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Abstract

Healthy ageing is associated with marked effects on sleep, including its daily amount and architecture, as well as the specific electroencephalogram (EEG) oscillations. Neither the neurophysiological underpinnings, nor the biological significance of these changes are understood, and crucially the question remains whether ageing is associated with reduced sleep need or a diminished capacity to generate sufficient sleep. Here we tested the hypothesis that ageing may affect local cortical networks, disrupting the capacity to generate and sustain sleep oscillations, and with it the local homeostatic response to sleep loss. We performed chronic recordings of cortical neural activity and local field potentials (LFP) from the motor cortex in young and older male C57BL/6J mice, during spontaneous waking and sleep, as well as during sleep after sleep deprivation. In older animals, we observed an increase in the incidence of non-rapid eye movement (NREM) sleep LFP slow waves and their associated neuronal silent (OFF) periods, while the overall pattern of state-dependent cortical neuronal firing was generally similar between ages. Furthermore, we observed that the response to sleep deprivation at the level of local cortical network activity was not affected by ageing. Our data thus suggest that the local cortical neural dynamics and local sleep homeostatic mechanisms, at least in the motor cortex, are not impaired during healthy senescence in mice. This indicates that powerful protective or compensatory mechanisms may exist to maintain neuronal function stable across the life span, counteracting global changes in sleep amount and architecture.

Significance statement

The biological significance of age-dependent changes in sleep is unknown, but may reflect either a diminished sleep need or a reduced capacity to generate deep sleep stages. Since ageing has been linked to profound disruptions in cortical sleep oscillations, and since sleep need is reflected in specific patterns of cortical activity, we performed chronic electrophysiological recordings of cortical neural activity during waking, sleep and after sleep deprivation from young

and older mice. We found that all main hallmarks of cortical activity during spontaneous sleep and recovery sleep after sleep deprivation were largely intact in older mice, suggesting that the well described age-related changes in global sleep are unlikely to arise from a disruption of local network dynamics within the neocortex.

Introduction

Ageing is a strictly regulated biological process, that is thought to provide selective advantages over attaining immortality and therefore increases the evolutionary success of a species. Ageing refers to a variety of modifications which occur progressively as a function of preceding time spent alive, and can be observed at the molecular, cellular and system levels, 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). Some of these processes are entirely physiological, and reflect programmed ageing, while others may reflect unwanted, normally inevitable but possibly preventable, consequences of various stressors encountered throughout life (Meyer et al., 1999; Enzinger et al., 2005).

Despite notable species differences, numerous studies in both humans and laboratory animals suggest that waking and sleep show systematic changes with ageing; however, the biological significance of these changes is not well understood (Shiromani et al., 2000; Ohayon et al., 2004; Klerman and Dijk, 2008; Altena et al., 2010; Bano et al., 2012; Hasan et al., 2012; Klerman et al., 2013; Banks et al., 2015; Gu et al., 2015; Panagiotou et al., 2017). One possibility is that age-dependent changes in sleep are a reflection of anatomical or physiological changes, such as a loss of synaptic connectivity (Morrison and Baxter, 2012), altered Ca^{2+} homeostasis (Toescu and Vreugdenhil, 2010), a decline in the function of specific brain circuits (Porkka-Heiskanen et al., 2004; Wigren et al., 2009; Altena et al., 2010; Wang et al., 2011), increased susceptibility to cellular stress (Naidoo et al., 2008; Naidoo, 2009; Altena et al., 2010; Kourtis and Tavernarakis, 2011), or a progressive loss of circadian rhythmicity, such as a decline in the

74 rhythmic output of the central circadian clock, the suprachiasmatic nucleus (SCN) (Satinoff et
75 al., 1993; Watanabe et al., 1995; Aujard et al., 2001; Biello, 2009; Colwell, 2011). It is also
76 possible that the sleep changes that occur with ageing may represent compensatory responses, in
77 which sleep plays an active and increasingly important role in maintaining cellular homeostasis
78 and optimal waking functions as the organism gets older. Consistent with this, sleep is known to
79 play a crucial role in various restorative functions, including molecule biosynthesis, membrane
80 repair, synaptic remodelling and other cellular maintenance processes (Mackiewicz et al., 2008;
81 Vyazovskiy and Harris, 2013; Xie et al., 2013; Tononi and Cirelli, 2014; Krueger et al., 2016).
82 While numerous studies have provided important insights into the global age-dependent
83 alterations in sleep-wake architecture and electroencephalogram (EEG) across 24-hours in mice
84 (Vyazovskiy et al., 2006b; Hasan et al., 2012; Banks et al., 2015; Panagiotou et al., 2017),
85 surprisingly little is known about the effects of ageing on cortical neural activity. This is an
86 important omission, as numerous studies suggest that the events occurring at a single neuron and
87 local neuronal population level in the neocortex have important contributions to global sleep
88 regulation (Vyazovskiy et al., 2009b; Vyazovskiy et al., 2011; Grosmark et al., 2012; Fisher et
89 al., 2016; Krueger et al., 2016; Rodriguez et al., 2016; Watson et al., 2016; Siclari and Tononi,
90 2017). It is well established that sleep-wake history is reflected in the level of EEG slow-wave
91 activity (SWA, 0.5-4Hz), which increases as a function of preceding wake duration and
92 decreases during subsequent sleep, and is therefore used as a measure of sleep homeostasis
93 (Borbély, 1982; Tobler and Borbely, 1986; Franken et al., 2001). Slow waves recorded with
94 cortical EEG or local field potential (LFP) electrodes, reflect the synchronous occurrence of
95 population neuronal silence, corresponding to neuronal hyperpolarisation or down-states within
96 thalamocortical networks (Destexhe et al., 1999; Timofeev, 2013; Vyazovskiy and Harris, 2013;
97 Crunelli et al., 2015; Neske, 2016). These so-called OFF periods become increasingly more
98 frequent the longer the duration of wakefulness, and are most prominent during the initial deep
99 NREM sleep occurring immediately after periods of wakefulness. Despite being a defining

feature of NREM sleep, locally occurring OFF periods may also be detected during wakefulness, especially after sleep deprivation (Vyazovskiy et al., 2009b; Vyazovskiy et al., 2011), supporting the notion sleep may be initiated at the level of local cortical networks (Krueger et al., 2016). While the local dynamics of slow waves and cortical neural activity during spontaneous sleep and after sleep deprivation have been thoroughly characterised in young and adult animals, this has not previously been investigated in the context of ageing.

Existing evidence suggests that ageing may lead to specific changes in cortical activity during sleep. For example, a loss of grey and white matter with ageing has been noted in humans (Coffley et al., 1992; Ge et al., 2002; Marner et al., 2003; Enzinger et al., 2005; Ziegler et al., 2012), and a consistent loss of hippocampal synaptic connections has been identified in rodents (Burke and Barnes, 2006, 2010; Morrison and Baxter, 2012). Both animal and human studies have shown ageing to be associated with alterations in synaptic transmission and structural synaptic changes (Peters et al., 2008; Dumitriu et al., 2010; Morrison and Baxter, 2012; Petralia et al., 2014). Importantly, older animals have been shown to have larger synaptic field potentials in the hippocampus, suggesting that the reduction in synaptic contacts was partially compensated for by an increase in the electrical responsiveness of the remaining neurons (Barnes and McNaughton, 1980). In addition to these structural changes, previous studies have shown that ageing leads to dramatic changes in global sleep characteristics, with sleep becoming more superficial or fragmented (Vyazovskiy et al., 2006b; Hasan et al., 2012; Wimmer et al., 2013; Banks et al., 2015). Although there is abundant evidence showing both structural and functional neuronal modifications with ageing, it remains unknown whether the changes at the level of local cortical circuits is causally related to age-dependent changes in global sleep architecture.

