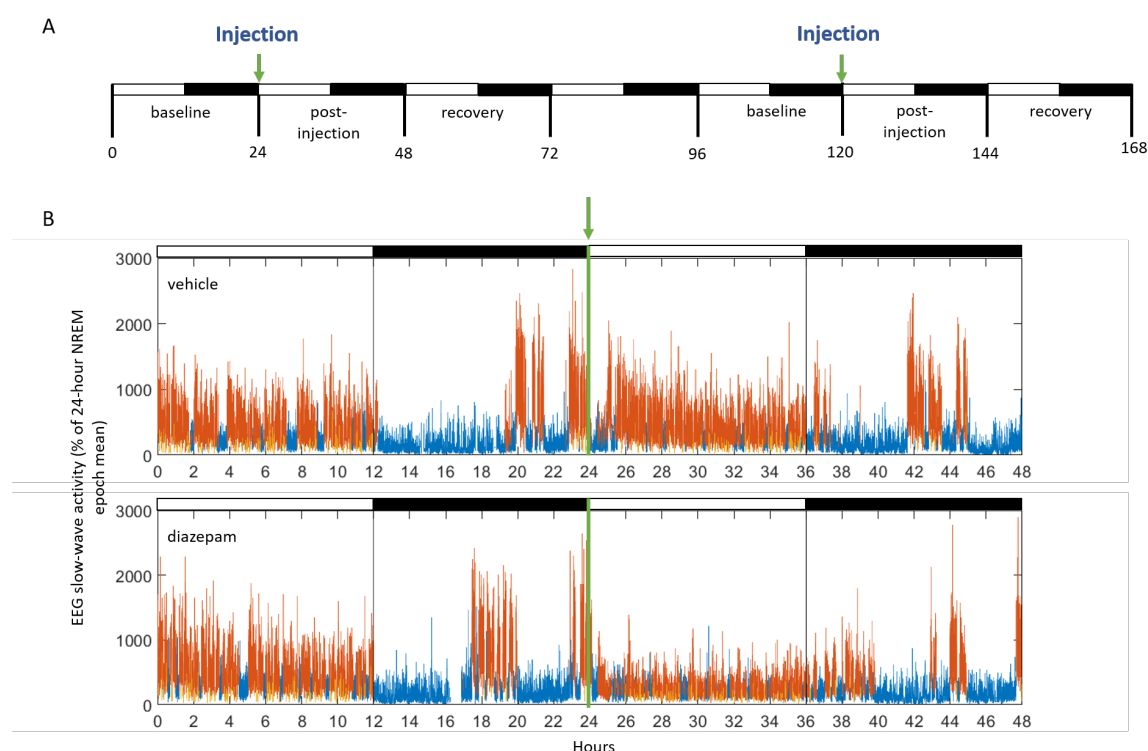


Figure 4-1:(A) A summary of the diazepam study protocol. The 24 hours prior to the injection was used as the baseline for each respective injection. The 24 hours after an injection was used to assess the effect of the injection, while a further 24 hours was also used in some analyses in order to assess the recovery from each injection. Each injection was separated by 96 hours. Each animal received both an injection of diazepam (3mg/kg) or vehicle (Saline with 0.3% Tween 80) dosed IP at a volume of 10mg/ml, with the order of injections randomised. Black and white bars depict the 12-h light and 12-h dark periods. (B) Hypnograms of an individual representative animal showing the profiles of EEG slow-wave activity (SWA, EEG power between 0.5-4.0 Hz, represented as a % of the 24-h mean of NREM sleep epochs only) recorded from the frontal cortex. Data are shown for the 24-hour hours pre- and post-injection, with the timing of each injection indicated by the green line and arrow. Top panel shows the vehicle injection day, bottom panel shows the diazepam injection day. Wake, NREM sleep and REM sleep are represented by blue, red and yellow colour-coding, respectively. The bars at the top of the panel depicts the 12-h light and 12-h dark periods. Note that SWA is greatly reduced after the injection of diazepam, but not vehicle.

In order to assess the effect of diazepam on the fragmentation of the sleep-wake cycle, the number and duration of individual episodes of waking, NREM sleep and REM sleep were quantified. For these analyses, a minimum duration of 16 seconds was required to be classified as an episode, allowing for brief awakenings (or interruptions of state) of 4 seconds. Next, EEG spectral analysis was performed according to our laboratories standard procedure, as described in section 2.3. Due to technical reasons, for the analyses on the frontal derivation only n=3 mice contributed to both EA and LA age groups, and for the occipital derivation n=3 and n=4 mice contributed to EA and LA age groups, respectively. Next, in order to account for variations in spectral power between animals, spectra for injection and recovery days per were normalised to the baseline day of the respective injection (vehicle or diazepam BL 12 hours) for each individual animal before averaging across animals. Finally, to investigate to time course of recovery after injections, data were subdivided into 3 hours intervals for both the injection and recovery day.

The novel aspect of this project was to characterise the cortical neuronal activity underlying the effect of diazepam. As a first step, overall average firing rates were calculated over the entire 12 hour recording period. However, the firing rates of individual neurons vary greatly, as previously discussed in Chapter 3: (McKillop et al., 2018). Therefore, spike sorting was performed to detect putative single units as previously described (section 2.4), however only for the first 2 hours and last two hours of each 12-hour light and dark period of the three recording days (baseline, injection and recovery), separately for vehicle and diazepam conditions. Spike sorting was only performed for the first 2 hours of each light period due to the high computational demand involved with spike sorting, which would have made running spike sorting on three 12 hour recordings difficult and time consuming. In addition, on some occasions, full data sets of neuronal activity were unavailable due to technical issues with the recording system. OFF periods were then detected based on the occurrence of slow waves, as described in Chapter 2:

(section 2.5). For OFF period analyses, one mouse from each age group was excluded for technical reasons (EA n=3, LA n=3). In order to better define the effect of diazepam on firing rates, the average firing rates during the 500ms prior to and after an OFF period were calculated. To allow comparisons between days and conditions, firing rates of each animal were normalised to the first 300ms of the baseline days (both vehicle and diazepam days averaged together to give a common reference for each animal) (Figure 4-11).

4.2.3 Statistical analysis

For analyses investigating the total amount of vigilance states, fragmentation of vigilance states, average firing rates, and the number of OFF periods, two-way ANOVAs were used to investigate the factors 'age', experimental 'day' and interactions between both. Where significant age effects were identified, either Tukey post hoc tests or unpaired t-tests were used to test for differences between EA and LA mice. For time course data, repeated measures ANOVA's (Bonferroni post hoc) were used to identify differences according to the factors 'time', baseline vs injection days ('BL-INJ'), experimental conditions ('veh-dzp'). Repeated measures ANOVAs (Bonferroni post hoc) were ran on log transformed (Figure 4-5 and Figure 4-6) or normalised (Figure 4-7 and Figure 4-8) power spectra to test for significant differences between 'frequency', 'age' and 'day', as well as interactions between each factor. For the recovery data in which spectra were assessed in 3-hour intervals, an additional factor 'hour' was used in repeated measure ANOVA analysis. Finally, for plots showing the MUA occurring during OFF periods, the average firing rates during the first 200ms of each condition and day were calculated for each animal. ANOVA with factors 'age', 'experimental condition' and 'day' were used to test for statistical differences between these averages.

4.3 Results

4.3.1 Effect of diazepam on sleep-wake architecture

Figure 4-1B shows representative plots of the SWA during each vigilance state, during baseline and injection days, used to visualise the overall effects of the injections. Consistent with previous studies, there was a reduction in SWA after diazepam injection, compared to both the baseline day before and the vehicle injection day. The reduction in SWA was mostly recovered by the dark period following diazepam injection. In order to assess this further, the total amount of wake, NREM sleep and REM sleep were calculated for the 24-hour baseline recording before vehicle and diazepam injections (veh BL, dzp BL), and the 24 hours post injection (veh, dzp) (Figure 4-2). Although the amount of wake was reduced and NREM sleep was increased during the diazepam day, no significant differences between the four recording days (Veh BL, Veh, dzp BL, dzp) were identified (wake: $F(3,24)=1.00$, $p=0.411$; NREM sleep: $F(3,24)=0.89$, $p=0.46$; REM sleep: $F(3,24)=1.36$, $p=0.28$). There were also no significant interactions between age and day for the amount of wake (age*day: $F(3,24)=0.03$, $p=0.992$), NREM sleep (age*day: $F(3,24)=0.16$, $p=0.92$) or REM sleep (age*day: $F(3,24)=0.56$, $p=0.65$) (Figure 4-2). ANOVA revealed that there was a significant effect of age on the total amount of wake ($F(1,24)=9.38$, $p=0.005$) and NREM sleep ($F(1,24)=9.57$, $p=0.005$), while the amount of REM sleep was not significantly different between age groups ($F(1,24)=1.91$, $p=0.18$). Unpaired t-tests comparing age groups for each condition identified a significant age difference for the vehicle day only for both the total amount of wake and NREM sleep (wake: $p=0.03$; NREM sleep: $p=0.02$, Figure 4-2). As overall the LA mice had a reduced amount of wake and an increased NREM sleep compared to EA mice, one-way ANOVAs were performed for each age group separately to identify possible differences relating to diazepam. However, no significant differences were found for either EA or LA mice.

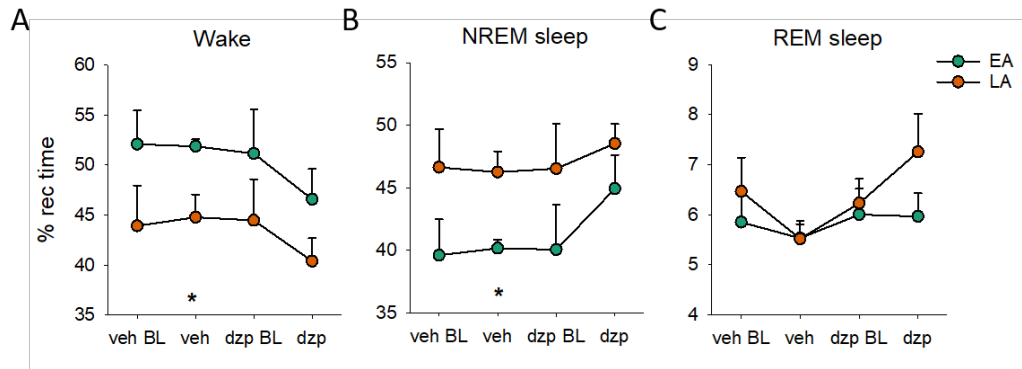


Figure 4-2: Total amount of wake (A), NREM sleep (B) and REM sleep (C) are plotted for the baseline 24 hours before vehicle injection (veh BL), 24 hours after vehicle injection (veh), baseline 24 hours before diazepam injection (dzp BL) and the 24 hours after diazepam injection. Data are mean and SEM. $n=4$ for both EA and LA mice. ANOVAs were used to test for significant differences between age groups and the four recording days. ANOVA testing revealed a significant age effect only. Unpaired t-tests were performed to determine which days showed significant age differences, as indicated on the figure $*p<0.05$.

Plotting the time course of each vigilance state in 2-hour intervals for EA and LA mice separately, revealed that the decreased amount of wake and increased NREM sleep was mostly accounted for by differences at the beginning of the dark period of the injection day (Figure 4-3). However, no significant differences between experimental days (baseline versus injection (BL-INJ)) or conditions (vehicle versus diazepam (veh-dzp)) were identified for the amount of wake (EA mice: BL-INJ: $F(1,12)=0.02$, $p=0.89$; veh-dzp: $F(1,12)=0.34$, $p=0.57$; BL-INJ*veh-dzp: $F(1,12)=0.20$, $p=0.67$; LA mice: BL-INJ: $F(1,12)=0.34$, $p=0.86$; veh-dzp: $F(1,12)=0.18$, $p=0.68$; BL-INJ*veh-dzp: $F(1,12)=0.22$, $p=0.65$). The amount of NREM sleep was significantly different between baseline and injection days (BL-INJ: EA mice: $F(1,12)=12.63$, $p=0.04$; LA mice: $F(1,12)=11.45$, $p=0.005$). In addition, LA mice also had a significant interaction between time and baseline and injection days (time*BL-INJ: $F(4,53)=2.87$, $p=0.03$). The amount of NREM sleep was not significantly different between conditions (veh-dzp, EA mice: $F(1,12)=1.71$, $p=0.22$; LA mice: $F(1,12)=0.50$, $p=0.49$). Finally, the amount of REM sleep showed significant interactions between time and BL-INJ for both EA and LA mice (time*BL-INJ; EA mice: $F(4,53)=3.12$, $p=0.02$; LA mice: $F(11,132)=3.02$, $p=0.001$). LA mice also had a significant interaction between BL-INJ and experimental conditions for REM sleep (BL-INJ*veh-dzp: $F(1,12)=4.71$, $p=0.05$). Neither age group showed significant differences between BL-INJ (EA mice: $F(1,12)=2.45$, $p=0.14$; LA mice: $F(1,12)=2.93$, $p=0.11$) or

veh-dzp days (EA mice: $F(1,12)=1.00$, $p=0.34$), LA mice: $F(1,12)=3.32$, $p=0.09$), for the amount of REM sleep. No further significant differences or interactions were identified for the amount of wake, NREM or REM sleep.

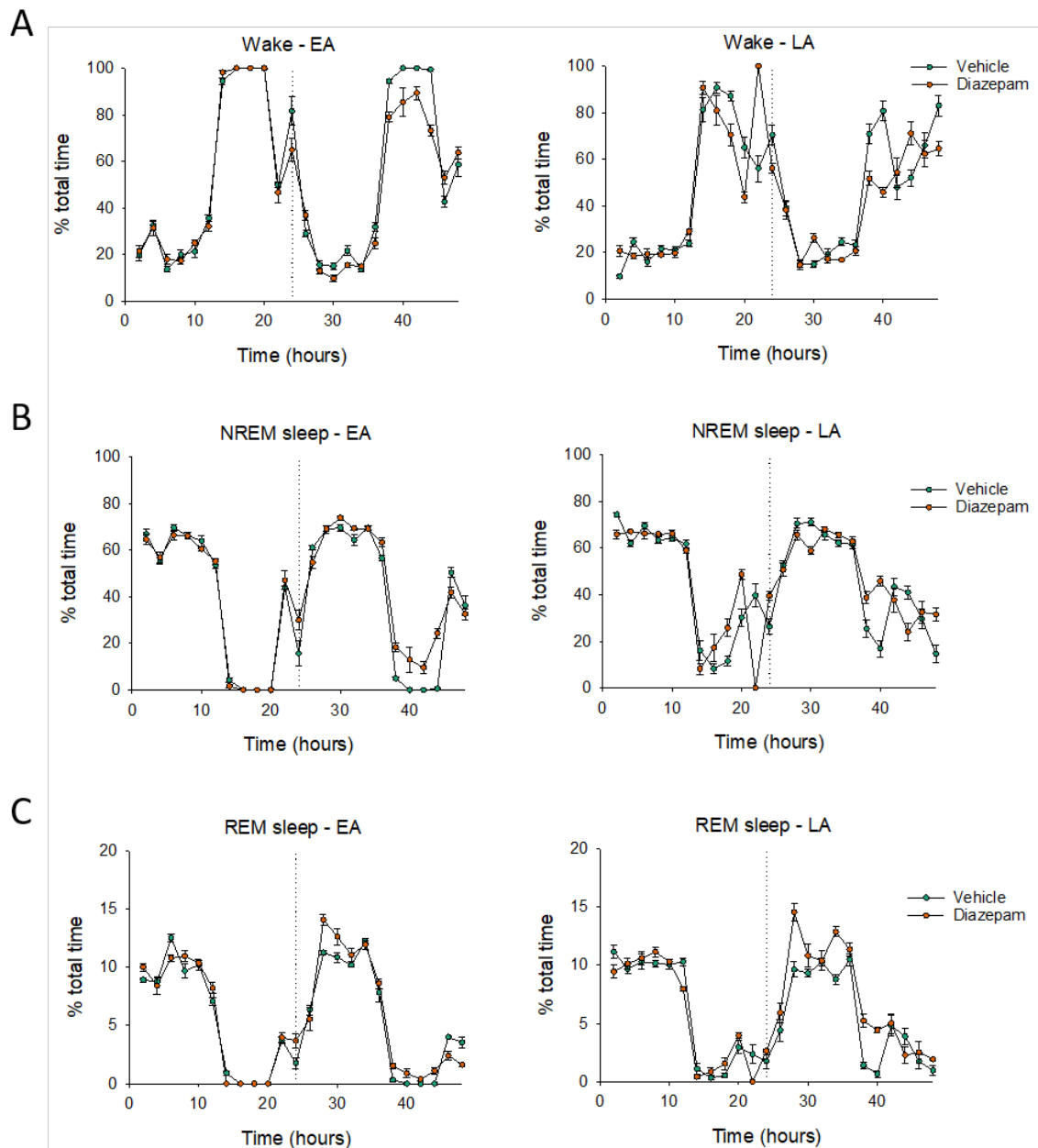


Figure 4-3: Time course of waking (A), NREM sleep (B) and REM sleep (C) during the 24-h baseline day and following injection day, averaged in 2-h intervals. The amount of each vigilance state is represented as % of the total recording time. Mean values, SEM. Data are shown separately for EA mice ($n=4$) and LA mice ($n=4$) to allow for comparisons between experimental conditions (vehicle and diazepam injections). Repeated measures ANOVAs were used to test for significant differences between baseline and injection days (BL-INJ) and between experimental conditions (vehicle and diazepam: veh-dzp).

In order to investigate the effect of diazepam on the fragmentation of the sleep-wake cycle, both the number and duration of wake, NREM sleep and REM sleep episodes longer than 16 seconds were quantified (Figure 4-4). Statistical analysis was performed using ANOVAs with factors 'age' and 'day', therefore 'day' effects represent general effects across both age groups. The number of waking, NREM sleep and REM sleep episodes were increased after diazepam, compared to dzp BL days (Waking: $F(3,24)=7.31$, $p=0.001$, dzp BL vs dzp $p=0.002$, Figure 4-4A; NREM sleep: $F(3,24)=4.62$, $p=0.01$, dzp BL vs dzp $p=0.01$, Figure 4-4B; REM sleep: $F(3,24)=3.35$, $p=0.04$, veh BL vs dzp $p=0.07$, dzp BL vs dzp $p=0.06$, Figure 4-4C). Additional age differences were also identified for the number of waking ($F(1,24)=37.35$, $p<0.0001$) and NREM sleep ($F(1,24)=29.59$, $p<0.0001$) episodes. Specifically, LA mice had a higher number of waking episodes during the vehicle and diazepam days (vehicle: EA 32.00 ± 1.68 , LA 65.50 ± 10.09 , $p=0.02$; dzp: EA 38.25 ± 3.57 , LA 82.00 ± 5.82 , $p=0.001$, unpaired t-tests), and a higher number of NREM sleep episodes during vehicle BL, vehicle and diazepam days (vehicle BL: EA 89.75 ± 6.28 , LA 125.5 ± 11.74 , $p=0.04$; vehicle: EA 101.75 ± 6.29 , LA 143.75 ± 11.76 , $p=0.02$; dzp: EA 105.75 ± 4.71 , LA 169.75 ± 20.39 , $p=0.02$, unpaired t-tests). No further significant effects or interactions were identified for the number of episodes.

With regards to the duration of episodes, ANOVA testing did not reveal any significant differences between experimental days, for NREM sleep ($F(3,24)=0.22$, $p=0.88$, Figure 4-4B) or REM sleep episodes ($F(3,24)=0.28$, $p=0.84$, Figure 4-4C). However, there was a significant effect of experimental day on the duration of waking episodes ($F(3,24)=4.08$, $p=0.02$, Figure 4-4A), explained by differences between the diazepam day and its respective baseline ($p=0.01$, Tukey post hoc; EA: dzp bl: 27.15 ± 4.06 secs, dzp: 18.34 ± 2.99 secs; LA: dzp bl: 15.14 ± 3.19 secs, dzp: 7.25 ± 0.82 secs). The average duration of waking episodes was shorter in LA mice compared to EA mice ($F(1,24)=42.91$, $p<0.0001$, Figure 4-4A). Unpaired t-tests revealed that this was accounted for by differences during the veh BL (EA: 22.13 ± 2.34 secs; LA: 13.66 ± 1.21 secs;

$p=0.02$), vehicle (EA: 23.46 ± 0.97 secs; LA: 10.34 ± 1.52 ; $p=0.0003$) and dzp days (EA: 18.33 ± 2.99 secs; LA: 7.25 ± 0.82 secs; $p=0.01$). A significant age affect was also identified for the duration of NREM sleep episodes ($F(1,24)=15.93$, $p=0.001$, Figure 4-4B), which were significantly lower in LA mice during the dzp day only (EA: 6.36 ± 0.15 secs, LA: 4.51 ± 0.43 secs, $p=0.007$, unpaired t-test). Therefore, diazepam injection overall increased the fragmentation of the sleep-wake cycle by increasing the number of waking, NREM sleep and REM sleep episodes, and decreasing the duration of waking and NREM sleep episodes.

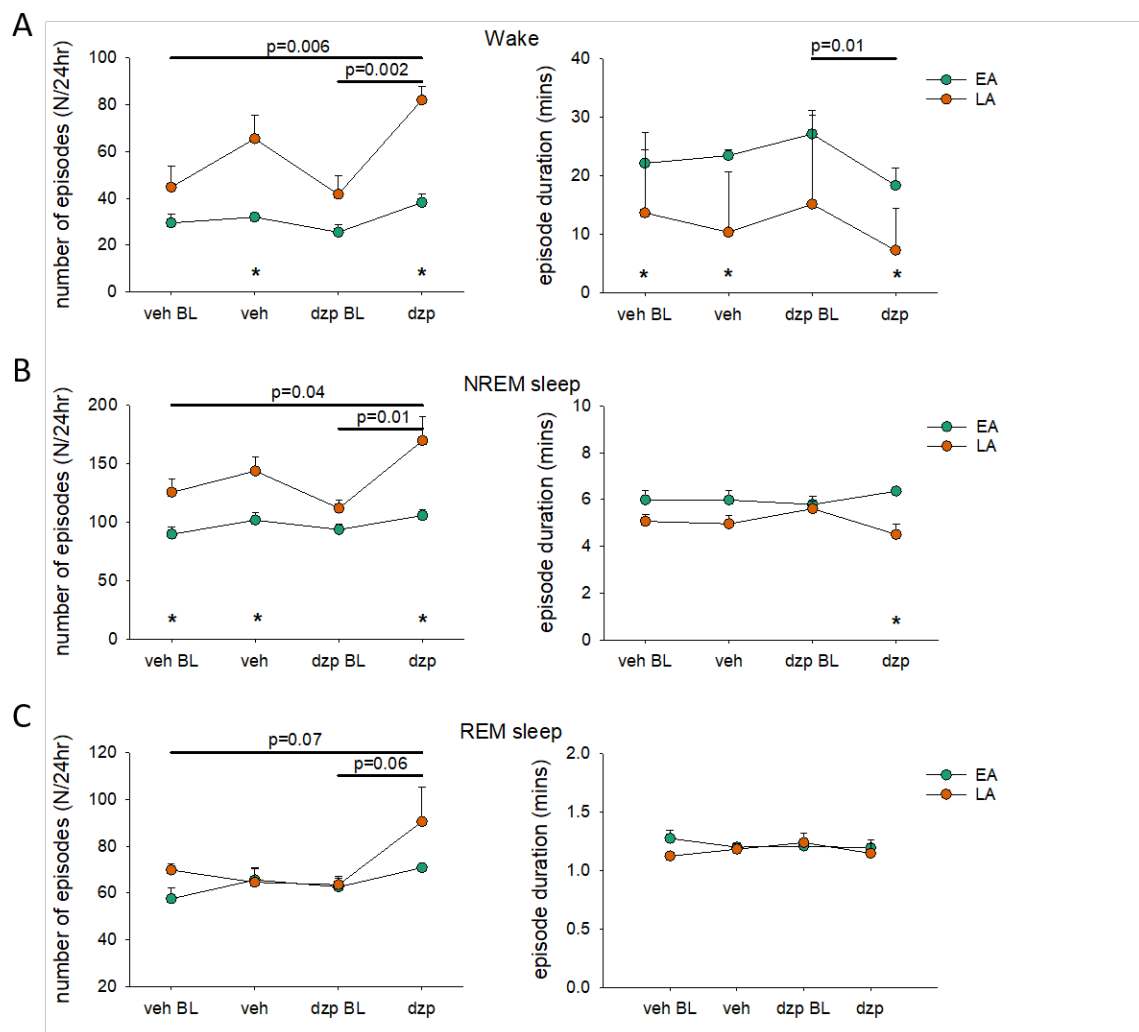


Figure 4-4: **Fragmentation of sleep-wake cycle.** The number (left panel) and duration (right panel) of wake (A), NREM sleep (B) and REM sleep (C) episodes were quantified for the four experimental days (24 hours each): vehicle baseline (veh BL), vehicle (veh), diazepam baseline (dzp BL) and diazepam (dzp). Data are mean values, SEM. EA and LA mice shown separately ($n=4$ per group). ANOVAs were used to test for significant differences between experimental days and age groups: Tukey post hoc test used to identify specific day differences, unpaired t-tests used to identify specific age differences. Lines above traces indicate significant differences between experimental days. Asterisks below traces indicate significant differences between age groups. $*p=0.05$.

4.3.2 Spectral power

As one of the main known effects of diazepam is to cause a reduction in spectral power in the slow-wave activity (SWA) range, power spectra were next calculated for the frontal and occipital EEG derivations (Figure 4-5, Figure 4-6).

4.3.2.1 Frontal EEG

Corresponding with previous studies, frontal EEG SWA spectral power during NREM sleep was reduced during the diazepam injection day (DZP), for both EA and LA mice (Figure 4-5). Repeated measure ANOVAs with factors 'frequency', 'recording day' and 'age', were first performed for each vigilance state. This confirmed that spectral power was significantly reduced during the diazepam day compared to all three other days (rec day: $F(3,16)=9.73$, $p=0.001$, Bonferroni post hoc: Veh BL vs DZP $p=0.01$, day Veh vs DZP $p=0.004$ and day DZP BL vs DZP $p=0.001$, Figure 4-5B). The frequency*recording day interaction, revealed that the differences were specific to the frequency range 1-7.75Hz (freq*rec day: $F(7,18)=6.84$, $p<0.0001$). There were also significant age differences identified during NREM sleep (age: $F(1,16)=32.06$, $p<0.0001$; freq*age: $F(2,38)=8.49$, $p=0.001$), which was apparent during each recording day (Veh BL: $p=0.01$; Veh: $p=0.009$; DZP BL: $p=0.004$; DZP: $p=0.05$). As both day and age were significant factors during NREM sleep, further repeated measures ANOVAs were performed for EA and LA mice separately, in order to determine which frequencies were different between the recording days. Both EA mice and LA mice showed significant effects of day (EA: $F(3,8)=5.73$, $p=0.02$; LA: $F(3,8)=4.70$, $p=0.04$). Importantly, a significant frequency*day difference was only identified for LA mice, as shown in Figure 4-5B ($p<0.05$ for frequency range 3-6HzHz). EA mice had a trend towards significance for the frequency*day interaction ($F(5,13)=2.52$, $p=0.085$).

Although REM sleep showed no significant effect of experimental day (REM sleep: $F(3,16)=1.39$, $p=0.28$), there was a significant interaction between frequency bin and day ($F(5,29)=3.38$,

$p=0.014$, Figure 4-5C). Once again, significant differences between age groups were observed for all four recording days (age: $F(1,16)=31.03$, $p<0.0001$; freq*age: $F(2,29)=5.22$, $p=0.013$; Veh BL: $p=0.01$; Veh: $p=0.008$; DZP BL: $p=0.006$; DZP: $p=0.04$). Therefore, further repeated measure ANOVAs were performed for EA and LA mice separately, however, no significant day or frequency*day interactions were identified for either EA mice (day: $F(3,8)=0.25$, $p=0.86$; freq*day: $F(4,12)=1.31$, $p=0.325$) or LA mice (day: $F(3,8)=1.67$, $p=0.25$; freq*day: $F(6,16)=2.41$, $p=0.076$).

Finally, waking showed no significant day effect or interaction (day: $F(3,16)=1.24$, $p=0.33$; freq*day: $F(3,18)=1.40$, $p=0.28$, Figure 4-5A). However, spectra during wake were significantly different between age groups (age: $F(1,16)=11.58$, $p=0.004$), though only during the DZP BL day ($p=0.04$). Repeated measure ANOVAs ran separately for EA and LA mice, revealed a significant day difference for LA mice ($F(3,8)=4.86$, $p=0.03$), but not for EA mice ($F(3,8)=0.17$, $p=0.91$). Neither age groups had a significant interaction between frequency*day (EA: $F(3,8)=0.33$, $p=0.81$; LA: $F(3,9)=1.64$, $p=0.25$).

4.3.2.2 Occipital EEG

In contrast to the frontal derivation, no significant differences were identified for the occipital derivation for either experimental day or age during waking (day: $F(3,20)=0.20$, $p=0.90$; age: $F(1,20)=1.17$, $p=0.29$, Figure 4-6A), NREM sleep (day: $F(3,20)=0.77$, $p=0.33$; age: $F(1,20)=2.14$, $p=0.16$; day*age: $F(3,20)=0.003$, $p=1.00$, Figure 4-6B) or REM sleep (day: $F(3,20)=0.05$, $p=0.99$; age: $F(1,20)=0.82$, $p=0.38$, Figure 4-6C). There was a significant frequency bin*age interaction identified during NREM sleep only ($F(2,31)=3.70$, $p=0.05$). No other significant interactions were identified between any of the measures for any vigilance state. Interestingly there was also a shift in the theta peak towards lower frequencies during REM sleep after diazepam administration. This effect was most apparent in the occipital derivation, where theta activity

predominates due to its proximity to the hippocampus where theta activity is thought to be generated.

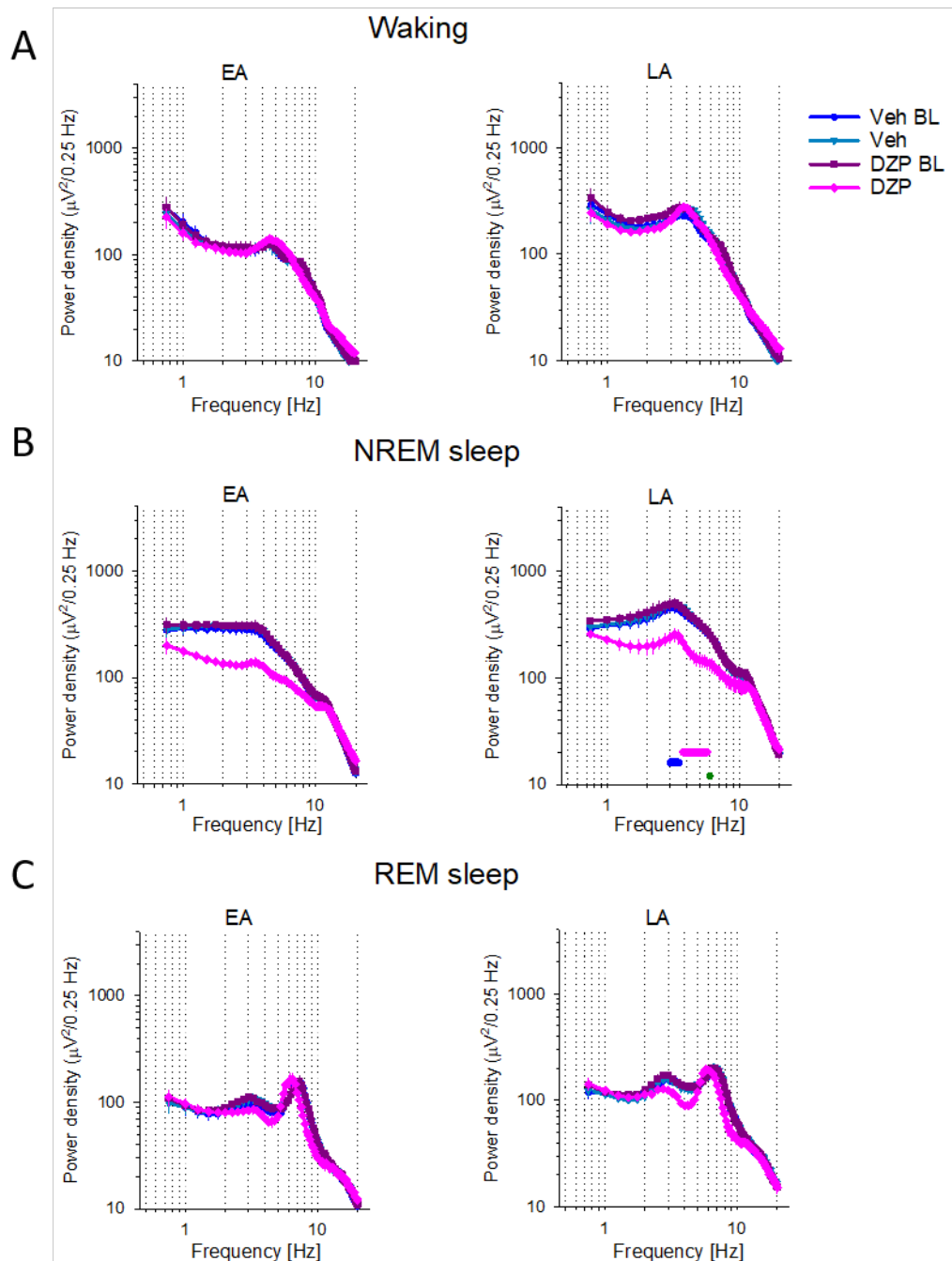


Figure 4-5: Frontal EEG power spectra during Waking (A), NREM sleep (B) and REM sleep (C). Data are plotted for the four recording days vehicle baseline (Veh BL), vehicle injection (Veh), diazepam baseline (DZP BL) and the diazepam injection day (DZP). Power densities are shown on a logarithmic scale for frequencies between 0-20, binned in a 0.25 frequency resolution. Mean values, SEM. EA n=3, LA n=3. Coloured lines below the plots indicate frequency bins where spectra differed significantly between the experimental days ($p < 0.05$, repeated measures ANOVAs (Bonferroni post hoc) on log-transformed values, ran separately for each age group. Pink: sig differences between Veh BL, Veh, DZP BL days and DZP. Blue: sig differences between Veh, DZP BL days and DZP. Red: sig differences between Veh and DZP days only. Green: sig differences between DZP BL and DZP only.

