

Neural signatures of tinnitus across the sleep-wake cycle



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Subjective tinnitus is an internally generated auditory percept, most commonly taking the form of hissing or ringing. Especially long-lasting tinnitus is associated with anxiety, depression, and sleep impairments. There is no effective treatment for tinnitus, although research has made progress in uncovering its neural basis, including identifying hyperactivity in the central auditory pathway and several cortical regions, including those involved in brain state control. We have proposed that the occurrence of aberrant neural activity associated with tinnitus has important implications for natural brain state dynamics across sleep and wakefulness and vice versa (Milinski et al., 2022).

Our first prediction was that the neural representation of tinnitus interferes with global sleep maintenance, while a strong sleep drive (such as high homeostatic pressure) overrides aberrant tinnitus-related activity. To test this empirically, a longitudinal assessment of behavioural and neural markers of tinnitus across the sleep-wake cycle was conducted in a ferret model of tinnitus after noise overexposure (NOE) over a period of ~6 months. The changes in behavioural performance and auditory evoked activity after NOE differed between individuals and suggest distinct degrees of tinnitus and hearing impairment with early or late onset. The emergence of tinnitus symptoms was associated with disrupted and reduced sleep, corroborating the possibility that noise-induced tinnitus is linked to sleep disturbance. In addition, neural markers of tinnitus were reduced during sleep, suggesting that sleep may transiently mitigate tinnitus.

Our second prediction was that besides the interaction between sleep and tinnitus, sleep might facilitate tinnitus emergence. To test this, we assessed in mice the effect of acute sleep deprivation after NOE on tinnitus development. Sleep deprivation mitigated behavioural and hearing impairment after NOE, which indicates that sleep may play a role in mediating the consequences of noise-induced peripheral trauma.

Overall, these results highlight the potential of further research on the interactions between natural brain states dynamics and tinnitus to uncover new avenues for future treatments.

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Table of Contents

Introduction	5
Chapter 1: Behavioural readouts for tinnitus in the ferret model.....	10
Background	10
Method Summary	18
Results	23
Discussion	36
Supplementary figures.....	41
Chapter 2: Behaviour and auditory processing after noise overexposure	44
Background	44
Method summary	48
Results	54
Discussion	81
Supplementary figures and tables	86
Chapter 3: The effect of noise overexposure on sleep-wake dynamics	100
Background	100
Method summary	104
Results	112
Discussion	163
Chapter 4: The effect of noise overexposure on auditory evoked activity across sleep and wakefulness	168
Background	168
Method summary	173
Results	176
Discussion	208
Supplementary figures and tables	213
Chapter 5: The role of sleep in tinnitus development	234
Background	234
Method summary	239
Results	245
Discussion	260
Supplementary tables.....	267
Discussion	272
Methods	282
Bibliography	305

Introduction

Sleep is a state of vigilance when experiences are generated internally while the brain is almost entirely disconnected from its sensory environment (Nir & Tononi, 2010; Oswald et al., 1960; Twyver & Garrett, 1972). Dreams of visual scenes and melodies have inspired magnificent art and musical pieces (Barrett, 2017; Grace, 2001), showing how impactful such experiences can be. Yet many of us may not spend a second thought on last night's dreams or, in fact, hardly remember them (Vallat et al., 2018). However, when we experience sensory percepts without evident relationship to environmental triggers during wakefulness, we immediately recognise them as disconcerting or even as part of a mental disorder. Despite our different attitudes towards them, both dreams and phantom percepts during wakefulness share common ground in that they are percepts driven entirely by internal processes (De Ridder et al., 2011; Elgoyhen et al., 2015a; Nir & Tononi, 2010). And while progress has been made in understanding how the brain represents the world around us, the question remains on whether natural processes during sleep can lead to insights about pathological processes that make up persistent phantom percepts.

Complex hallucinations are arguably the most striking phantom perceptions due to their vividness, yet the most prevalent phantom percept is subjective tinnitus, or tinnitus for short, which is affecting an estimated 15% of the population (Baguley et al., 2013a; McCormack et al., 2016). It is commonly defined as the constant perception of hissing or ringing, voices or even music without any identifiable acoustic source (Baguley et al.,

2013a). Tinnitus can be transient and only last for minutes or persist for months or years. Especially persistent tinnitus is often associated with depression, anxiety (Bhatt et al., 2017; Gomaa et al., 2014; Salazar et al., 2019) and disturbed sleep (Alster et al., 1993; Attanasio et al., 2013; Burgos et al., 2005; Folmer & Griest, 2000; Hallam, 1996; Hébert et al., 2011b; Izuhara et al., 2013a; Koning, 2019b; Schecklmann et al., 2015). A recent proposal in the field suggests considering two sub-types of tinnitus: on the one hand, tinnitus as the mere perception of a continuous phantom sound, and on the other ‘tinnitus disorder’, where the phantom percept is associated with additional suffering and therefore becoming a clinical entity in its own right (De Ridder, Schlee, et al., 2021). Yet, to date, there is no available cure for either tinnitus variant.

The cause of tinnitus is not always clear. Yet the most common triggers are considered to be hearing loss or the degeneration of cochlear nerve fibres (auditory neuropathy)(Kujawa & Liberman, 2009; Liberman & Kujawa, 2017) without changes in the audiogram thresholds, so called hidden hearing loss (Schaette & McAlpine, 2011), although even reversible hearing impairment has been shown to trigger tinnitus (Schaette et al., 2012). Hearing loss can be age-related (presbycusis, Shore et al., 2016), induced by noise overexposure (J. Turner et al., 2012) or caused by ototoxic drugs (Stolzberg et al., 2012). The loss of afferent input to the auditory brainstem in hearing loss is thought to cause a compensatory process to preserve neural activity levels, resulting in increased excitability and spontaneous activity along the central auditory pathway and beyond, which ultimately leads to tinnitus (Kalappa et al., 2014; Middleton et al., 2011; R. J. Salvi et al., 2000; Schaette & Kempster, 2006). Indeed, tinnitus is associated with functional changes in widely distributed brain regions, both auditory and non-auditory (Elgoyhen et al., 2015a; Leaver et al., 2011; Rauschecker et al., 2010a;

Schlee et al., 2009; Sedley et al., 2015; Vanneste et al., 2011). Many of these areas that show tinnitus-related activity, and especially cortical regions, exhibit dramatic changes in their activity dynamics across wakefulness and sleep (Milinski et al., 2022). This raises the possibility for an interaction between tinnitus and sleep-related activity.

Global spontaneous brain activity varies markedly across the sleep-wake cycle, and even during sleep different stereotyped global activity patterns can be observed (Adamantidis et al., 2019). The spatial overlap between areas affected by tinnitus and those showing sleep-dependent activity modulation may lead to a competition between pathological and physiological drives to determine network activity.

If tinnitus-related activity persists across vigilance states, it may result in a state of hyperarousal during sleep as is typical in some forms of insomnia and parasomnias (Castelnovo et al., 2018, 2019; Riemann et al., 2010), where the emergence of local and global activation prevents sleep onset or interferes with natural sleep–wake dynamics. For example, tinnitus-related hyperactivity may hinder the brain in initiating or maintaining the sleep state or prevent restful sleep. Yet, importantly, local and global changes in brain activity across vigilance states (Huber et al., 2000b; Vyazovskiy et al., 2011) may, in turn, interfere with local tinnitus-related activity. Especially high-intensity sleep, such as after a period of extended wakefulness (Huber et al., 2000b; Tobler & Borbély, 1986), may mitigate tinnitus temporarily. This could impose a dynamic modulation of the phantom sound across the sleep–wake cycle, depending on the relative weight of circadian and compensatory homeostatic drives (Milinski et al., 2022). Moreover, sleep-dependent processes may play a role in long-term tinnitus development. The significance of aberrant activity observed in tinnitus is thought to

depend on the formation and long-term maintenance of new functional neural networks (De Ridder et al., 2011). Indeed, neural plasticity following a triggering event plays a key role in tinnitus persistence (Engineer et al., 2011; Noreña & Eggermont, 2006a; S. E. Shore et al., 2016a). Given the well-described role of sleep in promoting neural plasticity and consolidating memory traces (Clawson et al., 2018; Frank et al., 2001; Gais et al., 2007; Káli & Dayan, 2004; O'Neill et al., 2010; Takashima et al., 2009) it is possible that sleep also plays a role in neural reorganisation as part of tinnitus development.

Therefore, the investigation of the relationship between tinnitus and sleep may provide valuable insights into the generation of the condition and its possible modulation by natural processes, as well as its role in mediating sleep disturbances so widely observed in tinnitus sufferers (Alster et al., 1993; Attanasio et al., 2013; Burgos et al., 2005; Folmer & Griest, 2000; Hallam, 1996; Hébert et al., 2011b; Izuhara et al., 2013a; Koning, 2019b; Schecklmann et al., 2015). The objective of this thesis was to investigate this relationship experimentally.

The following chapters will introduce the methodology and animal models used in this study to address particular aspects of the hypothesised interaction between sleep and tinnitus. First, to investigate the direct relationship between tinnitus and sleep, an animal model of noise-overexposure-induced tinnitus was established (Chapters 1 & 2). This study was performed using ferrets due to their similar hearing range to humans, their well characterised auditory cortical fields, and a long lifespan. To test the relationship between tinnitus and sleep disturbance, the time course of tinnitus development after noise overexposure was assessed in parallel with sleep-wake patterns (Chapter 3). Changes in neural markers of tinnitus were monitored across sleep

and wakefulness to investigate a potential sleep-dependent modulation of the condition. (Chapter 4). Finally, to assess the role of sleep in tinnitus development, the effect of sleep deprivation after noise trauma was evaluated in a mouse model (Chapter 5).

Noise overexposure in the ferret model was associated with distinct degrees of tinnitus and hearing impairment with early or late onset. The emergence of behavioural and neuronal signs of tinnitus was associated with disrupted and reduced sleep, corroborating the possibility that noise-induced tinnitus is linked to sleep disturbance. Hearing impairment, on the other hand, was associated with improved sleep maintenance, suggesting a protective function of elevated sensory thresholds. Furthermore, neural markers of tinnitus were reduced during sleep, indicating that sleep may transiently mitigate tinnitus. Experiments in the mouse model showed that sleep deprivation can alleviate behavioural and hearing impairment after noise overexposure, supporting the notion that sleep may play a role in mediating the consequences of noise-induced peripheral trauma.

These experiments corroborate the possibility of a bidirectional interaction between tinnitus and sleep on both short and long time scales. The results highlight the potential of further research into brain dynamics across sleep and wakefulness for the tinnitus field and suggest that, ultimately, the brain's natural processes to generate perceptual phantoms during sleep could be harnessed to understand and modulate those of pathological nature.

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Chapter 1: Behavioural readouts for tinnitus in the ferret model

Background

Tinnitus is the perception of an auditory phantom percept, most often in form of a hissing or ringing sound, without any associated psychiatric conditions and without a defined physical correlate (Baguley et al., 2013b). To date, there are no established clinical diagnostics based on objective measures of tinnitus. Assessments in humans are usually based on questionnaires addressing subjective loudness and intrusiveness of the percept in everyday tasks. The most widely used are the tinnitus handicap inventory (THI)(Kuk et al., 1990) and the tinnitus functional index (TFI)(Meikle et al., 2012). These questionnaires put little weight on the characteristics of the phantom percept *per se*, like loudness or frequency, but rather on its comorbidities, such as lack in concentration, sleep quality, hearing ability and general quality of life. Only the TFI includes questions on tinnitus loudness and occurrence, while its focus lies on assessing overall wellbeing of the patient.

Questionnaire-based assessments of tinnitus, therefore, allow for convenient diagnostics of clinically relevant symptoms – those that impact wellbeing of the patient living with tinnitus – on a large scale. Yet they pose important constraints on interpreting results in the context of tinnitus research. First, the subjective nature of questionnaire-based assessments renders them prone to participants' biases in self-reflection, misinterpretation of questions, and of inaccurate recall of symptoms, especially given

that many items on the TFI and THI ask for retrospective self-assessment. Objective measures of the tinnitus itself are needed to correlate tinnitus to changes in brain activity and use those to quantify the efficacy of therapies. Second, there can be a lack in temporal resolution of acquired data gathered through questionnaires, especially if items refer to effects the tinnitus had within the last week – such as in the TFI. It is well described that tinnitus is often a dynamic condition and can fluctuate in loudness, even within one day (Nicolas-Puel et al., 2002; Probst et al., 2017). For elucidating functional changes at the basis of the phantom percept it is crucial, therefore, not to rely on a single assessment but on repeated measurements per subject to capture dynamics in tinnitus characteristics over time.

While caveats such as subjective bias and no or very few repeated measurements per subject are common in questionnaire-based assessments of tinnitus, a general constraint of studies with human participants is the lack of personalised baseline data that could be used as references for each individual, respectively. For example, many studies assess tinnitus patients in comparison to a healthy group of subjects. This inevitably introduces variables that might differ between tinnitus and control groups beyond the characteristics of the tinnitus itself. Some can be controlled for, such as discrepancies in lifestyle. Yet the complex variety of disease history among tinnitus patients introduces many more variables: tinnitus is commonly associated with auditory disorders such as hearing loss or hyperacusis (Eggermont & Roberts, 2004a; Gold & Bajo, 2014; Schaette & McAlpine, 2011) or temporary hearing impairment (Schaette et al., 2012) but also with non-auditory conditions such as depression (Salazar et al., 2019), anxiety (Hou et al., 2020), mental stress (Ciminelli et al., 2018a) and disturbed sleep (Alster et al., 1993; Folmer & Griest, 2000; Hallam, 1996; Izuhara et al., 2013b; Koning,

2019b; Schecklmann et al., 2015). These comorbidities pose an additional challenge to finding control groups with matching characteristics except the tinnitus itself. Furthermore, not only disease history but also the tinnitus is specific to the individual. The expression of the phantom sound can vary in quality and be perceived as hissing, ringing or even be reminiscent of voices or music (Baguley et al., 2013b). Tinnitus can also vary in its perceived spatial origin, with some sufferers assigning its perceptual origin to one ear, to both, or to a more central location within the head (Baguley et al., 2013b). The objective of minimizing the impact of inter-subject variability on the assessment and diagnosis of tinnitus is another reason to base tinnitus assessment on repeated measurements before and after onset of tinnitus in each individual.

To this end, tinnitus research has been turning to animal models of the condition. Animals typically used in tinnitus research are common laboratory models such as mice (Middleton et al., 2011; J. Turner et al., 2012) and rats (Kalappa et al., 2014), but also other mammals with more similar hearing ranges to humans, such as cats (Noreña & Eggermont, 2003b), guinea pigs (Marks et al., 2018; Wu et al., 2016) and ferrets (Meredith et al., 2012). Animal models allow for control of tinnitus onset and most environmental factors, as well as for continuous long-term assessment over weeks or months after tinnitus triggering. In particular the ferret (*Mustela putorius furo*) with a lifespan of 6-8 years (Fox & Marini, 2014) allows for long-term assessment of auditory functioning in young adults (~1 year old) without expected age related hearing loss or other effects of ageing. In addition, ferrets are well-suited for auditory behavioural experiments (Gold et al., 2015) and their hearing range (36Hz – 44 kHz, with lowest thresholds for 8-12 kHz (Kelly et al., 1986)) widely overlaps with the human hearing range (20Hz to 20 kHz)(Purves et al., 2001). And yet, investigation of long-term

development of tinnitus under the controlled conditions that these models offer is still scarce in the field of tinnitus research.

To trigger tinnitus in animal models different paradigms that mimic the causes of the condition in human patients are used, most prominently exposure to high-intensity noise (Brozoski et al., 2002; Coomber et al., 2014; Kaltenbach et al., 2004; Noreña & Eggermont, 2003b; J. Turner et al., 2012) but also administration of ototoxic drugs such as salicylate (Stolzberg et al., 2012). In contrast to human studies, symptoms of tinnitus in animal models are assessed through behavioural paradigms that aim to provide objective readouts. These behavioural tests do not assess tinnitus directly but indirectly by measuring changes in performance due to the presence of a constant phantom percept.

Tinnitus assessment in animal models is mainly based on the detection of silent gaps in noise. Here, the animal indicates whether a presented auditory stimulus includes a silent gap. Such tasks can be based on operant conditioning (commonly in form of a two-alternative forced-choice paradigm, 2AFC) or based on reflex behaviour (prepulse inhibition of the startle response). It is thought that a persistent auditory phantom percept would make it more difficult to detect a silent gap. This could be due to literal perceptual filling of a silent gap by the phantom percept (J. G. Turner et al., 2006)(Fig.1A) or through impaired temporal resolution in auditory processing (Bowen et al., 2003). Importantly, even though tinnitus can be associated with reduced attention (Leong et al., 2020) it does not directly interfere with sounds different to the tinnitus.

The filling-in hypothesis has been questioned mainly based on data of human participants tested in operant variants of gap detection tests. For example, in operant

paradigms tinnitus patients showed identical gap detection ability to control subjects (Campolo et al., 2013; Zeng et al., 2020). However, it is not clear whether patients had stable tinnitus across tests and whether the used pure tone stimuli did sufficiently match the tinnitus pitch (Zeng et al., 2020) or whether small sample sizes and generally high performance across subjects could have masked subtle differences between tinnitus and control subjects (Campolo, Lobarinas and Salvi, 2013). Other experiments – using a GPIAS paradigm – did show impaired gap detection in tinnitus patients at both low and high background frequencies (Fournier and Hébert, 2013). While suggesting an impairment, the authors argue that rather than filling in silent gaps, tinnitus is associated with impaired central auditory processing, such as due to cortical map plasticity (Eggermont & Roberts, 2004a; Engineer et al., 2011), which in turn creates gap detection deficits (Fournier and Hébert, 2013). It remains to be established in how far the filling-in hypothesis explains results of behavioural tinnitus assessments, yet gap detection paradigms have been widely used in animal models and reveal robust behavioural changes after common triggers of tinnitus (Kalappa et al., 2014; M. J. Kim et al., 2021; J. Turner et al., 2012)

Most studies assessing tinnitus make use of the acoustic startle response: the most common paradigm is based on the fact that in normal hearing conditions a sound evoked startle response is changed if a brief silent gap within constant background noise precedes the startle sound and is detected by the animal (Kalappa et al., 2014; M. J. Kim et al., 2021; J. Turner et al., 2012)(Fig.1A,C). Gap detection leads mostly to inhibition of the startle response, but can also lead to facilitation (Longenecker et al., 2018). This can be determined by comparing trials with and without such a gap. Since performance of the animal is based on a reflex, this paradigm does not require a prolonged training

phase but can be assessed directly. Yet a caveat to this approach is rapid habituation to the startle stimulus, which reduces the informative power of the paradigm in repeated assessments. Habituation may be especially pronounced in carnivores (such as ferrets) as compared to rodents, since the latter are prey animals and therefore need to retain high sensitivity to potentially relevant sounds. This is evident, for example, in a lower startle response in cats as compared to rats (Gruner, 1989). Therefore, GPIAS paradigms can offer readouts unaffected by the motivational or training state of the animal and allow for continuous observation of tinnitus development with high time resolution (J. Turner et al., 2012), such as with daily measurements over a prolonged period of time, yet they are always prone to being affected by habituation.

A variant of the gap-in-noise detection paradigm where habituation effects are minimal is operant gap detection (Gold et al., 2015). Typically, animals are trained by positive conditioning in a 2AFC paradigm to discriminate between a continuous sound and a sound that includes at least one silent gap (Fig.1A). Correct responses are rewarded. While this paradigm requires both procedural and perceptual training to reach stable performance levels in baseline conditions, it is less prone to habituation than GPIAS paradigms, as performance is driven by appetitive motivation to gain the reward. 2AFC paradigms can also be based on negative conditioning, yet this is less well suited in the context of tinnitus assessment, as the alteration of limbic activity and altered emotional regulation in tinnitus (De Ridder et al., 2011; Elgoyhen et al., 2015a; Leaver et al., 2016; Rauschecker et al., 2010a; Sedley et al., 2015) may influence the reaction to negative reinforcement and thus bias operant performance beyond the effect of the animal's gap detection ability.

Reflex GPIAS and 2AFC positive conditioning of gap-in-noise detection are complementary in terms of their susceptibility to habituation and motivation of the animal. Yet neither paradigm allows to distinguish whether impairments in gap detection are due to constant perception of a phantom sound or due to impaired temporal resolution in auditory processing. The latter, in itself, can impact the ability to detect silent gaps of short durations (Bowen et al., 2003). A solution is offered by behavioural paradigms assessing the animal's ability to detect relatively long periods of silence (Stolzberg et al., 2013). Impairment in silence detection ability is thought to be affected by the presence of tinnitus but not by a change in temporal resolution in auditory processing. In the operant silence detection paradigm animals are trained to discriminate between constant and modulated sounds and presentation of no sound (silence). Once trained, animals perceiving a constant phantom sound will be biased to report a constant sound even in its absence (Fig. 1D).

Despite the caveats of these approaches individually, most previous studies in animal models of tinnitus only use one of these paradigms (most commonly GPIAS). In this study a combination of GPIAS, operant gap detection and silence detection was used to harness the complementary explanatory power of these readouts in assessing tinnitus over a prolonged period of time. This chapter will introduce these individual approaches and summarise the respective baseline performance in the ferret model.

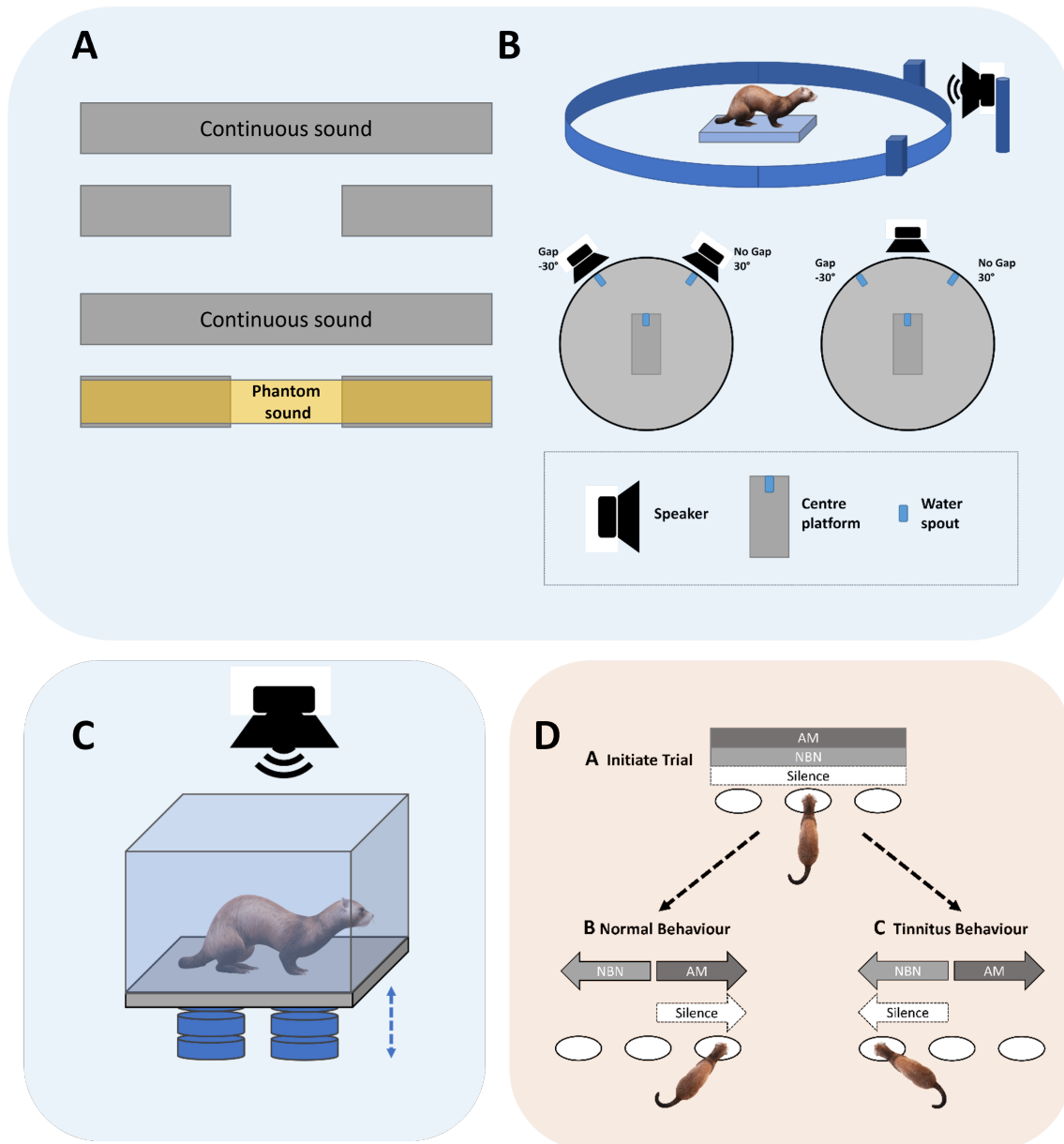


Fig.1: Behavioural assessment of tinnitus in an ferret model. **(A)** The persistent phantom percept in tinnitus fills the silent gap in a presented sound stimulus, biasing perception of the gap-stimulus towards perceiving a continuous stimulus. **(B)** Operant gap detection setup. A behavioural arena with a centre platform to initiate a trial, speakers in 30 and -30 degrees relative to the centre platform and water spouts in all three locations. Gap and non-gap sounds are presented by the -30° and 30° speaker, respectively, during the training phase, and from a centrally located speaker (0 °) during testing. **(C)** Setup to assess gap induced inhibition of the acoustic startle setup (GPIAS). A enclosure made of wire grid is positioned on a rigid platform on top of a load-cell sensor allowing to record changes in weight distribution of an animal inside the enclosure. A free-field speaker is located above the enclosure. **(D)** Silence detection paradigm. The task takes place in the same arena as shown in B. Animals are trained to activate the +30° sensor for NBN trials and the -30° sensor for AM and silence trials. With tinnitus, an animal is expected to mistake Silence trials for NBN trials and respond accordingly.