This omission is particularly relevant, as it remains to be established whether ageing is associated with a reduced homeostatic sleep need, or instead diminishes the capacity to generate and sustain deep NREM sleep and the associated network oscillations (Klerman and Dijk, 2008; Cirelli, 2012; Mander et al., 2017). Since both sleep need and sleep depth are mechanistically

and functionally related to the expression of slow wave activity within local cortical networks (Massimini et al., 2004; Nir et al., 2011; Vyazovskiy and Harris, 2013; Neske, 2016), we set out to address the above question by chronically recording neural activity and local field potentials (LFP) in mice aged approximately 5, 12 and 24 months. We hypothesised that if age-related changes in sleep arise, at least in part, from a disruption of network activity within the neocortex, it should manifest in specific changes within local cortical circuits, such as reduced spiking activity, local slow waves and the underlying OFF periods, or altered neural dynamics at state transitions. Furthermore, we posited that if ageing primarily targets the capacity to produce an adequate homeostatic response to sleep loss at the level of local neuronal populations, this should reduce the occurrence of local cortical slow waves and OFF periods after sleep deprivation. Surprisingly, we observed that ageing had little effect on neural activity within local cortical networks, and that the local homeostatic response to sleep deprivation, as measured by the occurrence of slow waves and OFF periods, was robust in older animals. Our findings suggest that the local cortical network mechanisms underlying sleep oscillations and sleep need are intact in older mice, and that the mechanisms underlying the profound global changes in sleep observed with ageing are distinct from those responsible for local sleep regulation.

Materials and Methods

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±0.2 months, n=11) and older age (OA, 24.6±0.4 months, n=10). Although the exact correspondence between age in mice and humans remains a topic of debate, we estimate that the age of the older group in our study corresponded to approximately 70 years in humans (Dutta and Sengupta, 2016). The number of animals used in this study was based on previous studies which investigated the effects of ageing and sleep-wake history on sleep characteristics, including its amount, fragmentation and EEG

dynamics (Welsh et al., 1986; Colas et al., 2005; Hasan et al., 2012; Wimmer et al., 2013; Panagiotou et al., 2017). Mice were group housed until they underwent surgery. The implantation of microwire arrays can cause an immune response which can deteriorate the electrophysiological signal and also destabilise the implant; we therefore opted not to perform longitudinal recordings. For this reason mice were implanted shortly before they were required for surgery. On average, there were 29.4±5.6, 22.8±1.2 and 31.5±3.7 days between the surgery date and baseline recording date, for EA, LA and OA mice, respectively (Welch F test: $F(2,14.2)=3.75$, $p=0.049$). Around two weeks post-surgery mice were transferred to custom-made clear Plexiglas cages (20.3 x 32 x 35 cm), where they were individually housed with free access to a running wheel (Campden Instruments, Loughborough, UK, wheel diameter 14 cm, bars spaced 1.11 cm apart inclusive of bars) throughout the experiment and food available ad libitum. Cages were housed in ventilated, sound-attenuated Faraday chambers (Campden Instruments, Loughborough, UK, two cages per chamber) under a standard 12:12 h light–dark cycle (lights on 0900, ZT0, light levels 120–180 lux). Room temperature and relative humidity were maintained at 22±1°C and 50±20%, respectively. All procedures conformed to the Animal (Scientific Procedures) Act 1986 and were performed under a UK Home Office Project License in accordance with institutional guidelines.

Surgical procedures and electrode configuration

Surgical procedures were carried out as previously described (Cui et al., 2014; Fisher et al., 2016), a summary of which is provided below. Surgical procedures were carried out under aseptic conditions using isoflurane anaesthesia (3–5% induction, 1–2% maintenance). One day before surgery animals received dexamethasone (0.2 mg kg, orally). 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. Before implantation, EEG screw electrodes were soldered to custom-made headmount connectors (Pinnacle Technology Inc. Lawrence, USA) and

unilaterally implanted into frontal (motor area, anteroposterior (AP) 2 mm, mediolateral (ML) 2 mm) and occipital (visual area, V1, AP 3.5–4 mm, ML 2.5 mm) cortical areas. Reference and ground screw electrodes were implanted above the cerebellum and contralaterally to the occipital screw, respectively. Two single stranded, 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. Mice were also implanted with a polymide–insulated tungsten microwire array (Tucker-Davis Technologies Inc (TDT), Alachua, FL, USA) implanted into deep layers of the primary motor cortex. Since this is the first study which aimed to characterise the effects of ageing on the cortical neural activity and local sleep homeostasis in mice, it was important to target an area that has been well studied in younger animals; therefore, we opted to perform LFP and MUA recordings from the frontal cortex (Vyazovskiy and Tobler, 2005; Vyazovskiy et al., 2011; Hajnik et al., 2013; Hayashi et al., 2015; Fisher et al., 2016). Furthermore, the frontal cortex was an obvious choice for investigating the effects of ageing on the local slow wave homeostasis, as sleep EEG slow wave activity has been shown to have frontal predominance in both rats and mice (Schwierin et al., 1999; Huber et al., 2000; Vyazovskiy et al., 2006a). The microwire array consisted of 16-channels (2 rows each of 8 wires), with a wire diameter of 33µm, electrode spacing 250µm, row separation L-R: 375µm and tip angle of 45 degrees. Due to the curvature of the neocortex in the area of interest the arrays were customised so that one row of electrodes was 250µm longer than the other (Fisher et al., 2016). 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 the arrays were lowered below the pial surface into deep layers of the motor cortex. A two-component silicon 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.

Animals were all monitored closely post-surgery and scored daily for measures such as grimace, appearance, natural behaviour and provoked behaviour, until they were deemed to have returned to baseline for a minimum of two days. Mice were provided with appropriate analgesia as necessary (dexamethasone 0.2 mg kg for 2 days and metacam 1–2 mg kg for a minimum of 3 days). The animals were closely monitored after surgery for on average 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=0.000, EA vs OA p=0.000, LA vs OA p=0.004).

Signal processing, vigilance state scoring and analysis

A Tucker Davis Technology (TDT, Alachua FL, USA) Multichannel Neurophysiology Recording System was used for data acquisition. 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). As an initial step, online spike sorting was performed using OpenEx software (TDT) by manually applying an amplitude threshold for online spike detection to eliminate artefactual waveforms caused by electrical or mechanical noise. Spikes that exceeded this predefined threshold (>2 x noise level, at least -25 μV) were stored as 46 samples (0.48 ms before, 1.36 ms after the threshold crossing) consisting of both voltage measures and time stamps. LFP, EEG and EMG data were then resampled offline at a sampling rate of 256 Hz. Custom-written Matlab scripts (The MathWorks Inc, Natick, Massachusetts, USA) were used for signal conversion. Data were then transformed into European Data Format (EDF) using open source Neurotraces software.

Recordings were subdivided into 4-s epochs and vigilance states scored offline by manual inspection of the signal (SleepSign, Kissei Comtec Co, Nagano, Japan). Two EEG channels

(frontal and occipital), EMG, two channels of MUA and running wheel (RW) activity were simultaneously displayed to aid vigilance state scoring. Vigilance states were classified as waking (low voltage, high frequency EEG with a high level or phasic EMG activity), NREM sleep (presence of EEG slow waves, a signal of a high amplitude and low frequency) or REM sleep (low voltage, high frequency EEG with a low level of EMG activity). Vigilance state artefacts in at least one EEG or MUA recording channel, resulting from contamination by eating, drinking or gross movements were also scored as artefacts so that they may be removed from appropriate analyses (percentage of total recording time: EA 8.7±3.1, LA 8.8±2.3, OA 7.9±3.5). After the data was scored EEG and LFP power spectra were computed by a Fast Fourier Transform (FFT) routine for 4-s epochs (fast Fourier transform routine, Hanning window), with a 0.25 Hz resolution (SleepSign Kissei Comtec Co, Nagano, Japan).

Experimental Design and Statistical Analysis

Since our primary aim 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, all experiments were carried out under standard laboratory conditions, where mice were kept under a 12:12 LD cycle in their home-cage environment. Mice were transferred to the recording chambers and habituated to both the cage and recording cables for a minimum of 3 days before recording, until patterns of activity showed normal entrainment, as previously described (Cui et al., 2014; Fisher et al., 2016). On the day following the baseline recording, a 6-hour sleep deprivation was performed in order to investigate the effect of prolonged wakefulness on cortical activity. This was performed based on previous evidence that older mice may have a reduced capacity to respond to an increased sleep pressure (Hasan et al., 2012). Sleep deprivation was performed for 6-hours starting at light onset using the well established gentle handling technique (Vyazovskiy et al., 2002). Mouse behaviour and polysomnographic recordings were constantly monitored and when mice showed signs of

sleepiness the experimenter provided the animal with novel objects. This method is thought to mimic naturalistic waking conditions in an ethologically relevant manner which is less stressful for the animals compared to other sleep deprivation methods. Novel objects included cardboard, colourful plastic and tissue paper. During sleep deprivation, the animals repeatedly attempted to initiate sleep, and EA, LA and OA mice slept on average 5.9±0.9, 7.4±2.7 and 12.3±2.1 minutes, respectively, during the 6-h period.