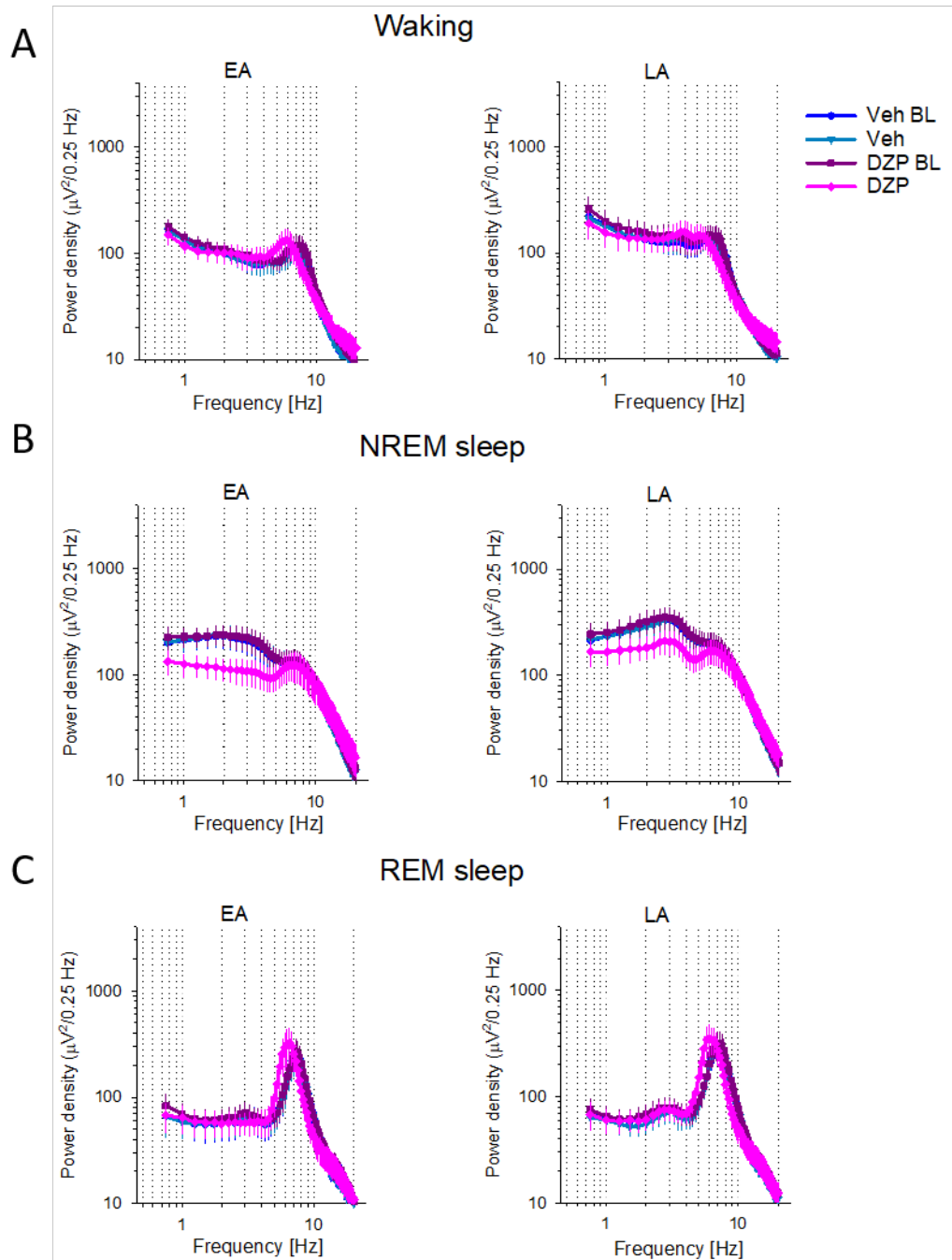


Figure 4-6: Occipital EEG power spectra during Waking (A), NREM sleep (B) and REM sleep (C), calculated for the occipital EEG. Data are plotted for the four recording days vehicle baseline (Veh BL), vehicle injection (Veh), diazepam baseline (DZP BL) and the diazepam injection day (DZP). Power densities are shown on a logarhythmic scale for frequencies between 0-20, binned in a 0.25 frequency resolution. Mean values, SEM. EA n=3, LA n=3. repeated measures ANOVA's did not identify any significant differences between the experimental days for EA or LA mice.

As individual animals have differences in spectral power, the light periods of both the injection experimental day and recovery day after injection (12-hour light period day following injection) were next normalised to their respective baseline light periods, for each animal before averaging (Figure 4-7). This was done for the frontal region during NREM sleep only as this was where the

most differences in absolute spectral power were observed. For the vehicle injection day, both EA and LA mice had power density values around 100% for both the injection and recovery days, indicating that these days were similar to that of their respective baseline day. As expected from the absolute spectra, the diazepam injection day was found to be significantly different to the vehicle day and the recovery day after vehicle injection ($F(3,16)=8.00$, $p=0.002$, Veh vs DZP $p=0.003$, Veh Rec vs DZP $p=0.012$, Figure 4-7). Importantly there were no significant age differences or interactions identified (age: $F(1,16)=0.90$, $p=0.36$; age*day: $F(3,16)=0.44$, $p=0.73$). No significant differences were identified over the frequency bins ($F(1,16)=0.77$, $p=0.40$), while there were also no significant interactions (freq*day: ($F(3,16)=1.54$, $p=0.24$; freq*age: $F(1,16)=1.17$, $p=0.30$; freq*day*age: $F(3,16)=0.95$, $p=0.44$). Due to the lack of differences according to frequency, no post hoc analyses were performed.

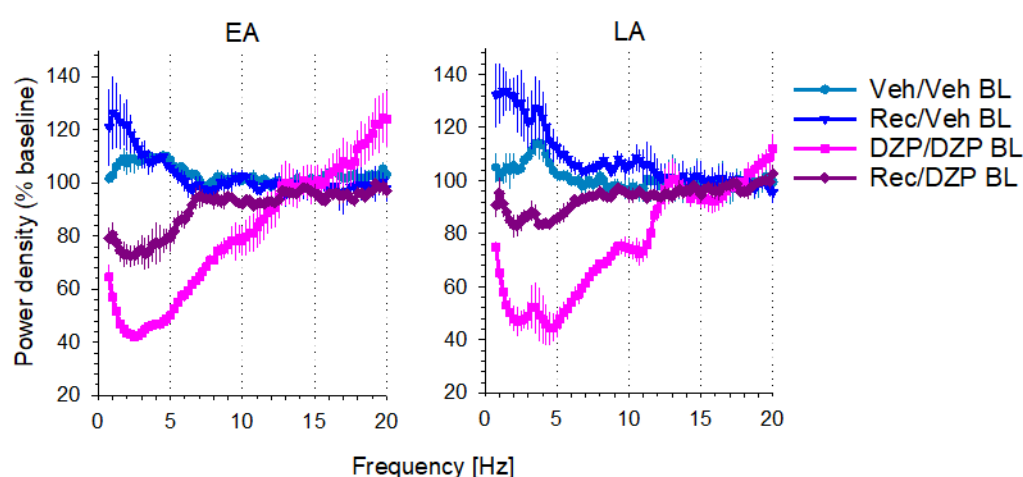


Figure 4-7: Changes in spectral power during NREM sleep relative to baseline recordings. Light periods of both the injection experimental day (Vehicle: Veh/BL, DZP/BL) and recovery day after injection (12-hour light period day following injection, Rec/BL) were expressed relative to their respective baseline light periods for each animal and then averaged across animals, for EA (left panel) and LA (right panel) mice separately. Data were recorded from the frontal cortical region. A value of 100% indicated no change relative to baseline. Mean values, SEM. EA $n=3$, LA $n=3$. Significance testing was performed using a repeated measures ANOVA comparing factors frequency bin, experimental day and age. Only factor day was significantly different.

Although no significant age differences were observed, visual inspection of these traces indicated that there may be age differences in the recovery after diazepam injection (Figure 4-7). Specifically, in EA mice spectral power in the lower frequencies remained reduced during the recovery day after diazepam treatment (Rec/DZP day), an effect that was less apparent in LA

mice. In order to address the time course of recovery after diazepam injection, the 12-hour light period following diazepam injection was subdivided into three-hour intervals and average spectra calculated (normalised to the same intervals during the diazepam baseline day) (Figure 4-8Ai). An overall effect of time interval was observed ($F(3,16)=5.45$, $p=0.009$), specifically between the first time interval (hours 1-3) and hours 4-6 ($p=0.02$), hours 7-9 ($p=0.02$) and hours 10-12 ($p=0.04$). There was also an interaction between frequency bin and time interval ($F(6,32)=4.51$, $p=0.002$), with significant differences depicted in Figure 4-8A ii (left panel ii). Importantly there was no significant effect of age observed ($F(1,16)=1.59$, $p=0.23$), however there was a significant interaction between frequency bin and age ($F(2,32)=3.36$, $p=0.05$). Most age differences were apparent in higher frequency ranges (see Figure 4-8A, right panel ii). To further assess this, repeated measures ANOVAs were next performed separately for EA and LA mice. Time intervals were only significantly different for LA mice ($F(3,8)=11.38$, $p=0.003$), between hours 1-3 and all three other intervals (vs hours 4-6 $p=0.01$, vs hours 7-9 $p=0.006$ and vs day 4 $p=0.009$). No effect of time interval was observed for EA mice ($F(3,8)=1.52$, $p=0.28$). A significant frequency* time interval interaction was identified for EA mice ($F(6,16)=2.81$, $p=0.05$), which were significantly different between hours 1-3 and 9-12 at 14.25 Hz only (Figure 4-8A). No significant effect of time interval was identified for LA mice ($F(5,13)=2.02$, $p=0.14$).

As the spectra had not returned back to baseline by the end of the 12-hour diazepam light period, the same was calculated for the recovery light period 24 hours following injection (DZP recovery day) (Figure 4-8B). All values had returned back to baseline level by the recovery day following injection, with no effects of time interval ($F(3,14)=1.22$, $p=0.34$), age ($F(1,14)=4.66$, $p=0.05$) or any interactions identified (Figure 4-8B). Due to technical issues, it was only possible to quantify this for the 12 hours after injection, rather than the dark period following injection.

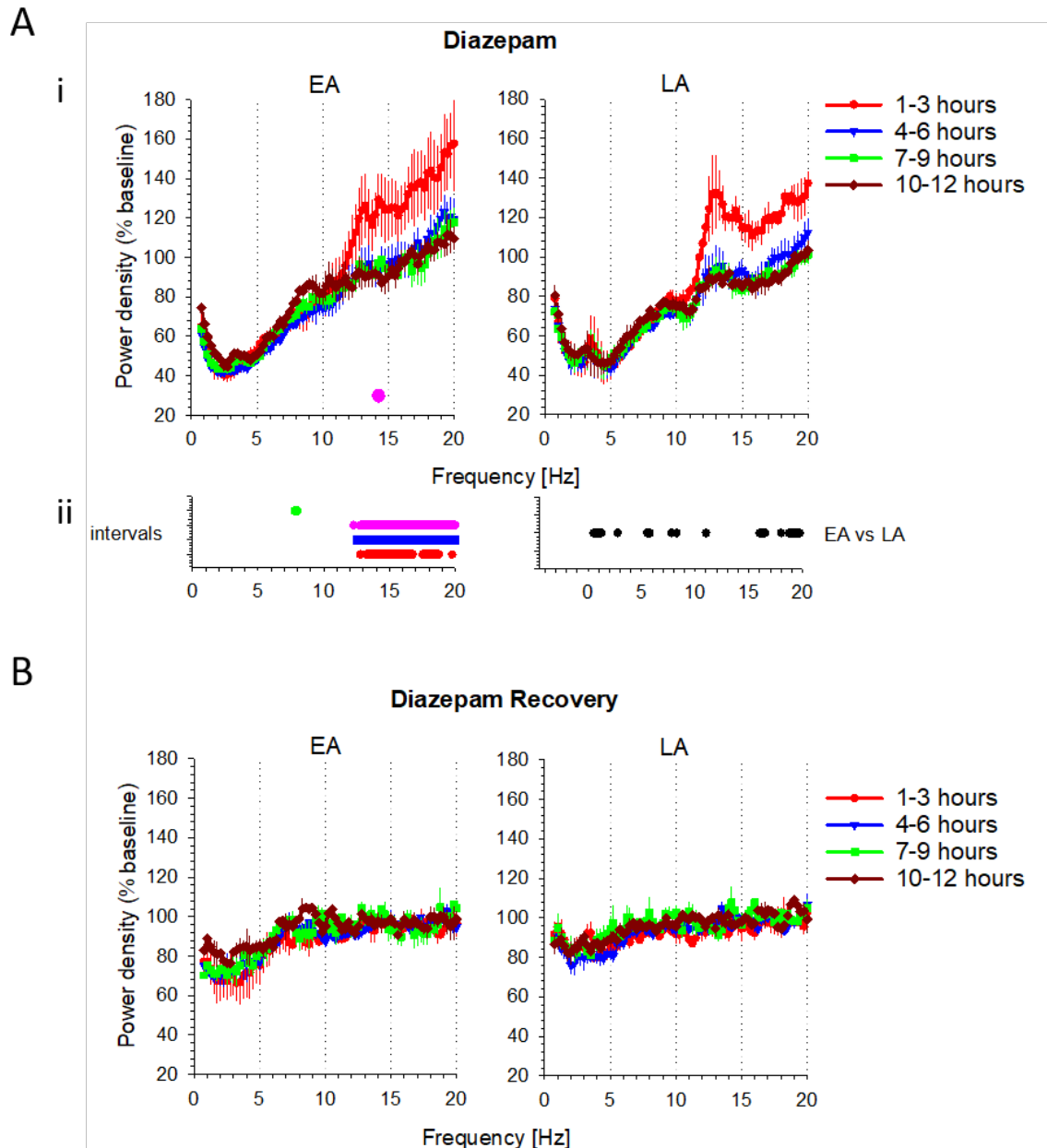


Figure 4-8: Recovery of spectral power after diazepam injection, subdivided into three-hour intervals. Data are shown for (A) the average over the immediate 12 hours after diazepam injection (Diazepam) and (B) the diazepam recovery day light period (24 hours after injection). Each interval was normalised to the same time interval of the baseline light period the day before diazepam injection, for each animal and then averaged across animals, for EA (left panel) and LA (right panel) mice separately. Data were recorded from the frontal cortical region. A value of 100% indicated no change relative to baseline. Mean values, SEM. EA n=3, LA n=3. For the diazepam recovery day n=2 for EA mice interval 7-9 hours and n=2 for LA mice interval 9-12, due to an absence of sleep during these periods. Significance testing was performed using a repeated measures ANOVA comparing factors frequency bin, experimental day and age. Overall significant differences (combined EA and LA) according to time interval are shown for hours 4-6 vs 9-12 (green), hours 1-3 vs 9-12 (pink), hours 1-3 vs 7-9 (blue) and hours 1-3 vs 4-6 (red) (panel A i, left panel). Overall significantly different frequency bins between EA vs LA are shown in panel A ii (right panel). Further repeated-measures ANOVAs were performed for EA and LA mice separately. The only significant difference was between hours 1-3 vs 9-12 for EA mice (as shown in pink on the EA plot for the diazepam day (A, top left panel).

4.3.3 Analyses of neural activity

The next aim of this study was to characterise the neuronal activity underpinning the effects of diazepam. Firstly, the average firing rates (FR) across the light periods of the baseline, injection and recovery days, were calculated for both vehicle and diazepam conditions, separated into EA and LA age groups (Figure 4-9). FR were not significantly different according to age, experimental days (exp: vehicle or diazepam) and recording days (rec: baseline, injection and recovery), for waking (age: $F(1,38)=2.74$, $p=0.11$; exp: $F(1,38)=0.003$, $p=0.96$; rec: $F(2,38)=0.15$, $p=0.86$, Figure 4-9A), NREM sleep (age: $F(1,38)=0.02$, $p=0.89$; exp: $F(1,38)=0.53$, $p=0.47$; rec: $F(2,38)=0.14$, $p=0.87$, Figure 4-9B), or REM sleep (age: $F(1,38)=0.60$, $p=0.44$; exp: $F(1,38)=0.40$, $p=0.53$; rec: $F(2,38)=0.16$, $p=0.85$, Figure 4-9C). In addition, no significant interactions were identified for any vigilance states.

Next, the average number and duration of OFF periods during the first 2 hours of the light period of baseline, injection and recovery days, were calculated for EA and LA mice (Figure 4-10). Interestingly, the number of OFF periods were reduced in the two hours immediately post diazepam injection (EA: BL: 23.45 ± 2.81 , dzp: 9.72 ± 2.95 ; LA: BL: 30.63 ± 4.47 , dzp: 13.83 ± 2.63 , Figure 4-10A). ANOVA significance testing (EA and LA combined) revealed significant effects of experimental condition ($F(1,26)=23.12$, $p<0.0001$) and recording day ($F(2,26)=3.32$, $p=0.05$, day 1 vs 2 $p=0.06$, day 1 vs 3 $p=1.00$, day 2 vs 3 $p=0.26$) on the number of OFF periods. There was also a significant effect of age ($F(1,26)=32.50$, $p<0.0001$), during all days except the diazepam injection day (post-hoc unpaired t-tests: veh BL: $p=0.08$, veh rec: $p=0.02$, dzp rec: $p=0.005$). Paired t-tests identified significant differences between vehicle and diazepam conditions for EA mice during the injection day (veh: 29.65 ± 0.45 , dzp: 9.72 ± 2.95 , $p=0.02$, Figure 4-10A, left panel) and LA mice during the injection day (veh: 35.98 ± 3.36 , dzp: 13.83 ± 2.63 , $p=0.005$) and recovery day (veh rec: 41.65 ± 4.17 , dzp rec: 30.02 ± 1.46 , $p=0.05$, Figure 4-10A, right panel).

The duration of OFF periods remained stable across the three days ($F(2,26)=0.02$, $p=0.98$) and between the two experimental conditions ($F(1,26)=0.08$, $p=0.79$). There was however a significant effect of age on the duration of OFF periods, with LA mice having longer OFF periods compared to EA mice ($F(1,26)=9.73$, $p=0.004$, average over three days: EA: $111.35\pm4.54\text{ms}$, LA: $130.00\pm2.65\text{ms}$).

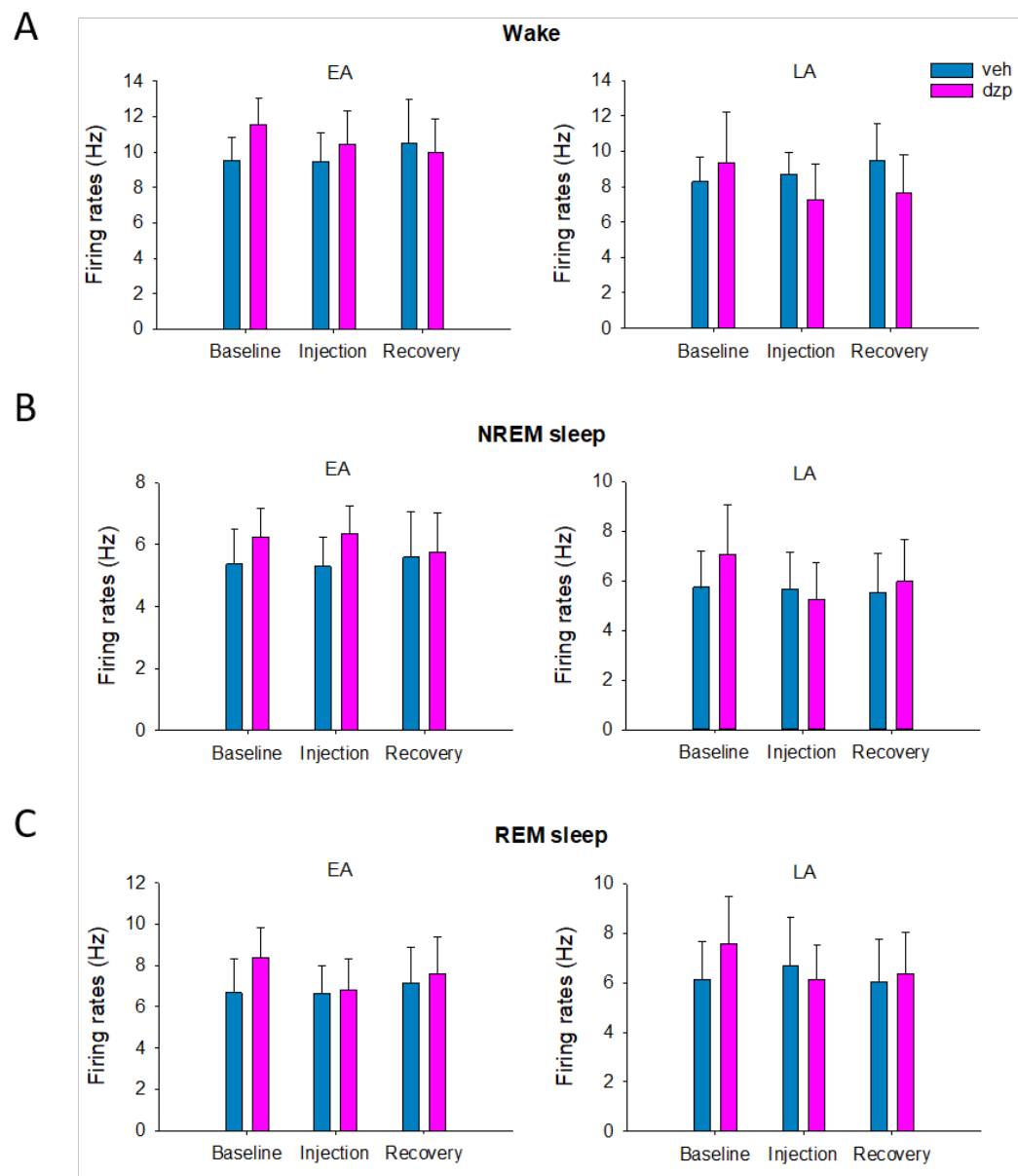


Figure 4-9: Average firing rates during vehicle and diazepam injection days, separated into baseline, injection and recovery days (12-hour light periods only). Data are shown for Waking (A), NREM sleep (B), REM sleep (C), separated into EA mice (left panels) and LA mice (right panels). Mean values, SEM. EA $n=4$, LA $n=4$. No significant differences were identified between ages, experimental days (vehicle or diazepam) and recording days (baseline, injection, recovery), for any vigilance state (ANOVA's).

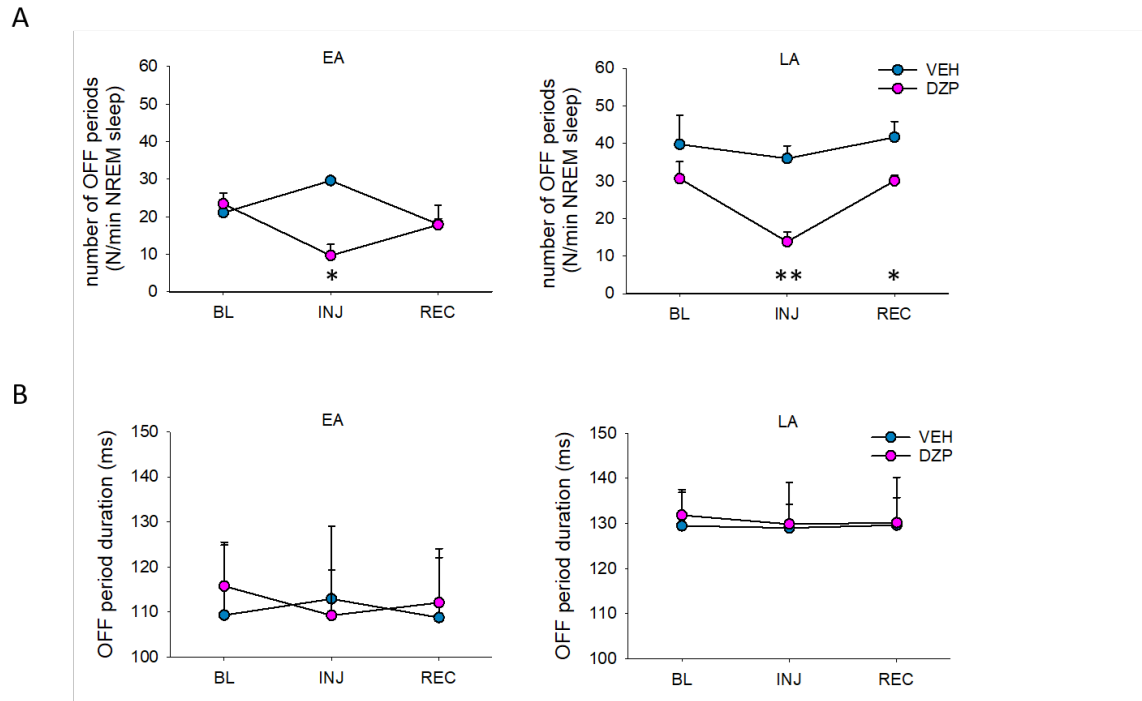


Figure 4-10: Average number (A) and duration (B) of OFF periods in the first 2 hours of baseline, injection and recovery light periods, for both vehicle and diazepam experimental conditions. Data for EA and LA mice are shown in left and right panels, respectively. Mean values, SEM. EA n=3, LA n=3. ANOVAs were used to determine significant differences between age, experimental days and recording days. Paired t-tests were used to identify significant differences between vehicle and diazepam conditions, with significant differences indicated on the figure. * $p < 0.05$, ** $p < 0.01$.

To investigate neuronal firing in more detail next the firing rates 500ms before and after the occurrence of slow-wave aligned OFF periods were plotted for the first two hours of baseline, injection and recovery days for both vehicle and diazepam conditions (Figure 4-11). This data was normalised to the first 300ms of the baseline days (combined vehicle and diazepam), to highlight differences between the conditions. For both conditions, on each of the recording days there is a clear suppression of MUA in association with the LFP slow waves. After diazepam injection there was a reduction in the average firing rates associated with OFF periods, which was at least partially recovered by the recovery day 24 hours after injection (Figure 4-11). Notably, this reduction in firing was most prominent in LA mice (Figure 4-11B). In order to statistically test this, the first 200ms of each condition was averaged and used in ANOVA statistical testing (factors: 'age', 'experimental condition' and 'day'). This confirmed a significant difference between diazepam and vehicle conditions ($F(1,26)=5.81$, $p=0.02$), and a trend towards significance between days ($F(2,26)=2.96$, $p=0.07$). Importantly, although visual inspection of the

data in Figure 4-11 suggested potential age differences, no significant age difference was identified ($F(1,26)=2.40$, $p=0.13$). Therefore, diazepam decreased average firing rates during ON periods (i.e. excluding OFF-states), which occurred similarly in both age groups.

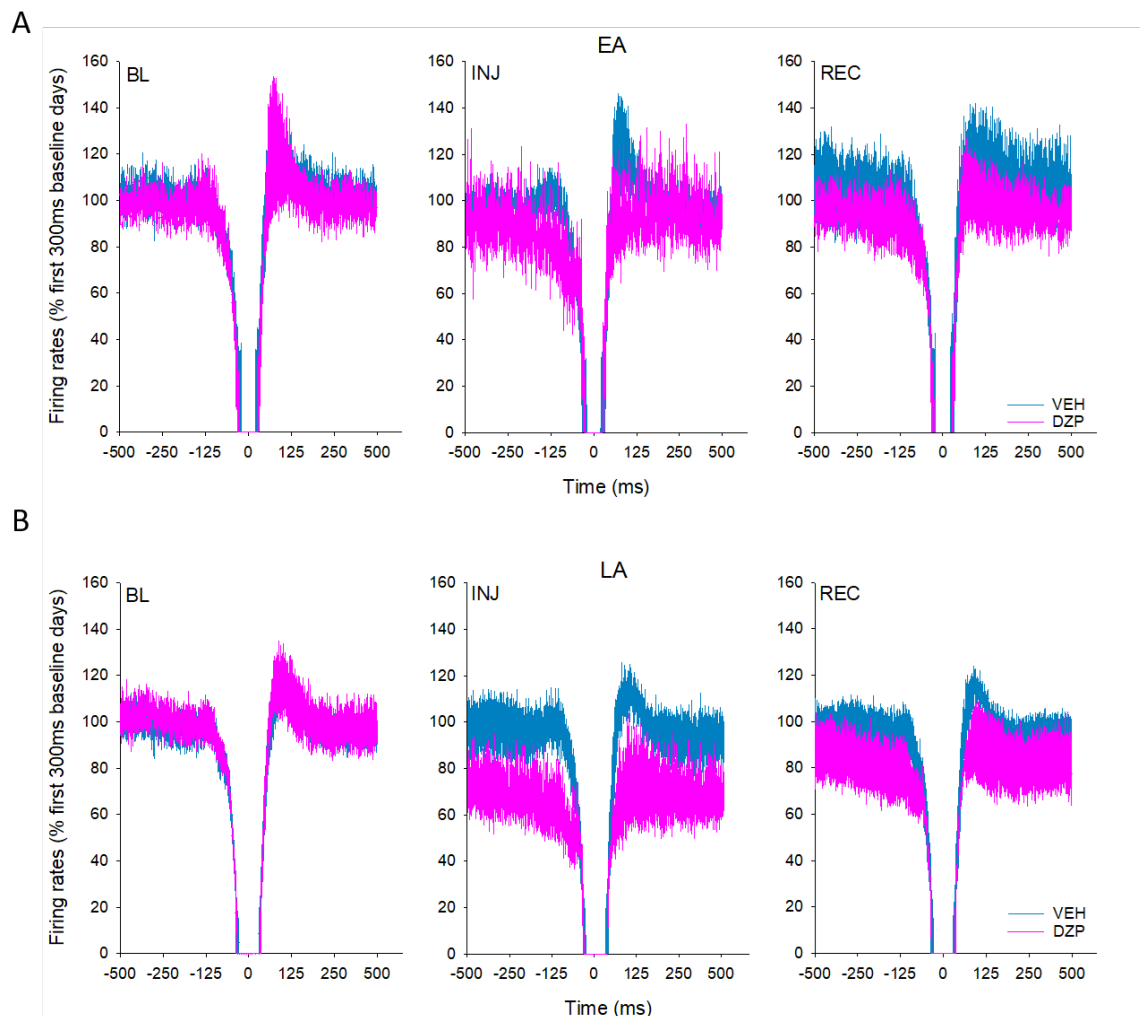


Figure 4-11: Slow-wave triggered suppression in MUA, defined as an OFF period, shown for the first two hours of baseline, injection and recovery days for both vehicle and diazepam conditions. Data are shown for EA (A) and LA (B) mice separately. The 500ms prior to and after an OFF period are shown, aligned by the occurrence of slow waves, from which they are defined. For each age group, the first 300ms of the baseline days (both vehicle and diazepam days combined) were averaged for each mouse and used as their common reference, for each of the conditions. Blue and pink traces represent vehicle and diazepam experimental conditions, respectively. Mean values, SEM. EA $n=3$, LA $n=3$.