Method Summary

Adult female ferrets (*Mustela putorius furo*, n=8) were assessed in the three different behavioural paradigms. Female ferrets were chosen over males due to markedly lower variability in body size and more homogeneous cranial dimension, allowing for consistency of cranial implants across animals. To reduce metabolic variability due to the oestrous cycle, ferrets were administered with Delvosteron (MSD Animal Health, Proligestone 100mg/ml) one month before change to the summer light cycle, if not stated otherwise. For a detailed description of all paradigms and animal husbandry, see the Methods chapter.

Gap induced inhibition of the acoustic startle (GPIAS)

GPIAS was assessed in a custom-made setup (Fig.1C). Animals were placed in a chamber within a sound isolated compartment and presented with continuous low intensity white noise (broad band noise, 0.5-30 kHz, intensity level 60 dB SPL). Once acclimatised to the chamber, 40 brief high-intensity white noise sound bursts (10ms, 120 dB SPL) causing a startle response were presented with a randomised inter-trial-interval of 3 - 13 seconds. The startle response was recorded using a load cell sensor positioned below the floor of the behavioural chamber. Half of the startle stimuli (20 trials) were preceded by 50 ms by a silent gap (gap length 50ms) in the background noise, serving as a predicting signal for the high-intensity sound burst. Therefore, in animals with normal hearing who are able to detect the gap, the startle response is commonly lower in 'gap trials' relative to 'non-gap trials' (Methods Fig.1) although gap induced facilitation of the startle response has also been reported (Longenecker et al., 2018). To capture effects of

habituation on the magnitude of startle responses within sessions, response magnitudes were assessed in blocks of 5 gap and non-gap trials, producing 4 blocks per assessment (Early, Mid1, Mid2, Late) as previously described by Longenecker and colleagues (Longenecker et al., 2018). Startle inhibition was then defined for each block as mean startle (gap trials) / mean startle (non-gap trials). This produces values ranging from 0 to 1 if the startle response in gap-trials is indeed inhibited relative to non-gap trials.

Operant gap detection

In the operant gap detection paradigm, animals performed in a custom-made behavioural arena (Fig.1B). Animals were trained to initiate a trial by activating infrared sensors on a centre platform of the arena ensuring a fixed head position of the animal. (See Methods for detailed conditioning protocol.) One of two types of sound stimuli was then presented through a single speaker facing the platform at 0 degrees. The two stimulus types were a continuous sound and the same sound but including silent gaps. In 'gap trials' (where a sound stimulus with silent gaps was presented), a water reward was administered if the animal approached a touch-sensitive valve located at -30 degrees relative to the initiation platform. In 'non-gap trials', a water reward was administered if the animal approached a location at +30 degrees relative to the initiation platform. Incorrect responses were not rewarded. Sound stimuli were broadband gaussian noise bursts (BBN, 30 kHz bandwidth) and one octave narrowband noise bursts (NBN) centred at 1, 4, 8, and 16 kHz, randomised across sessions. Within a given session sound stimuli were constant in terms of frequency composition, only gap lengths were randomised across trials. In every gap trial four equally spaced, identical silent gaps were introduced in the stimulus. Across trials, the length of these gaps differed and were of

either 3, 5, 10, 20, 50, 100 or 270 ms duration. Stimuli were generated *de novo* for each trial, cosine ramped with a 10 ms rise/fall time and had a total duration of 2080 ms. All stimuli were filtered using the inverse transfer function of the speaker to obtain stable intensity levels across included frequencies at 76 ± 5 dB SPL. Animals were assessed twice daily in blocks of 5 consecutive days separated by at least two days of constant access to water. Animals performed *ad libitum* in each session until ceasing to initiate trials. Animals were trained in this manner until they reached at least 80% correct responses in two consecutive sessions using only the longest gap length (270ms) only before being trained in the paradigm but now using all gap lengths. Here, animals completed approximately 600-1000 trials for each stimulus type.

Operant silence detection

Operant silence detection took place in the same operant arena as operant gap detection. The design of the paradigm has previously been described by Stolzberg and colleagues (Stolzberg et al., 2013). Here, initiation of a trial was followed by presentation of either of three stimulus types. A narrowband noise (NBN, one octave bandwidth with centre frequencies at 1, 4, 8, and 16 kHz and randomised across trials), amplitude modulated BBN (AM stimulus, 100% modulation depth, 5 Hz), and Silence (S, speaker off). During an initial training in the paradigm AM and Silence trials were rewarded for responses to a sensor located +30 degree relative to the initiation platform whereas NBN trials were rewarded for responses to the -30 degree sensor. After animals had completed this initial training in the paradigm (see Methods), the percentage of each type was adjusted to 50 % NBN, 30 % AM and 20% S stimuli. AM trials were rewarded for responses to a sensor located +30 degree relative to the

initiation platform, NBN trials were rewarded for responses to the -30 degree sensor whereas silent trials were never rewarded (Fig.1D). This design builds on the assumption that a persistent tinnitus will bias the animal to respond as if it perceived a NBN sound in Silence trials (Fig.1D). Similar to operant gap detection, animals were assessed two times per day for maximal 5 continuous days before a minimum of two days without assessment. Animals performed *ad libitum* in each session until ceasing to initiate trials. Animals completed a total of 1000 trials in this paradigm during baseline testing.

Data analysis and statistics

Performance in the operant gap detection paradigm was measured in form of the animal's hit rate (HR) in individual sessions. HR was assessed for the parameters of sound frequency (1, 4, 8, 16 kHz NBN stimuli), stimulus type (NBN or BBN), gap length (0, 3, 5, 10, 20, 50, 100, 270 ms) and session. Complementary to the HR, false alarm rates (FA) were defined as $1 - \text{HR}$, i.e. the percentage of trials the animal indicated the perception of a gap sound when, in fact, a non-gap sound (0 ms gap) was presented. Response time (RT) was defined as the time between stimulus onset and response of the animal (to either the +30 or -30 degree sensor). The sensitivity index (d') was not used in this analysis. d' increases with increasing HR and decreasing FA, and typically reflects whether an animal's cue detection ability improves or decreases when assessed in repeated measurements. In animals with tinnitus, HR in the gap detection paradigm is expected to be impaired, leading to a lower d' as compared to a healthy animal. At the same time, however, FA is expected to be reduced as well, as an animal experiencing a permanent phantom sound may be biased towards experiencing the continuous sound in this paradigm and thus be less prone to falsely indicate a gap stimulus. Therefore,

performance changes in an animal that develops tinnitus are not well reflected in d' . To account for this, the analysis focused on HR and FA individually without merging these measures into a common index. Main effects on hit rate were estimated by fitting a GLMM (target distribution: normal, link: identity) on approximately normally distributed HR data (repeated measures: gap length, testing session, stimulus frequency). The same procedure was followed for analysis of reaction times, except that the GLMM was fitted on log-transformed data in order to approximate a normal distribution.

Reflexive gap detection was assessed by evaluating the modulation of the behavioural startle response in gap trials as compared to non-gap trials. Startle modulation was assessed for the first 5 gap and first 5 non-gap trials, as well as the second, third and fourth block of five gap and non-gap trials, respectively. This block-based analysis has been suggested to capture effects of within-session habituation (Longenecker et al., 2018). For each block, the average startle response magnitude was calculated for gap and non-gap trials. Startle modulation was then defined as *mean startle (gap trials) / mean startle (non-gap trials)*. Main effects were estimated based on log-transformed data, with the repeated factor 'block' in a GLMM.

Operant silence detection ability was assessed by measuring hit rates and response times (defined as for operant gap detection, see above) in silence trials as compared to trials where auditory stimuli were present (AM or NBN). Main effects were assessed by fitting a GLMM on hit rates and log-transformed response times.

Results

Gap induced inhibition of the acoustic startle response (GPIAS)

To control for factors that could influence performance in operant paradigms, such as motivation and learning ability, animals were assessed in a gap detection paradigm based on reflexive behaviour: assessment of gap induced inhibition of the acoustic startle (GPIAS). Here, a silent gap in constant background noise predicts a high-intensity noise burst, which commonly leads to a reduced startle response in an animal as compared to an unpredicted noise burst. Thus, modulation of the acoustic startle response in gap versus non-gap trials provides a readout for gap-detection ability. Seven ferrets were tested in this paradigm, one animal was excluded from the experiment due to health reasons unrelated to the procedure. The startle signal was assessed by measuring the maximum peak to trough difference in the load-cell response within a fixed response window ranging from offset of the noise burst until 100ms after that (Fig.2A). The timing of startle responses was largely consistent across trials (Fig.2B).

To capture effects of habituation on the magnitude of startle responses within sessions, response magnitudes were assessed in blocks of 5 gap and non-gap trials, producing 4 blocks per assessment (Early, Mid1, Mid2, Late) as previously described by Longenecker and colleagues (Longenecker et al., 2018). For each block, the average startle response magnitude was calculated for gap and non-gap trials. Startle inhibition was then defined as *mean startle (gap trials) / mean startle (non-gap trials)*. This produces values ranging from 0 to 1 if the startle response in gap-trials is indeed inhibited relative to non-gap trials.

While three animals showed a GPIAS below 1, indicating inhibition of the startle response in gap trials, none of the other animals showed inhibition consistently throughout the testing session (Fig.3A). In fact, GPIAS between animals varied considerably (*effect of animal*, $F_{(6,20)}=8.385E+31$, $p<0.001$) suggesting that factors depending on the animal (such as general activity during the assessment) likely played an important role in driving intra-session variability in performance. This may also explain why no marked habituation could be observed throughout the testing session (*effect of block*, $F_{(3,23)}=0.37$, $p=0.77$, *n.s.*), even though animals showed the most pronounced GPIAS at the beginning of the session (Fig.3B).

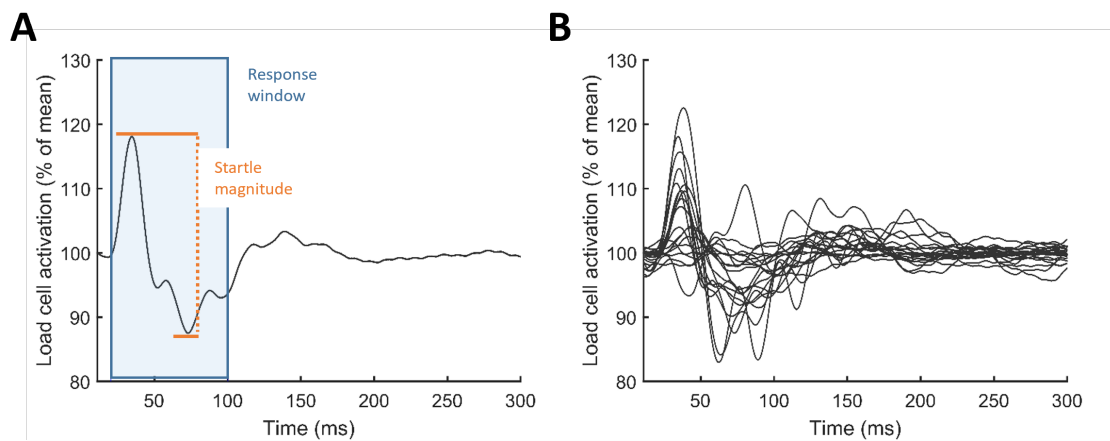


Fig.2: Gap induced inhibition of the acoustic startle (GPIAS). (A) Example of a raw startle response as recorded by the load-cell. The blue box marks the response window in which the startle response was measured. Orange lines mark the startle magnitude (maximum and minimum values of the trace). (B) Example of 20 trials in one animal, depicted as in A. Note that amplitude varies but the latency of first the positive peak is stable.

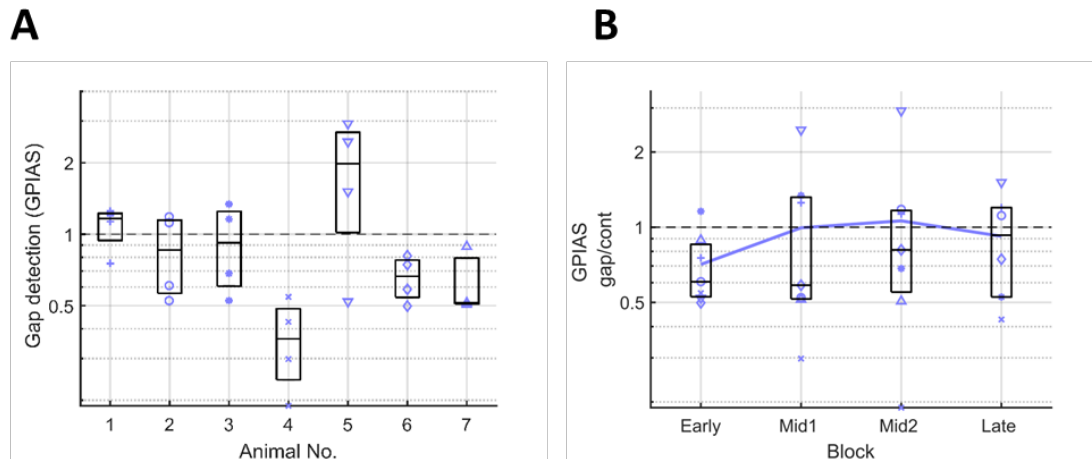


Fig.3: Gap induced inhibition of the behavioural startle (GPIAS). (A) Gap induced inhibition of the acoustic startle (GPIAS) for each animal. GPIAS is defined as startle magnitude in gap trials divided by startle magnitude in non-gap trials. GPIAS is calculated for 4 blocks per animal (each block includes 5 gap and 5 non-gap trials). Blue markers are GPIAS per block for each animal. (B) GPIAS across animals, separated by block within the assessment. Datapoints show the GPIAS for each block and animal, the blue line depicts animals averages across blocks. Note that for one animal the values for the 'Late' block could not be calculated as all 5 trials in the block were contaminated by artefacts.

Operant gap detection

Eight female ferrets were trained in the operant gap detection paradigm until they performed consistently at a rate above 80% correct responses per session where only the longest silent gap (270ms) was used. Subsequently, gap detection testing started, in which animals had to discriminate between non-gap and gap stimuli with gaps of 3, 5, 10, 20, 50, 100 and 270ms silent gaps and perform at least 1000 trials for each stimulus type (1, 4, 8, 16 kHz NBN and BBN). Here, false alarm rates decreased steadily over time (Suppl. Fig. 1) indicating a continuous training effect reaching beyond the animal's mere understanding of the paradigm, possibly due to perceptual training. To assess when FA would reach a stable level, a separate animal was tested over a prolonged period. Here, the persistent decrease in FA continued even after 170 days of training (Suppl. Fig. 2).

Therefore, the long-term dynamics of performance as assessed by FA may serve as an important proxy to disentangle the effects of an external intervention (such as noise exposure) and continuous training of gap-detection ability in this paradigm.

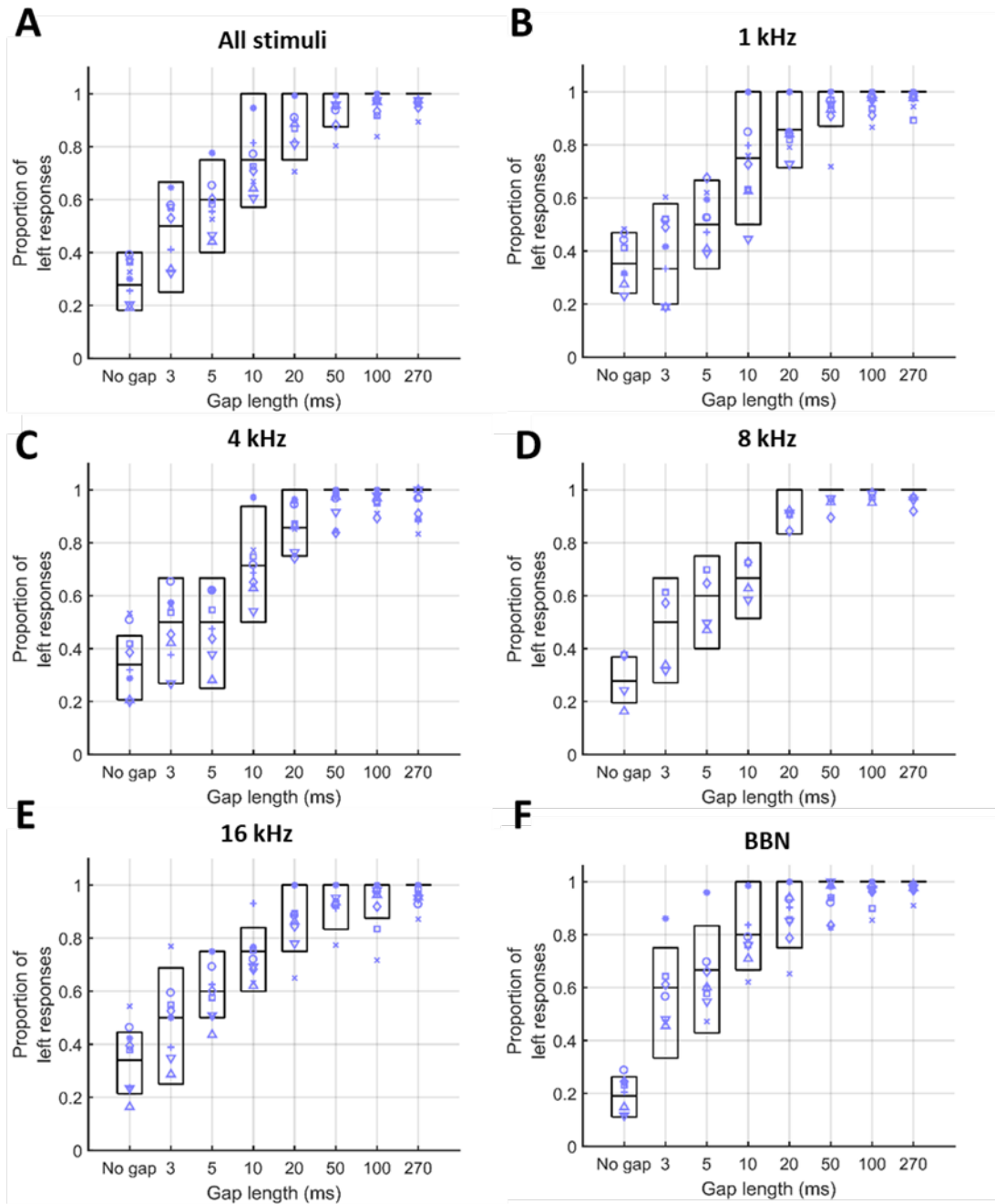


Fig.4: Operant gap-in noise detection in ferrets: hit rates by gap length (A) Behavioural performance: the proportion of left (-30°) responses by gap length. Data encompass all stimuli (NBN stimuli and BBN). Left responses correspond to correct responses for gap stimuli and to the false alarm rate for no-gap-stimuli. **(B-F)** Behavioural performance: proportion of left responses by gap length for stimuli 1 KHz NBN, 4 kHz, NBN, 8 kHz NBN, 16 kHz NBN and BBN. Blue markers depict animal averages based on average hit rate per testing session. Box plots are based on performance across all testing sessions and animals. Horizontal edges of the boxplots depict interquartile ranges, horizontal lines the median. Note that 8 kHz NBN stimuli were assessed for only 4 animals. Data include approximately 1000 trials per stimulus type for each animal.

All ferrets learned the task and, as expected, their ability to detect silent gaps strongly depended on the length of the gaps (*effect of gap*, $F_{(7,3544)}=301.85$, $p<0.001$) as hit rate improved with increasing gap length (Fig.4A). The three longest gap lengths were associated with performance of 80% or higher in all animals (Fig.4A). The modulation of performance by gap length was largely conserved across all tested stimulus types (Fig.4B-F).

In addition, performance was affected by whether stimuli did or did not contain a gap (*effect of trial type*, $F_{(1,3550)}=11.64$, $p<0.001$) in that animals showed significantly better performance for detecting gap stimuli rather than non-gap, continuous stimuli (*Non-gap vs Gap*, 0.70 ± 0.15 vs 0.78 ± 0.27 , Fig. 5A), suggesting a bias towards perception of a gap sounds, despite the balanced and equal reward criteria for both kinds of trials.

Irrespective of gap lengths or of whether the stimulus did contain a gap, hit rates were affected by the stimulus type ($F_{(4,3547)}=106.71$, $p<0.001$, *average ratios of correct responses for 1K NBN: 0.74 ± 0.28 , 4K NBN: 0.75 ± 0.27 , 8K NBN: 0.77 ± 0.26 , 16K NBN: 0.76 ± 0.26 , BBN 0.81 ± 0.23* , Fig.5B) with animals showing better gap detection ability for the BBN stimulus than for all NBN stimuli (Fig.5B)(*BBN vs 1 kHz NBN*, $\beta=-0.062$, $t_{(3551)}=-6.17$, $p<0.001$, *BBN vs 4 kHz NBN*, $\beta=-0.062$, $t_{(3551)}=-5.61$, $p<0.001$, *BBN vs 8 kHz NBN*, $\beta=-0.02$, $t_{(3551)}=-2.24$, $p=0.03$, *BBN vs 16 kHz NBN*, $\beta=-0.05$, $t_{(3551)}=-8.68$, $p<0.001$).

Animals also showed significantly better performance for 8 kHz NBN than for other NBN stimuli (*8 kHz vs 1 kHz NBN*, $\beta=-0.04$, $t_{(3551)}=-6.62$, $p<0.001$, *8 kHz vs 4 kHz NBN*, $\beta=-0.04$, $t_{(3551)}=-4.51$, $p<0.001$, *8 kHz vs 16 kHz NBN*, $\beta=-0.03$, $t_{(3551)}=-4.41$, $p<0.001$). Overall, animals were better at correctly detecting gap stimuli rather than non-gap stimuli and

longest gaps were associated with best performance across all stimulus types, yet animals performed best if the presented stimulus was BBN.

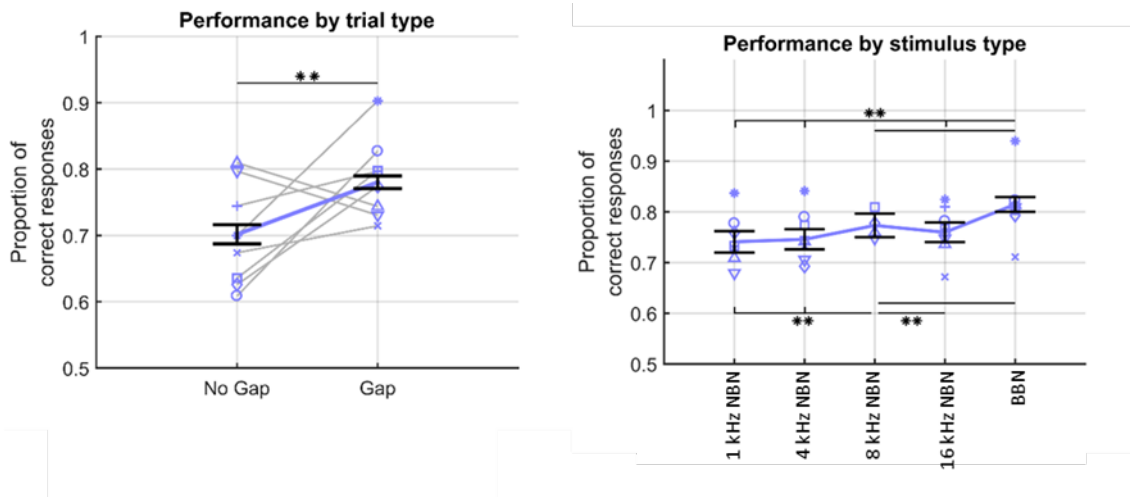


Fig.5: Operant gap-in noise detection in ferrets: hit rate by gap type stimulus type. (A) Hit rate by gap type: no gap vs gap stimuli. **(B)** Hit rate by stimulus type, i.e. stimulus frequency. Blue markers depict animal averages. Blue lines connect mean values across animals and sessions. Black markers depicts 95% confidence interval, based on performance in individual testing session of all animals. Blue markers are animal averages. Grey lines connect identical animal markers across conditions. Note that 8 kHz NBN stimuli were assessed for only 4 out of the 8 animals. Black horizontal bars indicate significant group differences of $p < 0.05$ (with ** indicating $p < 0.001$).