For specific analyses, some mice were excluded due to technical reasons, as stated in the figure legends. Data were analysed using Matlab (The Math Works, Inc., Natick, MA, USA) 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. In cases where data failed homogeneity testing a Welch F test with Games-Howell post hoc test was used instead. Where data failed normality testing, non-parametric Kruskal-Wallis tests, with Mann-Whitney post hoc tests were used. Critical p-values were adjusted for multiple testing (p/number of tests), and only those values which reached the more stringent criteria are reported. For time-course data, repeated measures ANOVAs were used to identify differences. In a few cases where the animals did not sleep during a specific interval, the data for corresponding time points were estimated using a multiple imputation technique within SPSS (5 imputations used). Details of the specific statistical tests used are provided in the appropriate figure legends. Effect sizes were estimated by calculating Cohen's D (Lakens, 2013) for main findings. All values reported are mean ± s.e.m, unless explicitly stated.

Analysis of extracellular neuronal activity

In order to investigate putative single unit activity, we performed offline spike sorting, as previously described (Fisher et al., 2016). For spike sorting we concatenated either a baseline light and dark period or a baseline light period and SD light period. An artefact removal procedure was used using custom written Matlab scripts, in order to eliminate remaining

281 artefactual waveforms. A principal component analysis (PCA) was then performed on a segment
282 of the spike waveform between 5th-35th time stamps as this segment is more informative about
283 the overall spike waveform. After performing PCA, each spike between samples 5 and 35 was
284 described by 31 variables, each being a linear combination of the original sampling values.
285 Clustering was then performed based on a k-means algorithm (Jolliffe, 2002), a partitioning
286 method which aims to divide n observations into k clusters in which each observation belongs to
287 the cluster with the nearest mean, serving as a prototype of the cluster. We used the k-means
288 function in Matlab, which was implemented according to Lloyd's algorithm (Lloyd, 1982). This
289 approach requires the user to a priori select the number of clusters. All selected clusters were
290 manually classified into k=1, 2, 3, 4 or 5 clusters and the best representation for that spike was
291 visually classified based on the average spike waveform with standard deviation, interspike
292 interval distribution, the time course of peak-to-peak amplitude across the recording period, the
293 corresponding time course of average firing rates and the autocorellogram of the spike train. This
294 was only performed for up to 5 clusters as it was unlikely that more than five clusters would
295 occur in the same MUA electrode. In order to ensure cluster quality and stability, clusters were
296 then further classified based on their signal to noise ratio, waveshape of the action potential,
297 stability of the amplitude across time and ISI distribution histogram, and spurious or unstable
298 clusters were excluded from the analysis as previously (Fisher et al., 2016).
299
300 Association between LFP slow waves and neuronal OFF periods
301 Population OFF periods were defined as periods of total neuronal silence across all 16
302 electrodes, which were consistently associated with LFP slow waves. To detect OFF periods, we
303 first concatenated all individual spikes and detected periods of silence lasting at least 20 ms and
304 not exceeding 1000ms. Next, we arranged all OFF periods into one hundred 1% percentiles and
305 calculated the average LFP wave (mean between all recording channels) triggered to the onset of
306 the corresponding OFF period (Fig. 1). This analysis revealed an exquisite sensitivity of the LFP

307 to the occurrence of neuronal silence, and the size of the resulting LFP slow wave increased
308 progressively as a function of longer OFF periods (Fig. 1). Subsequently, we defined OFF
309 periods as periods of network silence, which were associated with a slow wave at least 50% the
310 amplitude of the largest slow waves (100^{th} percentile) and were therefore associated with the
311 longest OFF periods in each animal (Fig. 1). To investigate the relationship between neuronal
312 activity and LFP slow waves, two complementary approaches were used. First, the LFP signal
313 was band pass filtered between 0.5-4 Hz (stopband edge frequencies 0.3-8 Hz) with Matlab
314 filtfilt function exploiting a Chebyshev Type II filter design (The MathWorks Inc, Natick,
315 Massachusetts, USA) (Achermann and Borbely, 1997; Vyazovskiy et al., 2009b; Fisher et al.,
316 2016), and waves were detected as positive deflections of the filtered LFP signal between two
317 consecutive negative deflections below the zero-crossing. Only LFP waves with a peak
318 amplitude larger than the median amplitude across all detected waves during baseline NREM
319 sleep were included in subsequent analyses. Subsequently, all slow waves were aligned to their
320 positive peak and the corresponding profile of neuronal spiking was computed. Second, we
321 detected OFF periods as described above, and calculated the average LFP aligned to the onset of
322 an OFF period. The incidence of OFF periods and slow waves, as well as the duration of OFF
323 periods were used in subsequent analyses to investigate age-dependent differences in neuronal
324 network activity.
325
326 Neuronal 'phenotyping' and vigilance-state dependency of cortical firing
327 Post spike sorting, we performed a characterisation of the firing rates of the identified putative
328 single units. The firing rates (i.e. the number of spikes per 1 second) of each neuron were
329 determined for each 4-sec epoch in artefact-free wake, NREM sleep and REM sleep, and their
330 distribution was plotted as a function of firing rate. This revealed a large variability in the
331 vigilance-state specificity of neuronal firing across putative single units; with some neurons
332 firing similarly across vigilance-states, whereas others fired at different frequencies according to

vigilance-state. In order to visualise this variability across putative neurons, the predominant firing frequencies for each putative neuron was extracted from their respective histogram of the distribution of their firing rates. Neurons were then sorted according to their peak frequency and plotted in ascending order. The distribution width of the firing rates histogram was calculated separately for each state and age group as previously (Fisher et al., 2016).

Histological verification of recording site

At the end of the study the electrode recording sites were confirmed using previously described histological methods (Fisher et al., 2016). Briefly, mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde solution. Brains were photographed to aid determination of electrode position, and then sectioned into 50µm coronal slices using a freezing Microtome. Sections were mounted onto slides and visualised using a fluorescence microscope. Prior to implantation, array electrodes were coated with a thin layer of DiI fluorescent dye (DiIC18(3), Invitrogen), to aid the identification of electrode tracts.

Results

The daily architecture of sleep is markedly altered in older mice

To investigate the age-dependent changes in cortical activity during waking and sleep, we performed continuous recordings of EEG, along with local field potentials (LFP) and extracellular neuronal activity (multiunit activity, MUA) from deep cortical layers of the primary motor cortex (M1) of freely moving C57BL/6J mice. Consistent with previous reports (Welsh et al., 1986; Colas et al., 2005; Hasan et al., 2012; Wimmer et al., 2013; Panagiotou et al., 2017), we found that the older mice had a reduced amount of wakefulness, (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 an increased amount of NREM sleep (repeated measures ANOVA: factor 'age': $F(2,28)=21.0$, $p<0.0001$, 'age x time interval': $F(11,153)=4.4$, $p<0.0001$), but not REM sleep

(repeated measures ANOVA: factor 'age': ns, 'age x time interval': $F(12,169)=3.8$, $p<0.0001$), during baseline recording days, particularly during the dark period (Fig. 2A,B). This resulted in a substantial increase in the total daily amount of sleep in older mice (% of recording time: EA, 47.0±1.0; LA, 50.6±1.5; OA: 57.1±0.8; Welch F test: $F(2,18)=31.3$, $p<0.0001$; Fig 2D). However, calculating the activity onset in each animal based on EEG/EMG defined wakefulness after lights off, revealed that 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±0.3min, n.s., Wilcoxon test).