4.4 Discussion

In this study I performed a characterisation of the effects of diazepam on the overall global sleep-wake characteristics and local neural dynamics in the neocortex during sleep in mice.

Firstly, overall sleep wake architecture remained fairly resistant to the effects of diazepam,

except for an increase in sleep-wake fragmentation, identified as an increase the number of wake, NREM sleep and REM sleep episodes, while the duration of waking episodes was also reduced.

One of the frequently reported effects of diazepam is to decrease EEG spectral power in the SWA frequency range (Kopp et al., 2003; Kopp et al., 2004; Lancel et al., 1996; Lancel and Steiger, 1999; Tobler et al., 2001), which was also replicated in this study. Diazepam also shifted the theta peak to slower frequencies, especially in the occipital derivation where theta activity is known to predominate due to its proximity to the hippocampus. It is possible that the shift in theta peak may reflect changes in body temperature, as has previously been shown (Deboer, 2002, 1998). This is supported by evidence that diazepam decreases body temperature (Mailliet et al., 2001). Interestingly, when average spectra were subdivided into 3 hour intervals this revealed enhanced power in higher frequencies during the first 3 hours after diazepam injection, corresponding with previous studies that showed an enhancement of broad band frequencies in the spindle frequency range and above after diazepam injection (Kopp et al., 2003; Kopp et al., 2004; Lancel et al., 1996; Lancel and Steiger, 1999; Tobler et al., 2001). The influence of diazepam on EEG spectra was more apparent in the frontal region compared to the occipital, corresponding well with similar previous reports (Kopp et al., 2003; Kopp et al., 2004). The reduction in SWA with diazepam injection was maintained across the 12 hours after diazepam but was recovered by 24 hours later. This is consistent with evidence in humans that showed the reduction in SWA persisted into the following drug-free night after injection of the benzodiazepines flunitrazepam, flurazepam or triazolam, whereas the effects on the spindle frequency range were recovered (Borbély et al., 1983).

In addition to the direct effects of diazepam, this study also replicated the well-established increase in the amount of sleep and increased sleep-wake fragmentation with ageing (see

section 1.2.2), which was also confirmed in this thesis (Chapter 3:). LA mice were also found to have significantly higher average absolute EEG spectral power recorded from the frontal region, for waking, NREM sleep and REM sleep. This corresponds well with the earlier chapter characterising the effects of ageing across 3 age groups of mice (Chapter 3:) and also previous studies (McKillop et al., 2018; Panagiotou et al., 2017). Although normalised EEG spectra suggested the possibility of age-differences in the time course of recovery after diazepam injection, subdividing the diazepam injection day into 3 hour intervals did not show any age differences in the recovery of power spectra. Therefore this suggests that the two age groups recovered similarly from diazepam injection. Importantly though, when ANOVAs were run for each age group separately, only LA mice had significant effect of time interval, suggesting that there was a bigger change in spectra across the intervals, in LA mice, compared to EA mice. Importantly though, I would like to reemphasise that as only small numbers of animals contributed to this study, age comparisons should be considered preliminary.

4.4.1 Neural characteristics

The most novel aspect of this project was the characterisation of the neuronal activity underpinning the overall global EEG effects of diazepam injection. There were no differences in the average firing rates across experimental days. However, OFF periods were less frequent after diazepam injection, while their duration was unaffected. This corresponds well with the well-established association between slow waves and their neuronal counterparts. Specifically that a lower SWA spectral power is associated with the occurrence of less slow waves and their associated OFF periods (Esser et al., 2007; Panagiotou et al., 2017; Riedner et al., 2007; Steriade et al., 1993a; Steriade, 2006; Vyazovskiy et al., 2007b, 2009b), as outlined in detail in the general introduction chapter (section 1.1.1). Importantly, the effects of ageing on general neural activity (including a higher number and longer duration OFF periods, along with an increased absolute

SWA power in LA mice) were also identified during baseline recordings in this study. However, despite these differences, only few age differences were identified in the response to diazepam.

Perhaps the most interesting and novel result of this study was that diazepam injection reduced average firing rates around OFF periods, which was most apparent in LA mice. Although not statistically significant, this suggests the intriguing possibility that differences in the effects diazepam across ageing may be detected as subtle changes in the neural activity surrounding OFF periods. If confirmed, the differences in neuronal activity may explain differences in the response to benzodiazepines with ageing, including treatments lacking efficacy or having a slow elimination resulting in unwanted prolonged drowsiness side effects or an increased sensitivity to the drugs (Borbély et al., 1983; Greenblatt et al., 1983; Nicholson et al., 1982).

4.4.2 Conclusions

This study replicated the well-established effect of diazepam of reducing SWA power and enhancing power in high frequency ranges. Furthermore, this study showed that cortical neural activity is affected by diazepam, as manifested in a reduction in OFF period incidence and reduced firing rates during ON periods. Importantly, ageing did not greatly influence the effect of diazepam on overall global characteristics of the sleep-wake cycle or power spectra. However, trends towards lower firing rates in LA mice suggest that diazepam may have a greater influence on local neural mechanisms in older individuals. These differences may account for the variability in the efficacy of benzodiazepines in older individuals. Further studies are necessary in order to improve understanding of these mechanisms and therefore improve treatment options for improving sleep in elderly populations.

Chapter 5: The effect of ageing and sleep on spatial learning in the Morris water maze task

5.1 Introduction

5.1.1 The Morris Water Maze (MWM)

The Morris water maze (MWM) was first developed in the 1980s by Richard Morris in order to study spatial localisation (Morris, 1984; Morris et al., 1982), and is now a common test of spatial learning and memory in rodents. The MWM involves placing rodents in a large pool of water, in which they have to use distal spatial cues to navigate to a submerged hidden escape platform. This maze is designed based on evidence that rodents find water aversive yet are natural swimmers so will swim to a hidden platform to escape water (Morris, 1984). Spatial learning can then be assessed over many training days and repeated trials. The directionality/path animals take, proximity to the platform and latency to locate the hidden platform can then be tracked using a video tracker software and used as a measure of spatial learning and navigation. One study used an automated algorithm to detect the various different searching strategies often used in the MWM, which are outlined in Figure 5-1. At the beginning of training, searching strategies can often be classified as non-spatial and instead involve techniques such as randomly searching the pool or thigmotaxis (Rogers et al., 2017). As animals learn the location of the escape platform strategies become more targeted and direct, as animals are more likely to utilise spatial cues (Rogers et al., 2017). This is reflected in a gradual decrease in the latency it takes to reach the escape platform. Once the location of the platform is learnt, a probe trial can also be performed, whereby the platform is removed from the maze and the animals spatial bias for the previous location of the platform can be assessed over a limited period of time, (for example 60 seconds). Measures of spatial memory including the time spent in proximity to the

platform can be automatically quantified by calculating the time spent in the quadrant which used to hold the platform, or by counting the number of crossings over the position of the platform. More details about the specifics of the maze used in this study can be found in the methods section of this chapter (section 5.2.2).

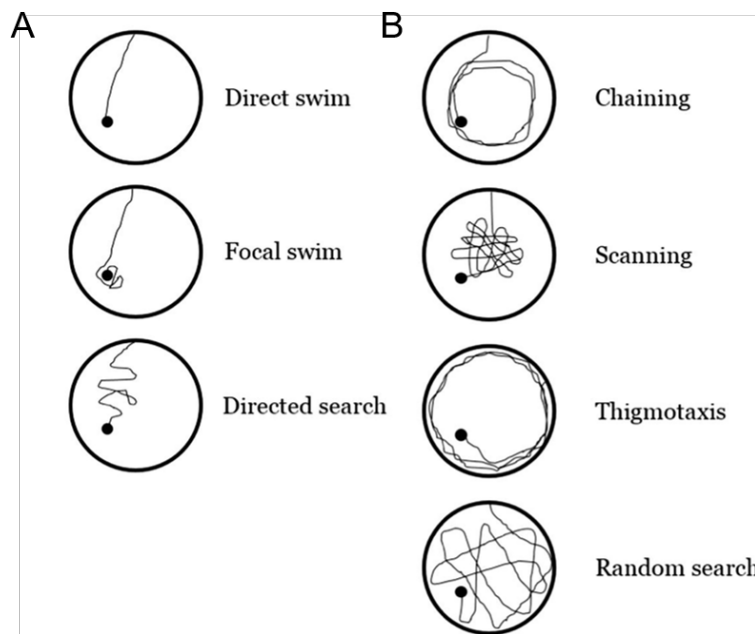


Figure 5-1: Searching strategies in the Morris Water Maze: strategies for finding the escape platform (black circle) can be spatial (A) or non-spatial (B), with spatial strategies reflecting more precisely learnt strategies. Strategies are listed from most to least precise. (Figure adapted from Rogers et al., 2017).

The MWM relies on subjects using spatial cues to continuously monitor and adjust their position in space, in order to navigate accurately to the escape platform (Morris, 1984). The MWM has been particularly useful for dissociating specific deficits in memory formation from sensory, motor and memory retrieval deficits. The MWM has a number of advantages over other spatial memory tasks such as; it does not require a great deal of training, does not rely on food deprivation, is easy to run (Vorhees and Williams, 2014). However, it should be noted that it may involve an increased stress compared to other tasks and may not be as sensitive for investigating working memory, as discussed in (Vorhees and Williams, 2014). In addition, extensive studies have shown that the spatial navigation involved in the MWM is hippocampal dependent, with both glutamatergic and cholinergic systems necessary for spatial learning, which is then

impaired by blocking NMDA receptors or by saturating long-term potentiation without impairing memory recall in the task (Bannerman et al., 1995; McNamara and Skelton, 1993; Morris et al., 1986; Moser et al., 1998). The reliability and replicability of the MWM across studies, as well as its utility across a range of species (rats, mice and even humans (virtual maze, Kallai et al., 2005)) have led to its extensive use in learning and memory research (Vorhees and Williams, 2006). The MWM has been greatly useful for investigating how specific factors, such as drug interventions, lesions, age, sex and animal models of disease (transgenic etc.) influence spatial learning (D'Hooze and De Deyn, 2001; Morris et al., 1982; Sutherland et al., 1983; Vorhees and Williams, 2006).

In order to assess whether performance in the task is specific to spatial distal cues or due to non-spatial factors, it is important that certain control measures are utilised efficiently. Cued trials involve making the platform visible to the animals to assist them in locating the platform. This is achieved either by elevating the platform above the surface of the water or by using a marker or 'flag' (Morris et al., 1982; Vorhees and Williams, 2006; Williams et al., 2003). Cued trials can, and are, commonly used to determine whether there are differences in the ability to use distal cues to locate the escape platform (e.g. due to differences in the visual system between the two groups), or whether non-spatial factors such as motivation to escape the water may be influencing performance (Morris et al., 1982; Vorhees and Williams, 2006; Williams et al., 2003). Importantly cued trials involve the same abilities utilised in the rest of the task, such as eyesight, swimming capacity, motivation to perform the task and strategies used to locate the platform, both with regard to swim paths and climbing onto the platform itself. However, these trials do not require the subjects to use distal cues to locate the platform and are therefore regarded as hippocampal independent (Lipp and Wolfer, 1998; Vorhees and Williams, 2006). Cued trials can be performed before or after the task, however, there is evidence to suggest that performing the cued trial prior to training is beneficial as animals are often highly activated when they are

first tested in this task and exposure to the maze in cued trials assists the animals in identifying the platform as an escape route (Vorhees and Williams, 2006).

In addition to cued trials, thigmotaxis behaviour, or an animals tendency to stick to the edge/walls of the maze, can be quantified and used as a measure of sensorimotor interference (Cain et al., 1996; Saucier et al., 1996). Animals often adopt this kind of behaviour when first exposed to a new environment, however, in most cases animals quickly learn to move away from the wall in order to seek alternative possible escape routes. It may be interpreted that those animals which consistently show this thigmotaxic behaviour may have sensorimotor deficits and so are not focussing on the task appropriately (Vorhees and Williams, 2006). Animals that do not climb onto the platform, do not stay on or instead swim over the platform, may also have increased levels of sensorimotor interference as these behaviours suggest a reduced association that the platform is an escape route (Vorhees and Williams, 2006). As previously mentioned, the use of cued trials may reduce the occurrence of these behaviours, especially if performed prior to spatial training, as it teaches the animals the basic task requirements (Vorhees and Williams, 2006).

5.1.2 Sleep and spatial learning

Many studies have investigated the link between sleep and neurobehavioural measures of spatial learning and memory. These studies can utilise either total sleep deprivation or sleep restriction techniques (reviewed in McCoy and Strecker, 2011). In one study total sleep deprivation 6 hours prior to behavioural testing, was found to have no effect on spatial learning, while it impaired spatial memory 24 hours after the initial training session (Guan et al., 2004). Similar effects were observed in a more extreme sleep deprivation study. Here rats were chronically sleep deprived for 18 hours a day for 5 days, with a 6 hour sleep opportunity provided from light onset followed immediately by training in the Morris water maze. Animals

had intact learning of the MWM but impaired recall in the probe trial (McCoy et al., 2013). Interestingly, while these studies did not observe effects on spatial learning, mild sleep restriction of either 6 hours SD for 4 days (Hairston et al., 2005) or 4 hours SD for 7 days (Yang et al., 2012) impaired spatial learning in the MWM task. Notably this was only identified in adolescent and not adult mice in the latter study (Yang et al., 2012).

The highly variable results identified in these studies highlight the complexity of the interaction between sleep and learning. Most studies to date utilised repeated sleep deprivation/restriction techniques, however these were variable in duration and as to whether behavioural testing occurred before or after sleep deprivation. In this study I performed sleep deprivation for 6 hours prior to testing in the MWM. 6-hours was selected based on previous studies ran by the research group, as well as to match the data presented in Chapter 3. In addition, there are a number of studies that have shown 6 hours of sleep deprivation is sufficient to cause a rebound in SWA, a well-established marker of sleep homeostasis (Chapter 3, and also (Cui et al., 2014; Fisher et al., 2016; McKillop et al., 2018; Vyazovskiy et al., 2007b, 2009b). Importantly 6 hours of sleep deprivation in rats has also been shown to increase the incidence of so-called 'local sleep' and have detrimental effects on a cognitive task, a pellet reaching task (Vyazovskiy et al., 2011).

The performance of rats in the Morris water maze has been shown to be highly reliable and individuals have consistent performance across trials, despite large variability between animals (Ingram, 1996; Lindner, 1997). The Morris water maze has been shown to be a useful model for assessing the age-related decline in spatial related changes in cognitive function (Lindner, 1997; Vorhees and Williams, 2014, 2006). Although it is generally thought that spatial learning is intact across ageing, there is also conflicting evidence that ageing may also lead to a gradual decline in spatial learning navigation accuracy in the Morris water maze task in rats, as determined by an increase in path length with increasing age (Lindner, 1997). Both human and rodent studies have

identified age-dependent deficits in spatial working memory, the latter utilising an adaptations of the MWM, and have shown the although perception of spatial relationships are often intact when a spatial memory component is added into the equation, older individuals have substantial deficits in their performance (Lester et al., 2017). This has led to suggestions that age-dependent differences in performance in the MWM may reflect a combination of deficits in learning, memory and attention, as well as in the processing of spatial information into searching strategies (Lindner, 1997).

5.1.3 Experimental aims

The aim of this chapter was to address objective 3 of the thesis: ***‘To determine the effect of ageing and sleep on behavioural performance in a hippocampal dependent task: the Morris water maze’***. Although studies have investigated the role of sleep in learning and memory, as well as how learning and memory is affected by ageing, few studies have linked the ageing and sleep effects. The overall aim of this experiment was to investigate the effect of ageing on spatial learning and memory and to determine the role sleep may play in this. In order to assess this, mice were sleep deprived and their spatial memory for the previously learned platform location was tested. The study aimed to address three main questions; 1: does ageing affect learning a spatial task? 2: can old mice remember the location of the platform when it is removed? 3: does sleep deprivation impair spatial memory, and is this age-dependent?

5.2 Methods

5.2.1 Experimental animals

Experiments were carried out in 50 male C57BL/6J (RccHsd) mice subdivided into two age groups as per previous conventions: early adulthood (EA) aged 9 weeks old and late adulthood (LA) aged 51.9 weeks old. The experiment was carried out in two batches of n=25 animals. Animals

were housed under a standard 12:12 h light–dark cycle (lights on 07:00, ZT0) with a stable temperature and humidity. Mice were group housed where possible, with the exception of cases where separation was required due to fighting. Food and water were available ad libitum for the duration of the experiment, however old mice were on a food restricted diet for the 6 months leading up to the experiment due to them being overweight. In the case of the 12-month-old mice, these were aged at Agenda’s Hatfield facility for 12 months, prior to their arrival at Eli Lilly. All procedures conformed to the Animal (Scientific Procedures) Act 1986 and were performed under a UK Home Office Project Licence in accordance with the Lilly UK Institutional Animal Care and Use Committee guidelines. All studies are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath and Lilley, 2015).

5.2.2 Water maze design

Experiments were performed in a designated procedure room in the animal facility at Eli Lilly. The water maze consisted of a white fibreglass circular pool 200cm in diameter and 60cm deep. The pool was filled with standard tap water up to approximately 50cm in depth (~1500 litres), and heated to $21\pm 2^{\circ}\text{C}$. Although substances are often added to the water to make it opaque, this has been shown not to be necessary and so was not done in this experiment. The pool was labelled with reference points, North, East, South and West, with spatial cues positioned at each compass point. These coordinates were used to subdivide the pool into four quadrants in analyses, NE, NW, SE and SW. Figure 5-2 shows a schematic of the room layout, with spatial cues labelled. During trials animals were monitored in an ante room separated from the maze room by a PVC curtain, in order to avoid providing additional spatial cues or distractions to the animals. A Sanyo CCD camera was positioned directly above the pool in order to track movement, which was acquired using Ethovision software (v12.0, Noldus). For the training phase of the experiment a platform approximately 15cm in diameter was placed into the pool one of four quadrants, moved at random to the various quadrants. The platform was centred in the

quadrant using lasers and a computer to visualise the exact location of the platform. This ensured that the platform was positioned the same across all trials.

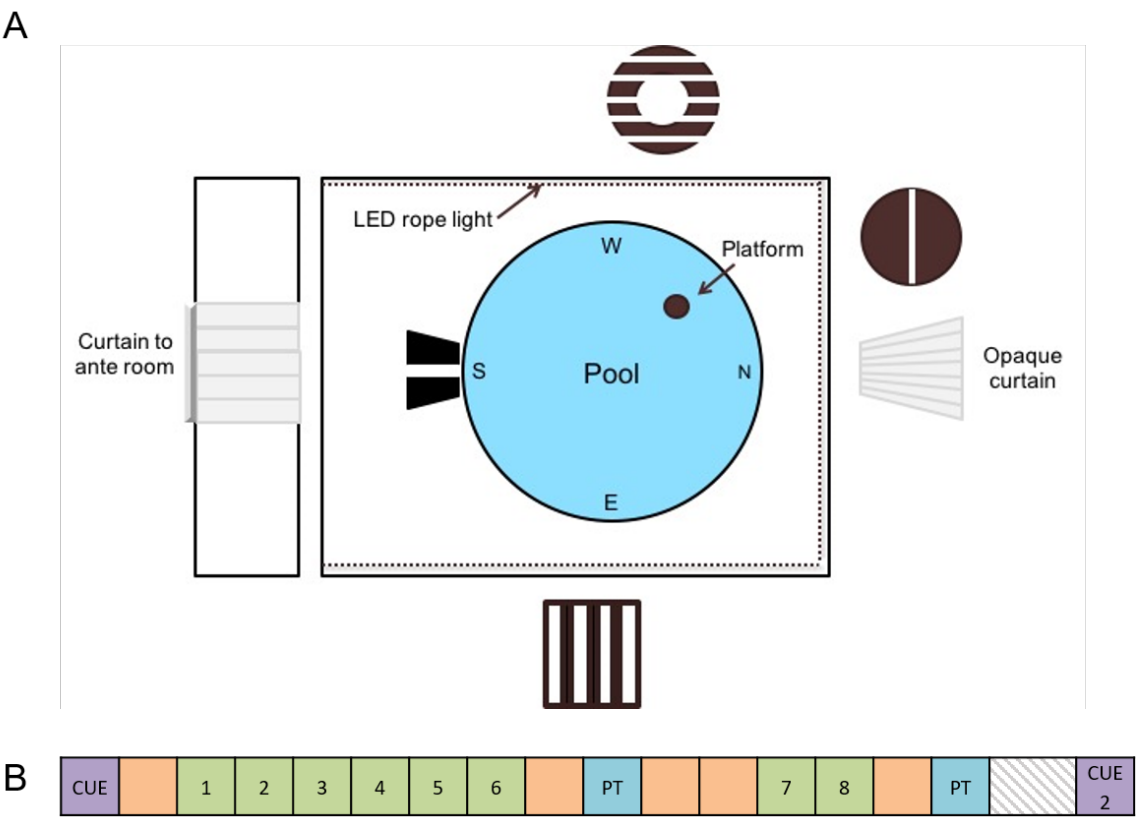


Figure 5-2:Experimental design. (A) Schematic of the room and maze design. Note: Spatial cues (made from foamex) are located on each wall around the maze, while a PVC curtain divides the maze and ante rooms. An additional opaque curtain is located on the Northern wall, though this was not used in this study. In this example the escape platform is located in the north-western (NW) position. (B) An overview of the experimental protocol. CUE (purple): visual cued trial where the platform is visible to the animals. Orange: rest days where no training or testing occurred. All animals had one day of rest prior to the probe trials and 2 days of rest after the initial probe trial to allow for recovery from sleep deprivation. Green: training days, where the number represents how many days training the animals have received. PT (blue): Probe trials, where the platform was removed. The main protocol (left of the gratings in grey) was carried out twice, once for each batch of animals. A second cue trial was performed one week after both batches had completed the full experimental protocol (right of gratings in grey).

5.2.3 Experimental protocol

The experiment consisted of three components; cued trials, a training phase and a testing phase. This is summarised in (Figure 5-2), with full details provided below. All parts of the experiment were carried out at approximately 1pm each day, in order to test at around the same circadian

time (ZT-6). This time was chosen to match the time at which the task would be completed after sleep deprivation (see testing phase below).

Cued trial

Prior to the start of training all mice underwent a single trial (60 seconds duration) in which the escape platform was elevated above water level by ~1-5mm (i.e. visible cued trial), as is a standard procedure in our group, and has been done previously (Williams et al., 2003). This was done to determine whether there are differences in the ability to use cues to locate the escape platform (e.g. due to differences in the visual system between the two age-groups), but also whether there were differences in motivation to escape the water. The cued trial was performed prior to the start of training with the aim to reduce stress associated with the task. Each animal only underwent one visual cued trial, as I did not wish to dramatically affect the learning of the task with the hidden platform.

As there was a low success rate (EA: 40.91%; LA: 34.78%) in finding the platform during the initial cued trial (see discussion for more details), a second visual cued trial was performed at the end of the experiment, after all training days and both probe trials were completed. This was crucial for enabling learning using spatial distal cues to be distinguished from non-spatial factors such as eyesight and motivation. In this trial the platform remained submerged but a flag raised above the water was instead used to mark the position of the platform, to increase the visibility of the platform, and with it the efficiency of locating the platform (Vorhees and Williams, 2006). As poor performance in the initial cued trial was unexpected, this second cued trial was not part of the initial protocol and was therefore performed one week after both batches of animals had completed the full experiment. Although the first batch had longer between finishing the experiment and having the second cued trial, both batches performed the cued trial with a high success rate (EA: 90.91%; LA: 100%).

Training phase

During training, the platform was placed in the pool so that it was just under the water level (i.e. hidden from view). Mice were trained to find the escape platform in the pool for a total of 6 days (4 trials per day, starting at each compass coordinate, pseudo randomly ordered). The target platform location was pseudo randomly assigned between groups in order to counterbalance for possible platform bias or quadrant effects, but kept the same for all trials for an individual animal in this experiment. During a trial the mouse was placed into the water at one of the compass points and learned to locate an escape platform in the water based on distal visual cues located around the room. In a single trial mice were placed in the water one at a time at one of the four compass points of the pool at random. Upon entering the water, movement was automatically tracked using the overhead camera. Mice were allowed to swim for a maximum duration of 90 seconds, or until they found the platform (trials automatically stopped tracking once mice remained on the platform for 3 seconds). If the platform was not found, animals were guided to the platform and left undisturbed for 10 seconds. This is based on evidence in rats that spatial learning is best acquired during the middle portion of the trial while en-route to the platform (Sutherland et al., 1987). Animals were left on the platform for 10 seconds at the end of each trial, regardless of whether they found the platform themselves or were guided there at the end of an unsuccessful trial. After completion of 4 trials, animals were placed in a heating chamber maintained at 30°C, and monitored until dry and able to return to their home cage.

Testing Phase – Probe Trial

After 6 days of training (4 trials in succession per day, per animal), all mice were given a rest day in which no training was performed but during which half the animals were transferred to cylindrical wheels where they were housed overnight. The other half remained housed in their home cages undisturbed. Assignment to sleep/sleep deprivation was stratified based on

performance during training days 4-6 (as it may be expected that animals have a good understanding of the platform location by this point). Those animals that were moved to the wheels then underwent sleep deprivation for 6 hours from light onset (7am-1pm). Sleep deprivation was performed by random, intermittent, wheel turning (see section 2.6 for details). After 6 hours of sleep deprivation, mice then underwent a probe trial in the water maze, in which the platform was removed from the pool. This therefore tested the spatial memory for the platforms previous location, using the spatial cues in the room. Each animal only had one probe trial, 60 seconds in duration. In contrast to the training phase, mice were placed in the tank in the opposite quadrant to the platforms previous position. As the study was a cross-over design, all animals also received the reverse sleep/sleep deprivation condition, 5 days after the initial test day (after 2 rest days followed by two days of retraining and a further rest day). Notably, although referred to as the sleep-condition it is not known how much the animals slept, just that all the animals in this condition had the opportunity to sleep.

5.2.4 Sleep deprivation procedure

Sleep deprivation was performed using a non-invasive enforced activity protocol in which mice were singly housed in a cylindrical chamber that randomly turned around its axis for 8 seconds to initiate the righting reflex and thus awaken the animals, as previously established (McCarthy et al., 2017). Full details of the sleep deprivation protocol are provided in the general methods chapter (section 2.6).

5.2.5 Specific analyses

For the spatial learning element of the task, I analysed the trial duration, latency to the platform, distance travelled, swim speed, thigmotaxis and immobility averaged across the four trials on

each of the 6 training days and two additional training days between the probe trials (days 7 and 8). Definitions of each measure are provided below:

Trial duration: time taken for subject to complete the task, starting from pool entry and ending after remaining on the platform for 5 seconds, measured in seconds.

Latency to platform: time taken for subject to reach the platform, starting from pool entry and ending immediately after reaching the platform, measured in seconds.

Distance travelled: the total distance travelled during a trial, measured as cm/trial.

Swim speed: the average speed travelled during a trial, measured as cm/seconds.

Thigmotaxis: total time spent within the border zone (10cm from the edge of the pool) during a trial, measured in seconds.

Immobility: total time during which the software has detected that the region of interest (mouse head) is greater than 10% of the average area travelled during that trial i.e. if the mouse is immobile it will not cover much distance and therefore the area of the head will be larger than 10% of the average area travelled during that trial.

As the escape platform is absent in the probe trials, alternative measures are instead used to quantify memory for the previous location of the platform. These often relied on dividing the tank into four equal quadrants: the target (where the platform was previously located), opposite, adjacent left and adjacent right quadrants. Definitions of the measures used to analyse the probe trial are provided below.

Time in target quadrant: total time spent within the target quadrant, measured in seconds.

Distance to zone (or quadrant): the cumulative proximity of each mouse to the previous platform position, captured at the video capture rate (12.5 fps), and summed per second across the trial, measured as mm/second.

Latency to platform: as above, but instead no platform is present and so the measure is based on the theoretical previous platform location.

5.2.6 Statistical analysis

Raw data was acquired using Ethovision software (v12.0, Noldus) and then imported to MS Excel for analysis. Data were analysed using Microsoft excel and SigmaPlot 14.0 and SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp) was used for statistical testing. All values reported are mean $\pm$ s.e.m. 5 animals were removed from analysis as they displayed distress during training, unwillingness to remain on the platform, extreme thigmotaxic behaviour or, in one case, did not complete the experiment. For training days, the 4 trials completed by each animal were averaged. Exact numbers of animals used in each analysis are provided in the appropriate figure legends. Repeated measures ANOVAs were used to determine differences across the 8 training days (days 1-6 before first probe and 7-8 between probe trials). Normality of data was checked prior to statistical testing. If sphericity testing was significant then Greenhouse-Geisser values are instead reported. Pearson's correlation analyses were used to identify correlations in the data, with Pearson r values and p values reported. In some cases paired and unpaired t -tests were used to test for significant differences between conditions and age groups, respectively. Two-way ANOVAs (univariate analysis of variance) were used to analyse the probe trials, where age was the between subjects factor, and sleep and sleep deprivation were the within subject variable.

5.3 Results

5.3.1 Spatial learning in the MWM task

As a first step, this study aimed to determine whether ageing has a significant effect on learning. Trial duration (or escape latency), significantly decreased across the initial 6 days of training (Factor Day= $F(7, 301)=34.92$, $p<0.0001$, repeated measure ANOVA, Figure 5-3A), with days 1 and 2 being significantly different to training days 4-8 ($p<0.0001$ for all except day 2 vs day 4 $p=0.001$). This decrease was not significantly different between age groups (Factor

Age= $F(1,43)=0.26$, $p=0.61$, repeated measures ANOVA), with both age groups reaching an average trial duration of 36.26 ± 3.56 and 34.67 ± 3.15 seconds at the end of the 6 days of training for EA and LA mice, respectively (Figure 5-3A). Importantly, there was no significant difference between the trial duration on days 5, 6, 7 and 8. As some younger animals jumped off the platform and so the trial did not automatically time out this could bias the data towards longer trial durations. In addition, older animals sometimes had difficulty getting onto the platform. As it is likely that in both these cases the animals did in fact know the location of the platform but for one reason or other could not get onto or stay on the platform, I instead calculated the latency to the platform, as this measure instead calculates the time it takes to initially make contact with the platform. Calculating the latency to reach the platform yielded very similar results to the average trial duration, with average latency decreasing over training days (Day= $F(7,301)=33.06$, $p<0.0001$, repeated measure ANOVA), but they were similar between age groups (Age= $F(1,43)=0.01$, $p=0.91$, repeated measures ANOVA, Figure 5-3B). The average latency to reach the platform was 30.49 ± 3.55 and 30.61 ± 3.32 seconds at the end of the 6 days of training for EA and LA mice, respectively (Figure 5-3B). The average latency to reach the platform by day 6 was significantly shorter than the average trial duration for both EA and LA mice ($p<0.0001$ for both). Due to the increased accuracy of the latency to platform measure, I have used this in further analysis rather than the trial duration.

Next I calculated the distance travelled per trial, as this is a common measure for quantifying learning in the water maze (Figure 5-3C). In this study there was a significant reduction in distance travelled over training days (Day= $F(7,301)=27.01$, $p<0.0001$, repeated measures ANOVA), which was accounted for mainly by significant differences between Days 1-3 and Days 4-8 by at least $p<0.05$ (day 1 vs 4, 5, 6, 7 and 8; $p<0.0001$). The reduction in distance travelled may be expected as mice learn the position of the platform and therefore travel a shorter distance to reach it. There was also an effect of age on the average distance travelled during a

trial in the maze ($\text{Age}=F(1,43)=7.40$, $p=0.009$, repeated measures ANOVA, Figure 5-3C).