While the ferrets were not trained to respond quickly, they tended to do so: in fact, mean response times (1663 ± 212 ms) were shorter than the overall stimulus duration (2080 ms). Therefore, analysing response times can be indicative of the animals' confidence or certainty about their response. Response times were similar between correct and incorrect responses for gap trials (*Gap trials: correct vs incorrect*, 1.68 ± 0.21 vs 1.66 ± 0.25 , $F_{(1,875)} = 1.91$, $p = 0.17$, Fig.6A) but in non-gap trials, animals showed longer response times when the trial outcome was incorrect (*Non-gap trials: correct vs incorrect*, 1.64 ± 0.18 vs 1.73 ± 0.26 , $F_{(1,884)} = 571.14$, $p < 0.001$, Fig.6B). This was not due to

animals responding quicker when approaching the sensor to indicate a gap-stimulus as overall response times were similar for right (+30°) and left (-30°) responses (*L vs R*, 1.7 ± 0.22 vs 1.65 ± 0.22 , $F_{(1,1761)}=1.26$, $p=0.26$, Fig.5C) but may rather be linked to the generally worse performance for detecting non-gap stimuli (Fig.5A). However, response times between gap and non-gap trials were similar in correct trials (*Non-gap vs Gap*, $1.64\pm0.18s$ vs 1.68 ± 0.24 , $F_{(1,3474)}=0.3$, $p=0.58$, Fig.7A) and in incorrect trials (*Non-gap vs Gap*, $1.73\pm0.26s$ vs $1.66\pm0.37s$, $F_{(1,2031)}=2.95$, $p=0.09$, Fig.7B).

The strong modulation of performance by gap length (Fig.4) suggests that the saliency of a particular gap could affect response times as well. Gap length did indeed affect response times for both correct trials ($F_{(7,3468)}=2.98$, $p=0.004$) and for incorrect trials ($F_{(7,2025)}=6.59$, $p<0.001$) (Fig.7C,D). However, the differences in response times here were consisting of fractions of seconds (*means correct trials, no gap: $1.64\pm0.18s$, gap 3ms: $1.71\pm0.2s$, gap 5ms: $1.71\pm0.27s$, gap 10ms: $1.66\pm0.21s$, gap 20ms: 1.67 ± 0.23 , gap 50ms: $1.66\pm0.21s$, gap 100ms: $1.66\pm0.23s$, gap 270ms: 1.68 ± 0.22 ; means incorrect trials, no gap: $1.73\pm0.26s$, gap 3ms: $1.64\pm0.29s$, gap 5ms: $1.62\pm0.27s$, gap 10ms: $1.66\pm0.36s$, gap 20ms: $1.71\pm0.42s$, gap 50ms: $1.78\pm0.59s$, gap 100ms: $1.68\pm0.47s$, gap 270ms: $1.61\pm0.41s$* , Fig.7C,D) and it remains questionable whether this effect is meaningful in the context of this paradigm where response times are affected by a multitude of factors (see Discussion section of this chapter).

Response times for different stimulus types (1,4,8,16K and BBN) showed a similar range and did not differ for correct trials ($F_{(4,3471)}=2.0$, $p=0.09$, **1K NBN** 1.69 ± 0.24 , **4K NBN** $1.66\pm0.23s$, **8K NBN** $1.76\pm0.19s$, **16K NBN** $1.66\pm0.21s$, **BBN** $1.68\pm0.26s$, Fig.8A) and only

minimally so for incorrect trials ($F_{(4,2028)}=11.91$, $p<0.001$, **1K NBN** $1.70\pm0.37s$, **4K NBN** $1.65\pm0.29s$, **8K NBN** $1.68\pm0.31s$, **16K NBN** $1.68\pm0.31s$, **BBN** $1.66\pm0.41s$, Fig.8B).

Thus, while performance in terms of hit rates was strongly affected by the length of a silent gap in a given stimulus, as well as by the frequency composition of a given stimulus, the response times of animals were largely stable.

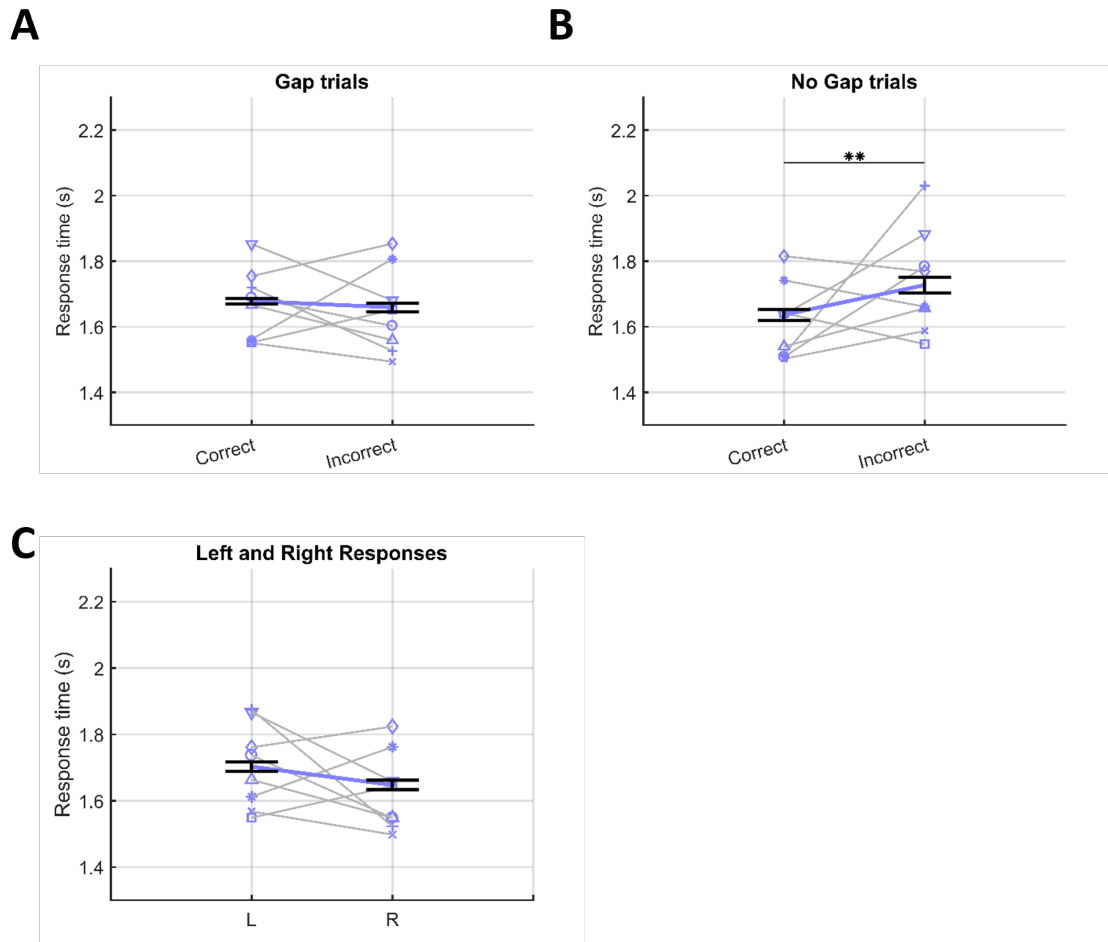


Fig.6: Operant gap-in noise detection in ferrets: response times by trial outcome and response location. (A) Response times for correct and incorrect gap trials in gap trials and (B) in non-gap trials. (C) Response times for left (-30°) and right (30°) responses. Blue markers depict animal averages. Blue lines connect mean values across animals and sessions. Black markers depicts 95% confidence interval, based on performance in individual testing session of all animals. Grey lines connect identical animal markers across conditions.

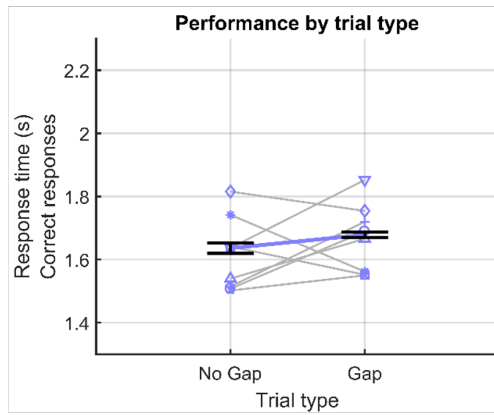
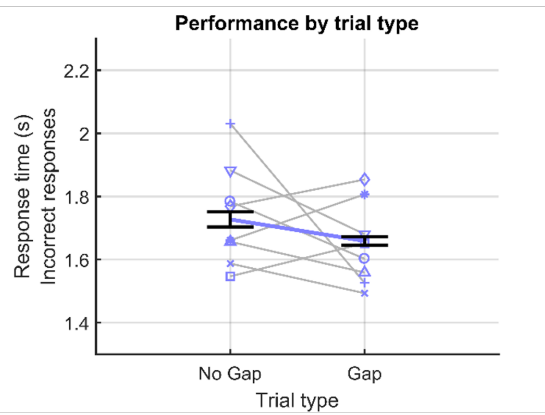
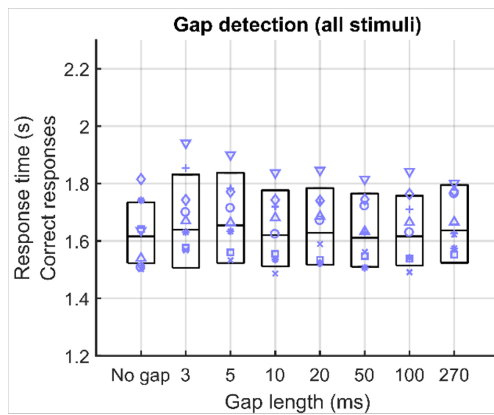
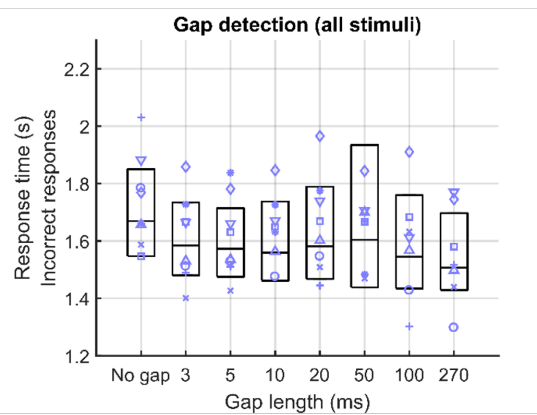
A**B****C****D**

Fig.7: Operant gap-in noise detection in ferrets: response times in gap and non-gap trials and by gap length. (A) Response times in correct trials for non-gap vs gap trials. (B) Response times in incorrect trials for non-gap vs gap trials. Blue markers depict animal averages. Blue lines connect mean values across animals and sessions. Black markers depicts 95% confidence interval, based on performance in individual testing session of all animals. Grey lines connect identical animal markers across conditions. (C) Response times by gap length for correct trials and (D) for incorrect trials. Blue markers depict animal averages. Box plots are based on performance in individual testing session of all animals. Horizontal edges of the boxplots depict interquartile ranges, horizontal lines in boxes the median.

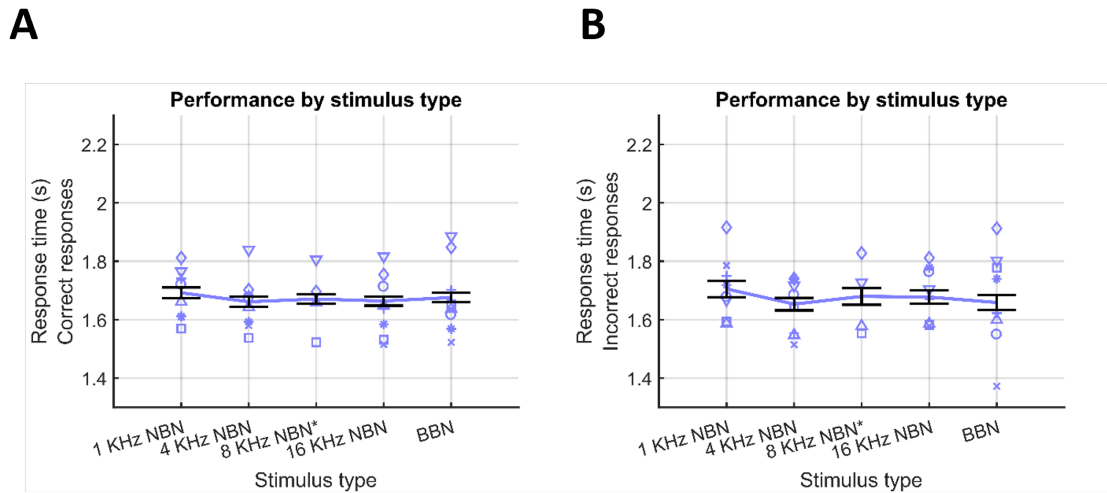


Fig.8: Operant gap-in noise detection in ferrets: response times by stimulus type. Response times are shown for (A) correct responses and (B) incorrect responses. Blue markers depict animal averages. Blue lines connect mean values across animals and sessions. Black markers depicts 95% confidence interval, based on performance in individual testing session of all animals.

Silence detection

In this paradigm (developed by Stolzberg et al. 2013, Fig. 9A) animals, which had previously been tested in operant gap detection (see above), were trained and tested to differentiate between amplitude modulated (AM) BBN trials, NBN trials and Silence (absence of a stimulus after successful trial initiation, see Methods for details). After reaching a stable performance level of >80% hit rate during the training phase for this paradigm, animals were assessed in at least 1000 trials.

Performance differed between trial types ($F_{(2,21)}=8.18$, $p=0.002$), as animals showed significantly lower performance in Silence trials as compared to AM (*Silence vs AM*, 0.91 ± 0.07 vs 0.99 ± 0.01 , $\beta=0.071$, $t_{(23)}=3.69$, $p=0.001$) and NBN trials (*Silence vs NBN*, 0.91 ± 0.07 vs 0.97 ± 0.04 , $\beta=0.06$, $t_{(23)}=3.94$, $p<0.001$)(Fig.9B). However, the high level of

performance (>90% correct) across all stimulus types suggests that animals were able to discriminate between silence and other stimuli.

Interestingly, animals' response times – the time between trial initiation and stimulus onset – was prolonged by multiple seconds in Silence trials, in both correct trials (*effect of trial type*, $F_{(2,21)}=79.66$, $p<0.001$) and incorrect trials ($F_{(2,18)}=21.82$, $p<0.001$). In both cases response times were significantly longer in Silence than in AM and NBN trials (**correct trials:** Silence vs AM, $3.16\pm0.54s$ vs $1.38\pm0.12s$, $\beta=-0.36$, $t_{(23)}=-12.56$, $p<0.001$, Silence vs NBN, $3.16\pm0.54s$ vs $1.43\pm0.1s$, $\beta=-0.34$, $t_{(23)}=-11.94$, $p<0.001$, **incorrect trials:** Silence vs AM, $4.22\pm1.98s$ vs $1.73\pm0.53s$, $\beta=-0.33$, $t_{(20)}=-6.18$, $p<0.001$, Silence vs NBN, $4.22\pm1.98s$ vs $1.46\pm0.27s$, $\beta=-0.44$, $t_{(20)}=-6.52$, $p<0.001$, Fig.9C,D). In line with the relatively low hit rate in silence trials this may indicate increased uncertainty, possibly due to inconsistency with the animal's expectation about presented stimuli (note that only 20% of trials were silence trials) as well as lack of saliency of the cue for trial onset in silence trials (only a visual but no auditory stimulus was present here).

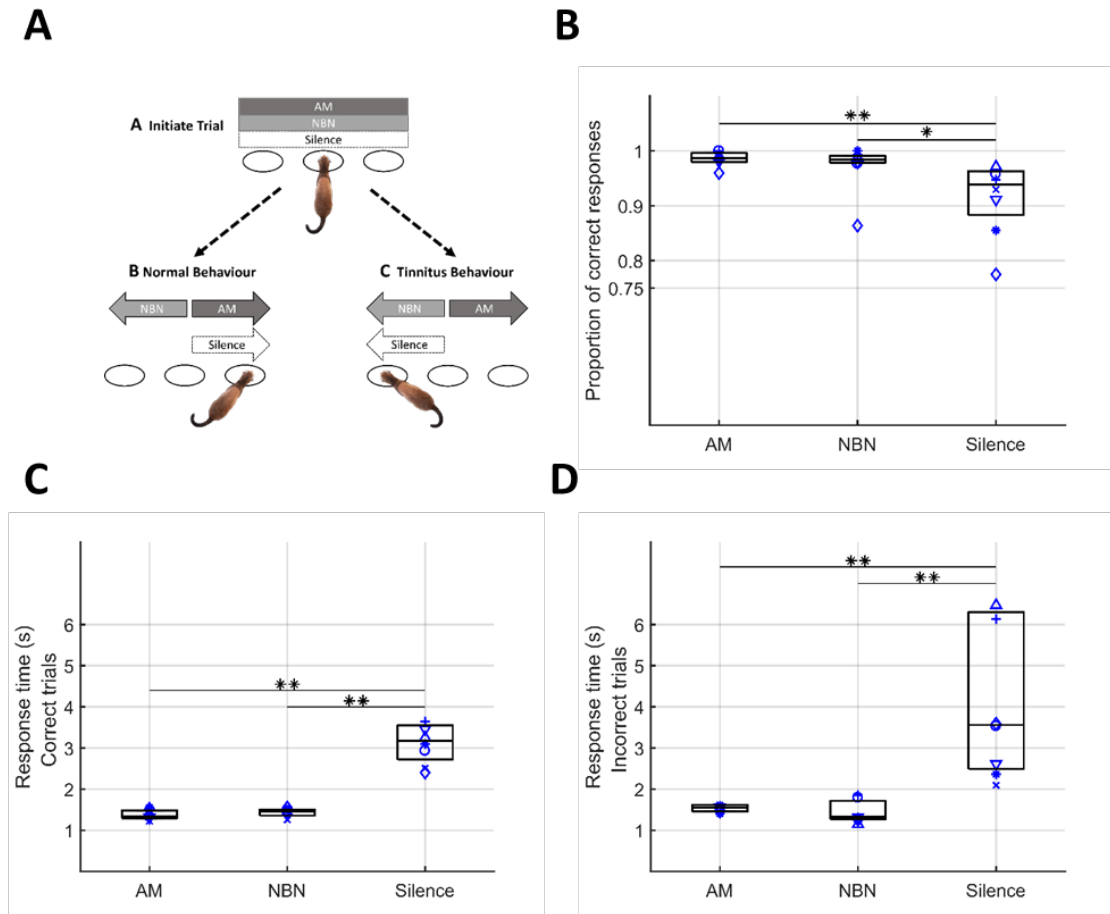


Fig.9: Behavioural silence detection. (A) The behavioural paradigm. **(B)** Proportion of correct responses in AM, NBN and Silence trials. **(C)** Response times in AM, NBN and Silence trials for correct trials and **(D)** for incorrect trials. Blue markers are animal averages, boxplots are interquartile ranges based on animal averages. Black horizontal bars indicate significant group differences between sound trials (AM, NBN) and Silence trials of $p < 0.05$ (with * indicating $p < 0.01$ and ** indicating $p < 0.001$).

Discussion

Ferrets were tested in several complementary paradigms which each allow for the assessment of behaviour affected by tinnitus. Both gap and silence detection paradigms build on the assumption that a persistent phantom sound interferes with the ability to distinguish silence from the presence of an auditory stimulus (Kalappa et al., 2014; M. J. Kim et al., 2021; Stolzberg et al., 2013; J. Turner et al., 2012).

Animals were assessed in two complementary gap detection paradigms. In a paradigm based on reflexive behaviour, some ferrets showed evidence for gap detection ability, although there was considerable variability in the startle measure between trials and animals. This may be partially due to animals' movement while in the experimental enclosure. While all animals were acclimatized to the testing enclosure, ferrets showed variable behaviour during testing sessions, in that some ferrets moved between trials (such as walking back and forth or turning), which may have conflated the signal. Movement of the animal, specifically locomotion, may also affect tone detection as has been described in mice (Schneider et al., 2018), which may influence the ability to detect auditory cues such as silent gaps. Animals showed negative GIAS values, suggesting gap induced inhibition of the startle response as well as positive GPIAS values. It is possible that instances of positive GPIAS signify startle induced facilitation. While facilitation has not previously been described in ferrets, it is a common observation in similar paradigms in mice (Longenecker et al., 2018; Plappert et al., 2004) and also occurs in rats (Reijmers & Peeters, 1994) and humans (Ludewig et al., 2003). Possible causes for facilitation can be high-intensity background noise of 70 dB SPL or higher (Davis, 1974) or varying frequency of the background noise (Gerrard & Ison, 1990). While none of these apply directly to the paradigm used in this study (background noise was presented at 40 dB SPL and did not vary in frequency) the movement of the animals in the testing chamber may have induced variations in subjective saliency of the background noise. It remains to be shown, however, whether this could drive facilitation of the acoustic startle and it cannot be ruled out that the variability seen in the data is due to uncontrolled noise or movement artefacts

In the operant variant of a gap detection paradigm ferrets learned to differentiate stimuli including silent gaps from continuous but otherwise identical stimuli. Here, gap detection ability was strongly modulated by the length of the silent gaps in the stimulus, as is expected in ferrets with normal hearing ability (Gold et al., 2015). Interestingly, animals showed best gap detection ability for BBN as compared to NBN sounds. This is likely due to the frequency composition of the used BBN stimulus. Since it covers a frequency range up to 30 kHz, the BBN activates the cochlea much more broadly than the NBN stimuli (Bear, 2020). The contrast between activation by the BBN stimulus and silence may therefore be more pronounced than in case of NBN stimuli, which could lead to inherently increased saliency of gaps in BBN as compared to NBN.

Animals did show better hit rate for gap than for continuous sounds. This was unlikely due to a side preference by the animals. First, animals were not significantly faster in indicating gap stimuli, neither for correct nor for incorrect trials. In addition, response times were not significantly different between the left and the right target sensor. Apart from this, any side preference would reduce the animals' efficiency in the task. While this might affect learning ability during initial training, it is expected to be compensated during the course of conditioning. Nevertheless, it is possible that animals tend to develop a bias towards perceiving gap sounds due to the evidently low success in detecting stimuli with particularly short gaps. Hence, from the perspective of the animal, there are 'easy' gap stimuli (namely the stimuli with the longest gaps) but there are no 'easy' non-gap stimuli as some of the subjective non-gap stimuli may in fact be stimuli with very short (non-audible) gaps. It is possible that, as a result of this, animals chose the gap target sensor slightly more often than at chance level. This would lead to higher

hit rate for gap trials overall, at the cost of hit rate for non-gap sound stimuli. This, however, remains to be addressed.