Body weight also increased with age (EA, 26.4±0.9; LA, 31.5±0.6; OA: 34.1±1.3; Welch F test: $F(2,17)=15.7$, $p<0.0001$; Fig. 2C) and notably, both age and body weight showed a significant positive relationship with the total amount of sleep during 24h (Fig. 2D). Since waking activities have an influence on the amount and distribution of sleep across 24-h (Vyazovskiy et al., 2006a; Vyazovskiy and Tobler, 2012; Fisher et al., 2016), we calculated the amount of running wheel (RW) activity in the three age groups. As expected, while all animals had free access to a running wheel throughout the experiment, younger mice used it more extensively than 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: $F(2,15)=15.0$, $p<0.0001$). Therefore, the pronounced age-dependent reduction in the amount of waking during the dark period (EA: 9.1±0.3 hours; LA: 8.0±0.5 hours; OA: 6.3±0.2 hours, Welch F test: $F(2,17)=29.9$, $p<0.0001$), could merely reflect the reduced tendency for older animals to engage in continuous wheel running (Vyazovskiy et al., 2006a; Fisher et al., 2016). Consistently, the amount of waking was positively associated with RW-activity across all ages ($r=0.84$, $p<0.001$). It should be noted, however, that causality and the directionality of the relationship between RW-activity and the capacity to sustain consolidated waking is difficult to determine using correlation analyses. Furthermore, since spontaneous wheel running is associated with substantial changes in cortical neuronal firing (Fisher et al., 2016), and may reflect a shift from preferentially goal-directed to automatic, habit-like behaviours (Vyazovskiy et al., 2017), the possibility remains that the global

385 change in sleep-wake architecture with ageing is mechanistically associated with specific
386 changes in cortical circuit activity. If this were the case, it may be expected that such changes
387 would be most readily detected in spontaneous patterns of network oscillations during sleep.
388
389 The number of LFP slow waves and population OFF periods is increased with ageing
390 As is well established, EEG and LFP slow waves during NREM sleep are associated with
391 characteristic changes in the membrane potential of cortical neurons, which give rise to
392 synchronous transitions across cortical neuronal populations between periods of activity (ON
393 periods) and silence (OFF periods) (Destexhe et al., 2007; Nir et al., 2011; Vyazovskiy and
394 Harris, 2013; Lemieux et al., 2015; Rodriguez et al., 2016). Based on this, our first question was
395 to determine whether these neuronal correlates of LFP slow waves are altered in older animals.
396 In contrast to previous human studies showing that EEG slow waves are markedly reduced with
397 ageing, we observed that the LFP and MUA signals were similar across age groups, and visual
398 inspection of the signals alone was not sufficient to differentiate between the age groups (Fig.
399 3A, representative recordings of LFP and MUA signals from individual mice from EA and OA
400 age groups are shown in Movies 1-2). In all three ages, LFP traces during NREM sleep were
401 characterised by pronounced positive slow waves associated with an unequivocal suppression of
402 MUA in the corresponding channels, typically encompassing most if not all recording channels.
403 Calculation of the average MUA triggered by individual slow-waves confirmed this and revealed
404 a suppression of neural spiking in association with slow waves in all age groups (Fig. 3B).
405 Consistent with a recent report (Panagiotou et al., 2017), LA and OA mice were found to have
406 significantly higher EEG spectral power density in slow frequencies (<10Hz) as compared to
407 young controls (Fig. 3C), yet the difference did not reach significance when we compared
408 spectral power of the LFP (Fig. 3C). This contradicts human studies (Dijk et al., 1989; Landolt
409 and Borbely, 2001; Mander et al., 2013), which instead report a decrease in EEG spectral power
410 in the SWA frequency range with age.

411 Since EEG and LFP are influenced by both distant and local sources, reflect volume
412 conduction and may also be entrained by oscillations occurring elsewhere in the cortex (Sirota et
413 al., 2008), we next focused on investigating the activity of cortical neurons. First, in order to
414 assess the relationship between LFP slow waves and the underlying local neural activity, all
415 detected OFF periods were aligned to their onset and the corresponding LFP signals were
416 averaged. LFP slow waves were exquisitely sensitive to the duration of corresponding OFF
417 periods in all ages, with the amplitude of slow waves progressively increasing with a lengthening
418 of OFF period duration (Fig. 1). Notably, the average amplitude of the slow-wave triggered by
419 OFF periods was reduced in both LA and OA mice as compared to EA mice (ANOVA:
420 $F(2,28)=4.9$, $p=0.015$, Fig. 3D,E), despite similar average OFF period durations (EA: 133.9ms;
421 LA: 141.9ms; OA: 140.0ms, n.s.). This suggests that the spiking activity and silence of
422 individual cortical neurons may, to some extent, be uncoupled from the slow network LFP
423 oscillation in older animals. To further address this, we calculated the average incidence of LFP
424 slow waves and population OFF periods during an undisturbed baseline 12-h light period.
425 Interestingly, the incidence of both increased significantly with age (slow wave incidence: Welch
426 F test: $F(2,18)=24.9$, $p<0.0001$, OFF period incidence: one-way ANOVA: $F(2,28)=3.9$,$p=0.031$,
427 Fig. 3F), with the majority of effect sizes corresponding to large effects as per Cohen's
428 convention (Lakens, 2013) (slow wave incidence: $d=-3.20$, $d=-0.84$, $d=-0.96$; OFF period
429 incidence: $d=-1.15$, $d=-1.17$, $d=0.07$; for EA vs OA, EA vs LA and LA vs OA, respectively).
430
431 <u>The response of local cortical network activity to sleep deprivation is intact in older animals</u>
432 It has been well described that in laboratory rodents EEG slow-wave activity during NREM
433 sleep decreases progressively during the light period and is higher after prolonged spontaneous
434 wakefulness or sleep deprivation (Tobler and Borbely, 1986; Vyazovskiy et al., 2009b;
435 Vyazovskiy et al., 2011). These dynamics are associated with a higher incidence of slow waves
436 and neuronal OFF periods, which are considered markers of increased physiological sleep

437 pressure at the network level (Vyazovskiy et al., 2007; Vyazovskiy et al., 2009b; Rodriguez et
438 al., 2016). Therefore, we next investigated the dynamics of both measures of network activity
439 across the baseline 12-h light period, when mice are predominantly asleep, and then performed
440 sleep deprivation the following day to determine the effect of prolonged wakefulness on these
441 measures. We found that the incidence of both slow waves and OFF periods, as well as the
442 duration of OFF periods decreased significantly across the baseline light period in all three age
443 groups (repeated measures ANOVAs: slow wave incidence factor 'time interval' $F(5,96)=19.6$,
444 $p<0.0001$, Fig. 4C; OFF period incidence factor 'time interval' $F(4,74)=12$, $p<0.0001$, Fig. 4D;
445 OFF period duration factor 'time interval' $F(6,116)=13.6$, $p<0.0001$, Fig. 4E), however only the
446 time course of OFF period incidence was significantly different between age groups (repeated
447 measures ANOVA: factor 'age' $F(2,21)=7$, $p=0.005$; EA vs LA $p=0.009$, LA vs OA $p=0.01$).
448 Previous studies have suggested that older humans and rodents have a reduced capacity
449 to generate a rebound in SWA in response to sleep deprivation, with enhanced age-dependent
450 differences found to occur after sleep deprivation (Munch et al., 2004; Lafortune et al., 2012;
451 Wimmer et al., 2013). Interestingly, in our study we observed a robust increase in SWA after
452 sleep deprivation in all three ages (individual plots: Fig. 4A), with no age-differences identified
453 for the time-course of SWA in the 6 hours of recovery sleep after sleep deprivation, for either the
454 frontal EEG or LFP (Fig. 4B). The initial levels of SWA in the frontal EEG derivation were
455 attenuated in OA mice (EA 218.1±8.5; LA 197.8±7.3; OA 181.1±8.3, ANOVA factor 'age'
456 $F(2,18)=5.4$, $p=0.015$, Fig. 4B), while no significant age differences were identified for the LFP
457 SWA. Effect sizes were calculated for the first hour after SD, a time point at which the main
458 compensatory effect of SD is observed. Large effect sizes were observed for comparisons of EA
459 vs OA (EEG: $d=1.55$; LFP: $d=1.01$) and EA vs LA (EEG: $d=1.00$; LFP: $d=1.00$), while small or
460 medium effect sizes were observed for LA vs OA comparisons (EEG: $d=0.74$; LFP: $d=0.04$).
461 The incidence of slow waves and OFF periods, as well as the duration of OFF periods were
462 significantly higher after SD as compared to the same 6 hours of the baseline recording day in all