Significant age-dependent differences were only present on Days 1 ($p<0.0001$) and 3 ($p=0.002$) of training. During the first three days the average distance travelled per trial was $1340.82\pm49.89\text{cm}$ for EA mice, compared to $1016.42\pm41.39\text{cm}$ for LA mice (Figure 5-3C). By day 4 both age groups had reduced to near identical distances (EA: $872.31\pm86.16\text{cm}$; LA: $839.99\pm57.96\text{cm}$) which remained for the rest of the experiment.

As distance travelled would be greatly influenced by swim speed, which in itself has been shown to be affected by ageing, I next quantified swim speed. Swim speed was found to be significantly different across training days ($\text{Day}=F(5,219)=4.36$, $p=0.001$, repeated measures ANOVA, Figure 5-3D), however pairwise comparisons revealed that significant differences were only apparent for comparisons between Day 3 vs 6 ($p=0.001$), Day 4 vs 6 ($p=0.04$) and Day 5 vs 6 ($p<0.0001$). There were no significant differences between the swim speed on Day 6 and during Days 7 and 8. Importantly in this study, a considerable, and significant reduction in swim speed was identified with ageing ($\text{Age}=F(1,43)=47.67$, $p<0.0001$, repeated measures ANOVA). Swim speed was on average $14.20\pm0.56\%$ lower in LA compared to EA mice when averaged across the 8 training days (EA: $18.51\pm0.18\text{ cm/s}$, LA: $15.88\pm0.14\text{ cm/s}$, $p<0.0001$, unpaired t-test, Figure 5-3D). As swim speed was significantly reduced in older mice, this warrants caution when interpreting data involving distance calculations, as when mice are swimming slower it would be expected that they may cover a smaller distance in the same maximally allowed time.

In addition to EA mice having a higher swim speed, they also spent more time in thigmotaxis ($\text{Age}=F(1,43)=4.8$, $p=0.035$, repeated measures ANOVA), which again reduced across training days ($\text{Day}=F(2,105)=51.7$, $p<0.0001$, repeated measures ANOVA) as mice learnt the location of the platform (Figure 5-3E). On Day 1 EA mice spent on average 31.45 ± 4.46 seconds in thigmotaxis while this was only 26.02 ± 3.38 seconds in LA mice (equating to a 17.26% difference

between age groups). This was reduced to 6.21 ± 1.15 seconds and 2.16 ± 0.86 seconds by day 6 of training, for EA and LA mice respectively (equating to a 65.18% difference between age groups). Coinciding with this, the total amount of time spent immobile was not significantly different between age groups (Age= $F(1,43)=2.73$, $p=0.11$, repeated measures ANOVA, Figure 5-3F). A significant day effect was noted for immobility (Day= $F(4,176)=2.80$, $p=0.03$, repeated measures ANOVA), however this could be explained by differences between day 3 and days 5 ($p=0.001$) and day 6 ($p=0.012$). It should be noted that this data was not normally distributed so data were first log transformed before running the repeated measures ANOVA. Values quoted and graphically represented correspond to non-logged values.

An important confound of this study is that the two age groups of mice had significantly different body weights (EA: 22.02 ± 0.29 g; LA: 35.45 ± 0.44 g; $p < 0.0001$ unpaired ttest). Notably, body weight was significantly negatively correlated with swim speed (Figure 5-4A) both at the beginning of training on day 1 ($r=-0.70$, $p < 0.0001$, Pearson's correlation), and also after 6 days of training ($r=-0.56$, $p=0.0001$, Pearson's correlation). However, when correlations between weight and speed were calculated for the two age groups separately, a significant correlation was only found for the LA mice during day 1 (Day 1: EA $r=-0.36$, $p=0.10$, LA: $r=-0.49$, $p=0.02$; Day 6: EA $r=-0.19$, $p=0.40$, LA: $r=0.09$, $p=0.69$). As older animals were found to swim at a slower speed than the younger mice, it may be expected that they also travel less distance. There was a strong positive correlation between swim speed and distance travelled in a trial (Figure 5-4B) both on day 1 of training ($r=0.52$, $p=0.0003$, Pearson's correlation) and after 6 days of training ($r=0.57$, $p=0.0001$, Pearson's correlation). Therefore, differences identified between age groups may be the result of age-dependent weight differences. However, it should be noted that the differences in swim speed may also be the result of other age-related factors not tested here, such as muscle strength.

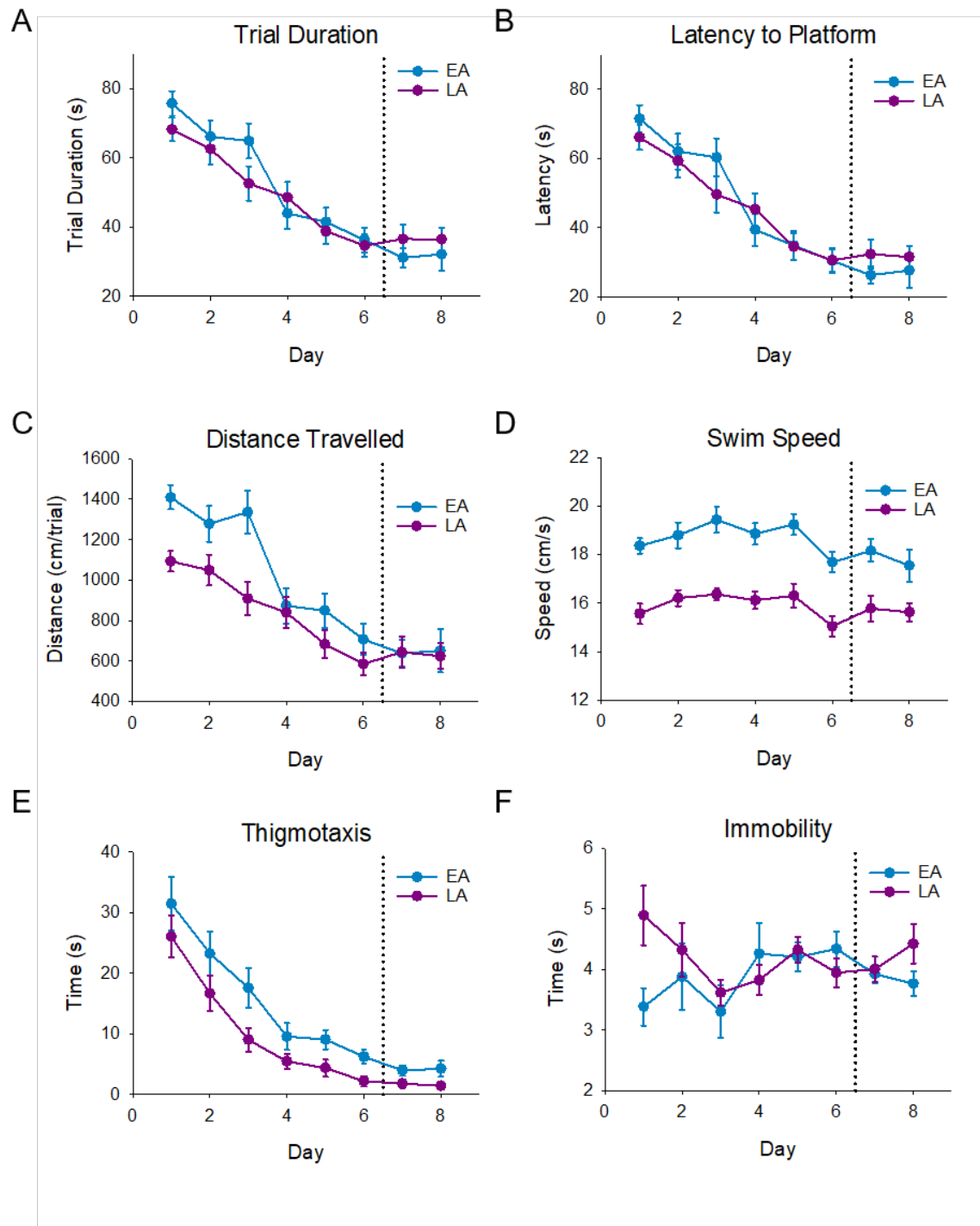
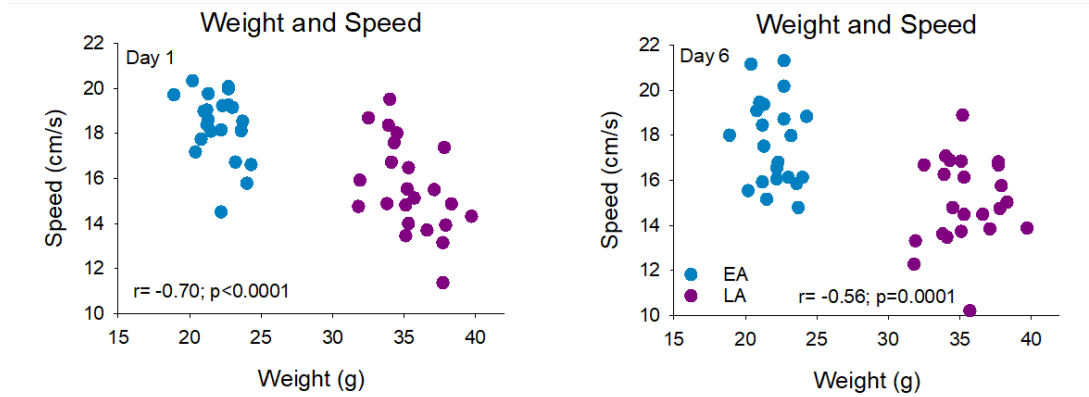


Figure 5-3: **The effect of ageing on spatial learning.** The average trial duration (A), latency to platform (B), distance travelled (C), swim speed (D), thigmotaxis (E) and time immobile (F) are plotted for the 6 days of training in the water maze task, and the two additional days of training (days 7 and 8) between the probe trials. For each day, the 4 trials are averaged per animal and then across animals within an age group. Data are mean values, SEM, $n=22$ (EA) and $n=23$ (LA).

A



B

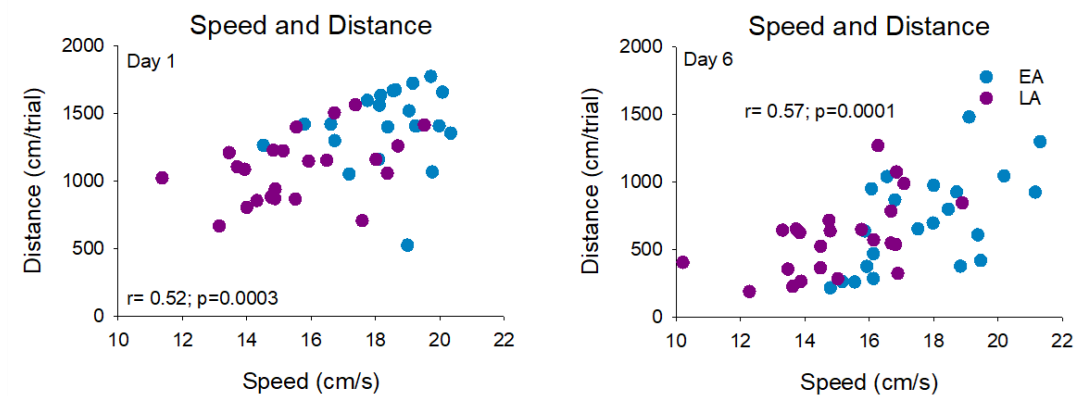


Figure 5-4: **Correlation between weight, speed and distance.** (A) Correlation between weight and speed. (B) Correlation between speed and distance travelled. Left panels represent data from day 1 and right panels represent data from day 6 of training. Data are mean values, SEM. Pearson correlation coefficients and p values are reported within each panel. EA n=22 LA: n=23.

5.3.2 Spatial memory in the MWM task

Next the probe trials were investigated. Firstly, trials were separated by age and according to whether the animals were allowed to sleep or were sleep deprived prior to the probe trial, and the two probe trials were then compared (Figure 5-5). Therefore, there were four groups used for analyses; early adulthood and allowed to sleep (EA_S), early adulthood and sleep deprived (EA_SD), late adulthood and allowed to sleep (LA_S) and late adulthood and sleep deprived (LA_SD). A one-way ANOVA comparing the two probe trials for each of the four groups, did not reveal any significant differences for the time to target quadrant (EA_S= $F(1,21)=0.220$, $p=0.644$; EA_SD= $F(1,23)=0.059$, $p=0.397$; LA_S= $F(1,22)=0.059$, $p=0.811$; LA_SD= $F(1,22)=0.277$, $p=0.604$,

Figure 5-5A) and the distance to zone (EA_S= $F(1,21)=1.034$, $p=0.321$; EA_SD= $F(1,23)=1.877$, $p=0.186$; LA_S= $F(1,22)=0.003$, $p=0.959$; LA_SD= $F(1,22)=0.109$, $p=0.744$, Figure 5-5B). As the latency to platform data was not normally distributed, due to the high number of trials in which the mice did not cross the exact platform location, a Kruskal Wallis non parametric test was instead used for this measure, which did not identify any significant differences between the two probe trials (EA_S = $\chi^2(1)=0.743$, $p=0.415$; EA_SD = $\chi^2(1)=2.216$, $p=0.144$; LA_S = $\chi^2(1)=1.031$, $p=0.331$; LA_SD = $\chi^2(1)=0.486$, $p=0.506$, Figure 5-5C).

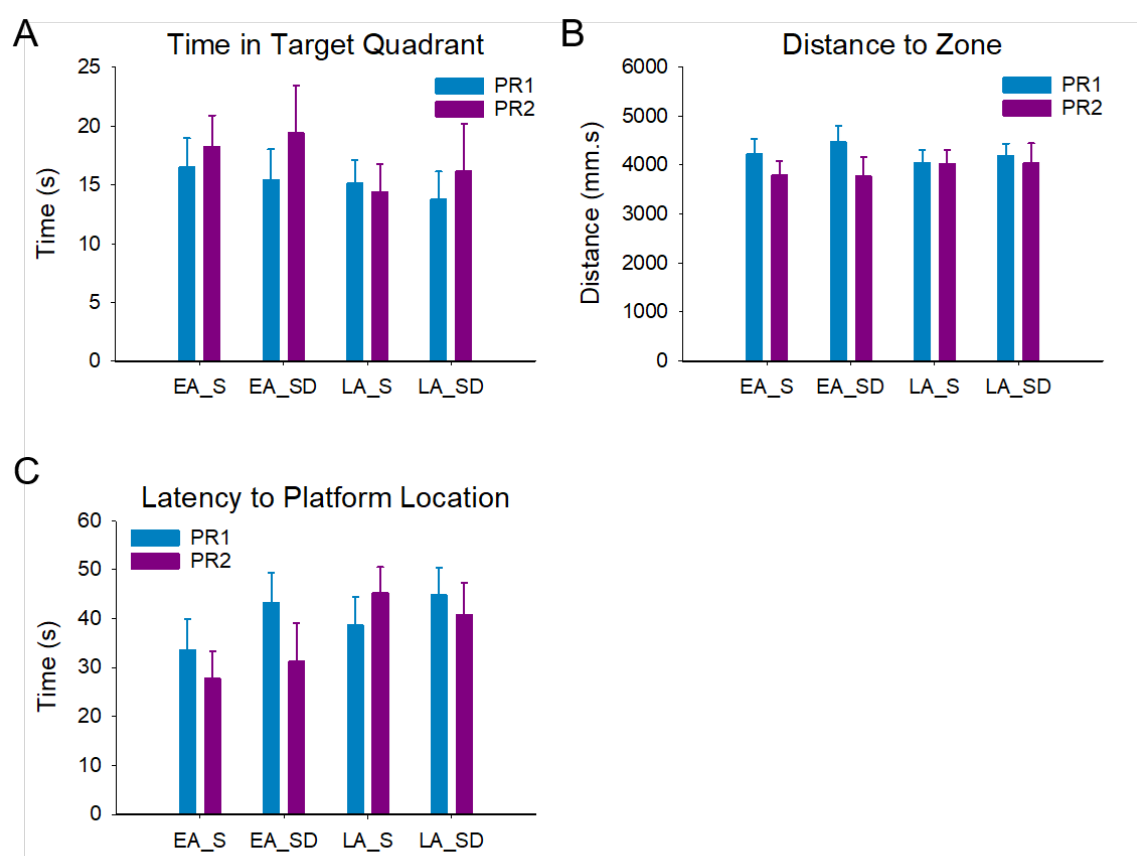


Figure 5-5: **Differences between the probe trials.** The time in the target quadrant (A), distance to zone (B) and latency to platform (C) are plotted for both probe trials. Groups were separated by age (EA or LA) and experimental condition (S: sleep, SD: sleep deprivation). Data are mean values, SEM. EA_S: $n=10$ and 12 , EA_SD: $n=12$ and 10 , LA_S: $n=11$ and 12 , LA_SD: $n=12$ and 11 for probes 1 and 2, respectively.

This study was ran in a crossover design, to enable the two probe trials to be combined and therefore increase the numbers of animals in each group. Although some variability may be expected, no significant differences were detected between the probe trials in this study (as

predicted by previous data collected by other members of our group) and so probe trials were combined giving group sizes of EA_S: n=22, EA_SD: n=22, LA_S: n=23 and LA_SD: n=23.

Sleep deprivation had no influence on the time in the target platform quadrant (Figure 5-6A), resulting in near identical times for both conditions, in both age groups (EA_S: 17.46 ± 1.80 ; EA_SD: 17.23 ± 2.29 ; LA_S: 14.72 ± 1.56 ; LA_SD: 14.90 ± 2.26 seconds; age= $F(1,86)=1.61$, $p=0.21$, condition= $F(1,86)=0.00$, $p=0.99$, age*condition= $F(1,86)=0.01$, $p=0.92$, two-way ANOVA). There were also no significant differences in the distance to the zone which was EA_S: 3988.55 ± 212.38 mm.secs; EA_SD: 4149.65 ± 260.74 mm.secs; LA_S: 4024.36 ± 192.54 mm.secs; LA_SD: 4119.36 ± 228.12 mm.secs (age= $F(1,86)=0.00$, $p=0.99$, condition= $F(1,86)=0.33$, $p=0.57$, age*condition = $F(1,86)=0.02$, $p=0.88$, two-way ANOVA, Figure 5-6A).

Previous evidence suggests that quadrant preference decreases after the first 30 seconds in rats (Blokland et al., 2004), therefore I next analysed the time spent in the target quadrant and the distance to the platform for the first 30 seconds of the probe trial only. In the first 30 seconds of the probe trial, the time spent in the target quadrant was not significantly different between age groups or experimental conditions (age= $F(1,86)=0.10$, $p=0.76$, condition= $F(1,86)=0.59$, $p=0.44$, age*condition= $F(1,86)=0.03$, $p=0.87$, two-way ANOVA), which was on average: EA_S: 8.13 ± 1.28 ; EA_SD: 7.12 ± 1.23 ; LA_S: 7.43 ± 1.02 ; LA_SD 6.94 ± 1.12 seconds (Figure 5-6B). Note a folded log transformation was performed on this data prior to running the ANOVA as the data was not normally distributed. The cumulative distance to the zone (target quadrant) in the first 30 seconds was also not significantly different between age groups or experimental conditions (age= $F(1,86)=0.01$, $p=0.93$, condition= $F(1,86)=1.57$, $p=0.21$, age*condition= $F(1,86)=0.01$, $p=0.93$), which was on average: EA_S: 1988.26 ± 120.87 ; EA_SD: 2154.00 ± 139.03 ; LA_S: 1987.16 ± 105.90 ; LA_SD 2131.60 ± 127.38 mm.sec (Figure 5-6B).

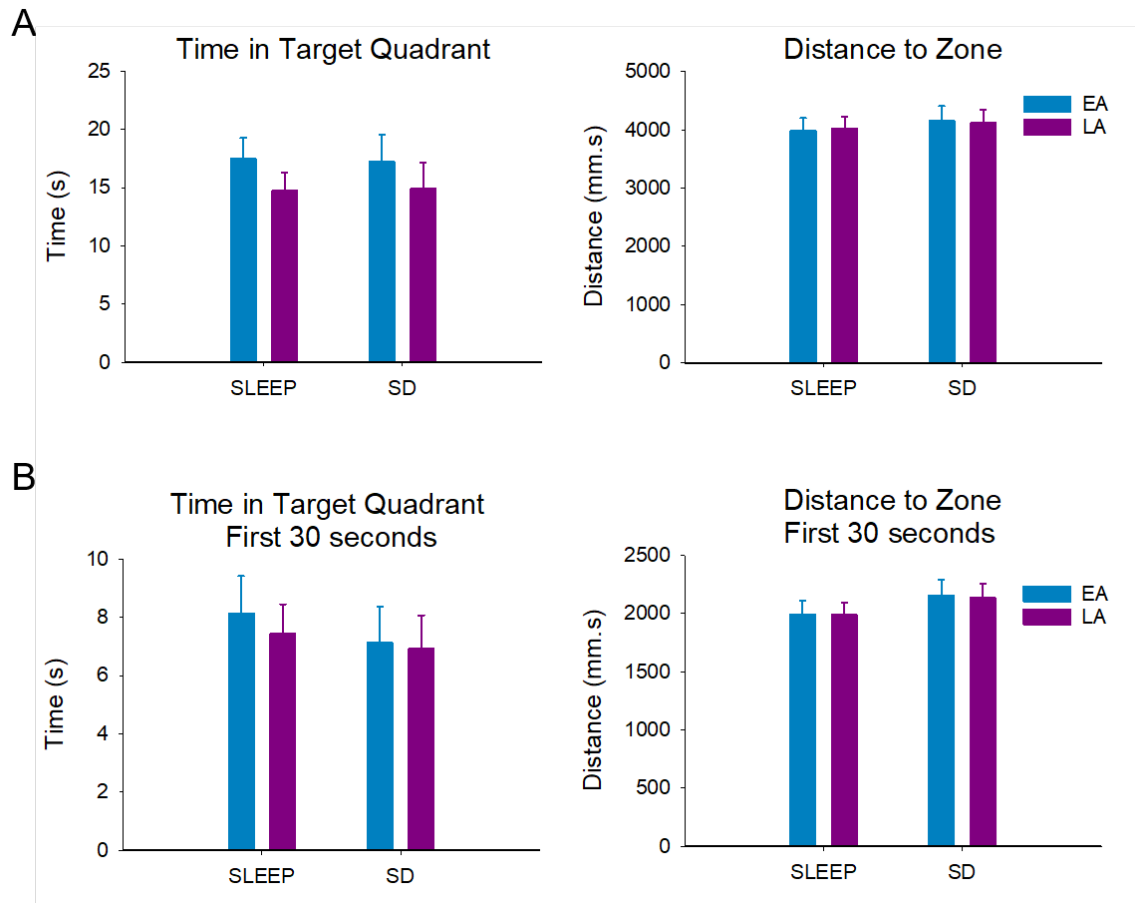


Figure 5-6: **The effect of ageing and sleep deprivation on spatial memory.** The average time in the target quadrant and distance to zone (quadrant) calculated separately for sleep and sleep deprivation conditions, are shown for full 60 seconds of the probe trial (A) and for the first 30 seconds of the probe trial (B). Data are mean values, SEM. EA_S: n=22, EA_SD: n=22, LA_S: n=23, LA_SD: n=23.

The latency to the exact previous platform location is also commonly calculated as a measure of spatial navigational memory. For EA mice the latency to the exact platform location was higher after SD compared to the sleep condition (EA_S: 30.34 ± 4.21 seconds, EA_SD: 37.75 ± 4.91 seconds, Figure 5-7A), though this was not significant (paired t-test $p=0.23$). The latency to platform was undistinguishable in both experimental conditions for the LA age group (LA_S: 42.04 ± 3.89 seconds, LA_SD: 42.80 ± 4.27 seconds, paired t-test $p=0.88$, Figure 5-7A). Interestingly a significant age difference was found for latency to the platform during the sleep condition only, which was on average 11.69% higher in LA mice (unpaired t-test: Sleep $p=0.05$, SD $p=0.36$, Figure 5-7A).

As merely calculating measures of learning for the probe trials does not take into account how well each animal performed during the training days, next the difference between the last training day and the following probe trial (day 6 and probe 1 or day 8 and probe 2) was calculated for the four groups (Figure 5-7: B). As the training trials were a maximum of 90 seconds, whereas this was only 60 seconds for the probe trial, in those cases where the average latency to the platform exceeded 60 seconds these were instead set to 60 seconds to match the probe trial (7 cases). These animals were not removed from analyses as this would lead to a misrepresentation of the data. This data was not expressed as a percentage of total time as this would overestimate the latency during the probe trials where the maximum trial length was shorter. There was a significant difference in the latency to the platform between the last training day and the probe day for LA mice for the SD condition only (LA_S: $p=0.10$, LA_SD: $p=0.04$, t-tests corrected for multiple testing) but not for EA mice (EA_S: $p=1.17$, EA_SD: $p=0.45$, t-tests corrected for multiple testing). However, calculating the difference between the last training day and probe day did not identify any overall effects of age or condition (EA_S: 5.08 ± 4.92 , EA_SD: 4.80 ± 4.84 , LA_S: 10.27 ± 4.60 and LA_SD: 12.35 ± 4.79 seconds, $\text{age}=F(1,86)=1.61$, $p=0.21$, $\text{condition}=F(1,86)=0.19$, $p=0.66$, $\text{age} \times \text{condition}=F(1,86)=0.01$, $p=0.94$).

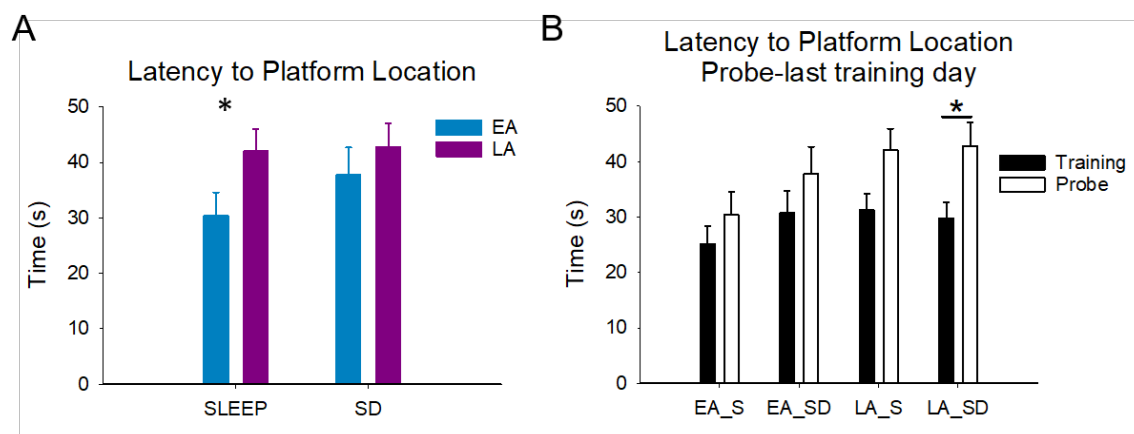


Figure 5-7: The effect of ageing and sleep deprivation on memory for the exact platform location. (A) The average latency to the previous platform location, expressed in seconds. Data are mean values, SEM. Unpaired t-tests used to test for significant age differences, $*p<0.05$. **(B)** The average latency to the platform location on the last training day before the probe trial and the probe trial (day 6 and probe 1 or day 8 and probe 2). EA_S: $n=22$, EA_SD: $n=22$, LA_S: $n=23$, LA_SD: $n=23$. $*p<0.05$, paired t-test.

Next, probe trials were subdivided according to performance, where a single crossing over the exact previous location of the platform counted as a successful trial (i.e. a latency to platform below the maximum 60 seconds duration). On average 77.27%, 54.55%, 60.87%, and 47.83% of the trials were successful for EA_S, EA_SD, LA_S and LA_SD groups, respectively (Figure 5-8A). This suggests that there was a reduced number of successful trials after sleep deprivation, compared to the sleep condition, though a chi squared test did not identify a significant effect of condition ($\chi^2(1)=2.963$, $p=0.085$) or age ($\chi^2(1)=1.252$, $p=0.263$). As the success rate of crossing the previous platform location was fairly low, trials in which the previous platform location was not crossed were removed from analysis, leaving 17, 12, 14 and 11 probe trials to contribute to further analysis, for EA_S, EA_SD, LA_S and LA_SD groups, respectively. Calculating the time in the target quadrant for only successful trials did not reveal any significant differences between age groups or experimental conditions (age= $F(1,50)=0.53$, $p=0.47$, condition= $F(1,50)=0.93$, $p=0.34$, age*condition= $F(1,50)=0.13$, $p=0.72$), which was EA_S: 19.55 ± 1.96 seconds, EA_SD: 22.73 ± 2.77 seconds, LA_S: 18.65 ± 1.51 seconds, LA_SD: 20.11 ± 3.48 seconds (Figure 5-8B). Similarly, the distance to zone (target quadrant) did not reveal any significant differences between age groups or experimental conditions (age= $F(1,50)=0.01$, $p=0.91$, condition= $F(1,50)=0.75$, $p=0.39$, age*condition= $F(1,50)=0.17$, $p=0.69$), which was EA_S: 3720.12 ± 213.61 , EA_SD: 3404.34 ± 291.01 , LA_S: 3592.14 ± 210.14 , LA_SD: 3478.67 ± 284.72 mm.sec (Figure 5-8C). The average latency to the previous platform for only successful trials was EA_S: 21.6 ± 3.01 , EA_SD: 19.14 ± 3.90 , LA_S: 30.44 ± 3.95 and LA_SD: 23.94 ± 3.97 seconds (Figure 5-8D). This was not significantly different between experimental conditions or for the interaction between age and condition (condition= $F(1,50)=1.69$, $p=0.20$, age*condition= $F(1,50)=0.01$, $p=0.93$). Interestingly, a significant age difference was identified for the latency to the first platform crossing (age= $F(1,50)=4.54$, $p=0.04$), with latencies generally higher in older mice. Importantly, this may be explained by a reduction in swim speed with age both during training but also present during the probe trials (age= $F(1,86)=77.42$, $p<0.0001$, condition= $F(1,86)=0.42$,

$p=0.519$, $\text{age} \times \text{condition} = F(1,86) = 0.662$, $p=0.418$), and during only those trials in which the previous location of the platform was crossed (successful trials, $\text{age} = F(1,86) = 77.42$, $p < 0.0001$, $\text{condition} = F(1,86) = 0.42$, $p=0.519$, $\text{age} \times \text{condition} = F(1,86) = 0.662$, $p=0.418$). However, if that were the case age-differences may also be expected when looking at latency to the platform in all probe trials, not just the successful trials.

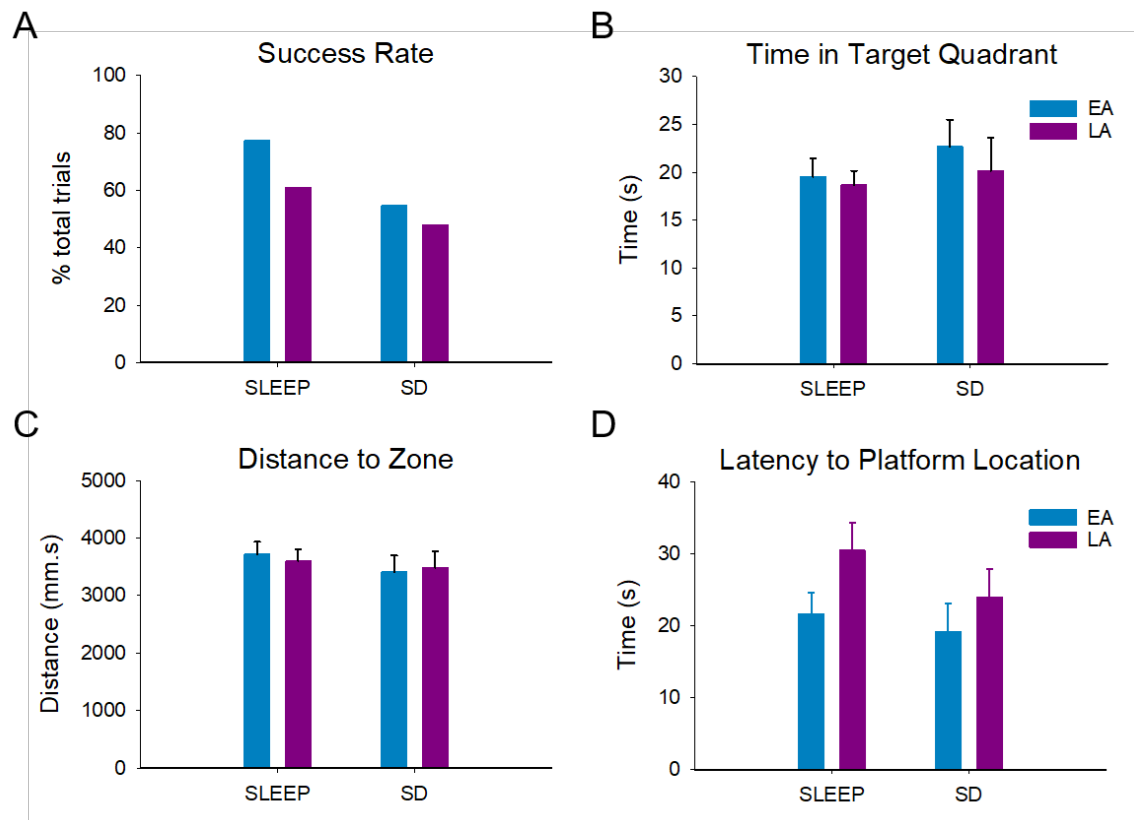


Figure 5-8: Measures of spatial memory were calculated for successful trials only. (A) The success rate of crossing the exact platform location. **(B)** The time in the target quadrant. **(C)** The cumulative distance to zone. **(D)** The latency to reach the previous platform location. Data are mean values, SEM. EA_S: $n=22$, EA_SD: $n=22$, LA_S: $n=23$, LA_SD: $n=23$.