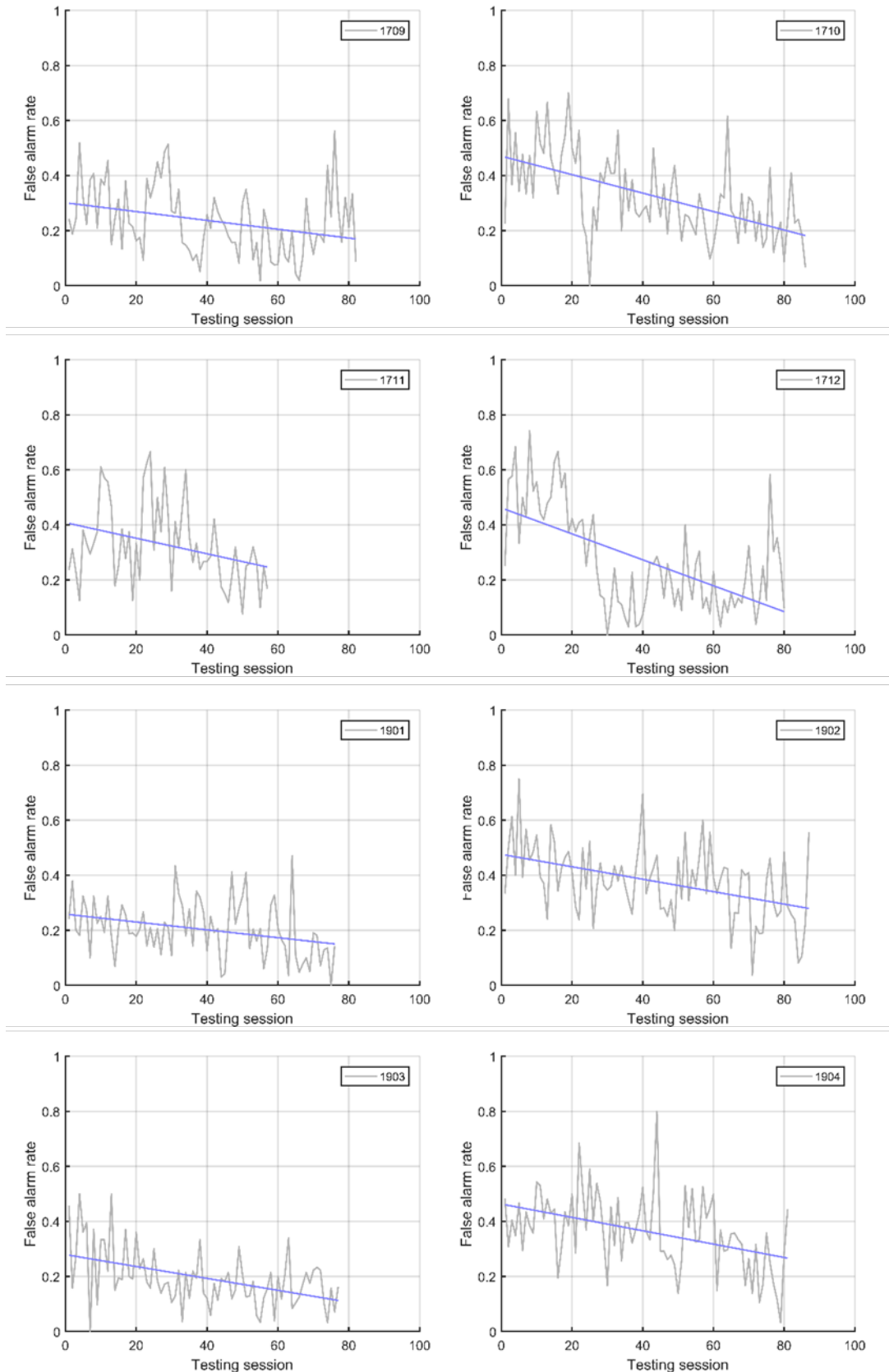
Response times in this paradigm were affected by length of gaps in presented stimuli yet varied only in fractions of seconds between stimuli with different gaps. To interpret the behavioural relevance of this effect, it is important to take into account which variables are likely to affect response times in the context this paradigm. Importantly, ferrets had to voluntarily engage in the task and position themselves on the central platform in order to initiate a trial, which may prompt task-specific attention (Otazu et al., 2009) consistent across trials. Yet, although previous experiments in this setup show that ferrets tend to have similar head angles relative to the activation spout across trials (Bajo et al., 2010) this was not controlled for in this study. Inconsistent head positions at trial onset could induce variability in the time the animal listens before approaching a target sensor. In addition, after having initiated a trial, animals did not always follow the same trajectory when approaching a sensor, which results in varying latencies between stimulus onset and target sensor activation by the animal. Given that the observed modulation of RTs between gap lengths was lower than the effects these uncontrolled factors alone could induce, it is not possible to assign significant effects on response times observed here to a particular cause.

In line with the demonstrated gap detection ability of the animals, ferrets were able to differentiate a prolonged auditory stimulus from the absence of a stimulus or 'silence'. Importantly, this paradigm is little affected by lacking ability in detecting sound on- or offsets or by reduced auditory temporal resolution more generally, factors that would impact detection of shorter gaps-in-noise as in the above-described operant paradigm.

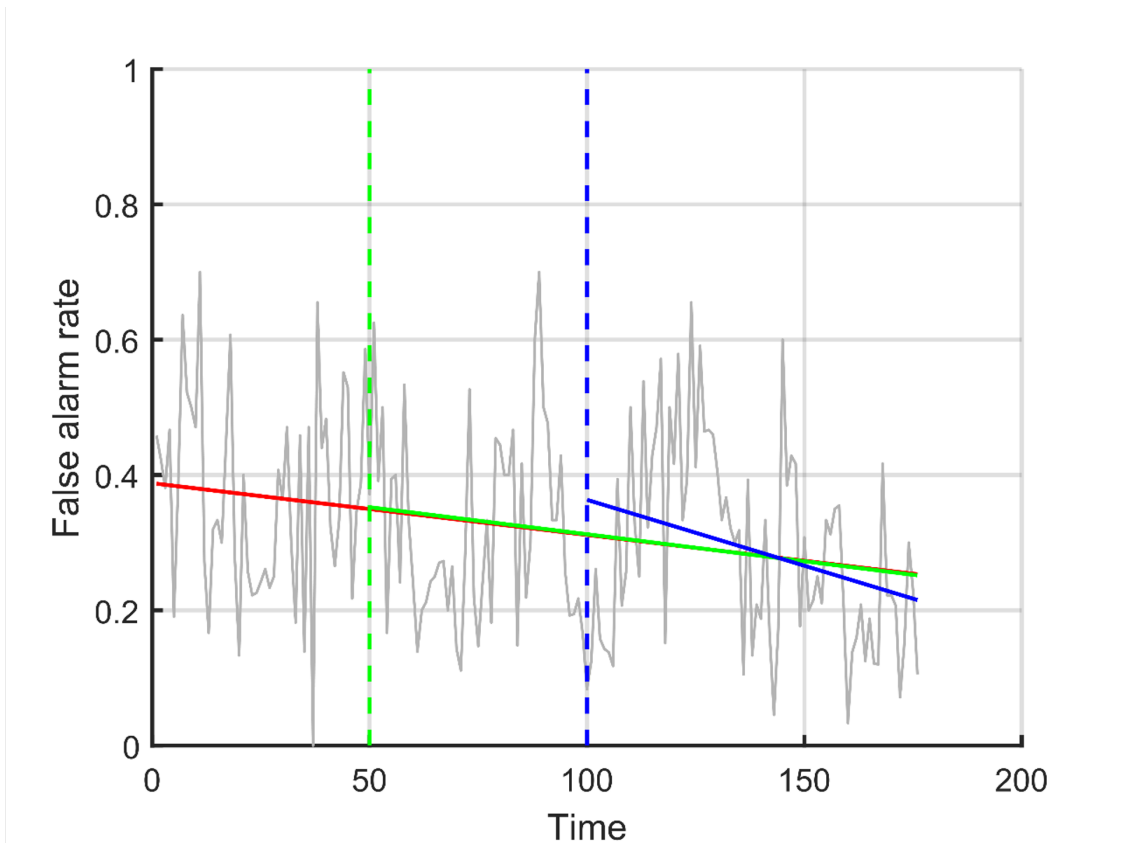
While, on average, animals successfully detected silence stimuli in more than 90% of cases, they still had a small but significant bias towards responding as if sounds were present. And markedly longer response times in Silence trials may suggest animals' uncertainty in line with that. Note that in contrast to the operant gap detection paradigm, response time differences between silence and auditory cues were on the scale of seconds. Next to uncertainty, this could indicate a general lack in response initiation in the face of reduced saliency of the sensory cue. Importantly, in this paradigm every trial onset was paired with a visual stimulus (including silence trials), yet the lack in additional auditory stimulation in silence trials may have led animals to hesitate and wait for additional evidence in form of a sound cue. This may have delayed the motoric response that, in other trials, is so clearly initiated by the auditory cue, and may therefore explain longer response times in silence trials.

All these measures – reflexive and operant gap detection and silence detection – assess behaviour that is affected by the development of a persistent phantom sound and, in combination, can control for various biases that are present in the individual paradigms. Assessment of reflexive gap detection led to inconclusive results, yet gap detection ability was extensively measured in an operant paradigm in which each animal readily performed about 5000 trials. Silence detection performance provided complementary information about behaviour sensitive to tinnitus. The demonstration of the feasibility and of the informative value of these paradigms in an experimental animal cohort presents the foundation for a model to study tinnitus development in the long term. Further, this study is based on objective measures with elaborate control over assessment timing and frequency, parameters that have not usually been controlled for neither in clinical trials nor in basic tinnitus research.

Supplementary figures



Suppl. Fig. 1: False alarm rate throughout the experiment. Data depict false alarm rates (i.e. falsely detected non-gap stimuli) of 8 animals over consecutive testing sessions. Blue lines depict a linear regression across sessions.



Suppl. Fig. 2: False alarm rate over a prolonged time of testing. Data depict false alarm rates (i.e. falsely detected non-gap stimuli) of an exemplary animal over 176 consecutive training sessions. Coloured plots depict linear regression curves fitted over the whole graph (red), or fitted after 50 (green) and 100 (red) sessions of testing, respectively.

Chapter 2: Behaviour and auditory processing after noise overexposure

Background

While tinnitus can be of short duration and cease after minutes or hours, it can also persist for years. It is thought that about 15% percent of the world population experience tinnitus (Baguley, McFerran and Hall, 2013). The latest assessment of Pan-European tinnitus prevalence estimates that 6% of people experience bothersome tinnitus (Baguley et al., 2013b; Biswas et al., 2022) which can be associated with comorbidities such as anxiety and depression (Bhatt et al., 2017), stress (Ciminelli et al., 2018a) and sleep alterations (Burgos et al., 2005; Crönlein et al., 2007; Hébert et al., 2011b). Yet it remains unclear how long-lasting tinnitus develops after a triggering event and, even less so, how this is paralleled by changes in underlying brain activity.

As described in the previous chapter, an animal model for chronic tinnitus in combination with regular assessments of objective metrics of the condition is well suited to address this. Chapter 1 introduced the behavioural paradigms, which, in combination, allow to study tinnitus long-term. An animal model of tinnitus, however, requires a controlled trigger to mark the putative onset of tinnitus.

The generation of tinnitus is not yet fully understood, and the trigger cannot be identified in all cases. Yet, most commonly, tinnitus is associated with hearing loss (Biswas et al., 2022; Nicolas-Puel et al., 2002), either ageing-related (presbycusis) or due

to damage to the cochlea or the auditory nerve (Liberman & Kujawa, 2017). Such damage can, for example, be caused by ototoxic drugs or high-intensity noise exposure (Baguley et al., 2013b). Indeed, damage to the cochlea or auditory nerve induces physiological changes that are thought to be directly involved in tinnitus generation (S. E. Shore et al., 2016a).

It is important to note, however, that tinnitus and hearing loss are not always linked. While the rate of tinnitus is considerably higher among those suffering from hearing loss than in normally hearing individuals, it is not a reliable predictor:

First, there are factors beyond sensory damage that can determine tinnitus emergence and its effect on wellbeing. In recent years, such factors have been linked to the mental health state of the tinnitus sufferer, in particular their stress levels (Ciminelli et al., 2018a). Stress has even been suggested to play fundamental role as an initial trigger for tinnitus (Sahley & Nodar, 2001). Yet, while the link between tinnitus and stress is indicated by many studies it remains unclear what the directionality of the interaction is and whether it is uniform across the diverse population of tinnitus sufferers (Elarbed et al., 2021).

Second, noise exposure can trigger tinnitus without having a lasting effect on hearing thresholds and tinnitus sufferers can present a normal audiogram (Sanchez et al., 2005; Schaette & McAlpine, 2011) – indicating that their hearing is not affected within the range tested in clinical diagnostics. Often, these tinnitus sufferers show impaired ability to follow conversations or to focus their attention in noisy environments, a condition also known as hidden hearing loss (Schaette & McAlpine, 2011). The most important cause of this is thought to be synaptopathy, where cochlear hair cells are intact but

synapses in the auditory nerve are lost (Kohrman et al., 2020; Liberman & Kujawa, 2017). Synaptopathy leads to incomplete afferent signals and a reduced signal to noise ratio in downstream targets that cause similar compensatory changes in gain as observed in hearing loss where the audiogram is altered. Therefore, even though in both hearing loss with altered audiogram and in hidden hearing loss without altered audiogram, the hearing impairment presents differently, in both cases sufferers have a lack in afferent auditory nerve signals. This may ultimately be at the basis of tinnitus formation (Schaette & Kempster, 2006; Sedley, 2019).

Due to the multitude of factors that can modulate tinnitus, ranging from hearing loss and ototoxic drugs to the mental health state, the putative tinnitus trigger is not always clear in individual cases. That said, a possible trigger that is among the most commonly observed in human tinnitus sufferers is chronic noise exposure or acute noise trauma (Nicolas-Puel et al., 2002), which patients can often trace back to specific events. Unlike ageing-related hearing loss (presbycusis) noise exposure can affect people of any age or physical state with the highest risk groups being those with regular or acute high-intensity noise exposure (NOE) such as musicians, pilots and builders. Under experimental conditions, NOE can be easily controlled and therefore presents an ideal experimental trigger for tinnitus commonly used in animal models (Bauer et al., 2008; Engineer et al., 2011; Kaltenbach et al., 2004; J. Turner et al., 2012). A caveat to NOE in animal models of tinnitus is the direct insult to the auditory system. This may, as described above, damage hair cells or cause synaptopathy causing a transient or permanent elevation of hearing thresholds. Importantly, many effects of NOE (hearing loss, hidden hearing loss, tinnitus) could each individually affect behavioural readouts of tinnitus, such as gap or silence detection ability. This would add a confounding factor

when treating performance in these paradigms as sufficient tinnitus readout. It is paramount, therefore, for an animal model of noise-induced tinnitus to assess the function of the auditory pathway and control for the impact of hearing impairment on behavioural assessments.

A technique that offers this control is the assessment of sound evoked auditory brainstem responses (ABRs). ABRs can be used to assess the sensory sensitivity of processing stages along the auditory pathway. Here, subcutaneous electrodes are placed over the midline and bilaterally over the petrous part of the temporal bone. The recorded signal is the summed potential of the auditory nerve and of further ascending processing stages in the central auditory pathway, such as the cochlear nuclei, superior olivary complex, lateral lemniscus nuclei or inferior colliculus (Akil et al., 2016; Suthakar & Liberman, 2019). Individual waves (i.e. waves at different latencies) of the response trace are thought to be related to the evoked activity at these stages, respectively, and can therefore inform about different types of impairment. For example, reductions in wave I are typical for a reduced response of the auditory nerve, whereas stable or increased later peaks can indicate elevated gain.

Next to the initial tinnitus trigger and modulating factors of tinnitus intensity, the consolidation of long-lasting tinnitus result in changes in both the auditory pathway and in non-auditory brain regions, such as in the prefrontal cortex, the hippocampus and the amygdala (Elgoyhen et al., 2015b) and, especially, the limbic system (De Ridder et al., 2011; Leaver et al., 2016; Rauschecker et al., 2010b). Yet the time course of such changes after a tinnitus trigger and their importance for tinnitus emergence remains unclear. An animal model for studying long-lasting tinnitus will allow to assess the time course of

tinnitus development, the underlying changes in brain activity and, building on that, open the possibility to test means to interfere with such changes subsequent to a tinnitus trigger.

Here, with the aim to establish an animal model for long-lasting tinnitus, a combination of behavioural measurements of tinnitus and auditory brainstem recordings was conducted regularly before and after noise exposure in a ferret model, spanning a monitoring period of 6 months after the putative tinnitus trigger.

Method summary

Effects of noise exposure (NOE) were determined in a group of female adult ferrets ($n=7$, the same group of animals as described in Chapter 1) by comparing their performance in three behavioural paradigms as well as ABRs recorded before and after NOE. Baseline assessment before NOE encompassed reflexive gap detection (GPIAS), operant gap detection, operant silence detection and auditory brainstem recordings (ABRs). Assessments after NOE consisted of the same paradigms and were conducted on two occasions: approximately 1 week after NOE (post NOE1 condition), and approximately 6 months after NOE (post NOE2 condition, Fig.1)

Auditory brainstem responses (ABRs) were obtained in anaesthetised animals using subcutaneous electrodes placed over the skull midline and bilaterally over the petrous part of the temporal bones. ABRs were assessed for click stimuli and one-octave width narrowband noise centred around 1, 4, 8 and 16 kHz (see Methods for a detailed description of the procedure). Assessments took place during the light phase. Baseline

ABRs were acquired after animals had completed baseline assessment in operant gap detection and operant silence detection over approximately 2 months. GPIAS (or reflexive gap detection) testing was conducted on a single occasion approximately 3 days after ABRs had been acquired. This completed baseline assessment of the animals.

Subsequent to baseline assessment of behaviour and ABRs, all animals were exposed for 2 hours to high-intensity (98 dB) noise under general anaesthesia (noise overexposure, NOE). Noise (1 octave narrowband centred at 8 kHz) was presented unilaterally via an earphone to the right ear while the left ear was plugged. NOE took place during the light phase, and animals were given at least 48hrs of recovery time before any other procedure.

Approximately one week after NOE, assessment of behaviour and ABRs was repeated (post NOE1 condition). Specifically, ABRs were assessed approximately 1 week after NOE. Within 2 weeks after NOE GPIAS was assessed (ensuring a minimal time of 14 days since the first GPIAS assessment shortly before NOE, to prevent habituation effects). Operant gap detection was assessed as in the baseline testing condition over a time period of approximately 2 months (until animals completed 1000 trials for each stimulus: 1, 4, 8, 16 kHz NBN and BBN). Subsequently, operant silence detection was tested over a time of approximately 2 weeks.

In order to capture later stages of possible tinnitus development a subgroup of ferrets (n=3) was tested again approximately 6 months after NOE (post NOE2 condition). GPIAS was assessed approximately 8 months after NOE. Two animals were assessed in all paradigms (ABRS, GPIAS, operant gap detection and silence detection), whereas one

was only tested in ABRs and operant gap detection (early termination of the experiment due to health reasons unrelated to the experimental protocol).

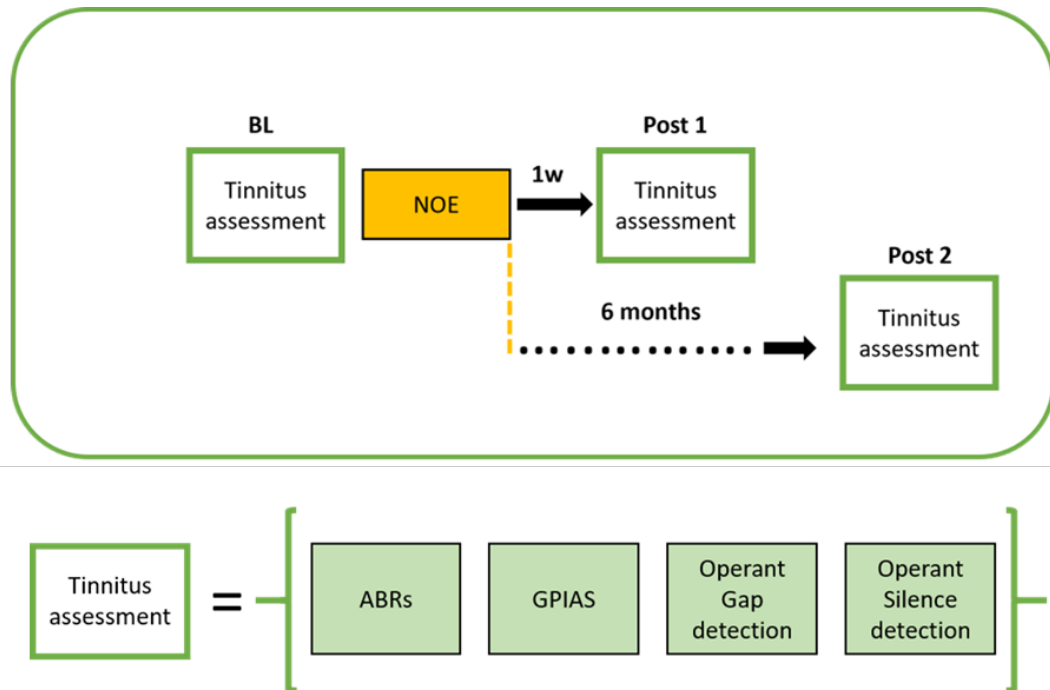


Fig. 1: Experimental timeline. Animals were assessed for tinnitus and hearing loss before and after noise over exposure (NOE). Assessments of all these metrics took place once under baseline conditions and on two occasions after NOE, the first assessment (Post 1) commencing 1 week after NOE and the second (Post 2) approximately 6 months after NOE. Animals were assessed in a battery of tests designed to estimate the presence of tinnitus and hearing ability. These consisted of testing operant gap detection, operant silence detection, gap pre-pulse inhibition of the behavioural startle (GPIAS) and of assessing auditory brainstem responses (ABRs).

Statistics and data analysis

Statistical analysis for all paradigms was conducted using generalised linear mixed models (GLMMs). Since animals were tested repeatedly over time, ‘time’ was always included in the model as a repeated factor.

ABR thresholds were determined through visual inspection of acquired signals by an experienced assessor under randomised and blinded conditions. For each sample, the lowest intensity where a response was visible was determined as the threshold. For further analysis of ABRs, peaks and troughs of signal averages across 700 repetitions were detected using a custom-written MATLAB algorithm (see Methods for details). Briefly, local maxima and minima were detected using the ‘findpeaks’ function (signal processing toolbox, MATLAB) and ABR waves I to V were defined depending on predetermined response windows for each sound level (40-90dB SPL). Based on the location and amplitude of the detected waves I to V the magnitude and latency was determined for each wave, respectively. The root mean square (RMS) of the signal was defined based on the MATLAB function ‘rms’ applied within a predetermined response window. Wave magnitude, latency and RMS, were compared over time. Specifically, measures were compared between BL and Post1 conditions for 7 animals and between BL and post2 conditions for 2 animals. Generalized linear models (GLMs) were applied to assess statistical significance (IBM SPSS).

Analysis of hit rates and response times in the operant gap detection paradigm was based on mean values per testing session and separated by the repeated measures ‘session’, ‘time’, ‘stimulus identity’ (‘frequency’) and ‘gap duration’. Testing sessions with less than 5 trials per gap length for at least 5 out of the 7 gap lengths tested were

excluded. Ferrets responded quickly (typically <3s) after stimulus presentation. Therefore, trials with response times longer than 10s were excluded as indicative of lacking engagement. Group comparisons were made by fitting a generalised linear model (IBM SPSS).

For analysis of gap detection thresholds, a sigmoid function was fitted on plots of hit rate (between 0 and 1) vs gap length, calculated for each animal, stimulus (1, 4, 8 and 16 kHz NBN and BBN) and condition (BL, Post1, Post2). Thresholds were defined as the gap length in closest proximity to a hit rate = 0.5 (50%) on the fitted function. Fits with slopes at threshold of more than 10 times the median slope across all samples were excluded from the analysis (2 out of 78 samples). Animal averages producing fits with slopes at threshold larger than 10 times the median slope across animals were excluded from the analysis (n=3).

Group comparisons in silence detection performance were conducted by fitting a GLM (IBM SPSS) with repeated measures 'session', 'time' and 'stimulus identity' ('frequency'). Trials with response times above 10 s were excluded from the analysis.

Data of GPIAS assessments after NOE were not included in the analysis, as data acquired under baseline conditions were inconclusive (see Chapter 1).