three ages (repeated measures ANOVA factor 'day': slow wave incidence $F(1,42)=108.6$, $p<0.0001$, Fig. 4C; OFF period incidence $F(1,42)=29.9$, $p<0.0001$, Fig. 4D; OFF period duration: $F(1,412=84.5$, $p<0.0001$, Fig.4E). This increase after SD was not significantly different between age groups (ANOVA, factor 'age'; slow wave incidence: $F(2,42)=0.185$, $p=0.832$, Fig. 4C; OFF periods incidence: $F(2,42)=0.99$, $p=0.38$, Fig. 3D; OFF periods duration: $F(2,42)=2.776$, $p=0.074$, Fig. 4E). Effect sizes were then calculated for the first hour after SD. For slow wave incidence, a large effect size was detected for EA vs OA comparisons ($d=1.02$), while medium effect sizes were detected for EA vs LA ($d=0.62$) and LA vs OA ($d=0.62$) comparisons. For OFF period incidence, medium effect sizes were detected for EA vs OA ($d=0.51$) and EA vs LA ($d=0.52$) comparisons, while LA vs OA comparisons only revealed a small effect size ($d=-0.09$). In contrast, OFF period duration only had small effect sizes for EA vs LA ($d=0.39$) and LA vs OA ($d=-0.46$) comparisons, while the EA vs OA comparison had a medium to large effect size ($d=-0.75$). Furthermore, all three age groups showed a comparable gradual decrease in slow wave incidence, OFF period incidence and OFF period duration over the 6 hours recovery after SD (repeated measures ANOVA factor 'time interval': slow wave incidence $F(3,53)=61.3$, $p<0.0001$, Fig. 4C; OFF period incidence $F(2,35)=50.0$, $p<0.0001$, Fig.4D, OFF period duration $F(3,56)=54.1$, $p<0.0001$, Fig. 4E; factor 'time interval'*'age': slow wave incidence $F(5,53)=1.6$, $p=0.179$, Fig. 4C; OFF period incidence $F(3,35)=1.7$, $p=0.142$, Fig.4D, OFF period duration $F(5,56)=0.654$, $p=0.67$, Fig. 4E). Therefore, in our study the response to sleep deprivation was not markedly different between age groups, apart from in the initial level of EEG SWA in the frontal derivation. The possibility remains that the pronounced baseline differences in the amount of sleep between ages could influence the homeostatic response after sleep deprivation. Indeed, correlation analyses revealed a negative relationship between the amount of NREM sleep during the baseline dark period before SD and the magnitude of the increase in the incidence of slow waves and OFF periods ($p<0.05$ for both).

488 It has previously been suggested that the higher absolute EEG SWA observed in older
489 animals may reflect higher sleep pressure (Panagiotou et al., 2017). One of the established
490 markers of higher sleep propensity is a faster build-up of SWA within NREM sleep episodes. To
491 address whether this increase is present in older animals, we quantified the incidence of LFP
492 slow waves and OFF periods in the first two minutes after the onset of a NREM sleep episode,
493 during both baseline recordings and after sleep deprivation. Interestingly, while EA mice showed
494 a robust increase in both parameters after sleep deprivation, this was largely attenuated or absent
495 in LA and OA mice (percentage change in SW incidence relative to baseline: EA +20.3±5.5; LA
496 +2.0±5.5; OA -3.9±4.7, one-way ANOVA factor 'age' $F(2,21)=5.8$, $p=0.01$; OFF period
497 incidence: EA +50.1±14.1; LA -7.6±7.6; OA -2.3±18.3; Kruskal-Wallis test $X^2(2)=8.214$,
498 $p=0.014$, with a mean rank score of 18.86 for EA, 10.86 for LA and 9.2 for OA mice). One
499 possibility is that this may reflect a reduced capacity to engage in deeper NREM sleep with
500 ageing, perhaps due to increased neuronal activity or excitability during NREM sleep (Klerman
501 and Dijk, 2008). On the other hand, as the older animals had a higher absolute number of slow
502 waves and OFF periods (Fig. 3F), there may be a ceiling effect taking place, in which no further
503 increase is possible.
504
505 The dynamic repertoire of the activity of single neurons during wake and sleep is largely stable
506 across the life span
507 The age-dependent changes in neuronal population activity we observed may arise either at a
508 single cell level or reflect larger scale processes such as global neuromodulation. Therefore, next
509 we investigated the effect of ageing on the vigilance state-specific discharge of individual
510 cortical neurons. On average, out of 16 microwire channels, 9.2±1.0, 9.3±1.0 and 9.2±1.0
511 channels showed robust MUA in EA, LA and OA animals, respectively, and subsequent spike
512 sorting resulted in 17.4±2.9, 15.6±1.7 and 16.7±2.5 putative single units detected per animal,

513 which was not significantly different between ages. Previous studies suggest that the distribution
514 of firing rates between individual neurons is best characterised by a lognormal distribution,
515 which renders calculating the mean firing rate or averaging between individual neurons
516 inappropriate (Watson et al., 2016). Therefore, we plotted the distribution of firing rates across
517 neurons, which revealed that the older groups of mice had a somewhat higher proportion of slow
518 spiking neurons (Fig. 5A). However, it is possible that this may be related to the higher amount
519 and presumably intensity of waking in EA animals, as compared to LA and OA mice.

520 It should be noted that the distribution of firing activity within individual neurons is also
521 highly variable (Fisher et al., 2016), and often deviates from normality (Fig. 5B). This therefore
522 also poses a problem for calculating average firing rates within individual neurons. Previous
523 studies suggest that cortical neurons change their firing characteristics depending on the global
524 behavioural state, although a great variability between individual neurons and cortical regions
525 has been noted (Hobson and McCarley, 1971; Vyazovskiy et al., 2009b; Fisher et al., 2016;
526 Niethard et al., 2016). For example, it is typically observed that neurons fire at a higher rate
527 during waking and REM sleep as compared to NREM sleep (Vyazovskiy et al., 2009b), although
528 this is not always the case, and this may be influenced by the recording technique or by the
529 cortical region being recorded (Hobson and McCarley, 1971; Niethard et al., 2016). The firing
530 phenotype of cortical neurons is determined by their electrophysiological characteristics,
531 connectivity pattern, ongoing behaviour or preceding sleep-wake history (O'Keefe and
532 Dostrovsky, 1971; Ascoli et al., 2008; Poulet and Petersen, 2008; Kropff et al., 2015; McGinley
533 et al., 2015; Fisher et al., 2016). We therefore performed neuronal 'phenotyping' by calculating,
534 for each putative single neuron, the distribution of their firing rates across all 4-second epochs of
535 NREM sleep, REM sleep and waking separately (Fig. 5B,C). Individual neurons were highly
536 idiosyncratic with respect to their state-dependent firing (Fig. 5B), and so we hypothesised that
537 neurons could be subdivided into distinct categories based on the distribution of their spiking
538 activity during waking and sleep, as is traditionally done for subcortical areas where sleep- or

539 wake-active neurons have been identified (Jones, 2005). To this end, we determined the peak
540 frequency for each putative single unit based on a histogram of its firing rates (examples shown
541 in Fig. 5B), and then plotted the histograms separately for each vigilance state (Fig. 5C).
542 Interestingly, we observed that cortical neurons could not be subdivided into distinct categories
543 based on their firing pattern, but instead they formed a continuum, where virtually any firing
544 phenotype could be observed within each vigilance state (Fig. 5C). Furthermore, the distribution
545 of cortical neurons as a function of their firing pattern was visually indistinguishable between
546 ages (Fig. 5C). On average 13.7±3.3, 15.9±6.6 and 14.5±4.5% of all putative single units, in EA,
547 LA and OA mice, respectively, discharged at a higher average firing rate during sleep (including
548 both NREM and REM sleep) as compared to during waking, and the proportion of such 'sleep-
549 active' cortical neurons was similar between ages. However, plotting the distribution of
550 individual neurons as a function of their spiking activity revealed that during wake faster spiking
551 neurons were more common in EA mice (Fig. 5D). This effect is likely accounted for by a higher
552 amount of waking and increased levels of arousal in general in younger animals. It should be
553 noted that although during wheel running the older animals did not attain the same speed as the
554 younger animals, we observed a negative relationship between the firing rates of individual
555 putative neurons and running speed in all three age groups, consistent with our previous study
556 performed in younger animals (Fisher et al., 2016). Furthermore, the distribution of neurons as a
557 function of their spiking activity during spontaneous NREM and REM sleep also did not reveal
558 any noticeable differences between ages (Fig. 5D). Finally, as previous studies have shown that
559 the distribution width of the firing rates during waking is influenced by behaviour (Fisher et al.,
560 2016), we next addressed whether this is also different between ages. No statistical differences
561 were observed in the firing rate distribution widths between age groups, for any vigilance state
562 (Fig. 5E). Therefore, as mice get older the overall composition of fast-spiking and slow-spiking,
563 as well as 'sleep-active' and 'wake-active' cortical neurons appears stable, and neurons largely
564 retain their vigilance state-dependent firing profile. This suggests that the dynamic repertoire of