5.3.3 Cued visual Trial

In order to determine whether the effects observed in this study were due to spatial learning or other non-spatial learning influences a cued trial was performed prior to the start of training. In this trial the escape platform was raised just above the level of the water so that it was visible to the mice. There was an unexpected low success rate of the animals locating the platform within the maximum 90 seconds, for EA (40.9%) and LA (34.8%) mice (t -test $p=0.58$, Figure 5-9A). This

may be due to it being the first time both the experimenter and all mice were exposed to the MWM. Taking into account all animals, regardless of whether they found the platform, did not reveal any age differences between EA and LA mice for the latency to platform (EA: 72.93 ± 5.96 secs; LA: 77.21 ± 4.92 secs; t-test $p=0.58$) and the average distance travelled within a trial (EA: 1542.49 ± 130.17 cm; LA: 1523.18 ± 75.90 cm; t-test $p=0.90$, Figure 5-9A).

As the success rate was low I repeated the visual trial after completion of the experiment. In the second visually cued trial the platform was left below the water surface (as in the training sessions), but a 'flag' was instead placed on the platform to increase its visibility. In this second visual trial the accuracy of finding the platform was dramatically increased to 90.91% (2 mice failed to locate the platform) and 100% for EA and LA mice, respectively (Figure 5-9B).

Interestingly, LA mice performed better in the cued trial, as evidence by a reduced latency to the platform (EA: 53.34 ± 7.61 secs; LA: 27.06 ± 3.30 secs; t-test $p=0.002$) and distance travelled during a trial (EA: 1142.29 ± 162.73 cm; LA: 508.31 ± 61.23 cm; t-test $p=0.0006$, Figure 5-9).

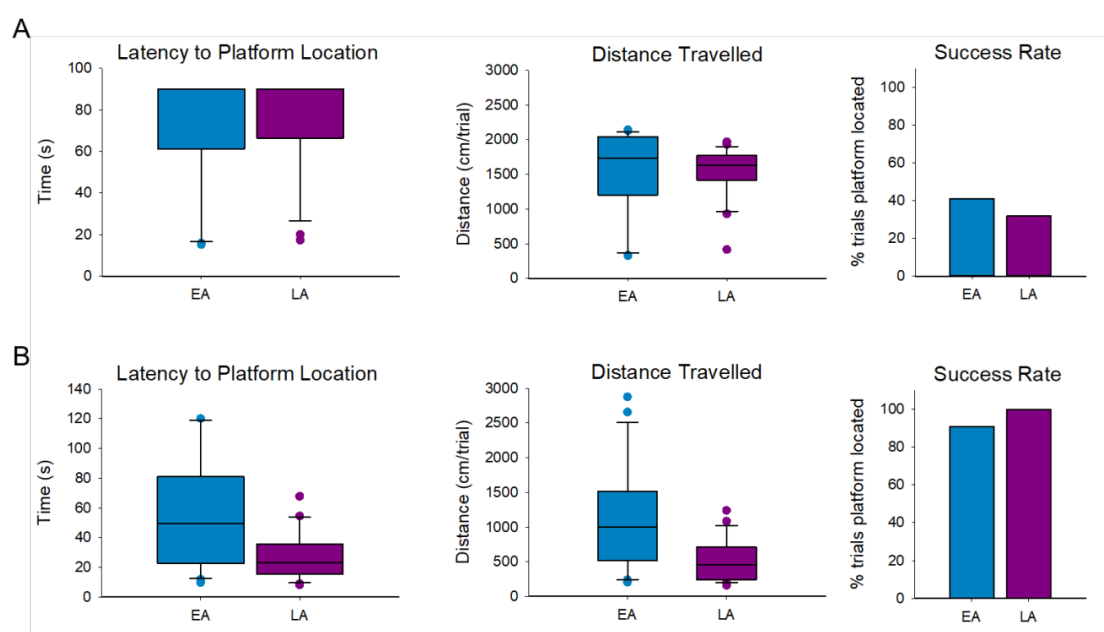


Figure 5-9: The effect of ageing on locating a visible platform in a cued trial. The latency to the platform location (left), distance travelled (middle) and overall success rate of finding the platform (right), are shown for the first cued trial before training commenced (**A**) and for the second cued trial after finishing the experiment (**B**). Note: the first cued trial was a maximum of 90 seconds while the second cued trial was a maximum of 120 seconds in duration. Data in left and middle panels are box plots showing median values. Data in the right panel are mean values. EA: $n=22$, LA: $n=23$.

5.4 Discussion

The first aim of this study was to determine the effect of ageing on spatial learning in the MWM. All measures of spatial learning investigated in this study showed significant improvement across the 6 training days, similarly for both EA and LA mice. Specifically, the trial duration, latency to platform and distance travelled significantly reduced across the training days, remaining constant across the two additional training days between the two probe trials. The only significant difference identified in these measures was for the distance travelled during the first 3 days of training only, where EA mice were found to swim further during a trial, compared to LA mice. This may be explained by the significantly slower swim speed in LA mice compared to EA mice across all training days, which, as would be expected, is significantly correlated with the distance travelled. As swim speed was significantly reduced in older mice, this warrants caution when interpreting data involving distance calculations.

The reduced swim speed in LA mice identified in this study is consistent with previous studies that showed ageing to be associated with a reduction in running and general locomotor activity (Possidente et al., 1995; Valentinuzzi et al., 1997; Welsh et al., 1986). As older mice are known to exhibit lower locomotor activity, both in the water maze, as well as generally in their home cage, it is possible that the lower initial distances may reflect this general reduction in activity.

However, it has been suggested that although animals may show hypo-activity in activity boxes, there may be no differences in swim speed in the MWM (Fitzgerald and Dokla, 1989), leading to suggestions that learning effects may be observed in the MWM regardless of differences in activity levels (Vorhees and Williams, 2006). Importantly, regardless of the highly significant differences in swim speed, no significant differences were identified in the common measures of spatial learning and memory quantified in this study, both during the probe trial but also at the end of the 6 days of training.

It is now well established that a number of species change their navigation strategies with age, often showing preferences for egocentric navigation strategies which utilises different environmental cues and often lead to navigational errors (Gallagher et al., 1993; Lester et al., 2017). For example, old rats are shown to adopt a more circling strategy which is less efficient than learning the exact location of the platform but increase the chance of finding the platform compared to just a random search strategy (Gallagher et al., 1993). This has led to suggestions that age-dependent differences in performance in the water maze may reflect a combination of deficits in learning, memory and attention, as well as the processing of spatial information into spatial search strategies (Lindner, 1997). The lack of age dependent differences identified in this study, suggest that at least at 12 months old, these previously reported age differences are not apparent. However, age differences in the distance travelled may be explained by differing search strategies between the age groups, with younger EA mice expressing a preference for generally increasing the amount of swimming, even in a thigmotaxic manner. Older LA mice instead adopt a technique in which they do not swim as far but remain active. By day 4 of training, the significant age-difference was no longer present, which may indicate that by this point both age groups are showing a similar level of learning and so take a more direct route to the escape platform. Importantly, despite swimming further, both age groups showed similar learning characteristics across training days. This suggests that spatial learning is not disrupted by 12 months of age, at least in mice.

As spatial learning was seemingly intact across ageing, I next investigated whether the spatial memory is affected by sleep deprivation. This was tested by running two probe trials, one after sleep deprivation and one after a sleep opportunity, in a crossover design. In this study the two probe trials were combined as statistical analysis comparing the two did not reveal any differences between the two probe trials. Previous studies have shown that 60 seconds after the

platform was removed rats spend approximately 50% of the total time in the training quadrant, however when the rats were re-tested 24 hours later animals only spent around 36% of time in the training quadrant, with the number of crossings also reduced (Morris, 1984). This may reflect true forgetting processes, where the animals can no longer remember the location of the platform, however it may also reflect extinction effects, whereby the absence of the platform decreases the strength of the previous memory (Morris, 1984). As the training days between the two probe trials (days 7 and 8) did not differ significantly to Day 6 of training, it seems unlikely that any extinction of learning occurred between probe trials in this study.

Time in the target quadrant is commonly used when assessing spatial memory in the MWM. In this study, the time in the target quadrant was not significantly different between age groups or experimental condition. As this measure relates to the entire quadrant, rather than the platform itself, this may lead to an overestimation of this measure, as not all time in the quadrant may be directly related to the platform (Blokland et al., 2004). As ageing is also associated with a reduction in swim speed and locomotor activity, it has been suggested that swim distance may be a more appropriate measure for assessing cognitive function compared to latency to the platform (Lindner, 1997). Therefore I also calculated the cumulative distance to the platform. This is thought to be one of the best measures of spatial learning and memory (Gallagher et al., 1993; Vorhees and Williams, 2006). Once again, there was no significant effect of age or experimental condition on the cumulative distance to the platform.

The latency to reach the previous platform location was not significantly different between sleep and sleep deprivation conditions, however LA mice were found to have a longer latency compared to EA mice for the sleep day only. There was a significant difference in the latency to the platform between the last training day and the probe day for LA mice but not for EA mice. However calculating the difference between the last training day and probe day did not identify

any overall effects of age or condition. The number of platform crossings is a commonly used measure, but crossings often do not occur very often during a probe trial leading to data that is often highly variable (Vorhees and Williams, 2006). For this reason, it has been suggested that the time or distance in the target quadrant or the distance to the target site may be more useful measures in the probe trial (Vorhees and Williams, 2006). Therefore, I focussed mainly on these measures and instead used the latency to crossing the platform to identify successful trials from unsuccessful trials for further analysis. It is thought that the decrease in the latency to reach the platform across a number of trials is due to the animals learning to swim away from the walls of the maze and so increasing the likelihood that the platform is found, while it also reflects true spatial learning of the platform location (Morris, 1984).

Most studies use probe trials that are 60 seconds in duration, however it has been suggested that probe trials that are 60 seconds long may lead to an underestimation of spatial ability in rats (Blokland et al., 2004), as measures of learning are often higher in the initial 30 seconds of a probe trial. This may be expected when you consider that animals may give up in searching the target platform after a certain amount of time and instead search for alternative escape routes. In order to address this, I calculated the time in the target quadrant and the distance to the target quadrant for the first 30 seconds of the probe trial only. However, once again no age or experimental condition differences were identified. Previous studies often have shorter latencies (often ~10 seconds (Blokland et al., 2004)) to reach the platform by the end of the training period, therefore the lack of differences between the time intervals in this study may be due to the fact that their performance was not as good as these studies.

As there were no significant differences in any measures of spatial memory during the full probe trial or the initial 30 seconds of the trial, I next hypothesised that age- or experimental condition- differences may lie in the difference between the last training day and the probe

trials. This would take into account the previous level of learning when assessing memory for the platform. Interestingly direct comparison of the final training day before the probe trial to the probe trial revealed a significant difference for the sleep deprived LA animals only. This suggests that although these mice reach a similar (or not significantly different) latency to the platform as the other groups at the end of training and during the probe trials, there was a significant increase in the latency to the platform location during the probe trial compared to the previous training, for the SD condition. This is supported by the low success rate of these animals (LA_SD) locating the platform during the probe trial (47.83% success) which would lead to a higher average latency. Though there were no significant differences in the success rates of the different groups. Importantly there were also no effects of age or condition in the differences between the last training day and the probe trial, which contradicts the significant difference identified for the LA_SD group. However, this may be expected when looking at group data rather than specifically within groups.

5.4.1 Further Considerations

This experiment works on the premise that mice will look for an escape route when placed in water. It has previously been identified that in some cases animals can express an over activation when first exposed to the maze (Vorhees and Williams, 2006). This can be expressed as a form of hyperactivity where animals jump off the platform and may not identify it as a possible escape route. Hyperactivity has previously been shown to result in deficits in the MWM (Morris et al., 1982). In this experiment, young (EA) mice showed hyperactive behavioural characteristics, whereby they would jump back into the water once finding the platform. In order to address this hyperactivity, careful assessment of trials was carried out and one animal was removed from all analysis due to it consistently showing this behaviour. It remains possible that the EA mice had an over activation as a result of the maze, which decreased their performance in the task.

However, the training data suggests EA mice still learnt the task well. Initial measures of trial duration overestimated the duration of the trial as this measure is reliant on the animals remaining on the platform for 5 seconds, therefore the latency to platform was the preferred measure in this study.

Cued trials prior to the spatial version of the task are thought to be useful for decreasing the over activation commonly identified in animals which are first exposed to the maze (Vorhees and Williams, 2006). In this study a cued visual trial was performed prior to the beginning of training, in an attempt to reduce this behaviour, however it was still detected in a number of animals. Importantly, analysis of this initial cued trial showed that only 40% of the animals located the platform, even though it was visible to the animals. This could be interpreted as these animals having poor vision, however as both age groups expressed this low success in finding the platform, it is more likely that additional factors such as it being the first time in the maze and the experimenters first time running the task. Therefore the low success in the cued trial may be an explanation for the reduced efficacy of the cued trial in reducing over activation in the maze, as has been previously suggested (Vorhees and Williams, 2006). Alternatively, another interpretation of this behaviour is that the water may not be as aversive to these animals, as they are willingly choosing to swim rather than remain on the raised escape platform. At the end of the experiment a second visual trial was performed, with the platform located in a different location to the training position. In contrast to the initial set of cued trials, in the second set 47/49 mice located the escape platform within the allotted 2 minutes (all within 1.5 mins). Therefore this suggests that vision did not affect performance in this study, which is consistent with the lack of age differences in all measures tested.

In contrast to the hyperactivity and lack of interest in the platform identified in a small group of the EA mice, some of the older (LA) mice were found to show increased shivering and a lack of

grooming after the task. Although previous studies in rats, showed that ambient temperatures in the range of 19-22°C did not cause significant fatigue or hypothermia, they did find that the age-dependent deficits they observed in the MWM may be confounded by a loss of thermoregulatory control with ageing (Lindner and Gribkoff, 1991). Therefore performance in the MWM task may be confounded by the fact that there is a loss of thermoregulatory control with ageing and therefore older mice may find the task more aversive compared to younger mice. It is important to reiterate that this behaviour (both jumping and shivering) was only apparent in a very small number of animals.

Although old animals had been on a restricted diet prior to the study (food was provided ad libitum throughout the study) their weight was still considerably higher than the EA mice. As mentioned earlier, this may be an explanation for the differences in swim speed identified here. Interestingly, despite dramatic differences in weight and speed, no other age-dependent differences were identified, suggesting that cognitive learning and memory processes remain undisturbed by ageing. The genetic background of mice has been shown to affect performance in the MWM (Lipp and Wolfer, 1998; Wolfer et al., 1998), however the chosen mouse strain, C57Bl/6J (RccHsd) mice, are known to be good swimmers, adopt a search strategy appropriate for the task, and have consistently been shown to learn the task well (Clapcote et al., 2005; Vorhees and Williams, 2006).

It should be noted that although the wheels used for sleep deprivation in this study will disrupt sleep, they will not completely eradicate sleep, and no quantifications of sleep could be attained using this technique. However, the sleep restriction protocol from which this studies protocol was adapted (Mccarthy et al., 2017), has been shown to induce functional deficits and increase the need for recovery sleep similarly to previous reports of sleep restriction in rodents (Baud et al., 2013; Leenaars et al., 2011; Mccarthy et al., 2017). It would therefore be expected that

sufficient sleep restriction also took place in this study. Previously, sleep restriction was performed for longer periods of time of around 11-12 hours (Baud et al., 2013; Leenaars et al., 2011; McCarthy et al., 2017), as compared to the 6-hours used in this study. 6-hours sleep deprivation was chosen for this study, to match with data in other studies contributing to this thesis, as well as 6-hours has been shown to be sufficient for inducing rebound effects during recovery sleep (Cui et al., 2014; Vyazovskiy et al., 2007b, 2009b). However, it remains a possibility that as these studies instead involved a more complete sleep deprivation and that the 6-hours sleep restriction in this study may not be sufficient for replicating these effects. Finally, as the sleep deprivation algorithm was modified to be used with mice (rather than its original purpose for use with rats), this methodology remains to be validated and so it is possible that the two age groups may have slept different amounts during sleep deprivation.

In this study it was not possible to determine the preceding sleep-wake history for each animal. Therefore particular behaviours during and before sleep restriction may have influenced the ongoing state and effects of SD, as has previously been shown (Brown, 2012; Cui et al., 2014; Dijk et al., 1987; Huber et al., 2007; Vyazovskiy et al., 2007a, 2014; Vyazovskiy and Tobler, 2012). In this study circadian factors were controlled for by performing the task at roughly the same time every day (approximately 6 hours after light onset). This time was chosen to match well with the beginning of the probe trial testing after the 6 hours of sleep deprivation probe trial days. In addition, although sleep deprivation ran for 6 hours from light onset, animals remained in the turning sleep deprivation wheels until shortly before their turn to be tested in the probe trial. Therefore those animals which had behavioural testing later would have been exposed to the wheels and therefore sleep deprivation for a longer duration than those tested towards the beginning of the testing session. In total the testing period after sleep deprivation only lasted roughly 1.5 hours though, which would be unlikely to have a great influence on the data.

5.4.2 Conclusions

It is important to interpret data acquired from the Morris water maze with care. This task cannot be considered a direct measure of cognitive function, but rather is an indirect measure of a psychological construct which is influenced by external, non-specific factors such as thermoregulation, stress and visual acuity to name a few (Lindner, 1997). In this study few differences between EA and LA mice and experimental conditions were identified, suggesting that ageing and sleep deprivation had little effect on spatial learning and memory in the MWM task. It is possible that the lack of differences observed in this study are due to the relatively young age of the oldest age group contributing to this study, therefore, it remains a possibility that the Morris water maze is a useful pre-clinical test for assessing spatial learning and memory across different conditions such as age and drug manipulations.

Chapter 6: The link between sleep, waking performance, cognitive function and ageing in two novel paradigms of the visual discrimination task

6.1 Introduction

Healthy ageing is well established to be associated with a number of molecular, cellular and system changes, including in behaviour and cognitive function (Burke and Barnes, 2006; Bishop et al., 2010; Kirkwood, 2010; Zoncu et al., 2010; Kourtis and Tavernarakis, 2011; Morrison and Baxter, 2012; Yeoman et al., 2012). Surprisingly, the mechanistic links underlying the relationship between age-related cognitive decline and sleep impairment are unclear. Clearly, there are a number of potential confounds in studying behaviour across ageing, such as a decline in visual acuity (Banks et al., 2015; Wong and Brown, 2007), decrease in contrast sensitivity (Alphen et al., 2009), decreased locomotor activity (Barreto et al., 2010; Ingram et al., 1981), and a decreased motivation to perform the task (Bordner et al., 2011; Brodaty et al., 2010). The rhythmicity of physiological measures such as drinking and body temperature are also altered by ageing (Ingram et al., 1981; Kopp et al., 2006). These factors may all lead to misinterpretations of deficits in performance or motor systems (Alphen et al., 2009), making it is notoriously difficult to design a task that is appropriate for studying cognition and ageing.

Touchscreen methodologies are becoming widely used for studying cognition in rodents (Bussey et al., 2008; Horner et al., 2013; Romberg et al., 2013), due to their resemblance to cognitive tasks used in humans which therefore increases the translatability between species. In rodents, these tasks involve visual stimuli being shown on a touchscreen which are then responded to by nose poking the stimulus. These touchscreen methodologies have a number of important

advantages. For example they are automated, have minimal input from the experimenter, properties of the visual stimuli can be strictly controlled and they use appetitive stimuli rather than the aversive stimuli (Horner et al., 2013). A number of tasks have been developed and validated for use with this platform, which can be used in combination to assess an animals cognitive profile or individually to study specific elements of neuropsychological constructs such as learning, working memory and executive function (Horner et al., 2013). The task has also been useful for studying cognitive decline associated with diseases, by using disease models such as mouse models of Alzheimer's disease (Horner et al., 2013; Romberg et al., 2013).

The visual discrimination task (VDT), used in this study, is a simple non-spatial associative learning task, which utilises an animal's ability to discriminate between two images displayed on a touchscreen. Over the course of a multi-day training programme the animals are conditioned so that they associate a conditioned stimulus (CS+) with a reward, and their preference for this CS+ over another non-rewarded stimulus (CS-) can be assessed as a measures of non-hippocampal associative stimulus-reward learning. This protocol is widely used and validated, while it has also been previously used in combination with *in vivo* electrophysiological recordings to investigate the role of corticostriatal system in choice learning and flexibility (Brigman et al., 2013). Importantly though, this has not previously been used to investigate the effects of insufficient or disrupted sleep on cognition, in older rodents. As the task is highly versatile this opens up great opportunities to adapt the task for use with ageing mice to target various aspects of learning and performance.

6.1.1 Experimental aims

This chapter aimed to address objective 4 of my thesis: '***To develop a novel behavioural paradigm to study sleep, sleep deprivation and cognitive function, which may be applicable to ageing studies in the future***'. Here I present two novel developments of the VDT task; extended

VDT and the alphabet task, that were designed to be used in combination with continuous recordings of brain activity to study different measures of learning and memory. Preliminary analyses were performed on these tasks to assess their suitability in studying behavioural performance. In this study, all animals were first subjected to daily behavioural training in the standard task and then one of two experiments were performed.

Experiment 1: extended VDT: aimed to study the effects of prolonged performance (4 hours) in a non-spatial associative learning task. One hypothesis is that continuous performance in this task for several hours would lead to a gradual decline in accuracy as a result of tiredness and increasing sleep propensity. While the link between sleep deprivation and a deterioration of cognitive and behavioural measures is well established, the underlying neural mechanisms remain unclear.

Experiment 2: The alphabet task: aimed to study cognitive flexibility, by exchanging the previously learned grating images for novel images (letters) and investigating the acquisition of new information. More specifically this task aimed to determine whether mice can adapt a schema to learn novel stimuli, and whether sleep plays a role in this process. It may be hypothesised that sleep-wake history affects memory acquisition and retention.

6.2 Methods

6.2.1 Pre-experiment training in the VDT task and surgical preparation

The visual discrimination task is a complex pairwise discrimination task, involving a number of components such as initiation, discriminating between two images, touching an image and collecting a reward (as described in Figure 6-1). Due to the complexity of the task, a number of training stages were carried out before animals were trained in the main experimental protocol of pairwise discrimination. The stages of training are outlined below:

1. Habituation phase (1 day): Palatable food is delivered every 10 seconds (10µl) regardless of response. Note: no images are shown on the screen during this phase. *Aim: animals learn to collect the food from the reward magazine/tray.*
2. Initial touch (1 day): One image is shown on the touchscreen (either on the left or right), and if the animals touch the screen they will immediately get the palatable food reward (10µl). If the mice do not touch the screen a reward is still provided after 10 seconds. *Aim: animals learn a contingency between touching the screen with the visual stimulus and the food reward.*
3. Must touch (~5 days): One image is shown on the touchscreen (either on the left or right). Animals must touch the screen in order to receive the food reward (10µl). The image will remain on the screen until it is touched. *Aim: animals learn that the specific screen must be touched in order to get the food reward.*
4. Must initiate (1-3 day): Animals must initiate a trial by entering their nose into the food magazine. Once initiated, one image is shown on the touchscreen (either on the left or right), and must be touched to receive the food reward. The image will remain on the screen until it is touched. *Aim: animals learn to initiate trials.*
5. Main Experimental Protocol: pairwise discrimination: initiation of a trial by placing their snout in the reward tray, initiates two images to be shown on the touchscreen behind the animals, one conditioned **correct** choice (45 degrees gratings, CS+), and another unconditioned **incorrect** choice (vertical or horizontal gratings, CS-). If the animals touch the correct choice, images will disappear and the palatable food is delivered (strawberry milkshake, 10µl) to the reward tray at the rear of the chamber; if they make the incorrect choice, images disappear, the chamber light turns on (5-sec) and no palatable food is delivered. If no choice is made after 10 seconds the images disappear and the chamber light turns on for a 5-seconds timeout period, this would count as an omission trial. Inter-trial intervals were set to 5 seconds, so animals had to wait 5 seconds before

they could initiate another trial. The pairwise discrimination protocol is summarised in Figure 6-1.

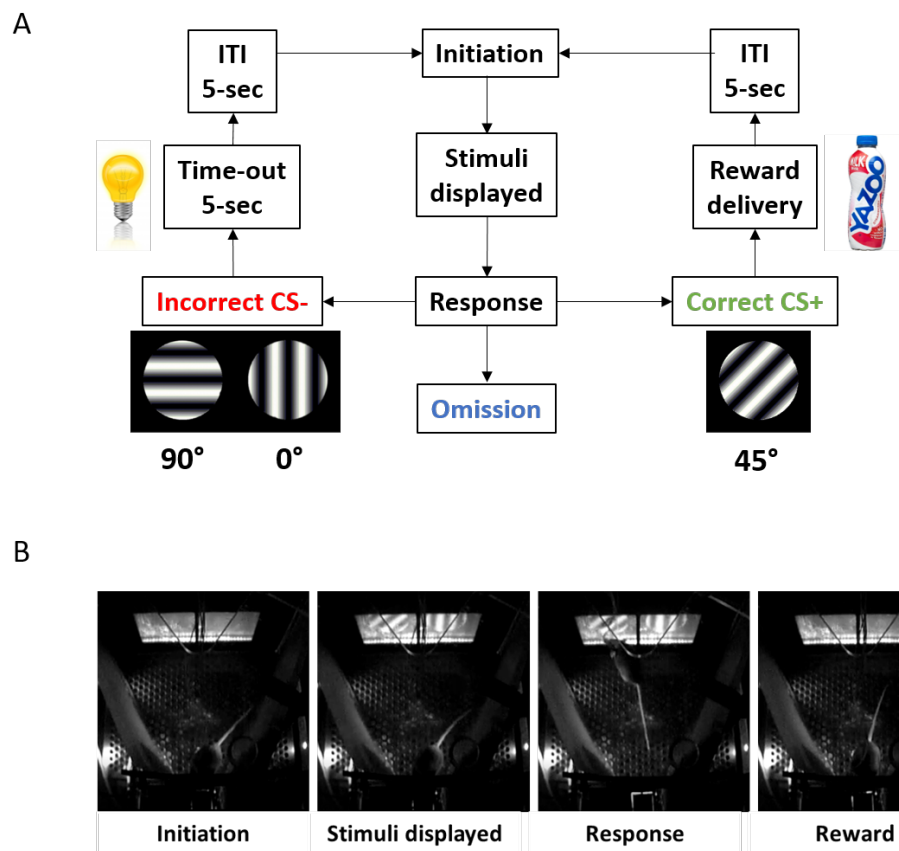


Figure 6-1: **Visual discrimination task (VDT) task: Pairwise discrimination protocol.** (A) A schematic of the protocol. (B) Photographs showing a mouse performing each stage of a single trial.

Mice were food restricted throughout both training and the experimental protocol. Mice were trained daily for 30 minutes (on occasion this was instead 60 minutes) in the pairwise discrimination task. Once the mice reached at least 80% accuracy in the visual discrimination task, they were provided with food ad libitum in preparation for surgery. Surgery was performed after initially training the animals in the task to ensure stability and longevity of the surgical implants for the duration of the study. After surgery mice performed one of two distinct experiments: ‘extended VDT’ and ‘the alphabet task’, as described in detail below. All animals underwent the same training followed by a surgical procedure to record either EEG alone, or in combination with cortical multiunit activity, however the experiments were performed in separate groups of animals, with both studies addressing distinct aims.

6.2.1.1 Surgical procedure

All mice contributing to this chapter underwent surgery to implant EEG screw electrodes, in frontal and occipital cortical regions, as well as a reference screw in the cerebellum (see general methods section 2.2 for details). Some of the animals contributing to the extended VDT chapter had an additional microwire array implanted into the motor cortex to record multiunit activity during the task. Electrophysiological signals were recorded as per our laboratory group's standard procedure, as stated in the general methods section of this thesis (section 2.3). This chapter includes a detailed analysis of behavioural data only, as the electrophysiological signals are currently undergoing detailed analyses and could not be included here.

6.2.1.2 Experimental animals

The experiments were carried out in male C57BL/6J mice. As the initial aim of this project was to develop a new behavioural paradigm for studying ageing in mice, in the extended VDT experiment animals were subdivided into early adulthood (EA, 27.33 ± 0.11 weeks, $n=7$) and late adulthood (LA, 51.86 ± 1.85 weeks, $n=9$) age groups. Only early adulthood mice were analysed for the alphabet task experiment (EA, 30.07 ± 0.71 weeks, $n=4$), and due to the low number of animals contributing to this part, the results are considered preliminary.

All environmental conditions were identical to that specified in the general methods chapter (Chapter 2), including a 12:12 light dark cycle (section 2.1). Mice were singly housed to enable the animals to be food restricted. All mice had water available ad libitum but were food restricted for the duration of this experiment. Body weight was monitored daily and food was adjusted accordingly to maintain weight at $>85\%$ free feeding weight. Food was provided at random intervals after completion of the task, to avoid entrainment to the food (Foster and Hankins, 2007; Mistlberger, 1994; Pendergast and Yamazaki, 2014).

6.2.2 Experiment 1: extended VDT

After surgery, mice were retrained for an average of 12 ± 0 days and 6.71 ± 1.51 days for EA and LA mice, respectively, until performance had improved to their pre-surgery level. Mice were then cabled to enable EEG recordings simultaneously with performance in the task and trained for an additional 7.25 ± 1.25 days and 8.00 ± 0.76 days, for EA and LA mice, respectively. Mice were first retrained without their cable based on pilot data that showed that animals did not relearn the task as well after surgery when cabled from the beginning. Animals then underwent testing in a new protocol that I helped develop, extended VDT, in which animals performed the standard pairwise discrimination task (as described above) continuously for four hours (Figure 6-2A). Testing was started four hours into the light period, based on previous evidence in our group that performance was typically low in the early light period, likely due to the highest sleep pressure during this time. The aim of this experiment was to determine how performance changed over the four hour training period, as well as to investigate how sleep after the task was affected by extended performance of a highly cognitively demanding task. Performance was limited to four hours to enable the effect on subsequent sleep to be determined. Interestingly, during the initial pilot experiments mice showed that they could perform the task for many hours, until they eventually had to be removed from the chamber. As the cohort of mice used in this task included two age groups, age comparisons were also made to determine whether cognitive performance was affected in this study by 12 months of age.

Performance in the task was assessed by calculating four main measures; the total number of trials (count of the number of trials during the 4-hour session), the proportion of correct trials (accuracy = correct/total number of trials), the proportion of incorrect trials (incorrect/total number of trials), and the number of omissions (omissions/total number of trials). The latency to perform both correct and incorrect trials (the time between trial initiation and the response)

was also used to assess changes in the speed of performing trials over the extended period. To assess the time course of learning the four hours were subdivided into 30 minute intervals for analyses.