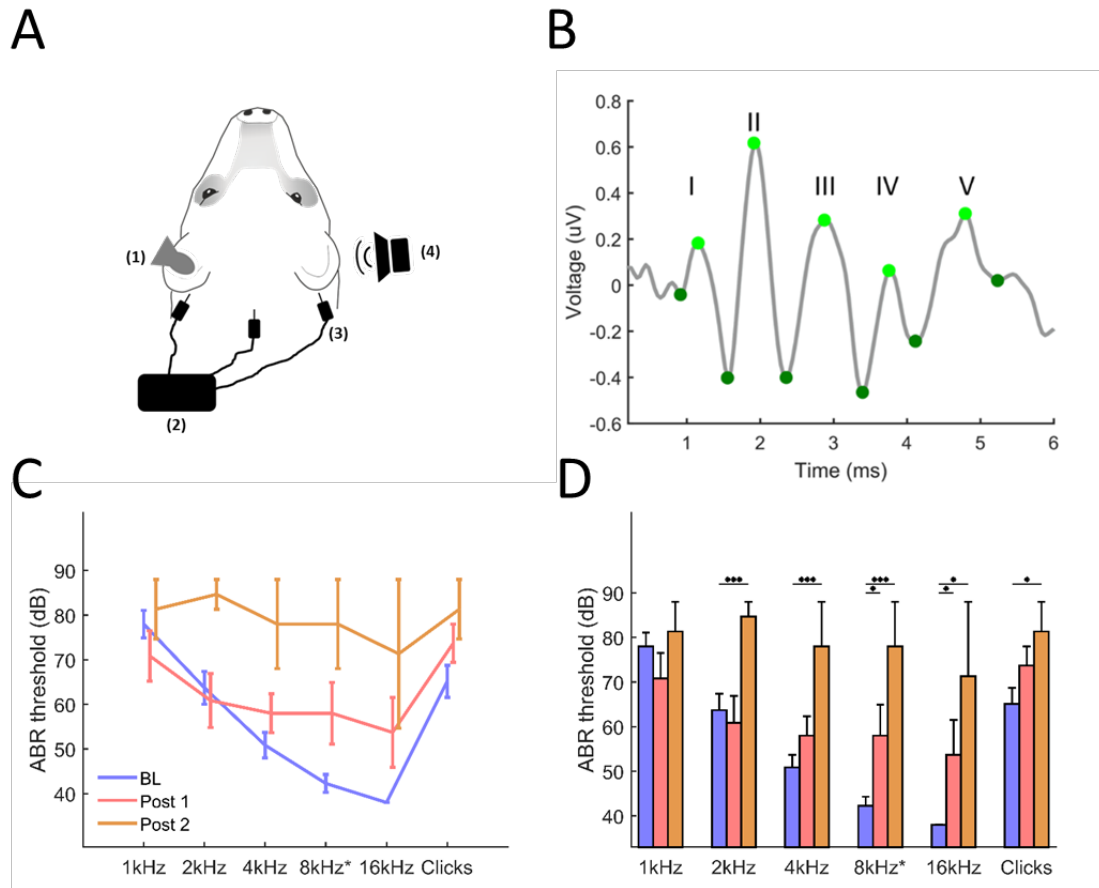


Fig. 2.1: Auditory brainstem response (ABR) measurements and thresholds before and after NOE. **(A)** Depiction of the ABR setup. The anaesthetised ferret is fitted with an earplug (1) in one ear. Subcutaneous electrodes (3) are placed and connected to an amplifier (2) and local PC, Sounds are presented via an earphone fitted to the other ear (4). **(B)** Example ABR average in one animal under baseline conditions. The signal is based on 700 trials, and evoked via with click stimulation (90 dB SPL). Waves I to V are labelled: light and dark green markers show detected peaks and troughs, respectively. For the analysis of ABR magnitude and latency, peak magnitudes were based on the difference between the peak and following trough for each wave. Wave latencies were based on the respective peak. **(C)** ABR thresholds, defined as the lowest intensity where a significant response – local peaks of waves I-IV – could be observed by a trained experimenter under blind conditions. Thresholds were assessed for 1, 2, 4, 8 and 16kHz centred NBN stimuli and a BBN stimulus. Note that the 8kHz NBN stimulus has identical frequency composition as the stimulus used for noise overexposure (NOE). Error bars are standard errors across animals.

Results

Noise exposure is associated with long-term effects on the auditory brainstem response

To assess the integrity of the auditory brainstem over time, ABRs were measured in all animals (Fig.2.1A, see Methods for details). Under normal hearing conditions (before NOE) a clear ABR signal was evoked by clicks with five waves readily identifiable for a sound level of 90 dB (Fig.2.1B). Thresholds were lowest for 16 kHz stimuli (40 dB SPL, Fig.2.1C,D) and increased with decreasing stimulus frequencies (Fig.2.1C), which is in line with the sharply tuned areas of maximum sensitivity in the 8-12 kHz frequency range in the ferret audiogram (Kelly et al., 1986). Following NOE, ABR thresholds tended to increase for stimuli in and adjacent to the frequency range of the NOE stimulus (Fig.2.1C). Initially, one week after NOE, thresholds significantly increased only for stimuli with centre frequencies at 8 kHz, matching the NOE stimulus (BL vs Post1, $\beta = 15.71$, $t_{(16)} = 7.24$, $p < 0.05$), and for 16 kHz NBN (BL vs Post1, $\beta = 15.71$, $t_{(16)} = 2.17$, $p < 0.05$, Fig.2.1C,D, Supp. Table 1). In the late assessment 6 months after NOE (Post2) thresholds increased for click stimuli and all tested NBN frequencies except for 1 kHz NBN (Fig.2.1D), suggesting a long-term degradation in the auditory brainstem.

Since distinct waves of the ABR signal could be differently affected by NOE, additional parameters were used to study the integrity of the auditory system over time: first, by measuring the amplitudes and latencies of waves I-V, the effect of NOE on each individual wave was assessed. This analysis was based on ABRs evoked using 90 dB SPL stimuli, the highest stimulus intensity, as only here all ABR waves were clearly discernible (see Fig.2.2A).

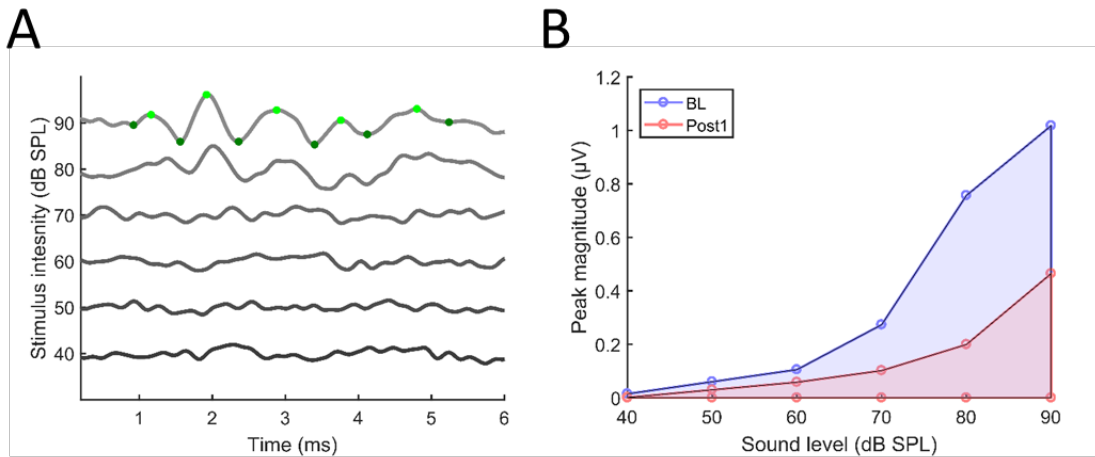


Fig. 2.2: Measuring the total magnitude of auditory brainstem responses (ABRs). (A) Example click-evoked ABRs of one animal under baseline conditions. Stimulus intensities ranged from 40 to 90 dB SPL. (B) Wave II magnitude plotted over sound level (example of one animal under baseline conditions). To estimate the total magnitude of a response component (here wave II magnitude) including ABRs to all assessed sound levels, the area under the curve was calculated for each condition, respectively. A reduced area characterises reduced total response magnitude across sound levels.

For click stimuli of the highest intensity (90 dB SPL) animals showed a change after NOE for multiple components of the ABR signal, specifically for wave I ($F_{(2,14)}=7.85$, $p=0.005$), wave II ($F_{(2,14)}=10.75$, $p=0.001$) and wave IV ($F_{(2,14)}=7.26$, $p=0.01$) (Fig. 3A). Many animals showed decreasing response magnitudes from BL already in the first assessment after NOE, Post1, yet there was no significant decrease at this point in time (wave I, BL vs Post1, $0.32 \pm 0.19 \mu V$ vs $0.18 \pm 0.11 \mu V$, $\beta = -1.358E-7$, $t_{(16)} = -1.82$, $p=0.09$, wave II, BL vs Post1, $0.81 \pm 0.51 \mu V$ vs $0.58 \pm 0.45 \mu V$, $\beta = -2.241E-7$, $t_{(16)} = -0.94$, $p=0.37$, wave IV, BL vs Post1, $0.042 \pm 0.34 \mu V$ vs $0.21 \pm 0.2 \mu V$, $\beta = -2.170E-7$, $t_{(16)} = -1.79$, $p=0.1$, wave V, BL vs Post1, $0.018 \pm 0.18 \mu V$ vs $0.26 \pm 0.25 \mu V$, $\beta = 7.918E-7$, $t_{(16)} = 1.4$, $p=0.18$) (Fig. 3A,B, see Supp. Table 1 for individual values).

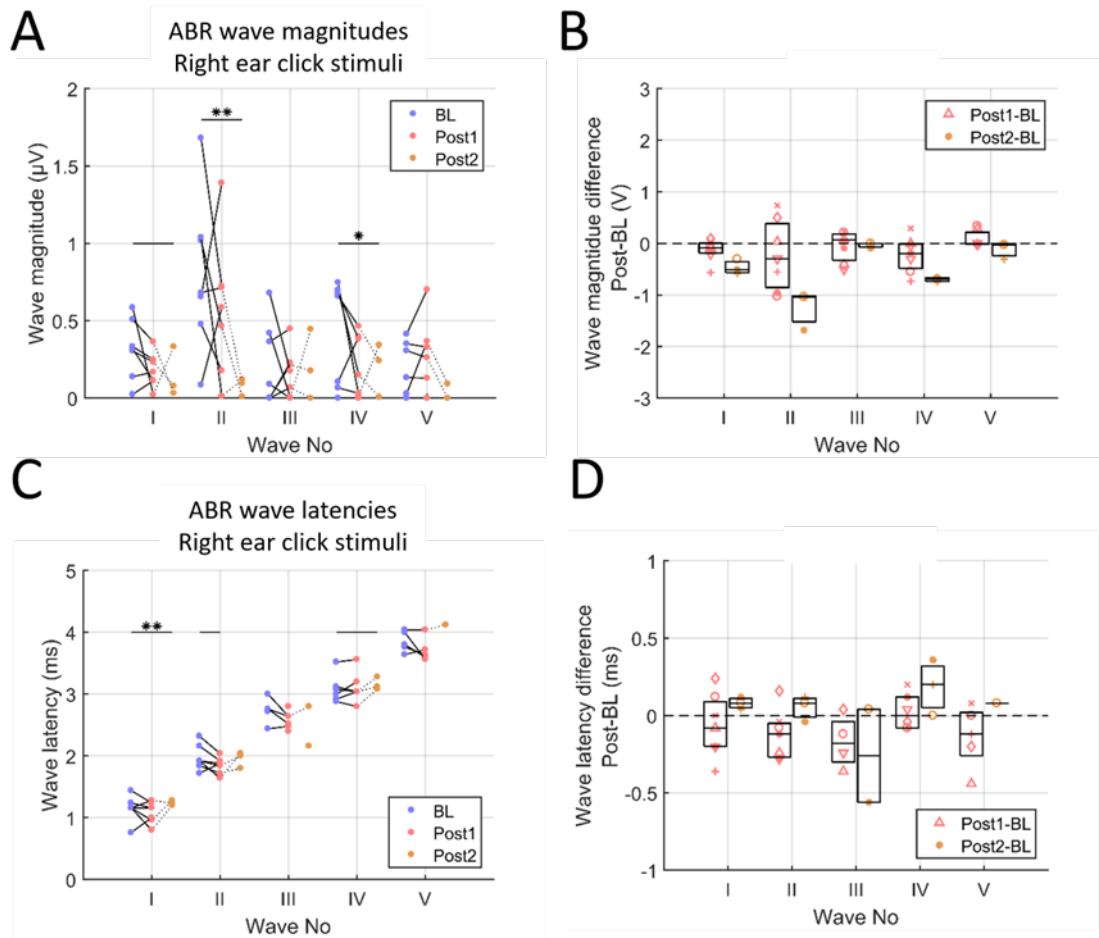


Fig.3: ABR magnitudes and latencies for waves I-V: click stimuli at 90dB SPL. (A) Animal averages of wave magnitudes (I-V) for baseline assessment (BL), and assessments after NOE (Post1 and Post 2). Lines connect data points of the same animal. **(B)** Differences in wave magnitude (Post-BL) for Post 1 and Post 2, respectively. Datapoints depict animal averages. Note that values below 0 indicate a decrease in wave magnitude from baseline to the respective 'post NOE' assessment. **(C)** ABR wave I-V latencies, data depicted as in A. **(D)** Differences in wave latencies from BL to post NOE, depicted as in B. Panels A,C: black vertical lines depict $p<0.05$, markers depict $p<0.01$ (*) and $p<0.001$ (**).

6 months after NOE (Post2), on the other hand, animals showed significantly reduced response magnitudes for the same ABR components: wave I (BL vs Post2, 0.32 ± 0.19 vs $0.15\pm0.17\mu V$, $\beta=-2.565E-7$, $t_{(16)}=-2.97$, $p=0.01$), wave II (BL vs Post2, $0.81\pm0.51\mu V$ vs $0.08\pm0.06\mu V$, $\beta=-8.600E-7$, $t_{(16)}=-4.25$, $p<0.001$), wave IV (BL vs Post2, $0.42\pm0.34\mu V$ vs $0.2\pm0.17\mu V$, $\beta=-3.671E-7$, $t_{(16)}=-3.79$, $p=0.002$), except for wave V (BL vs Post2, $0.18\pm0.18\mu V$ vs $0.03\pm0.05\mu V$, $\beta=-9.045E-8$, $t_{(16)}=-1.16$, $p=0.27$). It is of note that every single animal out of the 3 ferrets in the Post2 condition showed lower response

magnitudes in waves I, II and IV 6 months after NOE compared to BL (Fig.3B, orange markers & Suppl. Table 1).

There was no consistent change in latencies affecting the entire ABR, yet the same waves that showed decreases in amplitude also showed changed latencies: wave II showed a temporarily reduced latency in the first assessment post NOE ($F_{(2,14)}=3.93$, $p=0.04$, BL vs Post1, $1.97\pm0.2ms$ vs $1.85\pm0.13ms$, $\theta=-3.14$, $t_{(16)}=-2.26$, $p=0.04$)(Fig.3C,D). 6 months after NOE wave I showed increased latency ($F_{(2,14)}=10.6$, $p=0.002$, BL vs Post2, $1.15\pm0.2ms$ vs $1.24\pm0.04ms$, $\theta=2.4$, $t_{(16)}=4.34$, $p<0.001$) as did wave IV ($F_{(2,12)}=7.35$, $p<0.01$, BL vs Post2, $3.1\pm0.23ms$ vs $3.16\pm0.11ms$, $\theta=5.56$, $t_{(14)}=3.1$, $p=0.01$)(Fig.3C, Suppl. Table 2).

Finally, to test whether the effect of NOE was also reflected in a change in total ABR signal magnitude (i.e. across all sound levels) the total magnitude of ABR wave II and of the RMS of the signal across all tested click intensities was calculated. ABR wave II was chosen for this metric (Fig.2.1B) since this was the most clearly identifiable wave across intensities (Fig.2.2A) and also because it reflects activity in the cochlear nucleus (Henry, 1979) an early processing stage of the auditory brainstem, which is directly affected by loss of cochlear or auditory nerve activity. Note that ABR wave I, corresponding to activity in the auditory nerve directly, was not easily discernible and therefore not used for this analysis. In addition, to include all five waves in the ABR signal, signal magnitude was calculated based on the root mean square (RMS) of the signal within a predefined response window (1.6-4 ms, with a shift of 0.16ms for every step in decreasing sound level). To this end, values for wave II magnitude and RMS were plotted over sound level.

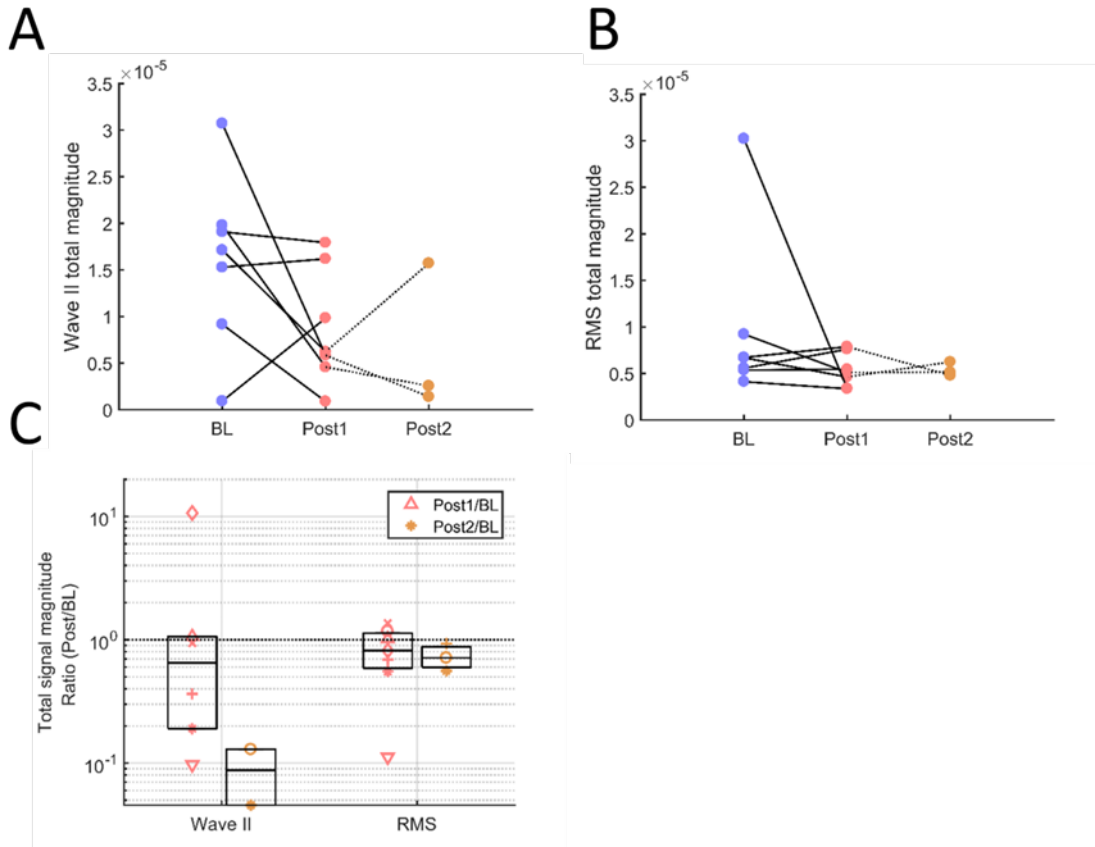


Fig.5: ABR total signal magnitude across intensities (click stimuli): BL vs. Post NOE. Depicted are the total magnitude for **(A)** ABR wave II and **(B)** the RMS across ABR waves I-V for sound stimuli of all levels (40-90dB SPL). Total magnitude is defined as the area under the graph for a linear function as shown in Fig.2D. **(C)** Change of wave II total magnitude and RMS total magnitude from BL to Post1 and Post2. Data points represent individual animals. Data are the ratio of Post1 or Post2 to the baseline measure. A value below 1 (dotted line) depicts a decrease in the respective measure relative to baseline. Panels A,B: Black vertical lines depict $p < 0.05$, markers depict $p < 0.01$ (*) and $p < 0.001$ (**).

Total magnitude was then defined as the area under the graph (for wave II magnitude and RMS, respectively) (Fig.2.2B) for each animal and condition. While the total response magnitude tended to decrease after NOE for both measures (Fig.5C), this did not reach significance, in terms of both wave II magnitude ($F_{(2,14)}=2.11$, $p=0.16$, BL vs Post1, $1.6 \times 10^{-5} \pm 0.93 \times 10^{-5}$ vs $0.88 \times 10^{-5} \pm 0.63 \times 10^{-5}$, $\beta = -7.229 \times 10^{-6}$, $t_{(16)} = -1.84$, $p=0.09$, BL vs Post2, $1.6 \times 10^{-5} \pm 0.93 \times 10^{-5}$ vs $0.66 \times 10^{-5} \pm 0.8 \times 10^{-5}$, $\beta = -1.090 \times 10^{-5}$, $t_{(16)} = -1.86$, $p=0.08$) (Fig.5A,C) as well as based on the RMS of the signal ($F_{(2,14)}=2.12$, $p=0.16$, BL vs Post1, $0.97 \times 10^{-5} \pm 0.92 \times 10^{-5}$ vs $0.53 \times 10^{-5} \pm 0.18 \times 10^{-5}$, $\beta = -4.356 \times 10^{-6}$, $t_{(16)} = -1.23$, $p=0.24$, BL vs Post2,

$0.97 \times 10^{-5} \pm 0.92 \times 10^{-5}$ vs $0.54 \times 10^{-5} \pm 0.08 \times 10^{-5}$, $\beta = -3.554 \times 10^{-6}$, $t_{(16)} = -1.76$, $p = 0.1$) (Fig.5B,C).

Note, however, that individual animals showed varying changes.

Overall, animals showed a reduction of click-evoked neural responses congruent with the hypothesis that NOE resulted in cochlear damage or in degradation of the auditory nerve. This was most prominently reflected in progressive threshold elevation soon after NOE, with the most pronounced impairment in sensitivity to clicks present 6 months after NOE. This impairment after NOE was only weakly reflected in the overall magnitude of the ABR, and only the most prominent ABR wave, wave II, was reduced in response to high-intensity (90 dB) stimulation.

To explore if the overall reduction of sensitivity to click stimulation was due to a lesion of a frequency specific part of the cochlea ABRs were also recorded based on stimuli with limited bandwidth centred at different frequencies.

Frequency specific ABRs responses are impaired early and long-term after NOE

When measuring ABRs evoked by stimuli with the same frequency composition as the NOE stimulus (8 kHz NBN) thresholds were significantly elevated already 1 week after NOE and even more so 6 months after NOE (Fig.2.1D), which is in line with frequency specific damage to the cochlea or auditory nerve. While this effect was stronger than observed for click stimuli, it was not reflected in more components of the ABR signal visible at 90 dB stimulation where ABR waves I to V were discernible: a consistent decrease in amplitude was only present for wave II ($F_{(2,14)} = 4.16$, $p = 0.04$) (Fig.6A,B, Supp. Table 5), in line with the wave II decrease for click stimuli (Fig.3). For 8K NBN,

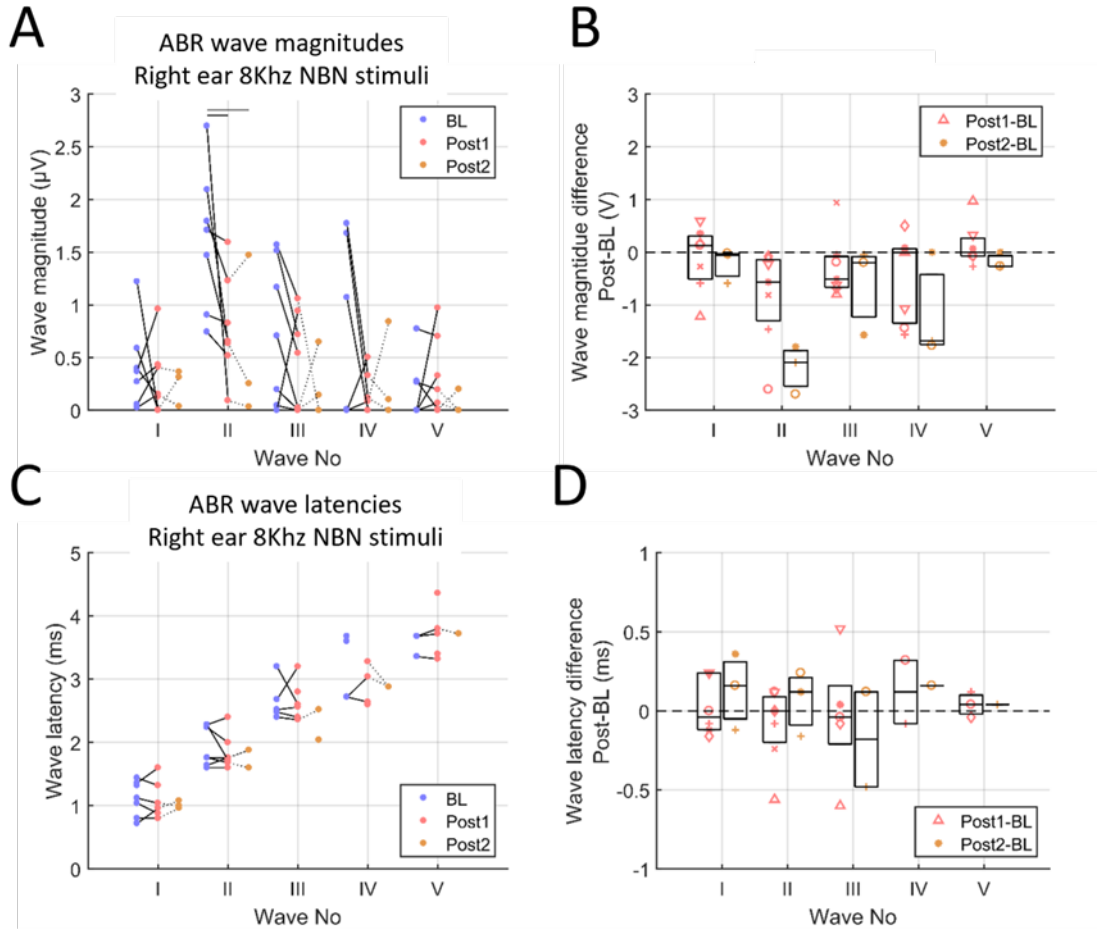


Fig.6: ABR magnitudes and latencies for waves I-V: NBN 8kHz stimuli at 90dB SPL. (A) Animal averages of wave magnitudes (I-V) for baseline assessment (BL), and assessments after NOE (Post1 and Post 2). Lines connect data points of the same animal. **(B)** Differences in wave magnitude (Post-BL) for Post 1 and Post 2, respectively. Datapoints depict animal averages. Note that values below 0 indicate a decrease in wave magnitude from baseline to the respective 'post NOE' assessment. **(C)** ABR wave I-V latencies, data depicted as in A. **(D)** Differences in wave latencies from BL to post NOE, depicted as in B. Panels A,C: black vertical lines depict $p < 0.05$, markers depict $p < 0.01$ (*) and $p < 0.001$ (**).

the decrease in wave II magnitude was also apparent much earlier than for clicks, already 1 week after NOE (*BL vs Post1*, $1.63 \pm 0.67 \mu V$ vs $0.79 \pm 0.49 \mu V$, $\beta = -8.375E-7$, $t_{(16)} = -2.61$, $p = 0.02$), in line with the early onset of threshold elevation for 8 kHz NBN, and this decrease persisted 6 months post NOE (*BL vs Post2*, 1.63 ± 0.67 vs $0.58 \pm 0.77 \mu V$, $\beta = -1.256E-6$, $t_{(16)} = -2.82$, $p = 0.01$). Response latencies did not significantly change throughout the experiment (Fig. 3C,D, Supp. Table 6).