565 the activity of single neurons during both waking and sleep is stable across the lifespan. This
566 observation is interesting as it suggests an intriguing possibility that the mechanisms underlying
567 the gross alteration of the daily sleep-wake distribution are distinct from those implicated in the
568 regulation of local cortical states.
569
570 Persistently firing cortical 'sleep-active' neurons are more abundant in older animals
571 We next asked whether the age-dependent changes in sleep and wake could be reflected in the
572 neuronal dynamics at vigilance state transitions, such as at the onset of individual NREM sleep
573 episodes (Fig. 6A). We suggest that this might be the case for two reasons. Firstly, the overall
574 amount of NREM sleep was substantially higher in older animals (one-way ANOVA:
575 $F(2,28)=23.7$, $p<0.0001$, Fig. 6B), while the number of transitions into NREM sleep was also
576 significantly higher in older animals (one-way ANOVA $F(2,28)=26.9$, $p<0.0001$, Fig. 6C). Since
577 it has been hypothesised that sleep is a cortical circuit phenomenon that may be initiated by local
578 networks (Pigarev et al., 1997; Krueger et al., 2008; Hinard et al., 2012; Lemieux et al., 2014;
579 Sanchez-Vives and Mattia, 2014), it is reasonable to assume that the neuronal dynamics involved
580 in sleep initiation may be different depending on the level of local sleep propensity. Previous
581 studies showed that as an animal transitions into NREM sleep, overall firing rates become
582 progressively slower, possibly due to the occurrence of neuronal silent periods (Vyazovskiy et
583 al., 2009b). At the same time, it has previously been shown that SWA, as well as the amplitude
584 of slow waves, increases progressively during the first 1-2 min after NREM sleep onset,
585 reflecting a progressive increase in sleep depth (Vyazovskiy et al., 2009a; Cui et al., 2014).
586 Consistently, we observed an increase in relative slow wave incidence across the first 2 minutes
587 of a NREM sleep episode, which was larger and more rapid in EA mice as compared to older
588 animals (Fig. 6D). One possible explanation for older animals having an attenuated build-up of
589 slow wave incidence is that the absolute incidence of both slow waves and OFF periods was
590 higher in older animals throughout NREM sleep (Fig. 3F). However, curiously, the gradual

591 increase in the number of OFF periods during the first 2 minutes of NREM sleep episodes did
592 not differ noticeably between ages (Fig. 6E).

593 We next plotted the neuronal firing activity of all individual putative neurons across all
594 animals during the first 2 min after the onset of a NREM sleep episode. Once again, neurons did
595 not fall into distinct categories based on their dynamics in the first few minutes after NREM
596 sleep onset. Instead we observed that even within a small cortical network, the recorded neurons
597 presented an entire spectrum of possible changes: with some neurons showing a progressive
598 increase in spiking in the initial minutes after sleep onset, while other neurons did not change
599 their spiking activity or showed a pronounced decrease (Fig. 6F). It was apparent, however, that
600 in OA mice a somewhat larger proportion of neurons increased their spiking activity in the initial
601 few minutes after the onset of NREM sleep episodes (Fig. 6F), which is consistent with the
602 observation that OA mice had a reduced relative increase in the incidence of slow waves as sleep
603 progressed into the second minute (Kruskal-Wallis H test χ^2 (2) = 9.979, p=0.007, with a mean
604 rank age score of 23.22 for EA, 12.73 for LA and 11.6 for OA mice, Fig. 6G). This may suggest
605 that even within individual NREM sleep episodes, the progression from a relatively more
606 superficial sleep to a deeper sleep is attenuated in older animals. This was confirmed by the
607 finding that older animals have more intense relative neuronal spiking in 2nd minute of NREM
608 sleep episodes (mean rank age scores 12.9 for EA, 13.86 for LA and 21.45 for OA mice,
609 Kruskal-Wallis H test χ^2 (2) = 6.4, p=0.04, Fig. 6H,I). Effect sizes calculated for the proportion
610 of neurons that increased firing by at least 30% at the onset of NREM sleep (Fig. 6I), revealed
611 large effect sizes for EA vs OA (d=-1.09) and LA vs OA (d=-1.06), with a small effect size for
612 EA vs LA (d=-0.07) comparisons. We hypothesise that this could affect sleep intensity, making
613 sleep relatively more superficial and easier to disrupt, or it could reflect local state instability
614 (Doran et al., 2001; Parrino et al., 2012), which may interfere with the restorative functions of
615 sleep. However, the high incidence of cortical neurons that increased their firing rates across
616 NREM sleep episodes is somewhat surprising, and appears to contradict the occurrence of OFF

periods, which are associated with reduced neuronal spiking, but were not altered or even enhanced, in absolute terms, in older animals.

Ageing does not affect the dynamics of cortical firing at NREM-REM sleep transitions

Transitions between NREM and REM sleep (Fig. 7A) are characterised by profound changes in cortical EEG, LFPs and neuronal activity (Vyazovskiy et al., 2009b; Funk et al., 2016; Niethard et al., 2016), as well as marked changes across several subcortical neuromodulatory systems (Fort et al., 2009; Van Dort et al., 2015; Weber et al., 2015). Once again, we observed substantial variability in the response of individual neurons to transitions between NREM and REM sleep (Fig. 7B,C), while the overall number of 'REM-sleep active' cortical neurons was not different between ages (Fig. 7D). Interestingly, a substantial proportion of neurons exhibited very similar patterns of activity between NREM and REM sleep. This supports the notion that both sleep states share important common characteristics (Funk et al., 2016), and further suggests that state-dependent changes in cortical activity during sleep are not affected markedly by the ageing process, at least in the motor cortex of mice.

Discussion

Intensive research over the past few decades revealed numerous effects of ageing on sleep amount and architecture, as well as on specific sleep oscillations in both humans and animals (Dijk et al., 1989; Bliwise, 1993; Landolt and Borbely, 2001; Carrier et al., 2011; Cirelli, 2012; Hasan et al., 2012; Lafortune et al., 2012; Rolls, 2012; Wimmer et al., 2013; Banks et al., 2016; Clawson et al., 2016; Mander et al., 2017). However, neither the origin, nor the physiological significance of age-dependent changes in sleep are fully understood, and the question remains whether ageing is associated with a decreased capacity to generate sufficient sleep or a reduced sleep need (Mander et al., 2017). Based on this, our study had two overarching aims: to determine whether the age-dependent changes observed at the level of EEG could be explained

643 by the differences at the level of underlying neural activity; and to determine the role of local
644 network mechanisms in the previously observed global sleep disruptions. We predicted that if the
645 ageing process targets neocortical circuits in the first place, this would manifest in a decreased
646 occurrence of network OFF periods during sleep, reduced homeostatic response at the level of
647 local neural activity or an overall reduced level of neural spiking. To our surprise, we
648 demonstrated that while ageing in mice is consistently associated with global changes in the
649 amount of sleep and its architecture, it does not profoundly affect cortical neural activity. The
650 only changes we observed were increases in the incidence of local LFP slow waves and
651 corresponding population OFF periods during NREM sleep with ageing, and that a larger
652 proportion of neurons discharged at a higher rate upon sleep onset. Otherwise, vigilance state
653 related patterns of cortical activity, and the homeostatic response to sleep deprivation measured
654 at the level of neuronal population activity during sleep, were intact in older animals. Our results
655 suggest that local cortical mechanisms of sleep regulation are not significantly impaired during
656 healthy ageing, making it unlikely that the global sleep disruptions identified with ageing arise
657 from changes in local cortical activity. We must therefore critically reconsider the notion that
658 local cortical mechanisms of slow-wave generation are deficient in older humans, at least until
659 intracranial recordings in older human patients can be performed. Furthermore, our results may
660 shed light on why earlier studies report conflicting results, specifically with respect to findings
661 obtained in humans and laboratory animals.