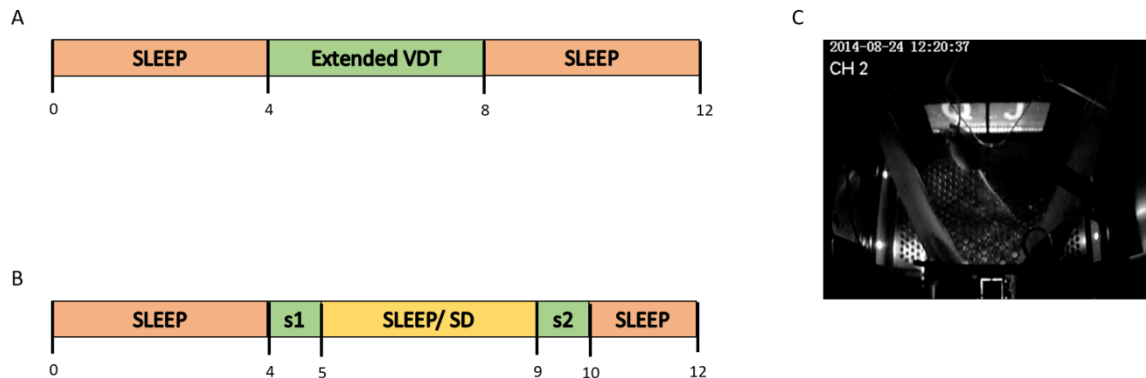


Figure 6-2: Protocols of the two experiments over the 12 hour light period. (A) Extended visual discrimination task (extended VDT). Animals were left undisturbed for 4 hours from light onset, were trained in the standard VDT protocol for 4 hours and were left undisturbed post training. (B) The alphabet task. Animals were left undisturbed for 4 hours from light onset. Animals then underwent 1 hour of training where they were first exposed to novel letters (session 1, s1), before being either left to sleep or sleep deprived for 4 hours. Animals were then retrained for a further 1 hour with the same images (session 2, s2), before being left undisturbed for the remainder of the light period. (C) An example image of a mouse performing the alphabet task with novel letters as images.

6.2.3 Experiment 2: the alphabet task

As in the extended VDT experiment, the animals were initially trained on the standard set of grating images, implanted with EEG/EMG electrodes, and retrained after surgery for an average of 30 ± 4.04 days post-surgery, depending on their performance. It took on average 20.25 ± 5.51 days to reach the 80% accuracy criterion and maintain this level of performance prior to the experiment. Mice were then cabled to enable EEG recordings simultaneously with performance in the task and given additional training in the standard pairwise discrimination task (8.5 ± 1.5 days). Once again, animals were first retrained without the cable so that performance in the task improved at a quicker rate.

In order to investigate the effect of sleep on the cognitive flexibility (in this case the ability to adapt schema to novel set of stimuli), as well as memory retention/consolidation over time, I developed an extension of the visual discrimination task, called the alphabet task (Figure

6-2B/C). On the experimental day of this task the animals were trained in the same pairwise discrimination task but were given novel images, in the form of letters rather than grating images (either capitalised S vs Y or G vs K), as can be seen in Figure 6-2B/C. Letters were chosen based on them having distinct shapes from one another, and to the previous grating images (excluding letters such as 'Z'). Animals completed two experimental days of training, each involving two experimental sessions one hour in duration. Each session was separated by 4 hours of either sleep opportunity or sleep deprivation in the home cage. Sleep deprivation was performed using the gentle handling technique (see section 2.6), or the animals were left undisturbed so that they could sleep ad libitum. The study was ran in a cross over design, so that each animal received both experimental conditions (sleep or sleep deprivation), with each separated by two days. On both days animals were exposed to a novel pair of letters for one hour, 4 hours after light onset. Letters were kept the same over the two training sessions in a day, but were changed across experimental days so that each animal was tested with both sets of letters. Importantly, during the sleep condition day although left undisturbed so that they may sleep, the mice slept for an average of only 63.41 ± 5.03 mins between training sessions, which was, however, significantly higher than during the sleep deprivation day (1.15 ± 1.02 mins of sleep during 4 hours, $p=0.001$ paired t-test). The low amount of sleep during the sleep condition may reflect the mice anticipating food, which could therefore increase arousal. To minimise this effect, a small amount of food (0.8g) was provided immediately after the first training session for both sleep and sleep deprivation conditions, with the aim of encouraging the mice in the sleep group to sleep.

To assess the time course of learning across sessions, data were subdivided into quartiles by taking the time between the first and the last trials and subdividing this into four equal time intervals (quartiles). The main aim of the experiment was to investigate how well mice can apply a well learnt rule to relearn which image is correct in a new set of images, and to determine

whether sleep plays a role in this. Comparisons were therefore made between training sessions, as well as between recording days to determine how well mice were able to relearn novel images and the potential role for sleep in consolidating learning between training sessions.

6.2.4 Statistical analyses

For both experiments data were analysed using Matlab (The Math Works, Inc., Natick, MA, USA), Microsoft Excel and SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp). All values reported are mean $\pm$ SEM, unless explicitly stated.

For the extended VDT, age comparisons between overall performance and latency measures in the task were performed using unpaired t-tests. For time-course data repeated measures ANOVAs were used to test difference across time as well as between age groups. For the alphabet task, ANOVAs were used to assess significant differences according to the sessions and experimental days (sleep or sleep deprivation). Repeated measures ANOVAs were used to test for significant difference in time-course data (factors 'experimental day' and 'sessions'). Due to the small numbers of animals contributing to this task, correlations between sleep and performance were calculated.

6.3 Results

6.3.1 Experiment 1 results: extended VDT

Plotting learning curves of the performance of each individual animal across the training days before extended VDT, revealed that there was an increase in the number of trials performed during a session (Figure 6-3), an improvement in the accuracy of performance (Figure 6-4) and a decrease or consistently low number of omission trials (Figure 6-5) across the training days, indicating an improvement of performance. Notably, while both age groups showed similar

trends, this was less pronounced in LA mice which showed larger variation between individuals. This was particularly apparent for the accuracy of performance, which remained low in LA mice and in most cases never reached the 80% accuracy criteria and did not show the same improvement over the initial training days (Figure 6-4B). Importantly, cabling the animals consistently resulted in a deterioration in performance in all animals, observed as a transient reduction in the accuracy of performance and an increased number of omissions (day of cabling indicated by the dotted vertical lines in Figure 6-3, Figure 6-4 and Figure 6-5). As accuracy reflects the number of omissions as well as the number of correct and incorrect trials, it is likely that the decreased accuracy as well as increased omissions may reflect increased difficulty in performing the task with the cable. However, this mostly improved with further training with the cables.

Next, the changes in performance across the four hours of the extended VDT task were investigated. Surprisingly, all mice performed the task for the full four hour period. Figure 6-6 shows example time course plots highlighting how the occurrence and latency of correct, incorrect and omission trials are distributed over the four hours of the extended VDT task in both EA and LA mice. Visual inspection of these plots suggests that although all animals continued to perform trials across the entire testing period, performance was variable over time. Specifically, these examples show an increase in the number of incorrect and omission trials in the last half of the task. There is also an indication that incorrect responses often have longer latencies, compared to correct trials (Figure 6-6).

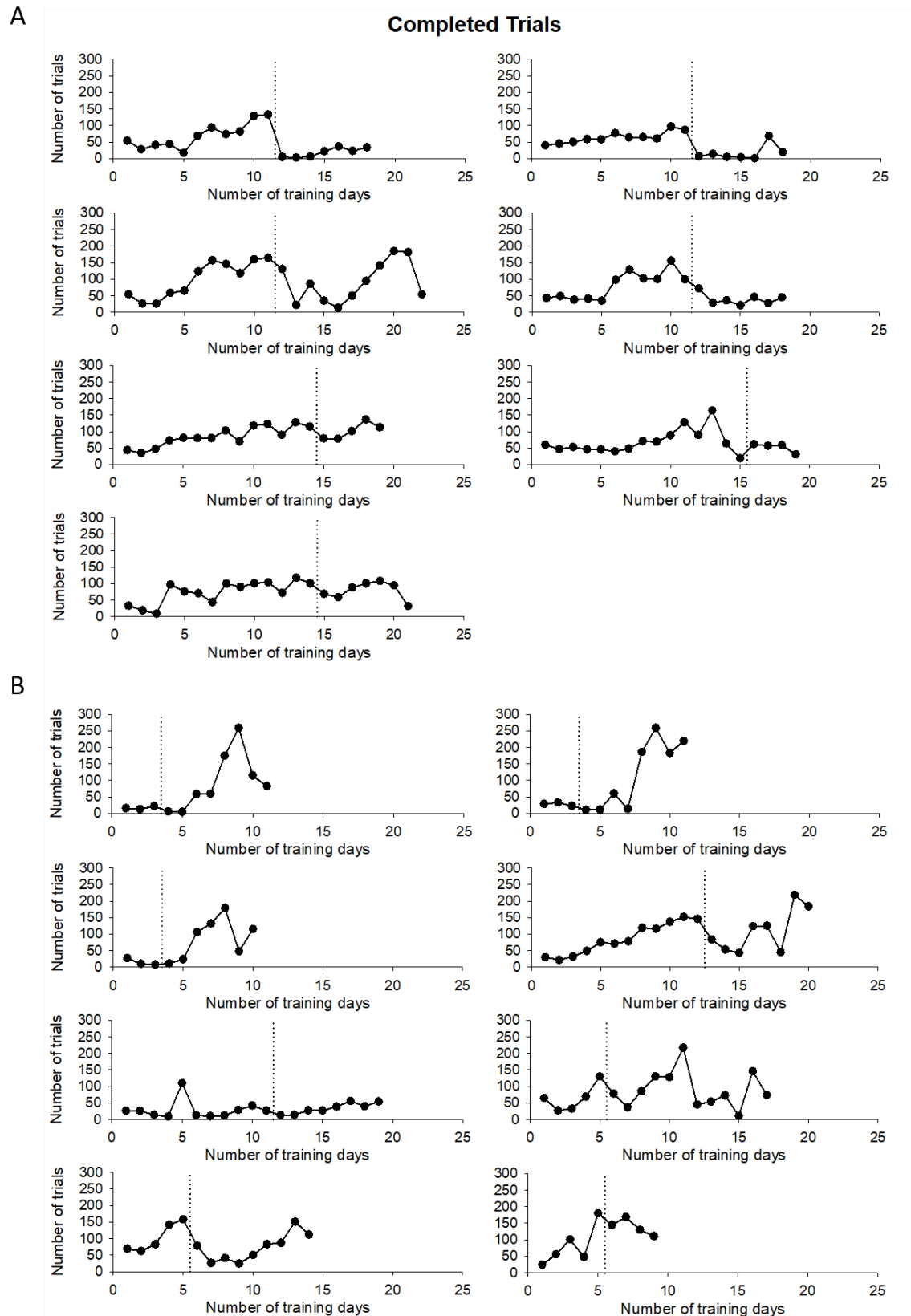


Figure 6-3: *Learning curves of the number of trials performed in the pairwise discrimination task (VDT) prior to the extended VDT protocol, shown for EA (A) and LA (B) mice. Each plot represents an individual animal. Dotted lines indicate the point at which the animals were cabled and further trained with the cable connected.*

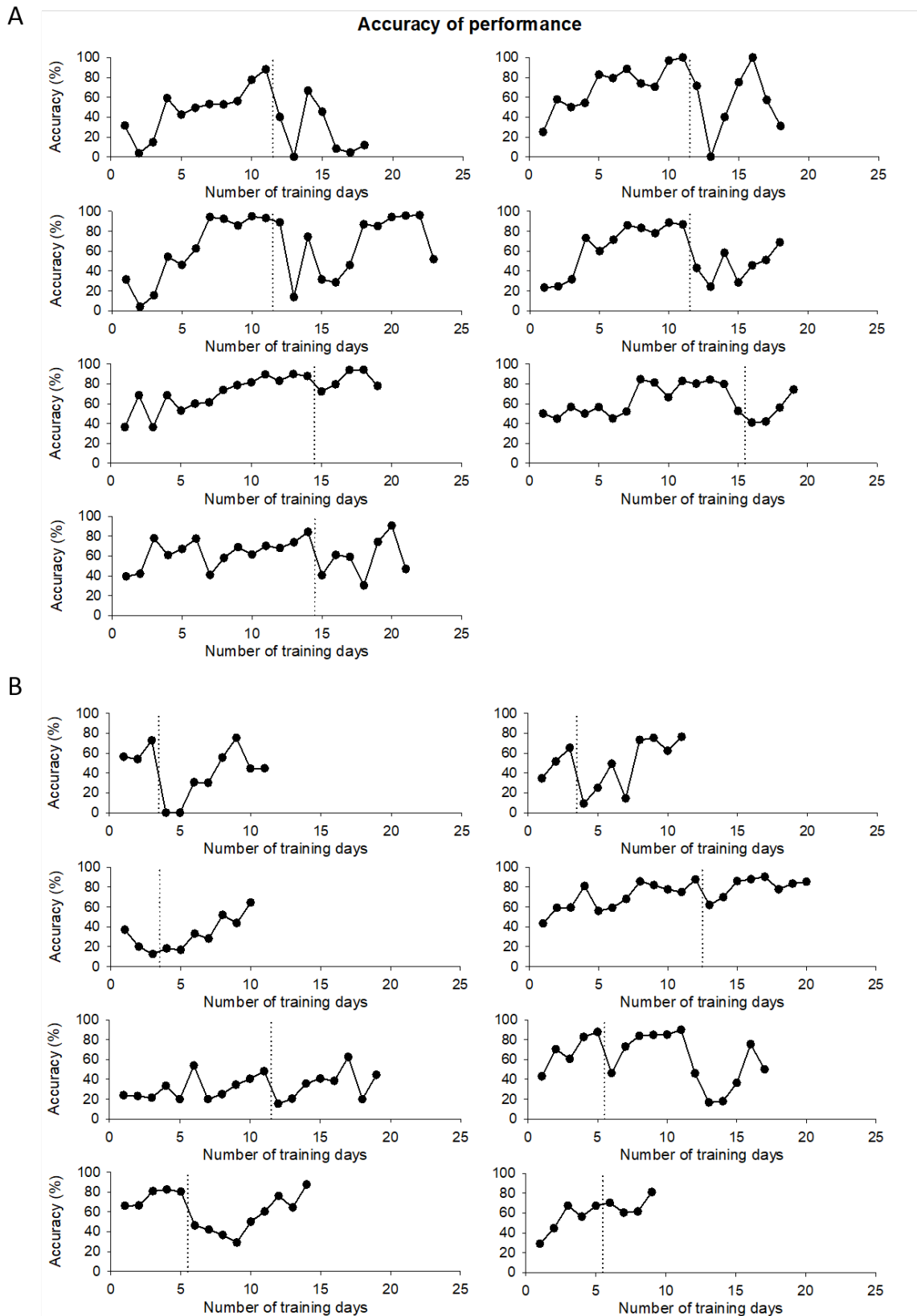


Figure 6-4: Learning curves of the accuracy of performance in the pairwise discrimination task (VDT) prior to the extended VDT protocol, shown for EA (A) and LA (B) mice. Each plot represents an individual animal. Dotted lines indicate the point at which the animals were cabled and further trained with the cable connected.

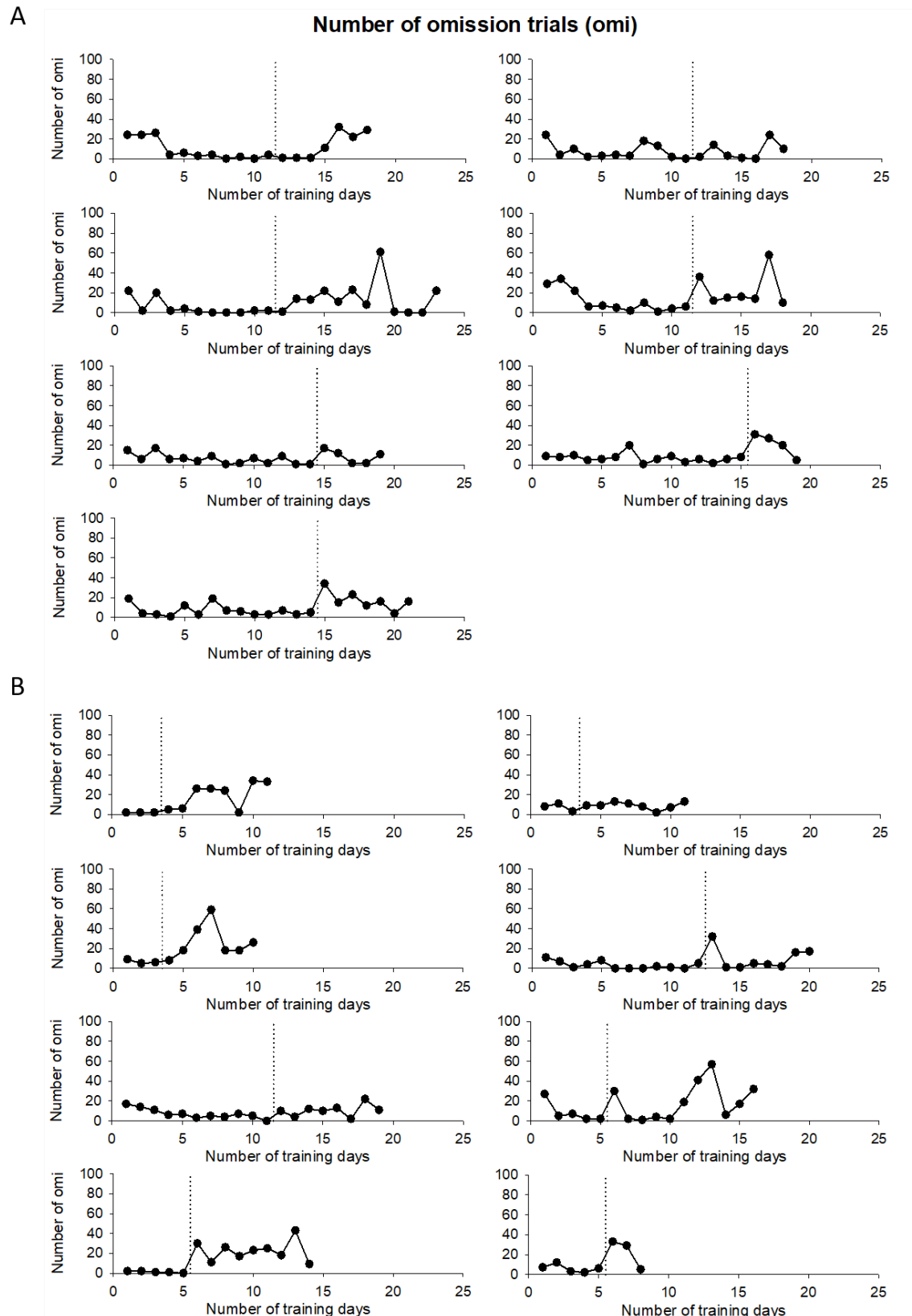


Figure 6-5: Learning curves of the number of omission trials performed in the pairwise discrimination task (VDT) prior to the extended VDT protocol, shown for EA (A) and LA (B) mice. Each plot represents an individual animal. Dotted lines indicate the point at which the animals were cabled and further trained with the cable connected.

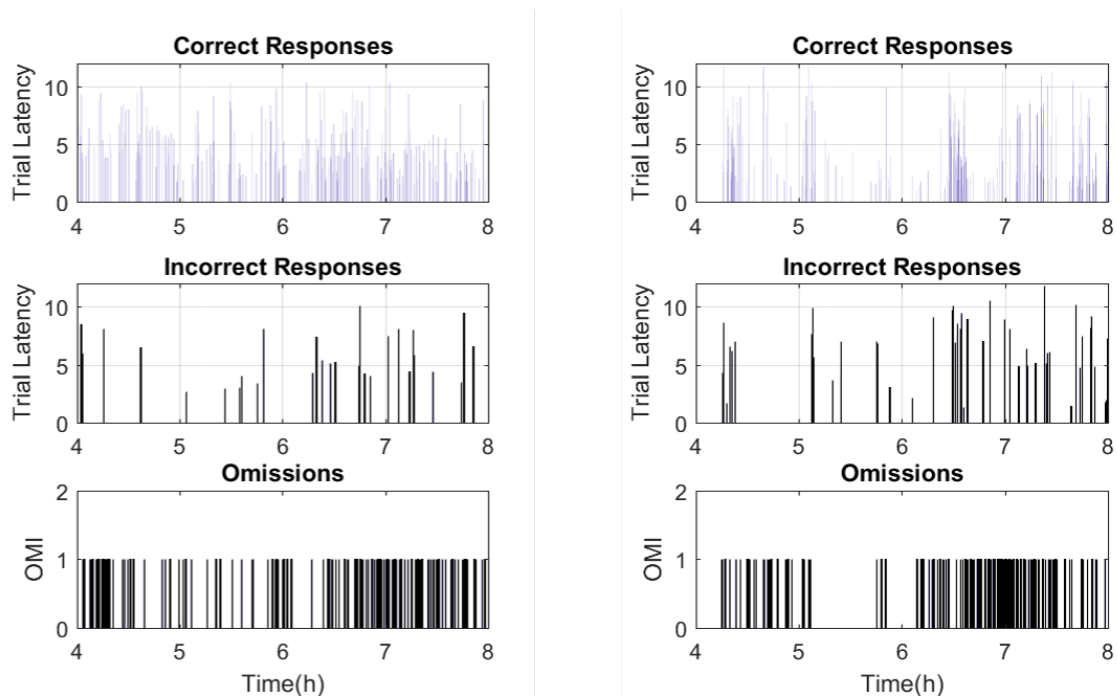


Figure 6-6: *The time course of correct (top), incorrect (middle) and omission (bottom) trials over the 4 hours of the extended VDT task. Examples are for an example EA (left panel) and LA (right panel) mouse. For both correct and incorrect trials data are plotted as time vs their respective latencies. As omissions trials indicated an absence of choice within the allotted 10 seconds, they were binary in their occurrence, with a score of 1 indicating an omission trial. Note that in both mice, there was an increase in the number of incorrect and omission trials towards the end of the task.*

As an initial step, a number of variables were quantified to investigate the effect of ageing on the overall performance in the VDT task (Figure 6-7). This revealed that the two age groups were not significantly different across the various measures quantified, except for LA mice having a higher number of incorrect trials (EA: 18.86 ± 3.30 , LA: 58.33 ± 10.35 , unpaired t-test $p=0.006$, Figure 6-7A), and subsequently an increase in the number of incorrect trials out of the total trials (EA: 5.92 ± 1.00 , LA: 14.58 ± 3.51 , unpaired t-test $p=0.05$, Figure 6-7B). Based on these initial analyses, the total number of trials (Tot), as well as the number of correct (cor/tot), incorrect (inc/tot) and omission (omi/tot) trials expressed as a proportion of the total number of trials were taken forward for further analysis.

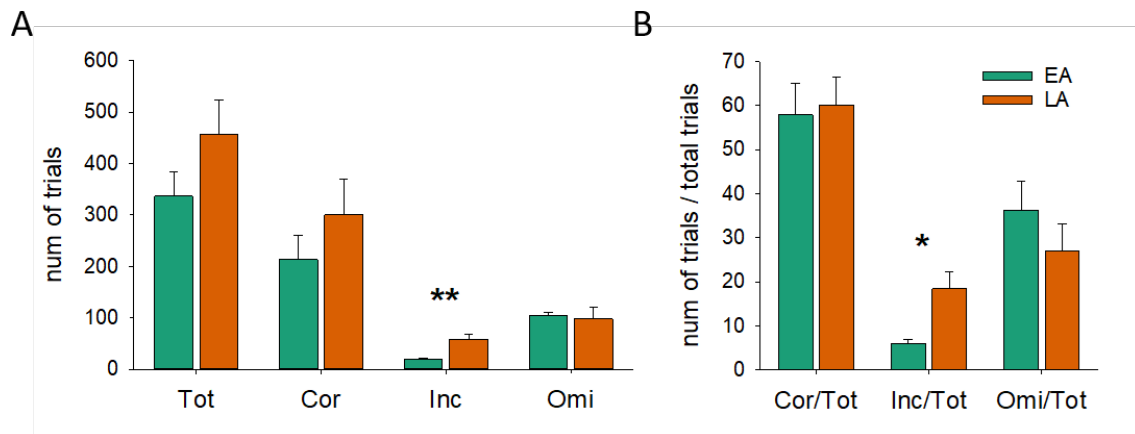


Figure 6-7: **Average performance in the extended VDT task.** (A) The mean total number of trials are shown separately for EA and LA mice (total number of trials, correct trials, incorrect trials, and omissions). (B) The number of correct, incorrect and omissions as a proportion of the total number of trials. EA $n=7$, LA $n=9$. Data are mean and SEM. Unpaired t-tests were used to test for significant differences between age groups. * $p<0.05$, ** $p<0.01$.

As the main aim of this experiment was to determine how performance changed over extended performance, i.e. time, I next looked at the change in 30 minute intervals. Unexpectedly, repeated measures ANOVA's did not reveal any significant changes in performance over time, for the number of correct ($F(3,42)=1.12$, $p=0.35$), incorrect ($F(3,42)=0.34$, $p=0.79$) or omission trials ($F(1,14)=0.12$, $p=0.74$) (Figure 6-8). These data suggest that performance in the VDT task remains fairly stable for at least four hours. Although significant age difference were identified in the total number of incorrect trials (Figure 6-7), no significant age-differences were identified for the number of correct ($F(1,14)=0.12$, $p=0.74$), incorrect ($F(1,14)=3.56$, $p=0.08$) or omission trials ($F(1,14)=1.45$, $p=0.25$), across time (Figure 6-8).

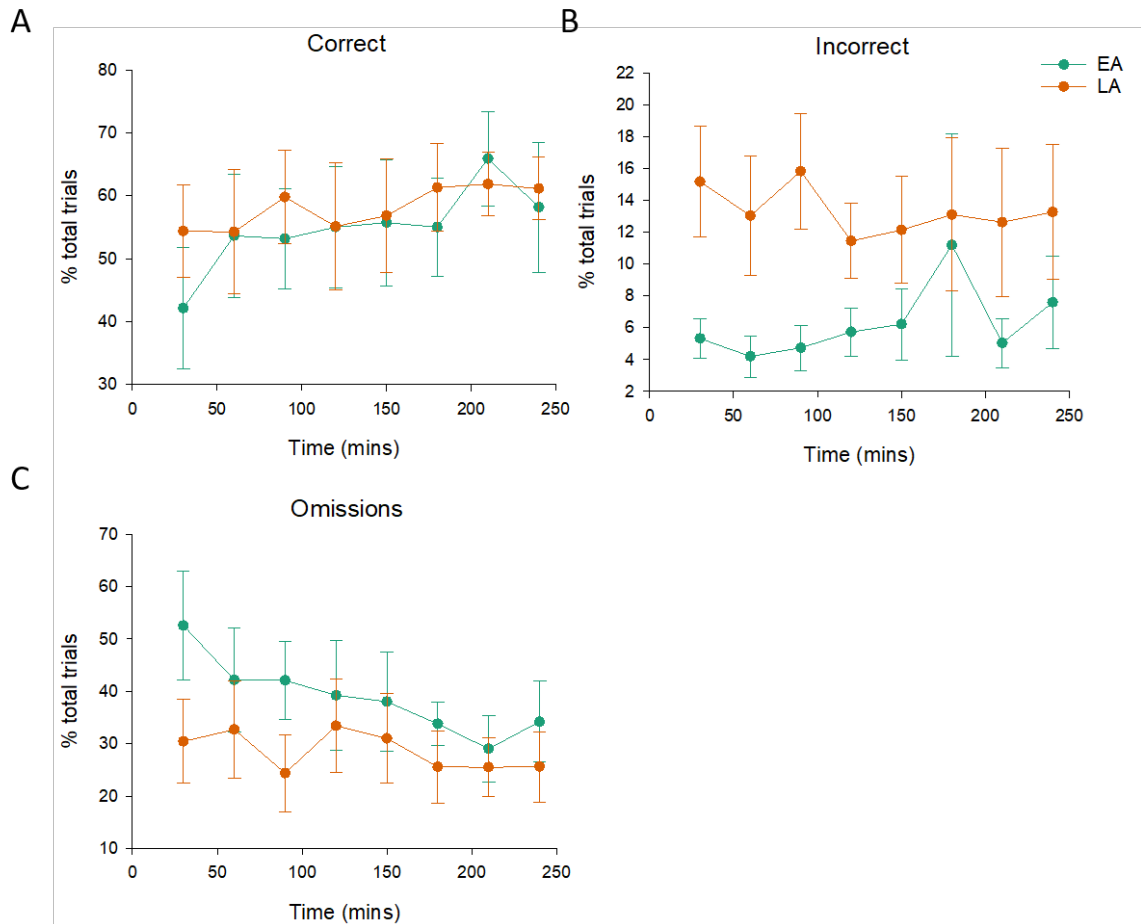


Figure 6-8: Performance is stable across the 4 hours extended VDT task. Data are shown for correct (A) incorrect (B) and omission (C) trials, expressed out of the total trials. Data are mean values (SEM), plotted in 30 minute bins. EA n=7, LA=9. No significant differences were identified across time intervals or between age groups (repeated measures ANOVA's).

In order to determine whether extended performance in the VDT task had an influence on the speed of response, the latency to make correct and incorrect responses was next calculated (Figure 6-9). The average latency to perform a trial was significantly longer for incorrect compared to correct trials for EA mice (correct: 5.33 ± 0.22 seconds; incorrect: 6.31 ± 0.56 seconds; paired t-test $p=0.04$, Figure 6-9A), but no difference was identified for LA mice (correct: 4.77 ± 0.17 seconds; incorrect: 4.88 ± 0.40 seconds; paired t-test $p=0.72$, Figure 6-9A). In addition, the latency to make incorrect trials was longer in EA mice compared to LA mice ($p=0.01$, unpaired t-test). The latency to make correct trials was not significantly different between age groups ($p=0.12$, unpaired t-test).

Interestingly, investigations into how the latency to perform correct and incorrect trials over the four hours of performance, revealed that the latency to perform incorrect trials showed characteristics of a possible ultradian rhythm. Specifically, this was visible as an increased latency to perform incorrect trials in the middle hour of the task, which was not significantly different between ages ($p=0.73$, unpaired t-test). In contrast, the latency to perform correct trials was fairly unaffected by extended performance, which was not significantly different between age groups ($p=0.35$, unpaired t-test).