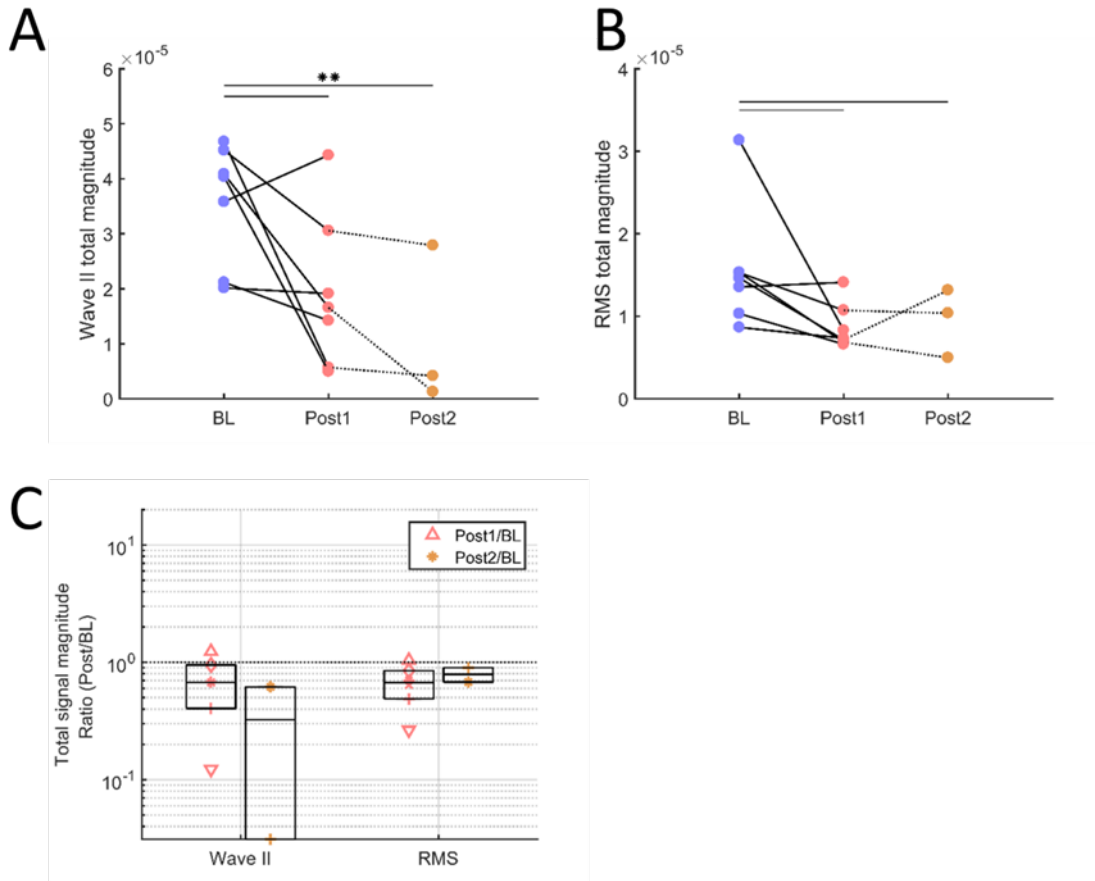


Fig.8: ABR total signal magnitude across intensities (8kHz NBN stimuli): BL vs. Post NOE. Depicted are the total magnitude for **(A)** ABR wave II and **(B)** the RMS across ABR waves I-V for sound stimuli of all levels (40-90dB SPL). Total magnitude is defined as the area under the graph for a linear function as shown in Fig.2D. **(C)** Change of wave II total magnitude and RMS total magnitude from BL to Post1 and Post2. Data points represent individual animals. Data are the ratio of Post1 or Post2 to the baseline measure. A value below 1 (dotted line) depicts a decrease in the respective measure relative to baseline. Panels A,B: Black vertical lines depict $p < 0.05$, markers depict $p < 0.01$ (*) and $p < 0.001$ (**).

The possible effect of NOE became further evident in the change in total response magnitude of the ABR signal: this was apparent from the first assessment post NOE (Post1) onwards for both wave II magnitude ($F_{(2,14)}=9.34$, $p=0.003$, BL vs Post1, $3.6 \times 10^{-5} \pm 1.1 \times 10^{-5}$ vs $1.9 \times 10^{-5} \pm 1.4 \times 10^{-5}$, $\beta = -1.641 \times 10^{-5}$, $t_{(16)} = -2.58$, $p=0.02$)(Fig.8A,C) and RMS ($F_{(2,14)}=3.62$, $p=0.05$, BL vs Post1, $1.6 \times 10^{-5} \pm 0.7 \times 10^{-5}$ vs $0.9 \times 10^{-5} \pm 0.3 \times 10^{-5}$, $\beta = -6.861 \times 10^{-6}$, $t_{(16)} = -2.51$, $p=0.03$)(Fig.8B,C, Supp. Table 8), and was still evident 6 months after NOE (wave II: BL vs Post2, $3.6 \times 10^{-5} \pm 1.1 \times 10^{-5}$ vs $1.1 \times 10^{-5} \pm 1.5 \times 10^{-5}$, $F = -2.718 \times 10^{-5}$, $t_{(16)} = -4.31$, $p < 0.001$; RMS: BL vs Post2, $1.6 \times 10^{-5} \pm 0.7 \times 10^{-5}$ vs $1.0 \times 10^{-5} \pm 0.4 \times 10^{-5}$, $\beta = -5.642 \times 10^{-6}$, $t_{(16)} = -$

2.28, $p=0.04$), further corroborating a prompt and lasting effect of NOE on sound processing in the NOE frequency range.

Overall, the changes in the ABR signal after NOE were much more profound than for click stimuli: both ABR sensitivity (based on thresholds) and its total signal magnitude (based on the entire ABR as well as wave II) significantly decreased early after NOE, and was even more pronounced 6 months after NOE. This prompt and lasting effect is supporting the possibility that noise exposure caused damage to frequency specific parts of cochlear, impacting the processing of stimuli in a similar frequency range as the NOE stimulus.

For high frequency NBN centred around 16kHz, thresholds, increased promptly and significantly after NOE, similar as for 8 kHz NBN stimuli, although not as pronounced (Fig.2.1D). Similarly, ABR signal magnitude also changed after NOE, but not as profoundly as for 8kHz NBN. Signal magnitude at 90dB sound level, where all ABR waves could be identified, decreased for both ABR wave I and wave II (Fig.9A,B, Supp. Table 9). While the change was only temporary in wave I ($F_{(2,14)}=5.17$, $p=0.02$, BL vs Post1, $0.69\pm0.39 \mu V$ vs $0.32\pm0.19 \mu V$, $\beta=-3.690E-7$, $t_{(16)}=-3.21$, $p=0.01$, BL vs Post2, $0.69\pm0.39 \mu V$ vs $0.4\pm0.11 \mu V$, $\beta=-2.506E-7$, $t_{(16)}=-1.44$, $p=0.17$), it appeared at the same time in wave II ($F_{(2,14)}=840.68$, $p<0.001$, BL vs Post1, $1.14\pm0.56 \mu V$ vs $0.65\pm0.48 \mu V$, $\beta=-4.902E-7$, $t_{(16)}=-2.61$, $p=0.02$) and was still evident 6 months post NOE (BL vs Post2, $1.14\pm0.56 \mu V$ vs $0.35\pm0.38 \mu V$, $\beta=-1.158E-6$, $t_{(16)}=-10.92$, $p<0.001$).

Latencies of the different components of the ABR signal remained stable throughout the experiment, with the exception of the latest component in the signal, wave V, where the peak of the wave was significantly later in Post2 than in BL ($F_{(2,11)}=4.57$, $p=0.04$, BL

vs Post2, $3.51 \pm 0.15 \text{ ms}$ vs $3.76 \pm 0.36 \text{ ms}$, $\beta = 5.672$, $t_{(13)} = 2.98$, $p = 0.01$) (Fig. 9C,D, Supp. Table 10). Interestingly, the same wave showed a trend towards increased magnitude in the Post2 assessment ($F_{(2,14)} = 5.52$, $p = 0.02$, *BL vs Post2*, $0.22 \pm 0.23 \text{ } \mu\text{V}$ vs $0.64 \pm 0.26 \text{ } \mu\text{V}$, $\beta = 2.922\text{E-}7$, $t_{(16)} = 2.11$, $p = 0.05$) (Fig. 9A,B).

The total magnitude of the ABR signal across sound levels was reduced after NOE, with evidence for marked change in the overall ABR signal (RMS) already one week after NOE ($F_{(2,14)} = 50.57$, $p < 0.001$, *BL vs Post1*, $1.57 \times 10^{-5} \pm 0.64 \times 10^{-5}$ vs $0.87 \times 10^{-5} \pm 0.2 \times 10^{-5}$, $\beta = -7.063\text{E-}6$, $t_{(16)} = -3.11$, $p = 0.01$) (Fig. 11B,C, Supp. Table 12). 6 weeks after NOE a significant decrease was evident for both the total magnitude of the RMS and wave II specifically (RMS total magnitude, *BL vs Post2*, $1.57 \times 10^{-5} \pm 0.64 \times 10^{-5}$ vs $0.75 \times 10^{-5} \pm 0.11 \times 10^{-5}$, $\beta = -8.605\text{E-}6$, $t_{(16)} = -6.34$, $p < 0.001$; total wave II magnitude, $F_{(2,14)} = 21.56$, $p < 0.001$, *BL vs Post2*, $3.1 \times 10^{-5} \pm 2.2 \times 10^{-5}$ vs $0.91 \times 10^{-5} \pm 0.67 \times 10^{-5}$, $\beta = -2.805\text{E-}5$, $t_{(16)} = -4.4$, $p < 0.001$) (Fig. 11A,C, Supp. Table 12).

It is interesting to note that both hearing sensitivity (based on ABR thresholds) and the overall signal magnitude (based on the total RMS) decreased soon after NOE. For the specific ABR component, wave II, total magnitude only decreased 6 months post NOE. This suggests that different components of the ABR response may be affected differently by noise overexposure.

Overall, it is interesting to note that initial threshold elevation after NOE did not reverse over time, a typical marker for hidden hearing loss (Lobarinas et al., 2017), but became more pronounced. The sensitivity to stimuli closely matching the NOE stimulus frequency range (8 kHz NBN) was impaired early after NOE and persisted long term, and similarly so for high frequency NBNs (16 kHz). Impairments in broader frequency ranges

were also evident but only 6 months after NOE. It has indeed been reported that noise overexposure does not only affect processing of sound frequencies in the same range as the NOE stimulus but also neighbouring frequency bands (Noreña & Eggermont, 2006a), although these effects are evident soon after NOE. Here, animals showed impairments even in click evoked ABRs after NOE, although these were less pronounced and total signal magnitude was not affected. As click stimuli contain a much wider frequency spectrum than the used NBN stimuli, evoked activity is more robust in the face of frequency specific lesions, as NOE might have induced in this paradigm. This, however, might still affect performance in the behavioural paradigms as part of this experiment (see the following sections).

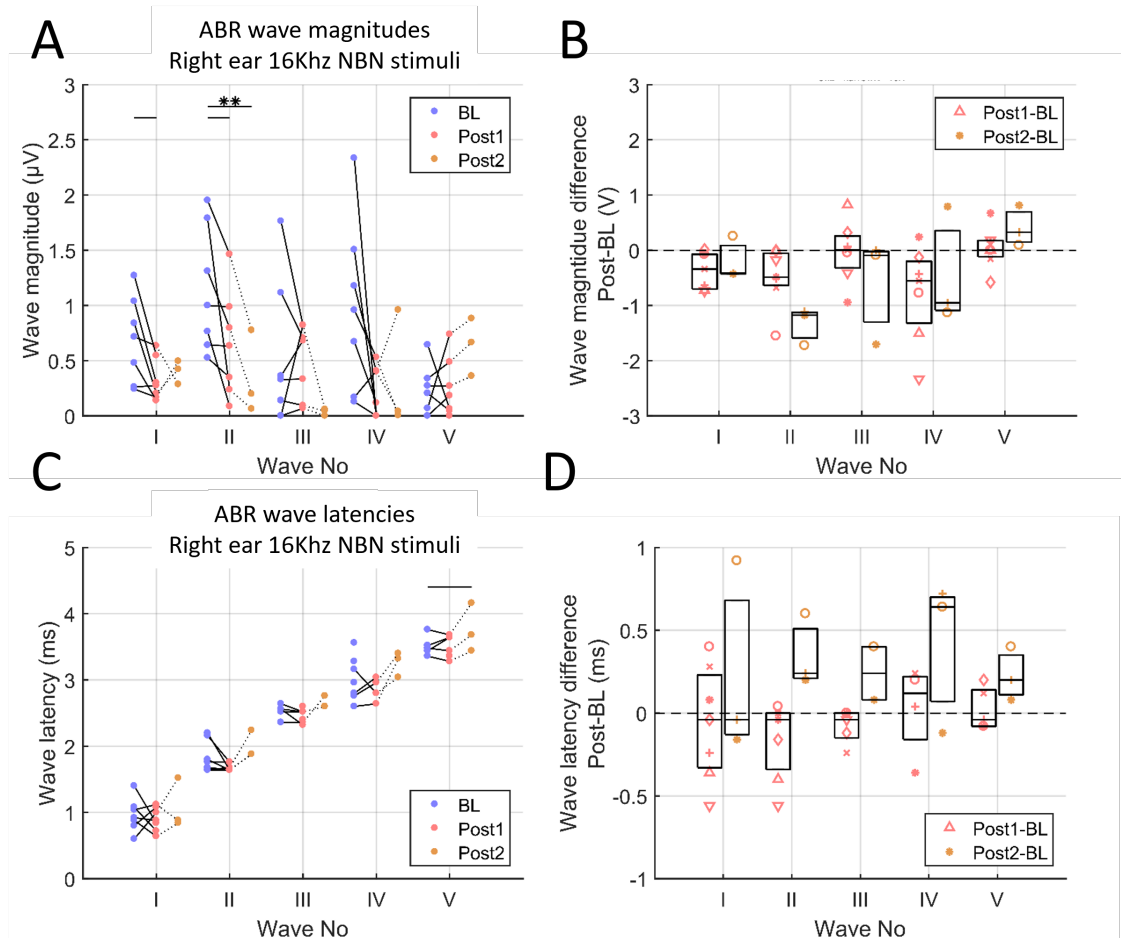


Fig.9: ABR magnitudes and latencies for waves I-V: NBN 16kHz stimuli at 90dB SPL. (A) Animal averages of wave magnitudes (I-V) for baseline assessment (BL), and assessments after NOE (Post1 and Post 2). Lines connect data points of the same animal. **(B)** Differences in wave magnitude (Post-BL) for Post 1 and Post 2, respectively. Datapoints depict animal averages. Note that values below 0 indicate a decrease in wave magnitude from baseline to the respective 'post NOE' assessment. **(C)** ABR wave I-V latencies, data depicted as in A. **(D)** Differences in wave latencies from BL to post NOE, depicted as in B. Panels A,C: black vertical lines depict $p < 0.05$, markers depict $p < 0.01$ (*) and $p < 0.001$ (**).

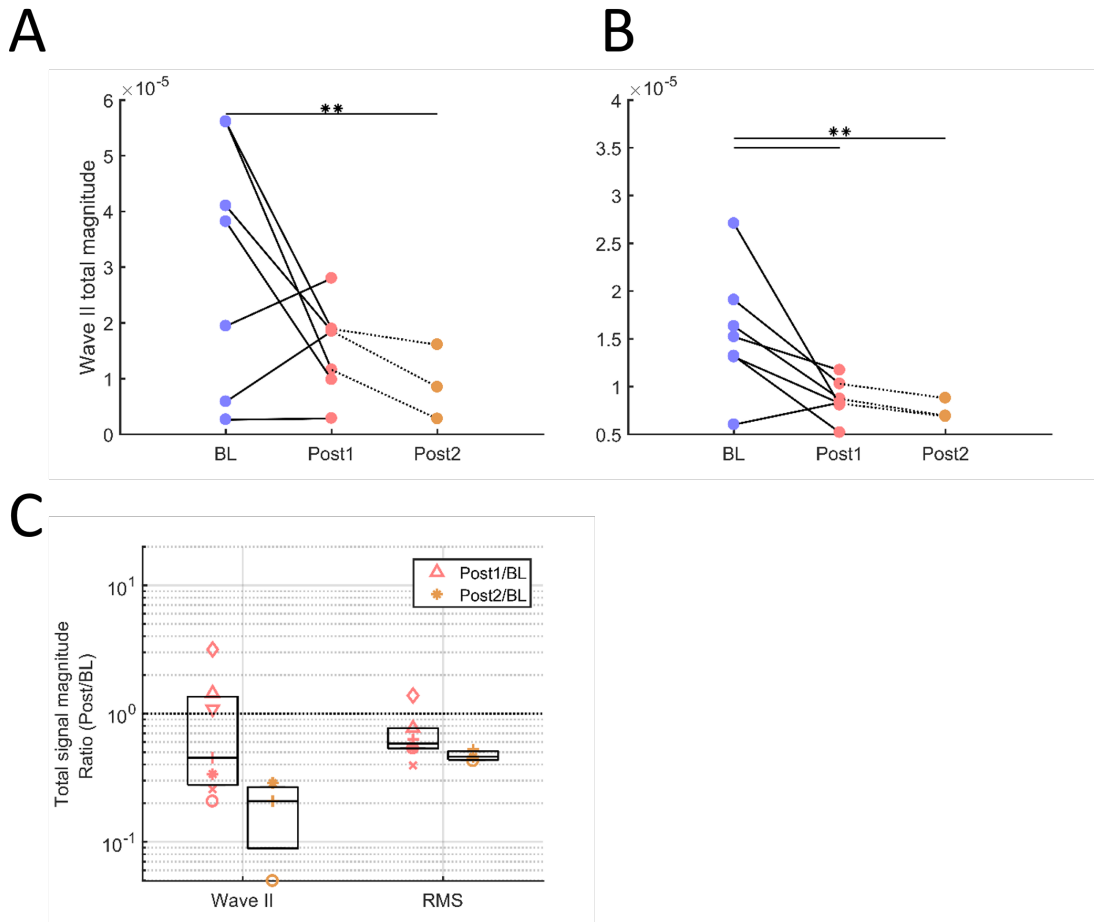


Fig.11: ABR total signal magnitude across intensities (16kHz NBN stimuli): BL vs. Post NOE. Depicted are the total magnitude for **(A)** ABR wave II and **(B)** the RMS across ABR waves I-V for sound stimuli of all levels (40-90dB SPL). Total magnitude is defined as the area under the graph for a linear function as shown in Fig.2D. **(C)** Change of wave II total magnitude and RMS total magnitude from BL to Post1 and Post2. Data points represent individual animals. Data are the ratio of Post1 or Post2 to the baseline measure. A value below 1 (dotted line) depicts a decrease in the respective measure relative to baseline. Panels A,B: Black vertical lines depict $p < 0.05$, markers depict $p < 0.01$ (*) and $p < 0.001$ (**).

Noise overexposure is associated with frequency specific impairments in gap detection ability

Under baseline conditions gap detection performance in ferrets depends on the duration of the silent gap in the presented stimulus, with lowest gap detection ability evident for the shortest gaps and progressively increasing performance for longer gaps (Fig.13A, blue markers, and Chapter 1). Ferrets maintained this modulation of gap detection ability by gap length throughout the experiment (*effect of gap length*, $F_{(7,6752)}=4489.37, p<0.001$), although, as visible by the shift of performance across most gap lengths over time (Fig.13A), gap detection changed after NOE: soon after NOE (Post1) gap detection performance worsened although this was not statistically significant (**Gap stimuli: effect of condition**, $F_{(2,5912)}=25.76, p<0.001$; BL vs Post1, 0.78 ± 0.27 vs 0.74 ± 0.29 , $\beta=-0.05$, $t_{(5914)}=-1.9$, $p=0.06$) (Fig.13B, 'Gap'). However, performance became significantly impaired 6 months after NOE (Fig.13B, **Gap stimuli: BL vs Post2**, 0.78 ± 0.27 vs 0.63 ± 0.34 , $\beta=-0.13$, $t_{(5914)}=-7.1$, $p<0.001$). Interestingly, performance for non-gap stimuli improved progressively after NOE (Fig.3B, 'No Gap', **No-gap stimuli: effect of condition**, $F_{(2,842)}=29.23$, $p<0.001$, BL vs Post1, 0.7 ± 0.15 vs 0.76 ± 0.13 , $\beta=0.064$, $t_{(844)}=2.2$, $p=0.03$; BL vs Post2, 0.7 ± 0.15 vs 0.86 ± 0.09 , $\beta=0.17, t_{(844)}=6.5$, $p<0.001$). This is in line with a decrease in false alarm rate (FA) over time in this paradigm that can be observed independently of noise exposure (see Chapter 1).

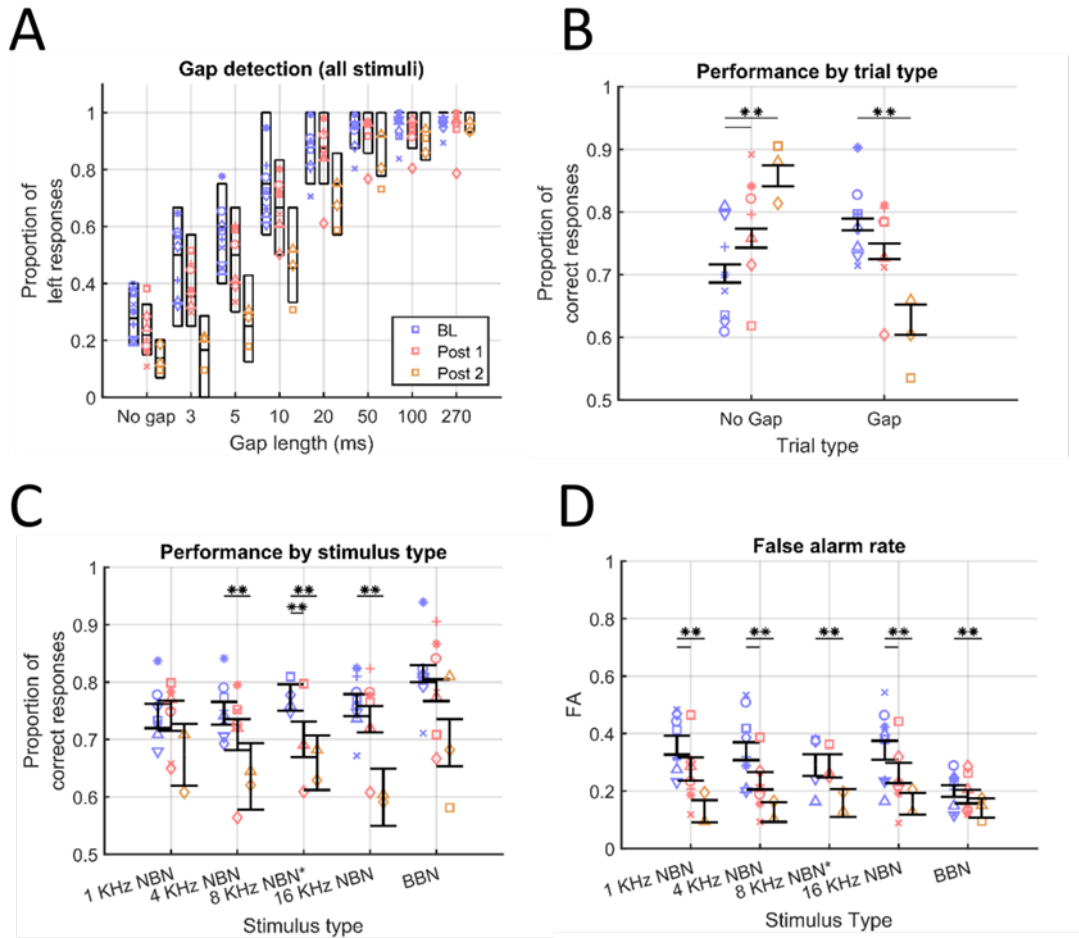


Fig.13: Operant gap-in noise detection in ferrets: BL vs Post NOE. (A) Gap detection performance (left responses) by gap length across time (Baseline, Post NOE 1, Post NOE 2). Left responses are correct responses in gap trials and incorrect responses in non-gap trials. (B) Proportion of correct responses by trial type (no gap and gap) across time. (C) Proportion of correct responses by stimulus type across time. Note that 8kHz NBN stimuli are in the same frequency range as the NOE stimulus. 8kHz NBN stimuli were assessed for 4 animals, of which in the Post2 condition 3 were tested for BBN and 2 for NBN stimuli. (D) False alarm rate by stimulus type across conditions. FA = 1 - proportion of correct responses in non-gap trials. Individual data points are animal averages. Box plots mark the interquartile ranges calculated across all sessions of all animals, vertical lines in box plots depict the median across session averages. Error bars depict the 95% confidence interval calculated across sessions. Panels B-D: Black horizontal lines depict sig. differences of $p < 0.05$, with markers for $p < 0.01$ (*) and for $p < 0.001$ (**).