662 One of the most intriguing observations of our study is that ageing has few effects on
663 cortical local neural activity despite profound effects on global brain activity and the daily sleep-
664 wake architecture, as identified in numerous studies (Dijk et al., 1989; Bliwise, 1993; Landolt et
665 al., 1996; Swaab et al., 2002; De Gennaro and Ferrara, 2003; Feinberg and Campbell, 2003;
666 Altena et al., 2010; Carrier et al., 2011; Colrain et al., 2011; Mander et al., 2013; Martin et al.,
667 2013; Mander et al., 2014; Dube et al., 2015; Clawson et al., 2016), as well as in this study.
668 Evidence suggests that sleep and slow wave oscillations emerge at the level of local neuronal

669 populations as a direct consequence of fine synaptic modifications or local changes in neuronal
670 connectivity arising from specific preceding waking activities (Krueger and Tononi, 2011;
671 Vyazovskiy and Harris, 2013). Therefore, it could be expected that if this regulatory mechanism
672 is impaired with ageing, it should manifest as local disruptions in cortical population activity.
673 However, we found that in older animals local neuronal networks are capable of entering and
674 sustaining consolidated OFF periods associated with local slow waves (Fig. 2), and so this basic
675 property of neuronal networks remains intact with ageing. Furthermore, we observed an increase
676 in the incidence of slow waves and OFF periods in older animals, which may be indicative of
677 increased sleep pressure (Esser et al., 2007; Riedner et al., 2007; Vyazovskiy et al., 2007), as has
678 been previously suggested based on EEG data (Panagiotou et al., 2017). Although the circuit
679 mechanisms behind the increased tendency of a network to enter OFF periods remains to be
680 determined, we speculate that altered network excitability may play a role (Chen et al., 2012;
681 Lemieux et al., 2015; Neske, 2016). Consistent with this hypothesis, it has been shown that
682 ageing is associated with a loss of synaptic connections or a reduction in their stability (Morrison
683 and Baxter, 2012; Grillo et al., 2013). It is possible that the higher incidence of local slow waves
684 we observed in older mice may also have a functional role, such as in the homeostatic
685 rebalancing or remodelling of synaptic networks which has been associated with sleep slow
686 waves (Chauvette et al., 2012; Krueger et al., 2013; Vyazovskiy and Harris, 2013; Tononi and
687 Cirelli, 2014; Watson et al., 2016; Sanchez-Vives et al., 2017). However, we also observed that
688 the homeostatic rebound of both slow waves and neuronal OFF periods did not manifest marked
689 differences between age groups in our study. This observation is intriguing and contrasts with
690 previous reports of reduced homeostatic rebound in EEG SWA in humans and mice (Wimmer et
691 al., 2013; Mander et al., 2017). It is possible that the direct comparison of changes observed at
692 the local network activity and 'global' EEG may be not adequate or precise enough to determine
693 the local mechanistic changes with ageing, as age-dependent changes may selectively target
694 specific levels of organisation, while sparing others. While we performed recordings of LFP and

MUAs from the primary motor cortex, it remains to be established whether associative areas, such as the prefrontal cortex, or primary sensory areas would show similar effects. We speculate that specific cortical and subcortical regions may be differentially affected by ageing, with some areas more sensitive than others.

Earlier studies have revealed numerous notable discrepancies between species with respect to the effects of ageing on sleep. For example, EEG SWA has been reported to be decreased in older humans (Landolt et al., 1996), whereas recent evidence suggests that in older mice SWA may instead be enhanced (Panagiotou et al., 2017), which was confirmed in our study. On one hand, this may reflect species differences in the effect of ageing on sleep need or the capacity to produce deep sleep stages (Klerman and Dijk, 2008). For example, differences between humans and mice may be related to their body and/or brain size and differences in their metabolic rates, which are associated with longevity and sleep (Zepelin and Rechtschaffen, 1974; Allison and Cicchetti, 1976; Siegel, 2005; Capellini et al., 2008; Herculano-Houzel, 2015). Given the major role of ecological factors, such as diet and the risk of predation, on the duration of sleep (Allison and Cicchetti, 1976; Siegel, 2005; Tobler, 2005; Capellini et al., 2008), it cannot be excluded that factors such as longevity and sleep duration also play an increasingly important role in ageing. At the same time, the capacity to engage cortical networks in global sleep may be differentially affected in organisms with different brain sizes, regardless of whether the rate of age-dependent synaptic loss is the same. Based on the evidence in our study we propose that different species may compensate for the inability to engage in deep consolidated sleep in different ways. For example, older humans may allow intrusions of sleep-like patterns of activity into the awake state, while older mice may increase the daily amount of global behavioural sleep. In addition, the spatial scale of the recording methods used to record brain activity in humans and mice differs substantially, with a single scalp electrode in human studies likely recording activity from a larger network than a microwire electrode implanted intracortically in mice. To the best of our knowledge, intracranial recordings from older humans

721 have not been performed during sleep, and so it remains to be determined whether the lack of age
722 differences in neuronal activity can be generalised across species. It is possible that older humans
723 may be perfectly capable of generating fully fledged *local* slow waves, and may in fact have an
724 intact *local* response to sleep loss.

725 An important conclusion of our study is that the mechanisms underlying local sleep
726 regulation are distinct from those changes implicated in 24-h sleep-wake control. The effects of
727 ageing on the amount and distribution of waking and sleep may be caused by a variety of factors,
728 such as a weaker entrainment to the light-dark cycle or disruptions to the circadian system
729 (Kondratova and Kondratov, 2012; Banks et al., 2015; Nakamura et al., 2015; Banks et al.,
730 2016), fundamental changes in waking behaviour (Gu et al., 2015; Fisher et al., 2016) or energy
731 homeostasis (Rolls, 2012). Notably, older mice typically have an increased body weight and
732 reduced locomotor activity (Kopp et al., 2006; Banks et al., 2015), which we also demonstrate
733 here, and which may be important in determining the overall sleep-wake architecture. It has
734 recently been shown that providing mice with running wheels restores the daily distribution of
735 waking and sleep and improves the capacity to maintain consolidated waking periods (Gu et al.,
736 2015). In our study all animals were housed with running wheels for the duration of the
737 experiment, and although we did not specifically manipulate the amount and speed of running,
738 we noted that the older animals not only ran in the wheels less but they also did not attain the
739 same running speed as younger mice. However, all three age groups consistently showed a
740 negative relationship between the firing rates of individual putative neurons and running speed,
741 as we described previously in younger animals (Fisher et al., 2016). Therefore, we surmise that
742 since the older animals were less able to sustain consolidated wake periods, possibly as a result
743 of a diminished propensity for running, this resulted in less time spent in the 'idling' default wake
744 mode with reduced cortical activity, as we previously suggested (Fisher et al., 2016).

745 In summary, we report that healthy ageing in mice does not lead to marked changes in
746 vigilance state related local neural activity, despite pronounced global changes in the daily

amount and distribution of waking and sleep. By and large, most basic features of cortical activity during sleep were not altered by ageing, suggesting that powerful protective or compensatory mechanisms may exist to maintain neural function in the neocortex across the lifespan. Crucially, our results suggest that older mice have an intact capacity to generate slow waves and a homeostatic response to sleep loss at the local cortical level, while the global sleep dynamics appear to be profoundly disrupted. Therefore, this study importantly highlights that sleep disruption in ageing cannot be adequately addressed or fully understood without taking into consideration the level of organisation and causal relationships or lack thereof between local and global aspects of sleep regulation.