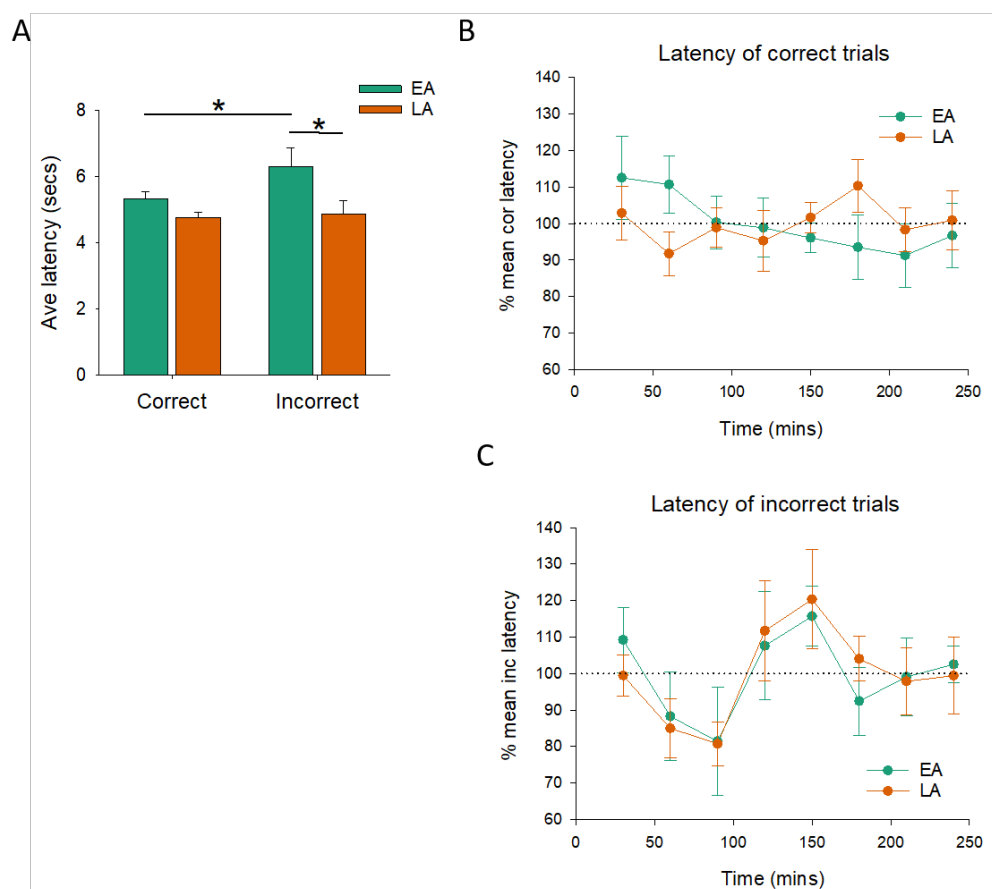
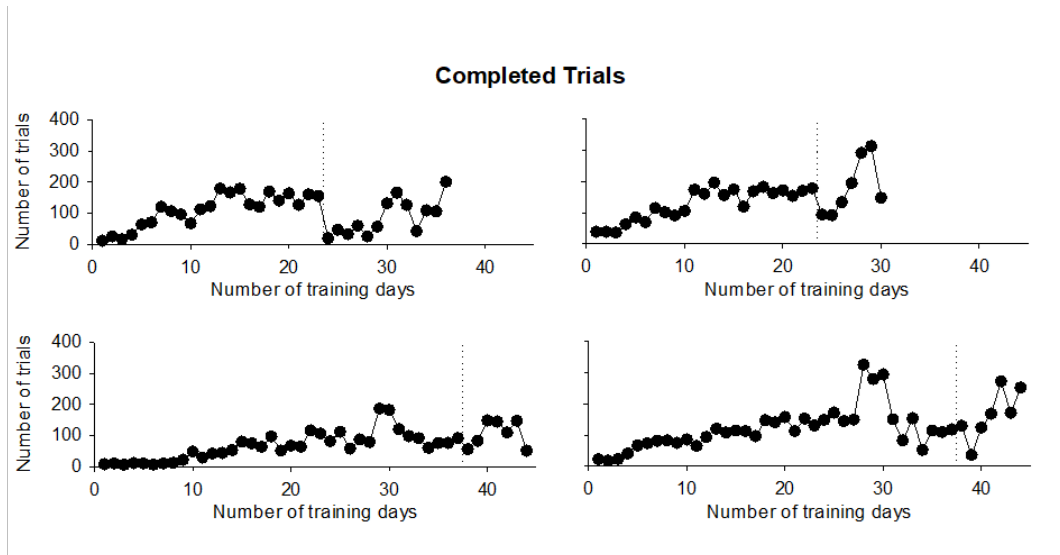


Figure 6-9: Latency of correct and incorrect trials. (A) The average latency to make correct or incorrect responses for the entire 4 hour period. Paired t-tests used to compare average latencies of correct and incorrect trials. Unpaired t-tests used to compare EA and LA age groups. Paired t-test used to compared correct vs incorrect trials within an age group. * $p<0.05$. (B) and (C) Show the average latencies to perform correct and incorrect trials, respectively. Data are expressed as percentage of the mean over all trials and plotted in 30 minute bins. All data are mean and SEM. EA $n=7$, LA $=9$. Dotted lines at 100% show the mean over the 4 hours, to which all data are normalised.

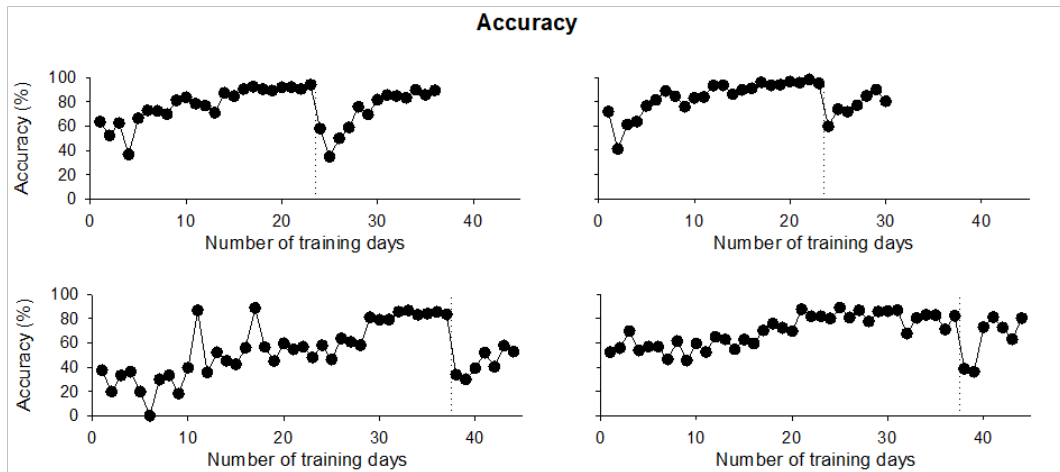
6.3.2 Experiment 2 results: the alphabet task

Similar to the effects reported for the extended VDT task, all animals contributing to this study also showed an increase in the numbers of trials and accuracy across training days, while the number of omissions reduced or remained low (Figure 6-10). As with the extended VDT task, cabling the animals (as indicated by the dotted lines in Figure 6-10) resulted in a drop in performance for all animals, observed as a pronounced reduction in the accuracy of performance and an increase in the number of omissions, which improved with further training with the cables (Figure 6-10). As mentioned previously, this may reflect an increased difficulty in performing trials while cabled or a distraction produced by the presence of the cable.

A



B



C

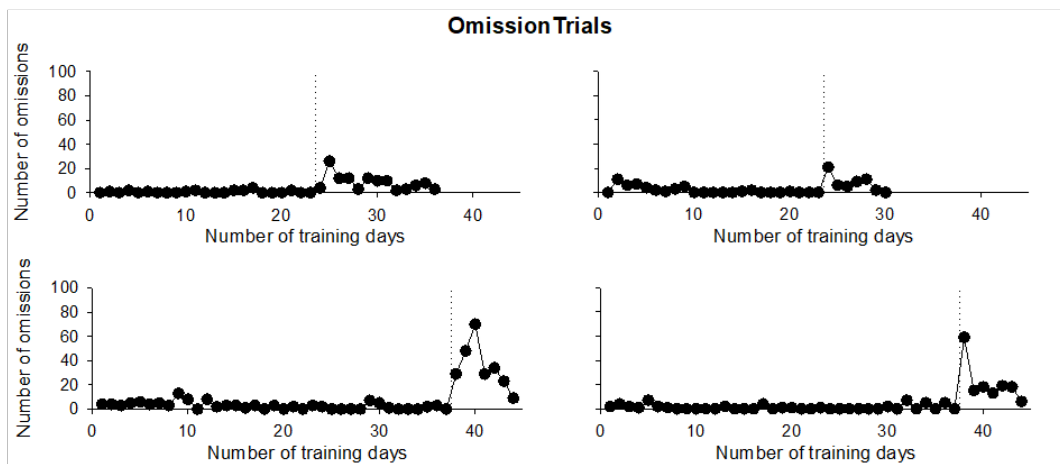


Figure 6-10: Learning curves of training in the VDT task prior to the alphabet task. Data are shown for the number of completed trials (A), accuracy (B) and omissions (C). Each plot represents an individual animal ($n=4$). Dotted lines indicate the point at which the animals were cabled and further trained with the cable connected. All animals showed a deterioration in performance after being cabled, as indicated by an increase in the number of omission trials and a decrease in accuracy.

Next, animals were tested in the alphabet task. As described in section 6.2.3, this consisted of two 1-hour long training sessions with novel images, separated by 4 hours of either sleep opportunity or sleep deprivation. To investigate whether sleep affects subsequent performance or memory retention, differences between the two alphabet task sessions were first investigated. Based on the results of the extended VDT task, the total number of trials and the number of correct, incorrect and omission trials out of the total number of trials were investigated in this study.

Both the number of incorrect and total number of trials were found to decrease from session 1 to session 2 for both sleep and sleep deprivation conditions though not significantly (ANOVAs; incorrect: $F(1,12)=0.37$, $p=0.55$, Figure 6-11B; total trials: $F(1,12)=0.61$, $p=0.45$, Figure 6-11D). The number of correct trials increased from session 1 to 2 for the sleep condition but showed little improvement for the sleep deprivation condition, though again there were no significant differences between session 1 and 2 (ANOVA $F(1,12)=0.31$, $p=0.59$, Figure 6-11A). Together this data suggests that although learning is occurring, this may be attenuated by sleep deprivation. There were no significant differences in the number of omissions between session 1 and 2 (ANOVA $F(1,12)=0.04$, $p=0.84$, Figure 6-11C). Notably, on the sleep day the number of omissions were lower in session 2 compared to session 1, while after sleep deprivation the number of omissions increased. However, there were no significant differences between sleep and SD conditions (correct: $F(1,12)=0.31$, $p=0.59$; incorrect: $F(1,12)=0.79$, $p=0.39$; omissions: $F(1,12)=1.14$, $p=0.31$; total trials: $F(1,12)=0.16$, $p=0.69$; Figure 6-11). Therefore, sleep did not have a significant effect on the improvement in performance between sessions in this study, as observed as no differences between sleep and SD days. As the number of omission trials were not normally distributed, data were first log transformed before undergoing ANOVA significance testing.

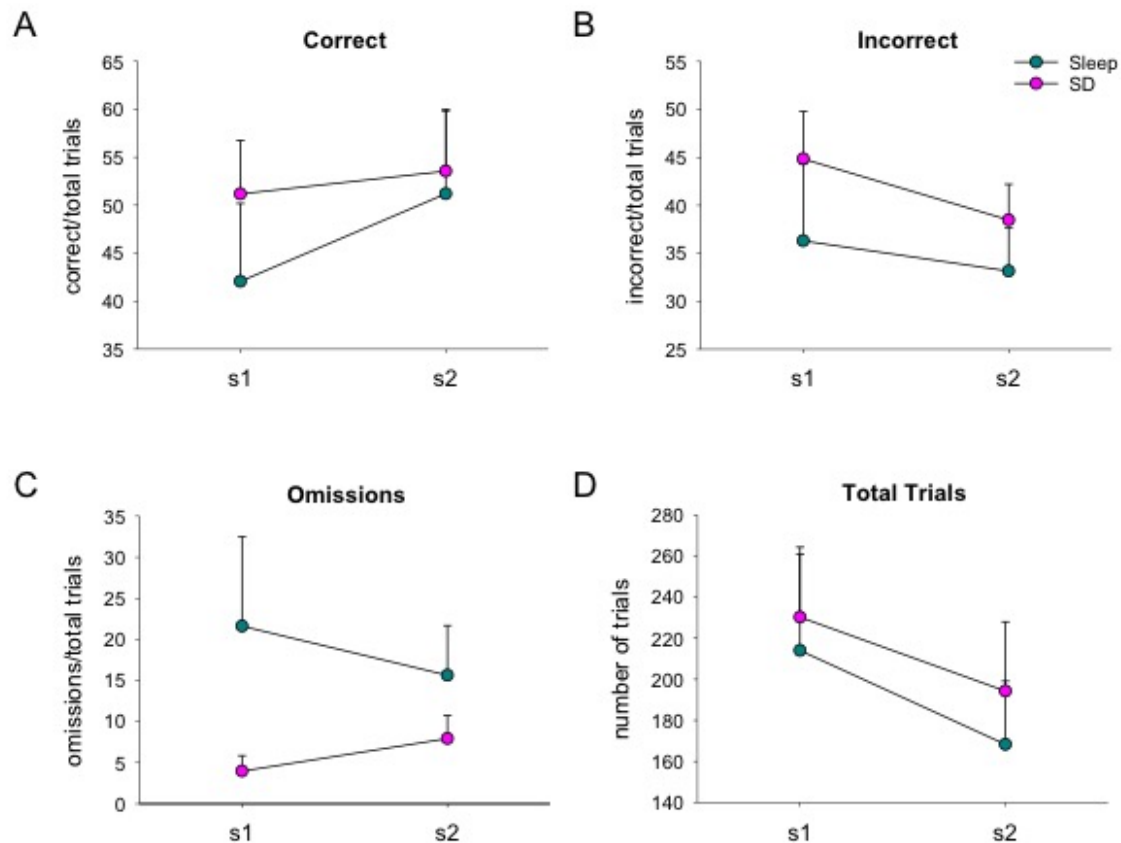


Figure 6-11: **Performance in the alphabet task.** Data are subdivided into the two training sessions, for both sleep and sleep deprivation days. (A) The number of correct/total number of trials, (B) incorrect/total trials, (C) omissions/total trials and (D) total number of trials are shown. Data are mean $\pm$ SEM. No significant differences were identified between sessions or across experimental sessions (ANOVAs factors 'sessions' and 'experimental conditions').

It is possible that these differences may be time dependent, reflecting the time it takes for the mice to adjust schema to learn the new images. Therefore, I next subdivided each session into quartiles according to the duration between the first and last initiation of a trial and plotted the number of correct, incorrect and omission trials out of the total trials as well as the total number of trials, for the two sessions within a day, separately for sleep and sleep deprivation days (Figure 6-12). Visual inspection revealed that the number of correct trials increased and incorrect trials decreased between quartile 1 and 4 in the first session, and remained stable across session 2, however no significant effects were identified for either correct (repeated measures ANOVA; factor quartiles: $F(2,20)=2.86$ $p=0.09$; day: $F(1,12)=0.13$, $p=0.73$; session: $F(1,12)=0.10$ $p=0.76$; Figure 6-12A) or incorrect trials (quartiles: $F(3,36)=1.08$ $p=0.37$; day: $F(1,12)=0.72$, $p=0.41$; session: $F(1,12)=0.23$ $p=0.64$; Figure 6-12B). This may be expected as this

is the first time the animals were exposed to the novel letters and so had to learn which image was correct. The learning of the new letters may also be reflected in the reduction in the number of trials across sessions (repeated measures ANOVA; factor quartiles: $F(3,36)=2.245$ $p=0.1$; day: $F(1,12)=0.166$ $p=0.69$; session: $F(1,12)=0.618$ $p=0.45$; Figure 6-12D) as animals may be performing more correct trials and so not need to perform as many trials. Importantly the number of omission trials were low across both sessions and days (repeated measures ANOVA; factor quartiles: $F(2,21)=2.85$ $p=0.09$; day: $F(1,12)=1.37$, $p=0.27$; session: $F(1,12)=0.17$ $p=0.69$; Figure 6-12C), suggesting that animals were motivated to perform the task and so were actively learning the task. Therefore, although there were visual trends, there were no significant changes in any learning measures across sessions or between days.

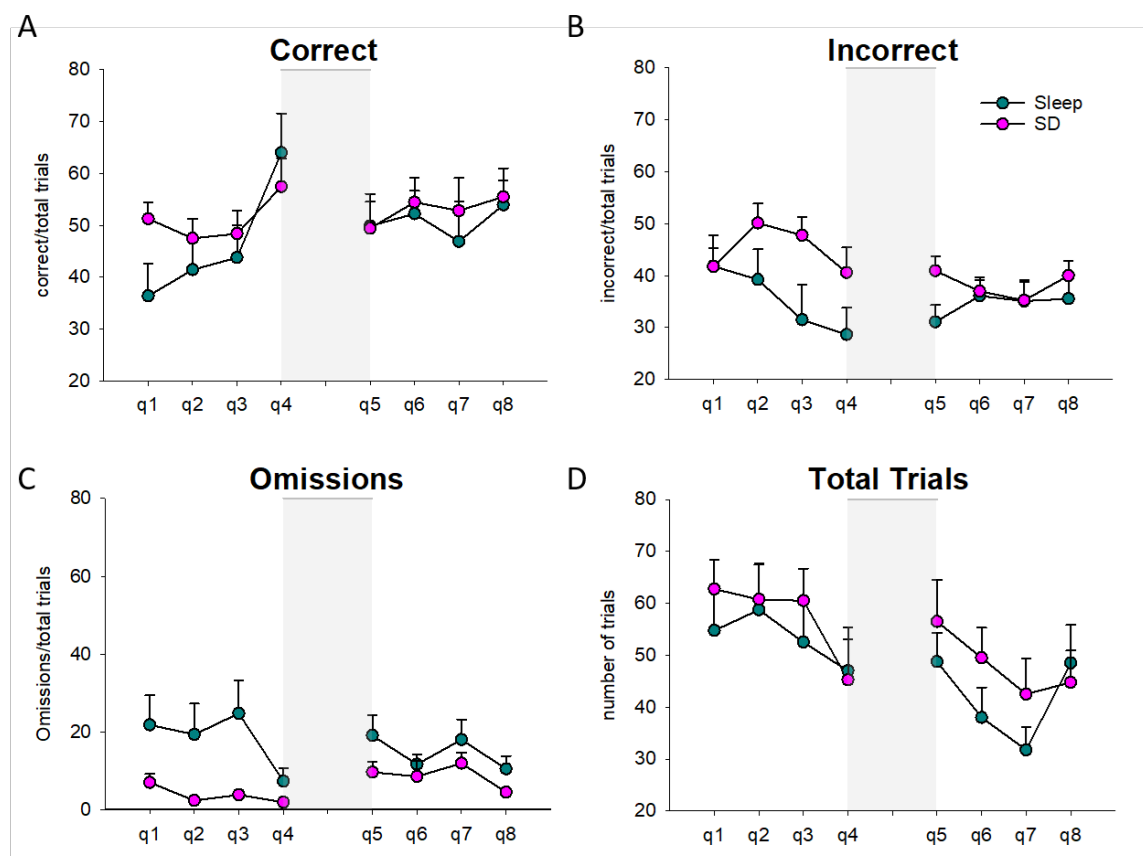


Figure 6-12: Time course of performance in the alphabet task. Each session was subdivided into quartiles according to the time between the first and last initiation in the task (session 1: q1-q4; session 2: q5-q8). Data are shown for the total number of trials (A), accuracy (correct/total trials) (B), and number of omissions (omissions/total correct) (C), for both sleep and sleep deprivation days. Shaded areas show the period in which animals were sleep deprived or allowed to sleep. Data are mean $\pm$ SEM. Repeated measures ANOVAs did not identify any significant differences across the quartiles or between the experimental days or sessions.

Next, the difference between the final quartile of the first session and the first quartile of the second session was quantified and plotted in Figure 6-13. No significant differences were identified between the two experimental conditions (correct: sleep: -14.14 ± 16.21 , SD: -8.06 ± 4.98 , $p=0.74$; incorrect: sleep: 2.43 ± 7.54 , SD: 0.36 ± 6.02 , $p=0.86$; omissions: sleep: 11.72 ± 9.03 , SD: 7.70 ± 3.59 , $p=0.67$; total trials: sleep: 1.75 ± 6.17 , SD: 11.25 ± 7.56 , $p=0.35$; paired t-tests).

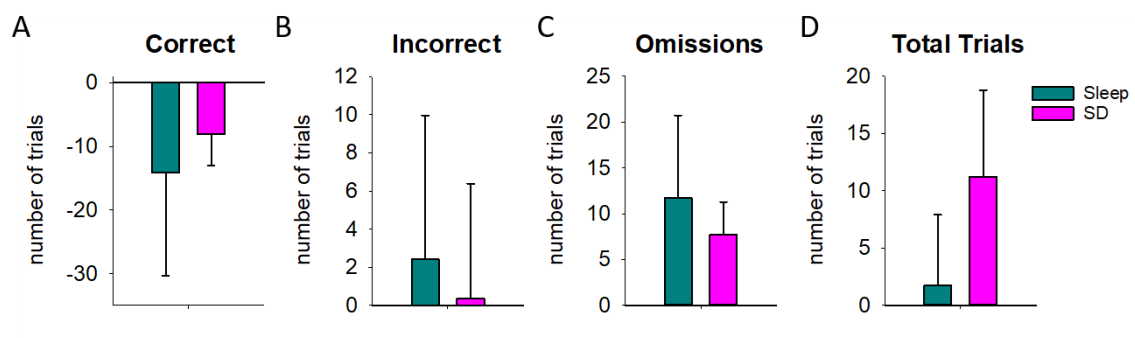


Figure 6-13: Difference in performance in the alphabet task at the beginning of the second session compared to the end of the first session. Differences were calculated by subtracting the performance in quartile 1 of session 2 from quartile 4 of session 1. Data are shown for the number of correct (A), incorrect (B) and omission trials (C) out of the total number of trials, as well as the total number of trials (D). Data are mean $\pm$ SEM. Paired t-tests were used to test for significant differences between experimental conditions. No significant differences were identified.

Finally, the next series of analyses were performed in order to determine whether sleep plays a role in learning in this task. Figure 6-14 shows the time course of the vigilance states across the 12 hour light period of the alphabet task recording days. Mice slept significantly more between the two sessions on the sleep day compared to the sleep deprivation day (SLEEP: 63.42 ± 5.03 minutes; SD: 1.15 ± 1.02 minutes; paired t-test $p=0.0008$). Noteworthy, some sleep still occurred during sleep deprivation, as is common in sleep deprivation studies.

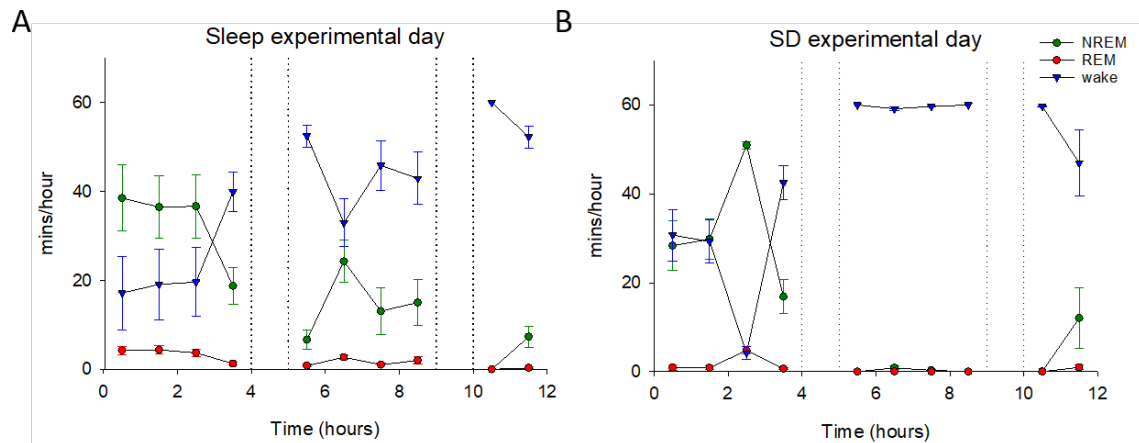


Figure 6-14: **Time course of vigilance states across the 12 hour light period.** Data are shown for sleep (left panel) and sleep deprivation (SD) (right panel) experimental days. NREM sleep, REM sleep and wake are shown in green, red and blue, respectively. Data are mean $\pm$ SEM. $n=4$.

The amount of sleep before session 1, between the two sessions and after session 2 on the sleep condition day were then calculated and used in further analyses. Specifically whether the amount of NREM or REM sleep correlated with the total number of trials or the accuracy of performance in the task (number of correct trials/total number of trials). There were no significant relationships between either the amount of NREM or REM sleep before session 1 and either the number of trials or the accuracy of performance during session 1 (Figure 6-15).

The amount of NREM sleep between the two sessions significantly positively correlated with the number of trials during the second session ($r=0.99$, $p=0.01$, Figure 6-16A), while the accuracy also showed a positive correlation, though this was not significant ($r=0.89$, $p=0.20$, Figure 6-16B). The amount of REM sleep was also positively correlated with both the number of trials and accuracy, however neither reached significance (number of trials: $r=0.69$, $p=0.31$; accuracy $r=0.61$, $p=0.39$; Figure 6-16).

Finally, the amount of NREM and REM sleep were both negatively correlated with the improvement in the accuracy between the sessions, which was only significant for REM sleep (NREM: $r=-0.75$, $p=0.25$; REM sleep $r=-0.98$, $p=0.02$; Figure 6-17).

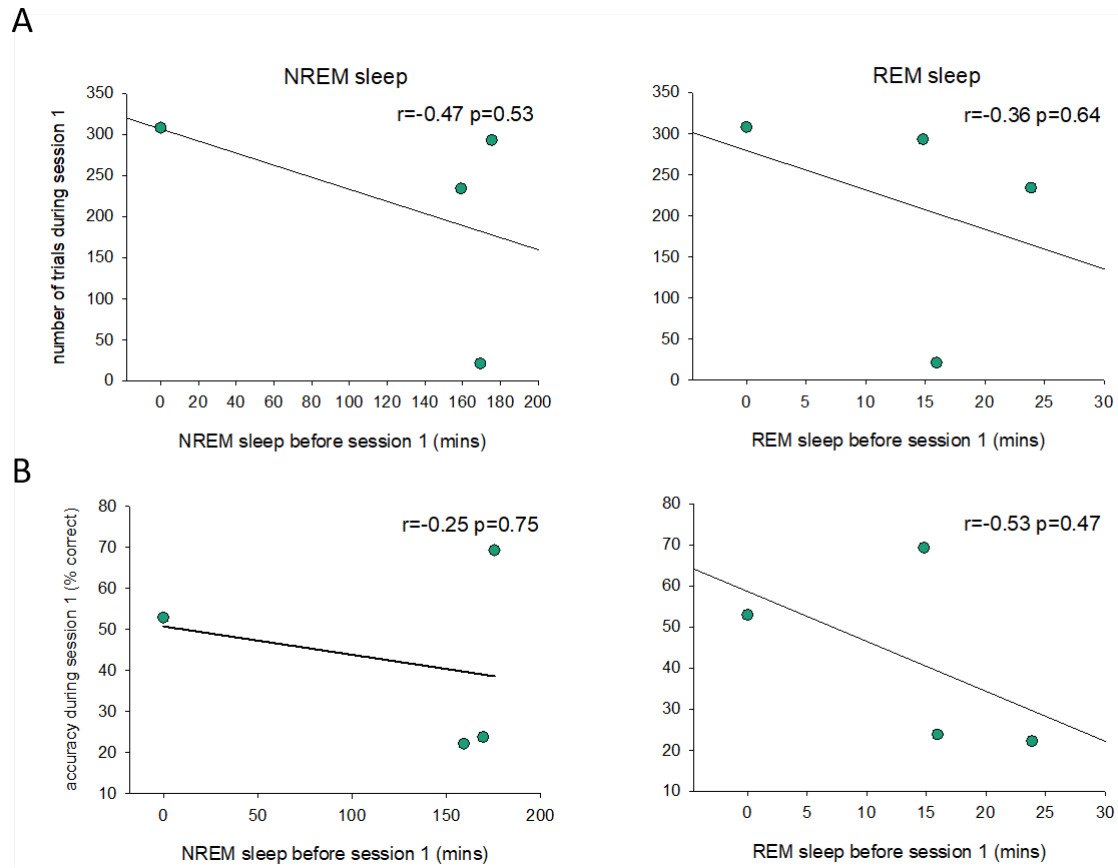


Figure 6-15: Correlation between the amount of sleep before and performance during session 1. Data are shown for the total number of trials (A) and the accuracy of performance (B). Left panels are correlations with NREM sleep, while right panels are correlations with REM sleep. Data points represent individual animals, with linear regression lines shown. Pearson correlation coefficient and *p* values are shown for each plot.

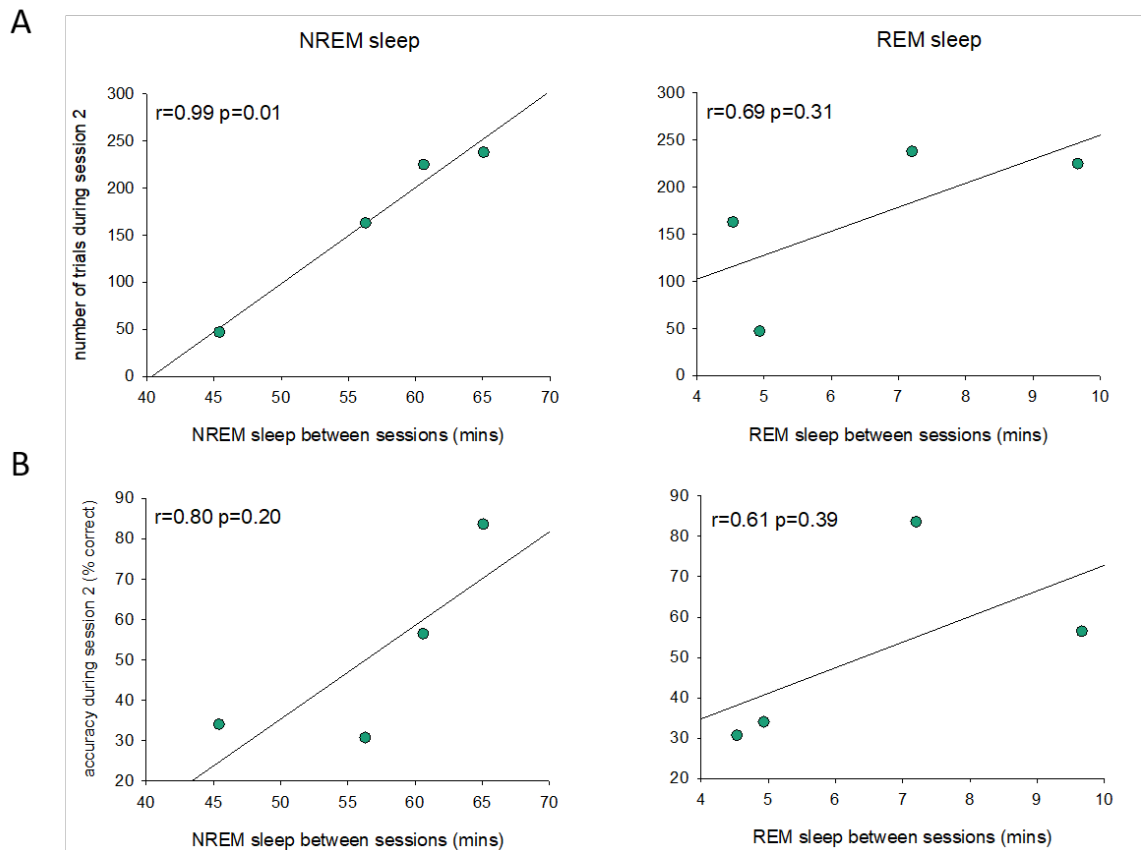


Figure 6-16: Correlation between the amount of sleep between sessions and performance during session 2. Data are shown for the total number of trials (A) and the accuracy of performance (B). Left panels are correlations with NREM sleep, while right panels are correlations with REM sleep. Data points represent individual animals, with linear regression lines shown. Pearson correlation coefficient and p values are shown for each plot.

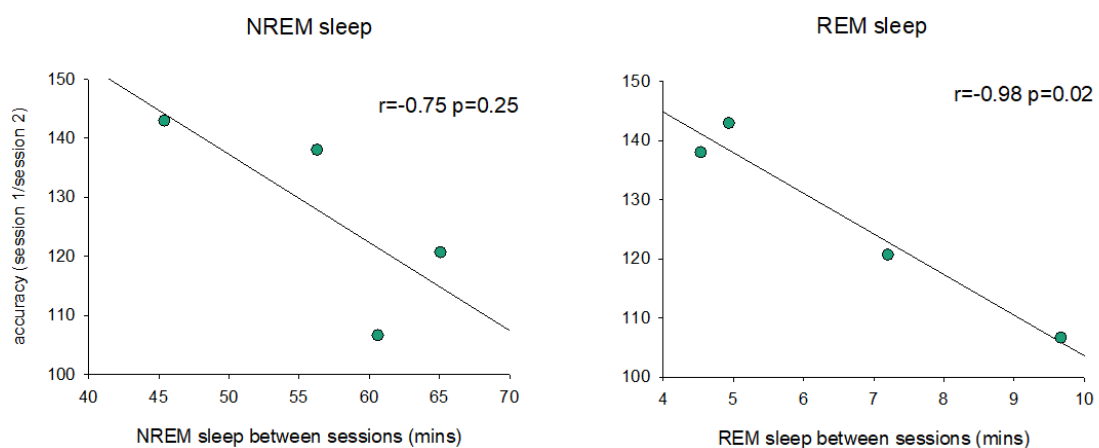


Figure 6-17: The influence of sleep on improvement in the accuracy of performance in the alphabet task between sessions. Both NREM sleep (left panel) and REM sleep (right panel) were negatively correlated with the improvement in accuracy between session 1 and session 2. Data points represent individual animals, with linear regression lines shown. Pearson correlation coefficient and p values are shown for each plot.