Yet, after NOE the improvement in FA depended on the stimulus type: specifically, ferrets showed improved (i.e. lower) false alarm rates early after NOE (Post 1, Fig. 13D) for 1, 4 and 16 kHz NBN stimuli (**1 kHz NBN**: effect of condition, $F_{(2,160)}=13940.11$, $p < 0.001$, BL vs Post1, 0.36 ± 0.15 vs 0.27 ± 0.15 , $\beta = -0.1$, $t_{(162)} = -2.66$, $p = 0.01$; **4 kHz NBN**:

$F_{(2,162)}=39.78, p<0.001$, BL vs Post1, 0.34 ± 0.15 vs 0.24 ± 0.12 , $\beta=-0.12, t_{(162)}=-2.84, p=0.01$, **16 kHz NBN**: $F_{(2,167)}=9866610697.7, p<0.001$, BL vs Post1: 0.34 ± 0.16 vs 0.26 ± 0.14 , $\beta=-0.08, t_{(169)}=-8.75, p<0.001$) but not for 8 kHz NBN, the noise overexposure stimulus (effect of condition, $F_{(2,115)}=1465.86, p<0.001$, BL vs Post1: 0.29 ± 0.14 vs 0.29 ± 0.12 , $\beta=0.006, t_{(117)}=0.27, p=0.79$) or BBN (effect of condition, $F_{(2,226)}=22.3, p<0.001$, BL vs Post1: 0.2 ± 0.11 vs 0.18 ± 0.11 , $\beta=-0.027, t_{(228)}=-1.1, p=0.28$). 6 months after NOE, FA improved significantly for the 8 kHz NBN stimulus as well (BL vs Post2: 0.29 ± 0.14 vs 0.16 ± 0.11 , $\beta=-0.08, t_{(117)}=-4.5, p<0.001$) and improved further for all other stimuli (**1 kHz NBN**: BL vs Post2, 0.36 ± 0.15 vs 0.13 ± 0.08 , $\beta=-0.24, t_{(162)}=-14.8, p<0.001$; **4 kHz NBN**: BL vs Post2, 0.34 ± 0.15 vs 0.13 ± 0.07 , $\beta=-0.19, t_{(162)}=-6.48, p<0.001$, **16 kHz NBN**: BL vs Post2: 0.34 ± 0.16 vs 0.16 ± 0.08 , $\beta=-0.19, t_{(169)}=-22.86, p<0.001$; **BBN**: BL vs Post2, 0.2 ± 0.11 vs 0.14 ± 0.09 , $\beta=-0.12, t_{(228)}=-5.83, p<0.001$). Note that proportions of correct responses were based on session averages (percentage of correct responses) for seven different gap types and one non-gap type, respectively. Therefore, the decrease in performance for most gap lengths (Fig.13A) led to a reduction in overall performance (Fig.13C), despite the improvement in detecting non-gap stimuli (i.e. the improvement in false alarm rates). Even though in each testing session the number of gap and non-gap trials was equal to not promote a side-bias of the animals, in order to inform the interpretation of the animals' behaviour, performance from all gap durations was included with even weighing as the performance could change for specific gap durations more so than for others. Therefore, the analysis was based on session means and performance for each gap type (including non-gap trials) contributed equally. Hence the improvement in non-gap trials only is not well reflected in the overall performance. For this reason, non-gap performance was also depicted separately as FA (Fig.13B,D). The

ferrets' performance after NOE did also depend on the stimulus type and was initially impaired only for stimuli of 8 kHz NBN (Fig.13C, **8 kHz NBN: effect of condition**, $F_{(2,941)}=32.31$, $p<0.001$, BL vs Post1, 0.77 ± 0.26 vs 0.7 ± 0.27 , $\beta=-0.1$, $t_{(943)}=-8.03$, $p<0.001$) while performance for other stimulus types remained stable (**1 kHz NBN, effect of condition**, $F_{(2,1301)}=2.17$, $p=0.12$, BL vs Post1: 0.74 ± 0.28 vs 0.74 ± 0.29 , $\beta=-0.001$, $t_{(1303)}=-0.02$, $p=0.98$; **4 kHz NBN**: $F_{(2,1317)}=6.74$, $p=0.001$, BL vs Post1, 0.75 ± 0.27 vs 0.71 ± 0.3 , $\beta=-0.022$, $t_{(1319)}=-1.05$, $p=0.29$; **16 kHz NBN**: $F_{(2,1357)}=69.498$, $p<0.001$, BL vs Post1, 0.76 ± 0.26 vs 0.74 ± 0.26 , $\beta=-0.03$, $t_{(1359)}=-1.18$, $p=0.24$; **BBN**: $F_{(2,1829)}=0.73$, $p=0.48$, BL vs Post1, 0.82 ± 0.23 vs 0.69 ± 0.32 , $\beta=-0.02$, $t_{(1831)}=-0.66$, $p=0.51$). This indicates that for 8 kHz NBN animals developed a deficit in gap detection ability without any improvement in detecting non-gap stimuli of the same type, which is in line with the observed hearing impairment in the range of the NOE stimulus. This impairment was maintained 6 months after NOE (Fig.13C, **8 kHz NBN: BL vs Post2**, 0.77 ± 0.26 vs 0.66 ± 0.31 , $\beta=-0.1$, $t_{(943)}=-8.03$, $p<0.001$) and performance dropped as well for NBN stimuli with adjacent frequency ranges relative to the 8kHz NBN (**4 kHz NBN: BL vs Post2**, 0.75 ± 0.27 vs 0.64 ± 0.35 , $\beta=-0.04$, $t_{(1319)}=-3.35$, $p<0.001$; **16 kHz NBN: BL vs Post2**, 0.76 ± 0.26 vs 0.6 ± 0.31 , $\beta=-0.14$, $t_{(1359)}=-11.79$, $p<0.001$). The stimuli arguably most different from the NOE stimulus in terms of frequency composition (1kHz NBN and BBN) were associated with stable performance throughout the entire experiment (**1 kHz NBN: BL vs Post2**: 0.74 ± 0.28 vs 0.67 ± 0.34 , $\beta=-0.03$, $t_{(1303)}=-0.94$, $p=0.35$; **BBN: BL vs Post2**, 0.82 ± 0.23 vs 0.79 ± 0.24 , $\beta=-0.05$, $t_{(1831)}=-1.19$, $p=0.23$). It is interesting to note, therefore, that the most affected stimuli (4 kHz NBN, 8 kHz NBN and 16 kHz NBN) showed differing dynamics after NOE: while the 8 kHz stimulus was associated with immediate impairment in gap detection and no change in FA after NOE, the reverse was true for 4 and 16 kHz NBN as animals

did show improvement in false alarm rate after NOE but developed gap detection impairment only late. FA did not seem to be tied to the animals' gap detection ability (it could stay stable or improve at the same time as gap detection ability decreased), which raises the question on whether more than one mechanism (such as hearing loss) is underlying the observed dynamics in performance.

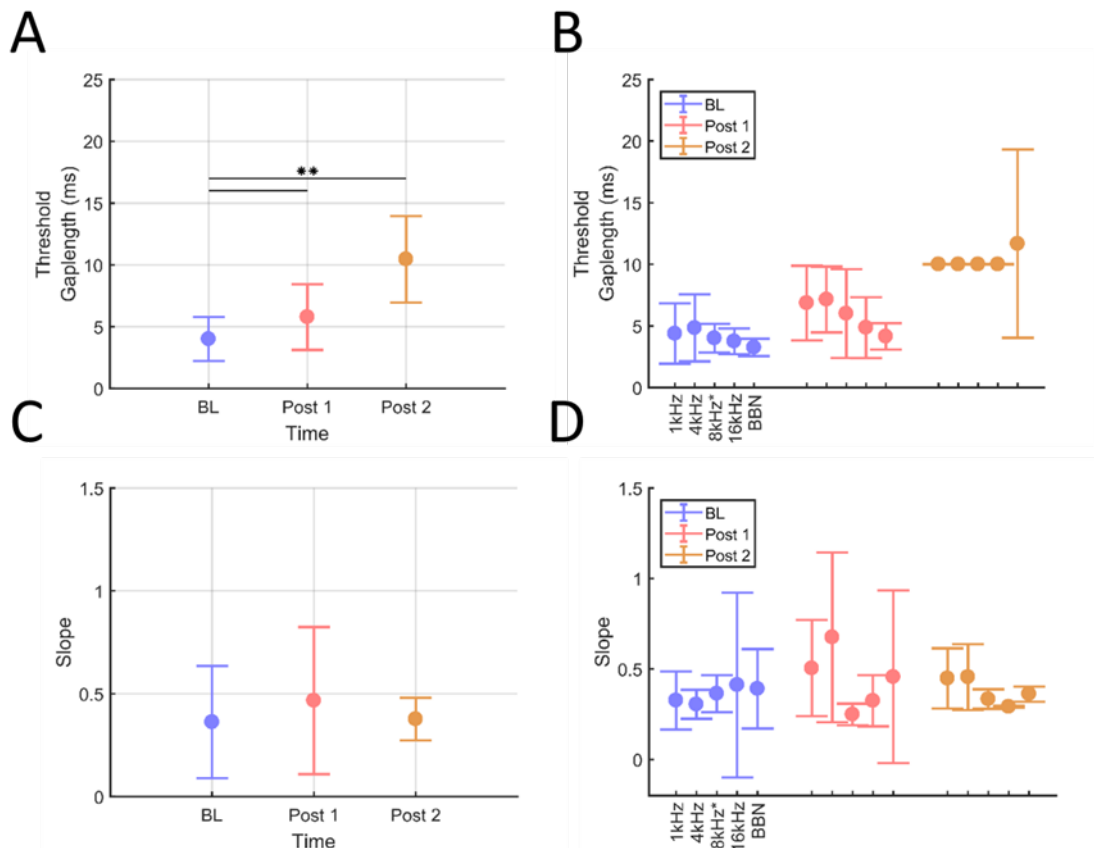


Fig.14: Gap detection thresholds and sensitivity (slope): BL vs Post NOE. (A) Average threshold (defined as gap length closest to 50% hit rate) for baseline, first assessment after NOE (Post1) and second assessment (Post 2). (B) Threshold as depicted in A but separated by stimulus type. (C) Slope of the psychometric function at threshold. (D) Slope as depicted in C but separated by stimulus type. Error bars are standard deviations across animals, central markers are animal averages. Panels A,C: Black horizontal lines depict sig. differences of $p < 0.05$ and $p < 0.001$ (**).

Next, to assess how the observed change in performance affected gap detection thresholds, a sigmoidal function was fitted on plots of percent correct over gap length and detection thresholds were estimated (defined as gap length corresponding to 50%

hit rate on a fitted sigmoid). There was a significant effect of condition on thresholds (*effect of condition*, $F_{(2,73)}=119.13$, $p<0.001$) and thresholds increased significantly already in the first assessment after NOE (Fig.14A, BL vs Post1, $4.0\pm1.78ms$ vs $5.77\pm2.68ms$, $B=1.69$, $t_{(75)}=2.41$, $p=0.018$) – although with marked variability between stimuli (Fig.14B). Thresholds increased even further 6 months after NOE (BL vs Post2, $4.0\pm1.78ms$ vs $10.45\pm3.5ms$, $B=5.95$, $t_{(75)}=11.68$, $p<0.001$, Fig.14A). The gap detection sensitivity (defined as the slope of the psychometric function at threshold) remained stable throughout the experiment (Fig.4C,D, BL vs Post1, 0.36 ± 0.27 vs 0.47 ± 0.35 , $p=0.15$, BL vs Post2, 0.36 ± 0.27 vs 0.38 ± 0.1 , $p=0.64$, Wilcoxon rank sum test). This suggests that the psychometric function shifted after NOE but largely retained its shape (see also Supp.Fig.1), indicating that performance was affected across a wide range of gap durations.

Response times increase after NOE

The impaired gap detection ability, especially evident for NBN stimuli covering a similar or adjacent frequency ranges as the NOE stimulus leaves open whether animals developed the impairment because of a form of hearing disability or due to a phantom percept. A further indication towards this could be given by assessing the animals' certainty in deciding whether they perceived a gap or a non-gap stimulus. In gap trials, a constant phantom percept may lead to high certainty for perceiving a non-gap stimulus instead. As a proxy measure for the animals' certainty, response times (between trial initiation and response) were assessed for all tested stimuli.

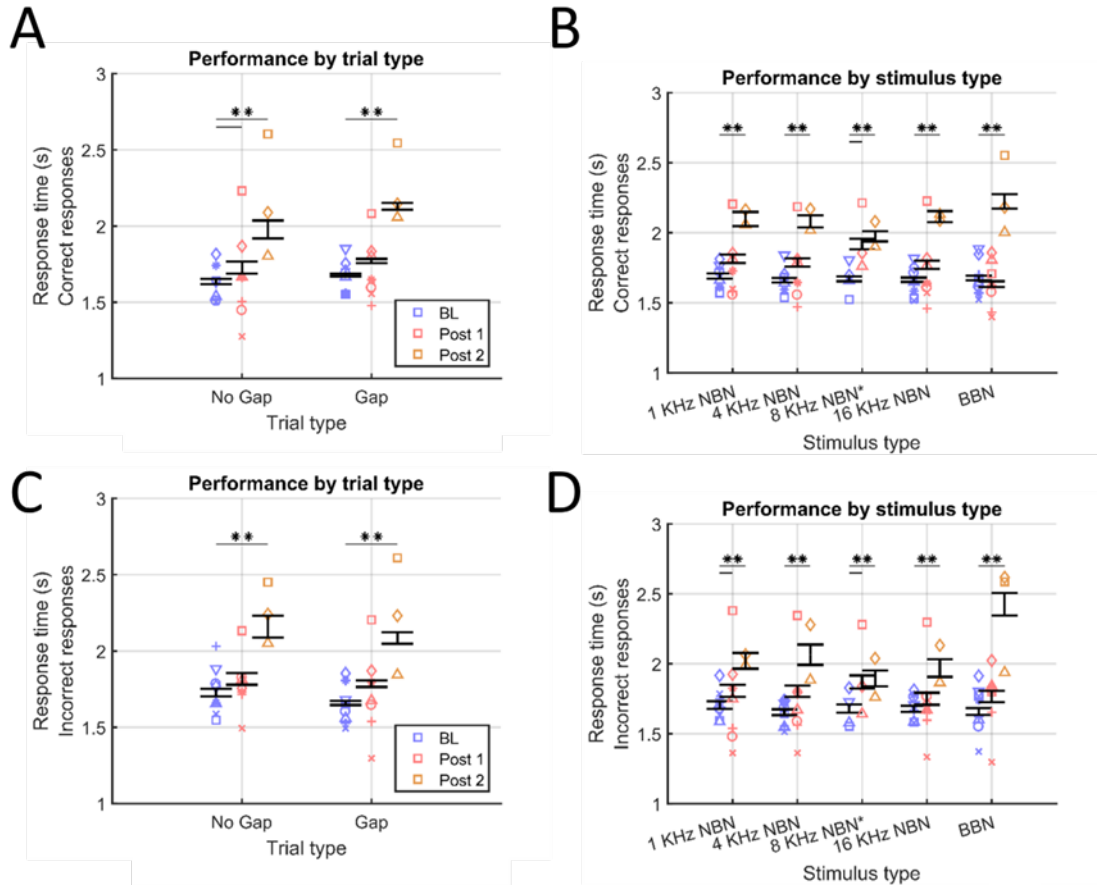


Fig.15: Operant gap-in noise detection: response times BL vs Post NOE. (A) Average response times in correct trials separated by gap type (continuous stimuli and gap stimuli), (B) separated by stimulus type (NBN stimuli and BBN) and (C) average response times in incorrect trials separated by gap type (continuous stimuli and gap stimuli) and (D) separated by stimulus type (NBN stimuli and BBN). Coloured markers are animal averages. Error bars depict the 95% confidence interval calculated across sessions. Black horizontal lines depict sig. differences of $p < 0.05$, with markers for $p < 0.01$ (*) and for $p < 0.001$ (**).

Response times changed significantly after NOE independent of the outcome of an animal's decision (Fig.15A,C). Response times showed a trend to increase already in the first assessment after NOE, yet this change was only significant for correct non-gap trials (Correct trials, **No gap**: effect of condition, $F_{(2,842)}=263548.24, p < 0.001$, BL vs Post1, $1.64 \pm 0.18s$ vs $1.73 \pm 0.34s$, $\beta=0.024, t_{(844)}=2.78, p=0.01$; **Gap**: $F_{(2,5719)}=129.97, p < 0.001$, BL vs Post1, $1.68 \pm 0.24s$ vs $1.77 \pm 0.32s$, $\beta=0.03, t_{(5721)}=1.8, p=0.07$; Incorrect trials, **No gap**: $F_{(2,828)}=105.34, p < 0.001$, BL vs Post1, $1.73 \pm 0.26s$ vs $1.81 \pm 0.33s$, $\beta=0.03, t_{(830)}=1.34,$

$p=0.18$; **Gap:** $F_{(2,3330)}=86.47$, $p<0.001$, BL vs Post1, $1.73\pm0.26s$ vs $1.82\pm0.33s$, $\beta=0.02$, $t_{(3332)}=1.26$, $p=0.21$). 6 months after NOE response times increased further and were significantly different from BL across all trials (Fig.15A,C) (*Correct trials, No gap:* BL vs Post2, $1.64\pm0.18s$ vs $1.98\pm0.31s$, $\beta=0.07$, $t_{(844)}=8.38$, $p<0.001$; **Gap:** BL vs Post2, $1.68\pm0.24s$ vs $2.13\pm0.32s$, $\beta=0.1$, $t_{(5721)}=7.81$, $p<0.001$; *Incorrect trials, No gap:* BL vs Post2, $1.73\pm0.26s$ vs $2.16\pm0.37s$, $\beta=0.1$, $t_{(830)}=8.15$, $p<0.001$; **Gap:** BL vs Post2, $1.66\pm0.37s$ vs $2.08\pm0.53s$, $\beta=0.08$, $t_{(3332)}=5.22$, $p<0.001$).

The prolongation of response times after NOE did not equally apply to all stimulus types, but in the first assessment after NOE (Post1) mostly to 8 kHz NBN, notably the stimulus associated with significant impairment in gap detection ability in the Post1 condition (Fig.13C). For the 8 kHz NBN stimulus animals showed significantly prolonged response times in all trials (Fig. 15C,D, *Correct trials, 8 kHz NBN: effect of condition*, $F_{(2,912)}=65.57$, $p<0.001$, BL vs Post1, $1.67\pm0.19s$ vs $1.92\pm0.32s$, $\beta=0.075$, $t_{(914)}=2.94$, $p=0.01$, *Incorrect trials*, $F_{(2,611)}=1585.56$, $p<0.001$, BL vs Post1, $1.68\pm0.31s$ vs $1.87\pm0.41s$, $\beta=0.05$, $t_{(613)}=2.14$, $p=0.03$). For other stimuli response times only increased in incorrect trials for 1 kHz NBN stimuli (Fig.15D, *Incorrect trials: 1 kHz NBN, effect of condition*, $F_{(2,784)}=72.22$, $p<0.001$, BL vs Post1, $1.71\pm0.37s$ vs $1.81\pm0.47s$, $\beta=0.04$, $t_{(786)}=2.29$, $p=0.02$). In incorrect trials of other NBN stimuli response times remained unchanged in Post1 (Fig.5D, *Incorrect trials: 4 kHz NBN, effect of condition*, $F_{(2,833)}=160.17$, $p<0.001$, BL vs Post1, $1.65\pm0.29s$ vs $1.80\pm0.46s$, $\beta=0.03$, $t_{(835)}=1.28$, $p=0.2$, **16 kHz NBN**, $F_{(2,882)}=764.36$, $p<0.001$, BL vs Post1, $1.68\pm0.31s$ vs $1.75\pm0.49s$, $\beta=0.02$, $t_{(884)}=0.95$, $p=0.34$) and, similarly, in correct trials (Fig.15B, *Correct trials: 1 kHz NBN*, $F_{(2,1249)}=42.93$, $p<0.001$, BL vs Post1, $1.69\pm0.25s$ vs $1.81\pm0.34s$, $\beta=0.04$, $t_{(1252)}=1.7$, $p=0.09$, **4 kHz NBN**, $F_{(2,1268)}=73.4$, $p<0.001$, BL vs Post1, $1.66\pm0.23s$ vs $1.79\pm0.32s$, $\beta=0.04$, $t_{(1270)}=1.87$, $p=0.06$, **16 kHz NBN**,

$F_{(2,1322)}=143.03$, $p<0.001$, BL vs Post1, $1.66\pm0.21s$ vs $1.77\pm0.32s$, $\beta=0.04$, $t_{(1324)}=1.57$, $p=0.12$). In BBN trials response times remained stable as well (Fig.5B,D, *Correct trials*, **BBN**, *effect of condition*, $F_{(2,1801)}=10.0$, $p<0.001$, BL vs Post1, $1.68\pm0.26s$ vs $1.63\pm0.26s$, $\beta=-0.002$, $t_{(1803)}=-0.23$, $p=0.82$, *Incorrect trials*, $F_{(2,1039)}=129.66$, $p<0.001$, BL vs Post1, $1.66\pm0.41s$ vs $1.77\pm0.52s$, $\beta=0.02$, $t_{(1041)}=1.85$, $p=0.06$). This suggests that a possible effect of NOE on how quickly animals responded applied initially on the NOE stimulus (8 kHz NBN) but did not generally affect other stimuli.

6 months after NOE response times were significantly longer for all stimulus types and across all trials (Fig.15B,D, *Correct trials*, **1 kHz NBN**, BL vs Post2, $1.69\pm0.25s$ vs $2.1\pm0.33s$, $\beta=0.09$, $t_{(1251)}=6.9$, $p<0.001$, **4 kHz NBN**, BL vs Post2, $1.66\pm0.23s$ vs $2.01\pm0.26s$, $\beta=0.1$, $t_{(1270)}=9.27$, $p<0.001$, **8 kHz NBN**, BL vs Post2, $1.67\pm0.19s$ vs $1.98\pm0.24s$, $\beta=0.09$, $t_{(914)}=7.34$, $p<0.001$, **16 kHz NBN**, BL vs Post2, $1.66\pm0.21s$ vs $2.12\pm0.25s$, $\beta=0.1$, $t_{(1324)}=9.35$, $p<0.001$, **BBN**, BL vs Post2, $1.68\pm0.26s$ vs $2.22\pm0.4s$, $\beta=0.1$, $t_{(1803)}=4.46$, $p<0.001$; *Incorrect trials*, **1 kHz NBN**: BL vs Post2, $1.71\pm0.37s$ vs $2.02\pm0.36s$, $\beta=0.08$, $t_{(786)}=8.89$, $p<0.001$, **4 kHz NBN**, BL vs Post2, $1.65\pm0.29s$ vs $2.06\pm0.44s$, $\beta=0.1$, $t_{(786)}=9.09$, $p<0.001$, **8 kHz NBN**, BL vs Post2, $1.68\pm0.31s$ vs $1.89\pm0.37s$, $\beta=0.06$, $t_{(613)}=4.98$, $p<0.001$, **16 kHz NBN**, BL vs Post2, $1.68\pm0.21s$ vs $1.97\pm0.39s$, $\beta=0.07$, $t_{(884)}=9.45$, $p<0.001$, **BBN**, BL vs Post2, $1.66\pm0.41s$ vs $2.43\pm0.63s$, $\beta=0.12$, $t_{(884)}=12.63$, $p<0.001$).