Figure legends

Fig. 1. Algorithm for OFF periods detection. (A) Average LFP slow waves calculated and plotted as a function of OFF period duration. All OFF periods were subdivided into 100 1% percentiles, and the corresponding average LFP signal aligned to the onset of an OFF period was calculated. Inset shows the effect of lengthening the minimal duration of OFF periods on their average duration. Short OFF periods were not associated with noticeable changes in the LFP, while the highest amplitude slow waves corresponded to the longest OFF periods. The horizontal line indicates the amplitude threshold used to define OFF periods (see panel B). **(B)** The relationship between the duration of OFF periods and the amplitude of corresponding average LFP slow waves in one individual animal. 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 (corresponding to the longest 1% of OFF periods). The corresponding thresholds are depicted as horizontal (slow wave amplitude) and vertical (minimal OFF period duration) lines. **(C)** The average LFP slow wave and corresponding profile of MUA centred on the mid-point of the OFF periods defined based on the above criteria.

773 **Fig. 2. The global alterations of sleep with ageing in mice. (A)** Hypnograms of individual
774 representative animals from each age group (EA=early adulthood, LA=late adulthood, OA=older
775 age). 24-h profile of EEG slow-wave activity (SWA, EEG power between 0.5-4.0 Hz,
776 represented as % of 24-h mean) recorded from the frontal cortex. Wake, NREM sleep and REM
777 sleep are represented by green, blue and red colour-coding, respectively. The bar at the top of the
778 panel depicts the 12-h light and 12-h dark periods. **(B)** Time course of waking, NREM and REM
779 sleep during 24-h baseline day, shown in 2-h intervals. The amount of each vigilance state is
780 represented as % of the total recording time. Mean values, SEM, n=10 (EA), n=11 (LA) and
781 n=10 (OA). Significant differences between ages are shown in blue, cyan and purple for EA vs
782 LA, LA vs OA and EA vs OA, respectively. **(C)** The relationship between age and body weight
783 across and within age-groups. The filled symbols correspond to individual animals. The straight
784 lines depict linear regression lines separately for the three age groups. **(D)** The relationship of
785 age (left) and body weight (right) with the amount of total sleep shown as % of recording time
786 over 24-h. (C)(D) Mean values, SEM, n=10 (EA), n=11 (LA) and n=10 (OA). R and p-values
787 correspond to Pearson's product moment correlation. Welch F test (Games-Howell post hoc)
788 used to compare age groups.
789
790 **Fig. 3. The relationship between LFP slow waves and cortical MUA in younger and older**
791 **mice (A)** LFP (top) and MUA (bottom) traces from a representative LFP channel in
792 representative animals from each age group. **(B)** Average LFP slow wave (top) and
793 corresponding average MUA triggered by slow waves (plots below) in the three age groups.
794 Note that in all three ages the positive LFP wave is associated with a clear-cut suppression of
795 neuronal spiking. **(C)** Frontal EEG (left) and LFP (right) spectral power density during NREM
796 sleep. Mean values, SEM. The triangles below depict frequency bins where EEG spectra differed
797 significantly between the age groups ($p<0.05$, unpaired t-test on log-transformed values, top: EA
798 vs LA, bottom: EA vs OA). **(D)** Average LFP slow wave triggered by the onset of generalised

neuronal silence (an OFF period) across all recorded neurons. Note that despite the average duration of OFF periods being similar between ages, the amplitude of the resulting slow wave was higher in EA animals, as compared to LA and OA mice. **(E)** The effect of ageing on the amplitude of the average LFP slow wave triggered by population OFF periods (as shown in panel D). EA: n=10; LA: n=11; OA: n=10. A one-way ANOVA (Tukey post-hoc test) was used to compare age groups. **(F)** The effect of ageing on the incidence of slow waves and OFF periods during baseline NREM sleep. EA: n=10; LA: n=11; OA: n=10. 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.

Fig. 4. 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, is colour-coded according to the vigilance state (wake = green, NREM sleep = blue, REM sleep = red). Sleep deprivation (SD) was performed for 6 hours from light onset. Note that after SD, a robust increase in EEG SWA is evident in all three animals, which is followed by a progressive decline during the subsequent recovery period. **(B)** Time course of EEG (top) and LFP (bottom) SWA during 6-h period after 6-h SD. Mean values, SEM (EA n=7; LA n=5-6; OA n=9). The time course of the decline in SWA was not significantly different between age groups for either the frontal EEG of LFP (repeated measures ANOVA). 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 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.

Fig. 5. Ageing and the vigilance state dependence of cortical neuronal firing. (A) The distribution of the firing rates of all putative individual neurons across all vigilance states, plotted as a proportion of the total number of neurons. On average, 17.4±2.9, 15.6±1.7 and 16.7±2.5 putative single neurons per mouse contributed to the analysis for EA, LA and OA mice, respectively. Inset shows the proportion of neurons that fire at slow rates (0-3 Hz), which was significantly different between age groups (one-way ANOVA, Tukey post hoc test). **(B)** Distribution of firing rates across 4-s epochs expressed as a percentage of the total number of epochs, for three representative individual putative single units. Wake is shown in green, NREM sleep in blue and REM sleep in red. Subplots show the corresponding average spike waveform (±std. dev) and autocorrelogram. Note that individual neurons are highly variable with regards to their vigilance-state specific firing. **(C)** The predominant firing rates for all putative neurons were extracted 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. Note that neurons did not fall into distinct categories based on their firing rates, but rather formed a continuum in which all possible peak firing rates could be observed. **(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. EA n=10; LA n=11; OA n=10. No statistical differences between age groups were identified (One-way ANOVA).

Fig. 6. Intraepisodic dynamics of cortical firing at the transition to NREM sleep. (A) A representative example of a cortical LFP recording at the transition from waking to NREM sleep. **(B)** The effect of ageing on the total amount of NREM sleep during 24-h. EA n=10, LA n=11,

OA n=10. One-way ANOVA (Tukey post-hoc test) was used to compare age groups. **(C)** The effect of ageing on the total number of NREM sleep episodes during 24-h. EA n=10, LA n=11, OA n=10. One-way ANOVA (Tukey post-hoc test) was used to compare age groups. **(D)** 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. Line indicates the 2nd minute of NREM sleep which undergoes further analysis in panel (G). **(E)** The same analysis as in (D) but for the incidence of OFF periods. **(F)** 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. The neurons are sorted as a function of the relative firing rates attained during the 2nd minute after the episode onset. **(G)** 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. 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). **(H)** Distribution of all putative neurons as a function of the change in their firing frequency within NREM sleep episodes. **(I)** The proportion of neurons, which 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 after the initiation of corresponding NREM sleep episodes. 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).

Fig. 7. The effects of ageing on the neuronal dynamics at the transition from NREM to REM sleep. (A) Representative example of cortical LFP at the transition from NREM sleep to

REM sleep. On average, 51.9±3.7, 45.8±2.0 and 45.3±2.5 N-R transitions contributed to the analysis below in EA, LA and OA animals, respectively. **(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 (±std. dev) and the autocorrelogram. Note that individual neurons show a great diversity in their state dependent firing within sleep. **(C)** The dynamics in firing rates during the last minute of NREM sleep and the first minute of subsequent REM sleep is 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. **(D)** The proportion of putative single neurons, discharging on average at a higher rate during REM sleep as compared to NREM sleep, expressed as a percentage of all neurons in the three age groups. EA n=10, LA n=11, OA n=10. One-way ANOVA did not identify any significant differences between age groups.

Multimedia legends

Movie 1. Example raw electrophysiological signals recorded from a representative early adulthood (EA) mouse during NREM sleep. Four channels of local field potentials (LFPs) and corresponding multiunit activity (pNeu) are shown. 5 seconds of recording is shown at any given time, at a playback speed 2x normal. Note the occurrence of synchronous silent (OFF) periods in the multiunit activity, corresponding to slow waves in the LFP.

Movie 2. Example raw electrophysiological signals recorded from a representative old age (OA) mouse during NREM sleep. Four channels of local field potentials (LFPs) and corresponding multiunit activity (pNeu) are shown. 5 seconds of recording is shown at any given time, at a playback speed 2x normal. Note the occurrence of synchronous silent (OFF) periods in the multiunit activity, corresponding to slow waves in the LFP, similar to those seen in the younger age groups.

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