6.4 Discussion

These initial studies were designed as proof-of-principle studies, to investigate the feasibility of recording performance in the VDT task while animals are cabled to record EEG and multiunit activity. For this reason, both experiments were only performed in small numbers of animals. Although this project ultimately aimed to determine the effect of ageing on behavioural performance and cognitive functions, as there were a number of unknowns relating to the experiment in general (e.g. can mice perform while cabled), most data presented here were recorded from young (EA) with some additional data from late adulthood mice in the extended VDT task.

6.4.1 Extended VDT

As the extended VDT was performed during the light period, when mice are usually asleep, the idea was that the task would provide a highly cognitively demanding form of sleep deprivation. Surprisingly, performance was largely stable throughout the task, as there were no changes in the correct, incorrect and omission trials over the four hour session. This suggests that the animals are able to sustain a high level of cognitive performance and overcome the circadian drive for sleep as well as sleep pressure when they are sufficiently motivated to perform the task. This therefore suggests the intriguing possibility that performance in this task overrides the need to sleep, perhaps due to food-anticipatory activity which is known to increase locomotor activity 2-4 hours before a scheduled feeding time via a food entrainable oscillator (Foster and Hankins, 2007; Mistlberger, 1994; Pendergast and Yamazaki, 2014). As sleep deprivation has been associated with dramatic changes both at the level of EEG and neural activity (Achermann and Borbély, 2003; Brown, 2012; Dijk et al., 1987), it would be interesting to investigate the effects of this task on similar measures. Although this task is in itself a form of sleep deprivation, it may be that other mechanisms such as neuromodulation, hunger drive etc. allow for EEG and

neural mechanisms to be maintained. Whether performance in this task leads to similar rebounds in measures of sleep homeostasis seen after classical sleep deprivation techniques, such as an increase in EEG SWA, remains to be established.

Plotting the time course of the latency to correct and incorrect responses showed that there were much larger fluctuations from the mean for incorrect trials, whereas the latency to correct responses remained fairly constant across the four hours. Specifically the latency to incorrect responses showed an increase in the middle hour of the task, perhaps reflecting an ultradian rhythm. Therefore the latency to make incorrect responses may be more sensitive to the effects of sleep deprivation, than the latency to perform correct trials. This may reflect the levels of attention, as when mice perform correct trials the speed does not vary which suggests the mice head straight towards to screens to make a decision. The delayed response to make incorrect responses may represent a lapse of attention or indecision in making a response before eventually making the wrong response. This could be reflective of so called 'state instability' in which sleep deprivation causes an increase increased variability in responses due to counteracting sleep-driving mechanisms and endogenous compensatory mechanisms that act to maintain attention and alertness across sustained attention performance (Doran et al., 2001; Jung et al., 2011).

With regards to age differences in the performance of the extended VDT task, LA mice were found to have an overall higher number of incorrect trials (and with it higher number of incorrect/total trials). Although no differences were found in the time course of the number of incorrect trials, the LA mice consistently initiated more trials, particularly during the first half of the task. Interestingly, LA mice were also found to have a significantly reduced latency to perform incorrect trials, suggesting that LA mice have fewer attentional deficits compared to EA

mice. However, once again, this age difference was no longer apparent in the time course of the latency to perform correct responses.

Although I ran statistical analyses on the data from the alphabet task, this must be interpreted with caution due to the low number of animals contributing to the data ($n=4$). Therefore the majority of results are descriptive in nature and preliminary. The data presented here suggest that learning in this task manifests as an increase in the number of correct responses and a decrease in the number of incorrect trials from session 1 to session 2, which is especially prominent across the quartiles of this first session when animals are first exposed to the new images and so must learn the correct response. Importantly these measures were fairly stable across the second session, indicating that the initial learning in session 1 is maintained across the 4 hours between session 1 and 2. In this study there were no difference between sleep and sleep deprivation conditions which suggests that sleep does not play a major role in the adapting memory schema to new rules in this task. Importantly, none of these measures were significantly different across sessions, despite visible trends. This may be reflective of the small numbers of animals used in this study ($n=4$) and so it is important to increase the numbers of animals before any conclusions can be made. The learning of the new letters may also be reflected in the reduction in the number of trials across sessions as animals perform more correct trials. Importantly the number of omission trials was low across both sessions and days, suggesting that animals were motivated to perform the task and so were actively learning the task.

In order to investigate the role of sleep in memory in this task, correlations between sleep and performance were calculated. The amount of sleep between sessions was found to positively correlate with measures of performance, which was significant between the amount of NREM sleep and the number of trials during session 2 (Figure 6-15B). This is consistent with evidence in

human studies that showed that sleep immediately after training in a visual discrimination task was important for improving performance (Stickgold et al., 2000a, 2000b). In contrast, sleep before the task did not greatly improve performance. This highlights the importance of sleep in consolidation of memory, as is well established (see section 1.1.4.1 for details). Alternatively sleep may provide restoration from previous performance, allowing animals to perform more trials. Rather surprisingly, in this study the amount of REM sleep between the two sessions was negatively correlated with the improvement in accuracy between sessions. Previous studies have suggested that REM sleep is beneficial to memory consolidation (Diekelmann and Born, 2010; Rasch and Born, 2013; Stickgold, 2005), therefore this finding is somewhat surprising.

6.4.2 Limitations of the study

The main limitation of this study was the low numbers of animals contributing to these data sets. For this reason it is difficult to draw strong conclusions and interpret statistical results. In particular, it was not possible to perform a comprehensive analysis on the link between performance and sleep in the alphabet task. I therefore instead used correlation analyses, however due to the low number of animals, the data set may not be an accurate representation of the studied population and are therefore difficult to interpret. Before firm conclusions are possible it would first be important to perform the task in larger numbers of animals.

However the central aim of this study, to determine whether these tasks could be utilised to study specific aspects of learning, was achieved, as animals were able to successfully perform these tasks. In order for the data to be validated it is important to run these tasks in more animals.

Plotting learning curves for the retraining days after surgery revealed that animals relearned the task well after surgery. However, in the extended VDT study LA mice had a reduced accuracy compared to EA mice, as indicated by few animals reaching the 80% threshold. It is possible

therefore that LA mice were more greatly affected by the surgery and cabling, resulting in deficits in performance after surgery. If deficits were present in the standard VDT protocol this could enhance the age differences in subsequent tasks. However, importantly, no major age differences were identified in the extended VDT protocol. In future studies it would be important to investigate the potential cause of these differences in more detail. It is possible that changing the cables or head stage orientation (positioned further back so as to not obstruct in the task as much) may aid the transition to performing the task cabled.

6.4.3 Overall conclusions

The main aim of this study was to develop a novel paradigm to study the relationship between sleep, waking performance, cognitive function and ageing. I developed two new approaches – extended VDT and the Alphabet task, which show high promise for addressing these aspects. In this chapter I report preliminary data that show that mice successfully performed both of these tasks. The main and most interesting finding was that mice performed consistently well for the duration of the task in the extended VDT paradigm. Furthermore, this preliminary data suggests that mice are able to learn to discriminate a completely new set of images within a few hours of training. However, before conclusions can be made about the role of sleep in the alphabet task, it would be crucial to perform this task in a larger cohort of animals. Finally, whether these tasks may be useful in studying the cognitive decline observed in ageing remains to be established, however the evidence provided here does not suggest any reasons as to why this would not be possible.

Chapter 7: Importance of findings and future directions

7.1 Main findings

The main aim of this thesis was to investigate the local cortical neural dynamics underpinning the effects of ageing on sleep and sleep oscillations in baseline conditions, after sleep deprivation and after an administration of a common hypnotic diazepam. Furthermore, as ageing has been associated with a memory and cognitive decline, I used behavioural tests to determine the link between ageing, sleep and cognitive processing.

Objective 1 was to investigate the spatio-temporal properties of cortical neural activity in freely moving young and older mice and to elucidate neurophysiological markers of ageing and sleep-wake history. In chapter 3 of this thesis I replicated many of the previously observed effects of ageing on sleep; including an increase in the amount of sleep, increased fragmentation of the sleep-wake cycle (predominantly an increase in the number and duration of wake episodes but also an increase in the number of NREM sleep episodes) and increased SWA power. The most important finding of this chapter was that healthy ageing in mice does not greatly affect vigilance state related local neural activity, despite pronounced global changes in the daily amount and distribution of waking and sleep. Specifically, no marked differences were identified in the capacity to generate consolidated network OFF periods (absence of spiking activity corresponding to slow waves) and local slow waves, in the overall vigilance-state specific firing profiles of individual neurons, or the local homeostatic response to sleep deprivation. Irrespective of age positive local field potential (LFP) slow waves were associated with an unequivocal suppression of multiunit activity (MUA). The most notable effects of ageing at the level of neuronal activity was an increase in the incidence of LFP slow waves and OFF periods, as

well as there being a higher proportion of neurons that increase their firing rates after sleep onset.

As hypnotics are commonly used to improve sleep and have pronounced effects on the EEG, **objective 2** was to investigate the effects of diazepam on cortical neural activity and to determine whether there are age-dependent differences in the neural responses to diazepam. In chapter 4 of this thesis, I showed that overall sleep wake architecture remained fairly resistant to the effects of diazepam, except for an increase in sleep-wake fragmentation. At the level of EEG, diazepam injection resulted in a profound reduction in SWA, that was maintained across the 12 hour light period. With regards to neural activity there were no differences in the average firing rates across experimental days. However, OFF periods during NREM sleep were less frequent after diazepam injection. Most importantly, diazepam injection reduced average firing rates around OFF periods. Despite replicating previously reported effects of ageing (increased sleep-wake fragmentation, increase in SWA, increase in the number and duration of OFF periods) there were no major age-differences in the response to diazepam in this study. However, our preliminary results suggest that the reduction of average firing rates around OFF periods was more pronounced in LA mice.

Objective 3 was to determine the effect of ageing and sleep deprivation on behavioural performance in a hippocampal dependent task: the Morris water maze (MWM). All mice showed a significant improvement in performance in the task across the 6 training days, which was not different between age groups. Subjecting the mice either to a 6-hour period of sleep deprivation or allowing them to sleep prior to a probe trial to assess spatial memory, did not identify any significant differences in performance in the water maze. In the water maze task few differences between EA and LA mice and experimental conditions were observed, suggesting that ageing and sleep deprivation had little effect on spatial learning and memory in the MWM task.

Objective 4 was to develop a novel behavioural paradigm to study sleep, sleep deprivation and cognitive function, which may be applicable to sleep and ageing studies in the future. In chapter 6, I report preliminary data that showed that mice were able to successfully perform both the extended visual discrimination task (VDT) and the alphabet task. Notably, performance in the extended VDT task was largely stable throughout the four hour testing period during the light phase. This suggests that the animals are able to sustain a high level of cognitive performance and overcome the circadian drive for sleep as well as sleep pressure when they are sufficiently motivated to perform the task. Furthermore, data from the alphabet task suggest that mice are able to learn to discriminate a completely new set of images within a few hours of training.

7.2 Novel observations

The findings I report in this thesis build on previous literature but contribute significant novel insights to the cortical and neural dynamics underpinning the effects of ageing on sleep in general as well as its link with hypnotics and cognition. The insights into the neural underpinnings of the slow oscillation provided in both the main ageing chapter (chapter 3) and the diazepam study (chapter 4) highlight the necessity to consider different levels of organisation together i.e. both local neural activity and global EEG, in order to fully determine the effects of ageing on brain function and sleep. As electrophysiological characteristics, connectivity patterns, ongoing behaviour and preceding sleep-wake history may all together determine the firing phenotype of specific cortical neurons (Ascoli et al., 2008; Fisher et al., 2016; Kropff et al., 2015; McGinley et al., 2015; O’Keefe and Dostrovsky, 1971; Poulet and Petersen, 2008), these factors may all play an increasingly important role in ageing.

Previous studies have focussed mainly on characterising the effects of ageing on global sleep including the overall structure of the sleep-wake cycle and associated EEG characteristics, and have shown that sleep becomes more fragmented and superficial with ageing (Altena et al., 2010; Banks et al., 2015; Bano et al., 2012; Gu et al., 2015; Hasan et al., 2012; Klerman et al., 2013; Klerman and Dijk, 2008; Ohayon, 2004a; Panagiotou et al., 2017; Shiromani et al., 2000; Vyazovskiy et al., 2006a; Wimmer et al., 2013), which was also replicated in this study. Importantly though, these previous studies did not investigate the neuronal dynamics that underlie cortical slow waves. As this was the first time chronic recordings of neuronal activity was performed in freely moving mice in the context of ageing, there were a number of unknowns involved with the study. First and foremost, it was not known whether 2 year old mice would cope with the surgical and recording procedure. However, I was successfully able to record neural activity from individual neurons from 2 year old mice over extended periods of several weeks. The main novel observation of this thesis was that at the local level, the network activity underpinning EEG/LFP slow waves was largely intact in old mice.

An interesting observation in this study was that individual neurons were highly variable in their state specific firing, yet the firing profiles of individual putative neurons during waking and sleep was largely stable across the life span. The lack of differences at the local neural level contrasts with the considerable differences observed at the global scale, such as in the EEG or sleep-wake architecture, and suggest that these disruptions are unlikely to arise from changes in local cortical activity.

Previous studies have shown a reduction in EEG slow-wave activity (SWA) with ageing in both humans (Agnew Jr. et al., 1967; Dijk et al., 1989; Feinberg et al., 1984) and mice (Banks et al., 2015; Colas et al., 2005; Hasan et al., 2012; Wimmer et al., 2013b). This led to conclusions that ageing may reduce the homeostatic sleep need (Mander et al., 2017; Wimmer et al., 2013b). In

contrast to these results, in this thesis SWA was found *to increase* with ageing, accompanied by an increase in the incidence of slow waves and OFF periods. In combination with another recent study that showed an increase in EEG SWA with ageing (Panagiotou et al., 2017), these new results suggest that older mice may instead have an increased sleep pressure. Once again, this data highlights the need to consider local neural dynamics, in addition to global EEG, when investigating the effects of ageing on sleep.

An alternative explanation for the effects of sleep on ageing is that ageing primarily diminishes the capacity to generate and sustain deep NREM sleep (Cirelli, 2012a; Klerman and Dijk, 2008; Mander et al., 2017). This is partially supported by the findings in this thesis, that the robust increase in the incidence of slow waves and OFF periods in the first 2 minutes of NREM sleep after sleep deprivation (compared to baseline values), was attenuated in LA and OA mice. It is possible that ageing may result in global state instability or disrupt the balance between excitation and inhibition in cortical networks, which prevents a smooth transition into a state with reduced activity typical for deep sleep. As a result, sleep in older mice may be less restorative, resulting in an increased sleep propensity, an inability to maintain consolidated periods of wake and an increase in SWA. Indeed evidence suggests that the homeostasis of firing rates and precisely regulated excitation/inhibition balance are essential to reduce metabolic costs of waking and neurotoxicity (Haider et al., 2006; Laughlin et al., 1998). The failure of cortical networks to maintain stable function may thus result in the inability to sustain longer periods of wakefulness with ageing leading to a fragmentation of the sleep-wake cycle, which is often reported with ageing (Colas et al., 2005; Hasan et al., 2012; Welsh et al., 1986; Wimmer et al., 2013b), and also shown in this thesis. Local and global state instability (Doran et al., 2001; Parrino et al., 2012) may in turn result in diminished behavioural performance and attentional and learning deficits.

The profound reduction in EEG spectral power in the SWA range with diazepam injection reported in this study is consistent with previous literature (Kopp et al., 2003; Kopp et al., 2004; Lancel et al., 1996; Lancel and Steiger, 1999; Tobler et al., 2001). While the molecular mechanisms of benzodiazepines action have been elucidated in great detail (see main introduction for details, section 1.3), previous studies have not looked at the neural activity underlying the response to diazepam in the neocortex. This is particularly important given that in elderly populations it is well established that benzodiazepines often have a lack of efficacy and numerous side effects associated with their use (Amanti, 2018; Borbély et al., 1983; Greenblatt et al., 1983; Nicholson et al., 1982). In this thesis, ageing did not greatly affect the effect of diazepam on overall global characteristics of the sleep-wake cycle or power spectra, however trends towards lower firing rates in LA mice suggest that the local neural mechanisms after diazepam injection may be more affected than global mechanisms. The subtle differences identified in the neural activity underpinning the effects of diazepam may, at least partially, account for differences in the efficacy of benzodiazepines in older individuals (Borbély et al., 1983; Greenblatt et al., 1983; Nicholson et al., 1982). Therefore, although both ageing and diazepam injection are associated with decreases in SWA, the neural underpinnings of these effects may be different and so it is crucial to consider the local neural dynamics together with global dynamics in order to elucidate how hypnotics are working across ages.

It has been long established that sleep plays an essential role in memory (Diekelmann and Born, 2010; Rasch and Born, 2013) and that disruptions in sleep, either with regard to the amount or timing of sleep, may be associated with psychiatric and neurodegenerative disorders, which often involve disruption in cognitive processes such as memory (Wulff et al., 2010). Given the numerous bidirectional links between sleep, ageing and memory, it is possible that the disruptions of sleep with ageing may contribute to the cognitive decline observed with ageing. However, currently the mechanisms underlying this possible link are unknown. In this thesis I

therefore investigated the effects of ageing and sleep on performance in the Morris water maze task and VDT.

In chapter 5 I used the Morris water maze task in combination with the use of sleep deprivation and aged mice, to investigate the link between sleep, ageing and cognition. Importantly, I found few age-differences in performance in the task, and sleep deprivation also did not have a large influence on performance. This data suggests that ageing and sleep deprivation had little effect on spatial learning and memory in the MWM task. It is possible that the lack of differences I observed in this study are due to the relatively young age of the oldest age group, therefore the Morris water maze remains a useful pre-clinical test for assessing spatial learning and memory across different conditions such as age and drug manipulations. It should however be noted that this task cannot be considered a direct measure of cognitive function, but rather is an indirect measure of a psychological construct which is influenced by external, non-specific factors such as thermoregulation, stress and visual acuity to name a few (Lindner, 1997).

Although touchscreens are widely used and validated for studying various different aspects of cognition including; learning, working memory and executive function (Horner et al., 2013), it has never been used in the context of ageing. As the task is highly versatile this opens up great opportunities to adapt the task for use with ageing mice to target various aspects of learning and performance. Therefore, in this thesis I presented data from two new approaches developed based on previous touchscreen methodology – extended VDT and the Alphabet task. These initial studies were designed as proof-of-principle studies, to investigate the feasibility of recording performance in the VDT task while animals are cabled to record EEG and multiunit activity. The main and most interesting finding was that mice performed consistently well for the duration of the task in the extended VDT paradigm. Furthermore, the preliminary data suggest that mice are able to learn to discriminate a completely new set of images within a few hours of

training. However, before conclusions can be made about the role of sleep in the alphabet task, it would be crucial to perform this task in a larger cohort of animals. Finally, whether these tasks may be useful in studying the cognitive decline observed in ageing remains to be established, however the evidence provided here does not suggest any reasons as to why this would not be possible.

Although performance was fairly stable across the extended VDT task the latency to make incorrect responses may be more sensitive to the effects of sleep deprivation, as compared to the latency to perform correct trials. This could be reflective of so called 'state instability' in which sleep deprivation causes an increased variability in responses due to counteracting sleep-driving mechanisms and endogenous compensatory mechanisms that act to maintain attention and alertness across sustained attention performance (Doran et al., 2001; Jung et al., 2011).

It has previously been suggested that ageing may be associated with a number of structural changes, such as a loss of synaptic connections, and a reduction in the stability of synaptic connections, while alterations in synaptic transmission have also been observed (Dumitriu et al., 2010; Grillo et al., 2013; Morrison and Baxter, 2012; Peters et al., 2008; Petralia et al., 2014).

This may explain why in both Chapter 3 and 4 of this thesis the local underpinnings of the slow waves may be distinct from the global dynamics. The fact that individual neurons may maintain their activity across the lifespan suggest that synchronisation across networks may be responsible for the changes at the level of EEG. It is also possible that the well-established effects of slow waves in the homeostatic rebalancing or remodelling of synaptic networks (Chauvette et al., 2012; Krueger et al., 2013; Tononi and Cirelli, 2014; Vyazovskiy and Harris, 2013; Watson et al., 2016), may be affected by ageing, observed as an increased incidence of local slow waves in older mice. Overall these may explain the differences in cortical dynamics observed in both ageing and in the response to diazepam treatment. The differential effects of

local and global dynamics associated with the decline in cognition remain to be established. However, the behavioural data reported here suggest that these tasks may be useful for investigating this relationship.

7.3 Is ageing different between humans and mice?

With the ever expanding ageing population there is becoming an increased need to determine the mechanisms underpinning the effects of ageing. Sleep is an ideal state to investigate the effects of ageing on brain functions; however the mechanisms underpinning the effects of ageing on sleep are largely unclear. This has increased the demand for appropriate animal models for studying the aetiology and mechanisms underlying the link between ageing and sleep. As species often vary with regards to their brain and body size, and often have differences in their metabolic rates, which are associated with longevity and sleep (Allison and Cicchetti, 1976; Capellini et al., 2008; Herculano-Houzel, 2015; Siegel, 2005; Zepelin and Rechtschaffen, 1974), it is also a possibility that species differences may exist in the effects of ageing on sleep characteristics (Klerman and Dijk, 2008). These differences may also have important implications in the regulation of global and local neural activity.

An important species difference for this study was that, in contrast to the reduced sleep with ageing observed in humans, mice in fact often show an increased amount of sleep with ageing, though mouse data is also inconsistent in these findings (see section 1.2.2). In addition, SWA has been shown to be reduced in humans whereas there is recent evidence to suggest that this is not the case in mice, which instead show an increased amount of SWA (Panagiotou et al., 2017), though evidence is conflicting (see section 1.2.2). This brings into question the viability of using mice as a model for studying human sleep. It is therefore important to address this question in more detail, in order to determine whether mice are in fact a useful model.

The discrepancy between humans and mice may be due to the majority of previous studies utilising only EEG recordings, which record from a larger area of the cortex. Therefore, these may not have had the resolution or precision to determine the more localised mechanistic changes, or lack of changes associated with ageing. In this thesis I did not perform a detailed analysis of occipital EEG recordings, such as characteristics of EEG slow waves, because EEG signals have relatively poor spatial resolution. This is especially relevant in the context of ageing, where this analysis has not been performed before and so the origin of potential differences may be difficult to establish. It is precisely for this reason I focused most of the analyses on the LFP recorded simultaneously with local MUA activity. In this thesis, I showed that the local dynamics in neural activity underlying the effects of ageing on sleep as well as diazepam, may be distinct from those that occur at the global EEG level. This highlights the importance of considering the level of organisation, as well as the species, when interpreting results. In addition, the spatial scale of EEG recordings varies greatly across species, with a single electrode recording from a larger area of the cortex in humans as compared to mice. As intracranial recordings during sleep have not been performed in older humans, it remains to be established whether the effects observed here may be generalised across species. Although, it is possible that older humans, as in the mouse data I report here, have an intact capacity for generating slow waves. Furthermore, there may be species-specific strategies to compensate for the effects of ageing. For example, in mice there may be an overall increase in sleep, however in humans there may be more local sleep-like activity occurring during wake.

It is becoming more popular to use genetic manipulations to investigate ageing processes and age-related diseases. However, these results are often complicated to interpret due to the effects often being dependent on the genetic background of the mice (Franken et al., 1998). For example, considerable strain-dependent differences in the age-dependent effects on sleep,

circadian, and light input parameters (Banks et al., 2015; Eleftheriou et al., 1975; Hasan et al., 2012) have been identified. Importantly, the most commonly used strain of mice, C57BL/6J, was also found to display higher activity measures, fewer sleep episodes in the dark phase, less fragmentation, shorter alpha periods, a higher running wheel activity and a higher circadian amplitude compared to C3 strains (Banks et al., 2015).

7.4 The role of extrinsic and intrinsic factors in the effects of ageing on sleep

There are numerous extrinsic and intrinsic factors that need to be considered, when studying the effects of ageing on sleep.

Ageing may lead to a weaker entrainment to the 24-hour circadian cycle, which could influence the distribution of the sleep-wake cycle over 24-hours (Banks et al., 2015; Kondratova and Kondratov, 2012; Nakamura et al., 2011). In particular, ageing causes a reduction in total activity levels and reduction in the amplitude of the activity/rest cycle, leading to a less distinct separation between light and dark phase activity (Banks et al., 2015; Nakamura et al., 2011; Valentinuzzi et al., 1997). It is thought that ageing may also lead to a weakening of the coupling between oscillators in the SCN (master circadian clock) (Farajnia et al., 2014, 2012; Ramkisoensing and Meijer, 2015). To ensure that no differences in circadian period or phase influenced the results presented in this thesis, mice were housed under entrained conditions (12:12 LD). As a result, these specific age-dependent circadian changes are unlikely to influence the results presented here. In order to minimise the effects of circadian time on behavioural performance in this study, both the water maze and VDT tasks were performed at roughly the same time every day (approximately 6 hours after light onset).

In mice, ageing leads to an increase in body weight which has been shown to reduce locomotor activity and running behaviour (Banks et al., 2015; Kopp et al., 2006; Possidente et al., 1995; Valentinuzzi et al., 1997; Welsh et al., 1986), which was also confirmed in this study. Access to a running wheel has been shown to lengthen the wake bout duration (Vyazovskiy and Tobler, 2012). This was also replicated in this study with the amount of running being highly positively correlated with wake bout length. As it has been shown that the activities undertaken during wakefulness, such as running wheel activity can have a substantial effect on subsequent amount and distribution of sleep (Fisher et al., 2016; Vyazovskiy et al., 2006b; Vyazovskiy and Tobler, 2012), it is possible that age-dependent differences in running wheel activity may, at least partially, contribute to the age-dependent alterations in global sleep-wake structure in this study. Since older mice run considerably less, but local cortical dynamics and local sleep homeostasis are not changed, it may be argued that it is unlikely that environmental enrichment or experimental conditions have a strong influence on the results. However, it cannot be excluded that running had an important influence, as running affects several aspects of brain, behaviour and neural function in various species (Meijer and Robbers, 2014; van Praag et al., 1999).

Studying ageing in behavioural tasks is notoriously difficult as there is a complex relationship between ageing and cognition, which varies greatly depending on the species, strain, and task used to assess behaviour. Clearly, there are a number of potential confounds in studying behaviour across ageing, such as increased body weight, decline in visual acuity (Banks et al., 2015; Wong and Brown, 2007), decreased contrast sensitivity (Alphen et al., 2009), decreased locomotor activity (Barreto et al., 2010; Ingram et al., 1981), and a decreased motivation to perform tasks (Bordner et al., 2011; Brodaty et al., 2010). The rhythmicity of physiological measures such as drinking and body temperature are also altered by ageing (Ingram et al., 1981; Kopp et al., 2006). These factors may all lead to misinterpretations of deficits in performance or

motor systems (Alphen et al., 2009), making it notoriously difficult to design a task that is appropriate for studying cognition and ageing. In the water maze task investigated in this thesis (Chapter 5), swim speed was significantly reduced in older mice and therefore these mice travelled a shorter distance in the task compared to EA mice. It has previously been suggested that differences in swim speed may reflect sensorimotor deficits, however as this was based on data from young animals only it is more likely that the differences observed in this study merely reflect age-dependent differences in locomotion, especially as no differences in spatial learning measures were identified between the groups. However, it has been suggested that although animals may show hypo-activity in activity boxes, there may be no differences in swim speed in the MWM (Fitzgerald and Dokla, 1989), leading to suggestions that learning effects may be observed in the MWM regardless of differences in activity levels (Vorhees and Williams, 2006).

7.5 Future directions

This was the first study to perform chronic recordings of neuronal activity from the neocortex in freely moving mice up to 2 years of age. The main aim of this thesis was to characterise neuronal activity in the context of ageing but I also performed several pilot experiments to explore a number of other aspects such as pharmacological responses and behaviour. As some of these studies were exploratory the numbers of animals were low, particularly for chapter 6 which reported results from the touchscreen task. As the numbers of animals used in this study were fairly low, it is difficult to make statistical comparisons. In order for these effects to be fully understood it is necessary to replicate these studies in more animals.

Previously, age-dependent differences in sleep have been identified in animals aged ~25 months old (Colas et al., 2005; Eleftheriou et al., 1975; Hasan et al., 2012; Welsh et al., 1986; Wimmer et al., 2013), which may become apparent at ages > 1 year (Hasan et al., 2012). In this study age dependent differences in global and local sleep characteristics were identified at 1 year old. The

exploratory nature of the behavioural studies in this thesis (both the water maze and VDT task) meant it was essential to start with relatively young animals (up to 12 months) to first determine whether animals could learn and perform the tasks. It is therefore possible that no cognitive decline may be observed at these ages. Therefore, it is important to run these tests in older animals now that the tasks have been shown to work in younger animals.

In this thesis I initially proposed that ageing may target specific local networks, however it remains to be established, whether the differences, or lack thereof, reported here may be generalised across other cortical and subcortical brain areas, such as associative and sensory areas. The motor cortex was selected for recordings in this thesis as it has been well studied in younger rodents during spontaneous sleep and after sleep deprivation (Fisher et al., 2016; Hajnik et al., 2013; Hayashi et al., 2015; Vyazovskiy et al., 2011; Vyazovskiy and Tobler, 2005). Our research group also has extensive experience in recording and analysing neural activity during waking and sleep from this region. This is especially important as this was the first study to investigate LFP and MUA dynamics during sleep in the context of ageing and so it would have been unwise to start recording from a cortical region that our laboratory group had limited experience with. The motor cortex was also chosen based on the well-established frontal dominance SWA is known to have during NREM sleep, both in rats and mice as well as humans (Finelli et al., 2001; Huber et al., 2000b; Massimini et al., 2004; Nir et al., 2011; Schwierin et al., 1999; Sirota and Buzsaki, 2005; Vyazovskiy et al., 2006b; Werth et al., 1996a). As slow waves have been recorded in every cortical region recorded to date, it is possible that the observations in the motor cortex could generalise to other cortical areas. As ageing is well established to involve disruptions in sensory processing (Alphen et al., 2009; Banks et al., 2015; Wong and Brown, 2007), it would be important to first investigate sensory regions and run basic tests on the integrity of sensory functions in older mice. The prefrontal cortex is also one of the main regions posited to be affected by ageing, both at a structural level (Peters et al., 1998) as well as

in the performance of tasks involving the prefrontal cortex (Banks et al., 2015; Mattay et al., 2006; Rajah and D'Esposito, 2005). Therefore this region would be an interesting target for future studies.

In this thesis I present data that suggest that animals up to 12 months of age are successfully able to perform the water maze and VDT tasks. Although EEG and neuronal data was recorded during performance of the VDT tasks, analysis of this data was beyond the scope of this thesis. As in the earlier chapters of this thesis I provided evidence that the effects of ageing may differentially affect the local and global dynamics of sleep, this would be important to investigate in the context of behavioural tasks. Therefore future studies should investigate the neural activity during and after performance in these tasks. This would be particularly interesting in the context of the extended VDT task as this task is a form of sleep deprivation which involves a highly cognitively demanding form of wake. As ageing is thought to involve changes in the homeostatic regulation of sleep, it would be crucial to understand the effects of extended performance on cortical neural activity.

Finally, it has been shown that the age-dependent reduction in synaptic connectivity may be compensated for by an increase in the electrical responsiveness of neurons to incoming stimuli (Barnes and McNaughton, 1980). Therefore, it is possible that local neural activity may be maintained over ageing, due to compensatory or protective mechanisms. Future studies should perform intracellular recordings to determine whether the cellular and ionic mechanisms underlying slow wave regulation may also undergo compensatory changes.

7.6 Overall conclusions

By integrating several state-of-the-art approaches from putative single unit electrophysiological recordings to behaviour and pharmacology, this thesis has provided novel insights into the neural mechanisms involved in the age-dependent changes in sleep and cognition. The differences between global and local mechanisms affected by ageing, and also interventions such as diazepam, suggest that in order to fully understand the effects of ageing, local and global mechanisms must both be taken into account. Only by considering these different levels of organisation will it be possible to determine whether ageing primarily affects homeostatic sleep need or may instead diminish the capacity to generate and sustain NREM sleep (Cirelli, 2012; Klerman and Dijk, 2008; Mander et al., 2017). It is possible that local neural activity may be maintained over ageing, due to compensatory or protective mechanisms. This is important for both basic neuroscience and for sleep-related issues of public health and safety. Only by understanding local cortical mechanisms will it be possible to improve current treatments aimed at helping with the unwanted effects of ageing, such as cognitive decline and sleep disruptions.

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