To assess whether the trial outcome affected response times differently after NOE, correct and incorrect trials were compared. For gap trials, response times between correct and incorrect trials were similar before NOE (Fig.16A, *Gap trials BL: correct vs incorrect*, 1.68 ± 0.21 vs 1.66 ± 0.25 , $F_{(1,875)}=1.91$, $p=0.17$) but were longer in incorrect trials in the first assessment after NOE (**Post 1: correct vs incorrect**, 1.77 ± 0.26 vs 1.79 ± 0.43 ,

$F_{(1,586)}=20.1$, $p<0.001$) but shorter 6 month after NOE (**Post 2: correct vs incorrect**, 2.13 ± 0.23 vs 2.06 ± 0.37 , $F_{(1,212)}=9801.04$, $p<0.001$), although inter animal variability was high in the last assessment (Fig.16A). For non-gap trials, animals showed longer response times in incorrect than in correct trials throughout the experiment (Fig.16B, *Non-gap trials*, **BL: correct vs incorrect**, $1.64\pm0.18s$ vs $1.73\pm0.26s$, $F_{(1,884)}=571.14$, $p<0.001$; **Post 1: correct vs incorrect**, $1.73\pm0.35s$ vs $1.82\pm0.33s$, $F_{(1,577)}=477.15$, $p<0.001$; **Post 2: correct vs incorrect**, $1.98\pm0.31s$ vs $2.16\pm0.37s$, $F_{(1,209)}=737178.93$, $p<0.001$), even though after NOE ferrets tended to respond faster when they triggered then non-gap sensor ('R' in Fig.16C)(**BL: L vs R**, $1.7\pm0.22s$ vs $1.65\pm0.22s$, $F_{(1,1761)}=1.26$, $p=0.26$; **Post 1: L vs R**, $1.79\pm0.3s$ vs $1.76\pm0.39s$, $F_{(1,1165)}=350.57$, $p<0.001$; **Post 2: L vs R**, $2.15\pm0.31s$ vs $2.02\pm0.34s$, $F_{(1,423)}=66031.54$, $p<0.001$) and generally improved their performance in detecting non-gap stimuli during the course of the experiment (Fig.13B).

In summary, early after NOE performance was most impaired for the NOE stimulus (8 kHz NBN), including gap detection ability and delayed response times. In the long term, also the most similar NBN stimuli used in adjacent frequency ranges (1 and 16 kHz) were associated with reduced gap detection ability. Even though this drop in performance did not affect 1kHz NBN and BBN stimuli, animals showed markedly increased response times across all stimulus types. It must be noted, that response times in this paradigm can be affected by many factors, such as different head positions at trial initiation or varying walking trajectories of the animals when approaching a target sensor (see also Chapter 1). Yet, in combination with the generally impaired performance in this paradigm, the observed prolongation of response times may nevertheless indicate a general uncertainty of the animals due to the increased proportion of failure and therefore generally raised caution.

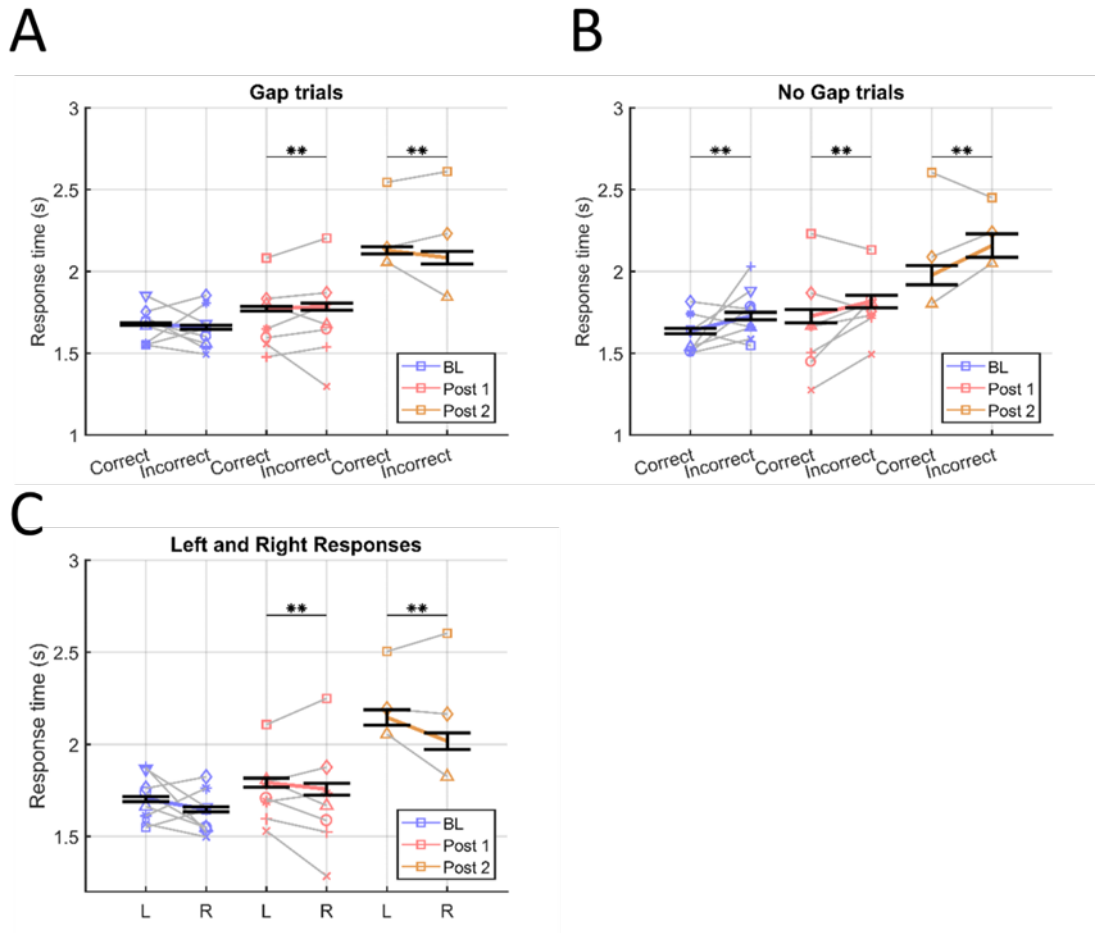


Fig.16: Operant gap-in noise detection: response times by trial outcome and sensor in BL, Post1 and Post2 conditions. (A) Response times in gap trials and **(B)** in non-gap trials depicted by trial outcome (correct vs incorrect response) during BL and Post NOE. **(C)** Response times for responses at the left vs the right sensor for BL and Post NOE. Error bars depict the 95% confidence interval calculated across sessions, coloured lines connect averages. Markers are animal averages. Black horizontal lines signify $p < 0.001$ (**).

The impaired gap detection ability for 8 kHz NBN stimuli and, later 4, 8 and 16 kHz NBN stimuli may be due to hearing loss in a wide range of frequencies around the NOE frequency (affecting 4, 8 and 16 kHz NBN stimuli) or, possibly, due to the animals experiencing tinnitus in one or more of those frequency ranges. Indicative of tinnitus is the improvement in detecting non-gap stimuli while gap detection was impaired. This could suggest a tendency of the animals to perceive stimuli as non-gap sounds irrespective of whether they did contain a silent gap. This is striking, since in baseline

conditions (before noise overexposure), most animals showed a tendency to detect gap sounds more so than non-gap sounds (Fig.13B, BL, blue markers). Interestingly, for 8 kHz, initially after NOE only gap detection performance was impaired while FA remained stable, which is in line with the observed initial ABR threshold elevation after NOE, whereas in the long term, the bias towards detecting non gap sounds is indicative of tinnitus.

It is worth noting here, that false alarm rates in this paradigm tend to improve over prolonged periods of testing even under baseline conditions (see Chapter 1). Yet, importantly, decreasing false alarm rates in parallel to progressively impaired gap detection is not in line with an improving learning effect in the paradigm. Rather, it suggests that animals developed a perceptual bias in favour of continuous sounds irrespective of trial outcome. To test whether this bias was indeed due to a phantom sound (tinnitus) or due to a lack in temporal resolution of processing short silent gaps, the animals' ability to detect relatively long periods of silence was assessed in a variant of operant silence detection (below).

Operant silence detection indicates perceptual ambiguity between silence and NBN stimulation after noise overexposure

In this paradigm, animals had to discriminate between an amplitude modulated (AM) sound, an unmodulated NBN stimulus (frequency bands centred around 1,4,8 and 16 kHz), and the absence of a sound stimulus in so called 'silent trials' (Fig.17A, see Methods for details). Ferrets showed similar performance for detecting NBN stimuli after NOE as compared to baseline (**NBN**: $F_{(2,14)}=2.53, p=0.12$, BL vs Post1, 0.97 ± 0.04 vs 0.97 ± 0.02 ,

$\beta=0.01, t_{(16)}=0.32, p=0.76$, BL vs Post2, 0.97 ± 0.04 vs 0.91 ± 0.1 , $\beta=-0.03$, $t_{(16)}=-2.19$, $p=0.05$) and showed a minimal decrease in performance (1% decrease) to detect AM sounds 6 months after NOE (**AM**: $F_{(2,14)}=6.57$, $p=0.01$, BL vs Post1, 0.99 ± 0.01 vs 0.99 ± 0.01 , $\beta=-0.001$, $t_{(16)}=-0.19$, $p=0.86$, BL vs Post2, 0.99 ± 0.01 vs 0.98 ± 0.01 , $\beta=0.004$, $t_{(16)}=2.39$, $p=0.03$). In silence trials ferrets showed impaired performance after NOE (Fig.17B) although that difference was not statistically significant (**Silence: effect of time**, $F_{(2,14)}=2.44$, $p=0.12$, BL vs Post1, 0.91 ± 0.07 vs 0.8 ± 0.83 , $\beta=-0.17$, $t_{(16)}=-2.15$, $p=0.05$, BL vs Post2, 0.91 ± 0.07 vs 0.83 ± 0.06 , $\beta=-0.06$, $t_{(16)}=-0.87$, $p=0.4$). Ferrets did, however, show longer response times in 'correct' silence trials (Fig.17C) initially after NOE, possibly suggesting increased uncertainty about the absence of a stimulus (**Silence**: $F_{(2,14)}=6.27$, $p=0.01$, BL vs Post1, $3.16\pm0.54s$ vs $3.95\pm0.95s$, $\beta=0.1$, $t_{(16)}=3.08$, $p=0.01$) although 6 month after NOE response times were again similar to BL conditions (**Silence: BL vs Post2**, $3.16\pm0.54s$ vs $3.18\pm0.06s$, $\beta=0.03$, $t_{(16)}=1.79$, $p=0.1$). In incorrect silence trials, response times did not significantly change throughout the experiment (Fig.17D, **Silence: effect of time**, $F_{(2,14)} = 0.64$, $p=0.54$, BL vs Post1, $4.22\pm1.98s$ vs $3.73\pm0.91s$, $\beta=-0.036$, $t_{(16)}=-0.42$, $p=0.68$, BL vs Post2, $4.22\pm1.98s$ vs $3.58\pm0.01s$, $\beta=-0.07$, $t_{(16)}=0.93$, $p=0.37$). Interestingly, response times in trials with a

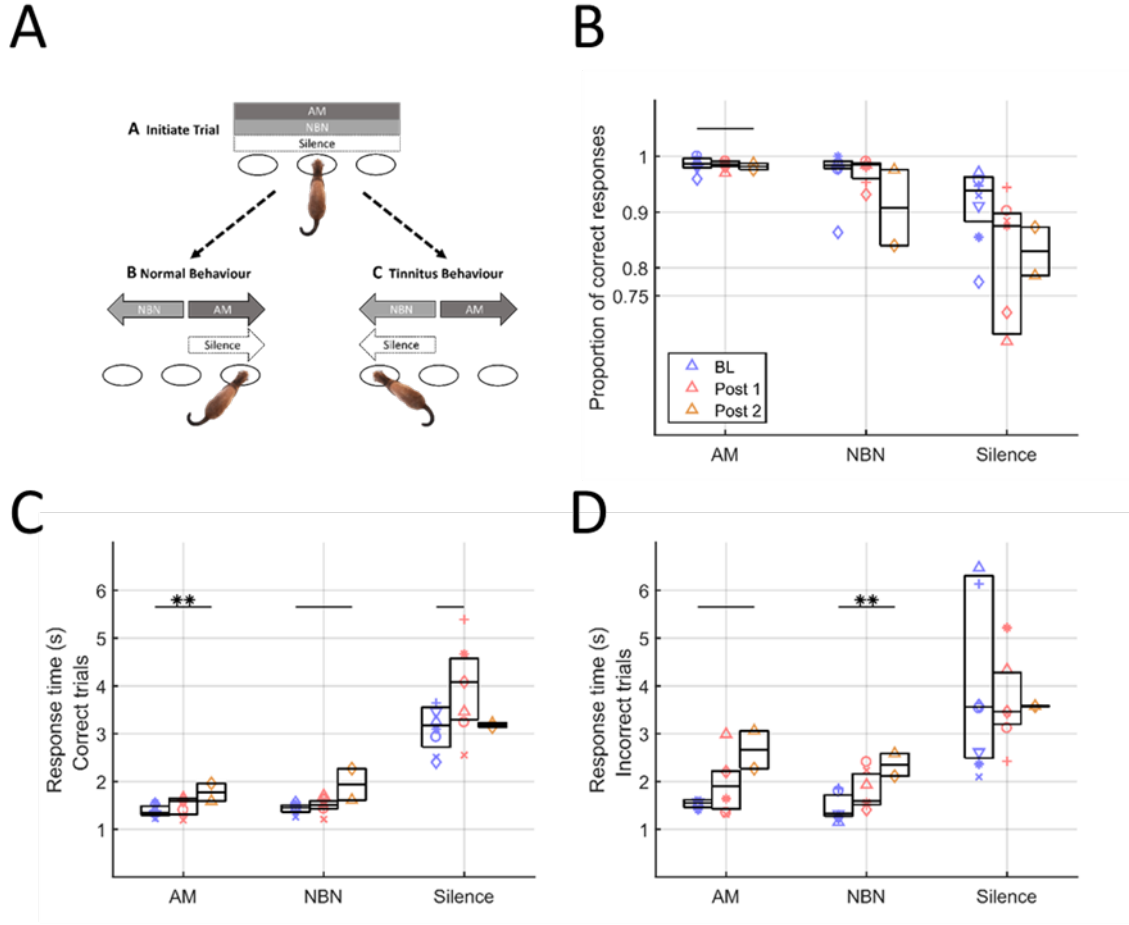


Fig.17: Behavioural silence detection: BL vs Post NOE. (A) The behavioural paradigm. (B) Percent correct in NBN, BBN and silence trials. (C) Reaction times in NBN, BBN and Silence trials for correct trials and (D) for incorrect trials. Individual data points are animal averages. Box plots mark the interquartile ranges calculated across all sessions of all animals, vertical lines in box plots depict the median across session averages. Horizontal lines depict sig. differences of $p < 0.05$ with markers depicting $p < 0.001$ (**).

sound stimulus, while stable initially after NOE, were significantly longer 6 month after NOE in correct trials (Fig.10c, *Correct trials*, **AM**: effect of time, $F_{(2,14)}=41.2$, $p < 0.001$, BL vs Post1, $1.38 \pm 0.12s$ vs $1.5 \pm 0.21s$, $\beta=0.03$, $t_{(16)}=2.2$, $p=0.05$, BL vs Post2, $1.38 \pm 0.12s$ vs 1.77 ± 0.26 , $\beta=0.09$, $t_{(16)}=6.61$, $p < 0.001$, **NBN**: $F_{(2,14)}=4.7$, $p=0.03$, BL vs Post1, $1.43 \pm 0.1s$ vs $1.49 \pm 0.16s$, $\beta=0.02$, $t_{(16)}=1.21$, $p=0.25$, BL vs Post2, $1.43 \pm 0.1s$ vs $1.94 \pm 0.47s$, $\beta=0.1$, $t_{(16)}=2.23$, $p=0.04$), and in incorrect trials (Fig.17D, *Incorrect trials*, **AM**: effect of time $F_{(2,12)}=13.95$, $p < 0.001$, BL vs post 1, $1.73 \pm 0.52s$ vs $1.95 \pm 0.6s$, $\beta=0.06$, $t_{(14)}=0.88$, $p=0.4$, BL vs Post2, $1.73 \pm 0.53s$ vs $2.66 \pm 0.56s$, $\beta=0.18$, $t_{(14)}=3.91$, $p=0.01$, **NBN**: $F_{(2,13)}=132.8$,

$p < 0.001$, BL vs Post1, $1.46 \pm 0.27s$ vs $1.81 \pm 0.39s$, $\beta = 0.09$, $t_{(15)} = 2.1$, $p = 0.05$, BL vs Post2, $1.46 \pm 0.27s$ vs $2.35 \pm 0.33s$, $\beta = 0.26$, $t_{(15)} = 8.7$, $p < 0.001$,). The increased tendency of animals to not detect silence after NOE – although not significant – may in combination with generally increased response times in trials where a sound stimulus was present indicate increased uncertainty about the animals' decision of whether to indicate presence or absence of a sound in their choice. It seems less likely that animals had difficulty discriminating between the sound stimuli (AM and NBN) as performance was not affected for NBN trials and only minimally so for AM trials. While this may not be unequivocal evidence for tinnitus, it is in line with that possibility.

Discussion

The immediate effect of noise insults has been widely described in animal models (Bauer et al., 2008; Engineer et al., 2011; Kaltenbach et al., 2004; J. Turner et al., 2012). In this study, noise overexposure had lasting effects on both central auditory brainstem processing and on behaviour-dependent auditory function.

Auditory brainstem responses were immediately after NOE impaired for 8 kHz NBN stimuli, and higher frequency stimuli (16 kHz NBN). Responses to these stimuli were reduced long term, visible for high-intensity stimulation but also across tested sound levels and indicating a general reduction in evoked activity. Six months after noise overexposure thresholds were significantly elevated in a wide range of frequencies, which suggests that animals developed lasting hearing impairment.

Impairments over time after noise exposure were also visible in the tested behavioural paradigms, most prominently in operant gap detection. Here, as in the ABRs, the impairment was most pronounced for 8 kHz NBN stimuli but, eventually, performance also decreased for stimuli in adjacent frequency ranges to the NOE stimulus (4 and 16 kHz NBN) – the same sound frequencies that were most affected by long-term hearing impairment. Note that while ABR thresholds were higher than the level of sound stimuli in the operant gap detection paradigm (76 ± 5 dB SPL) for most stimuli 6 months following NOE, animals still performed reasonably well (Fig.13). Animals did likely not suffer from bilateral hearing loss and could partially compensate for unilaterally reduced hearing ability when performing in the task. Remarkably, performance in detecting non-gap stimuli increased in parallel to deteriorating gap detection, suggesting an overall tendency towards detecting continuous sounds.

It is of note that a control animal never exposed to noise also showed a progressively improving false alarm rate, even after 150 testing sessions (Chapter 1, Suppl.Fig.2), yet without a progressive impairment in gap detection ability. Therefore, it is unlikely that the animals' degradation in gap detection ability is linked to the prolonged testing period of the experiment but may rather be due to noise-induced hearing loss or tinnitus. The animals' non-gap detection ability improved after NOE despite hearing and gap-detection impairment, which suggests that animals did not improve performance in this paradigm in general but rather developed a tendency to report non-gap stimuli independent of the present trial.

Such a tendency was further corroborated by performance in the silence detection paradigm. Here, most of the animals (5 out of 7) showed a decrease in silence detection

ability after NOE, even though the effect was overall not significant. Yet, the increased response times of animal in trials where a sound was present – even though animals' performance in detecting NBN and AM sounds correctly was largely stable over time – suggests a general uncertainty about whether a sound was indeed present. This can be seen as an indicator for the animals' perceptual ambiguity between the absence of external stimulation and the presence of an NBN-like stimulus (Stolzberg et al., 2013). In combination with the trend towards decreased performance in the silence trials, this is in line with the hypothesis that animals developed a positive symptom like tinnitus.

Impairments in operant gap detection for sound frequencies adjacent to the NOE frequency (4 and 16 kHz NBN), as well as the significant increase in response times in the silence detection paradigm both appeared only 6 months after NOE and may therefore be based on a common mechanism taking place on a longer time scale than acute hearing loss. It is likely that animals suffered cochlear damage in a NOE-specific frequency range around 8 kHz, as indicated by the impaired ABR responses and elevated thresholds for 8 kHz stimuli from the first assessment one week after NOE and onwards, which would also explain the immediate drop in operant gap detection performance for 8 kHz NBN after NOE. Yet, the effects for stimuli in adjacent frequency ranges are, due to their late onset and since they cover a more narrow frequency range than the long-term threshold elevation, possibly not explained by hearing loss alone.

It is well described that tinnitus can develop after noise overexposure (Baguley et al., 2013b) but is possibly consolidated only after the completion of neural plasticity in response to the original insult (Shore & Wu, 2019), which may be an important factor in late onset or late intensification of tinnitus relative to the auditory insult. Further,

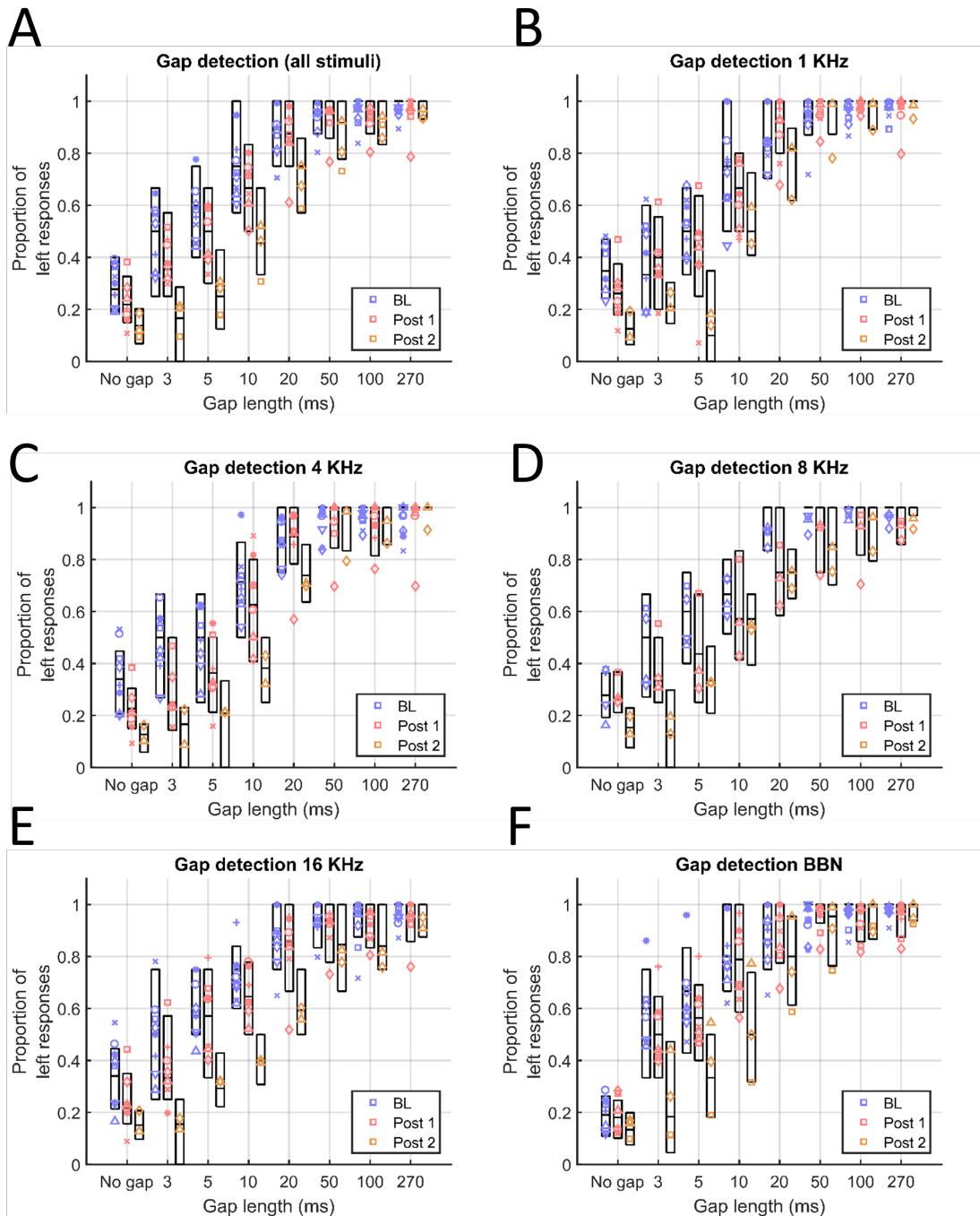
degeneration of spiral ganglion cells can often occur weeks or months after the original noise insult, prompting the consequences of deafferentation (such as tinnitus) much later than hearing loss due to damaged hair cells becomes evident (Liberman & Kujawa, 2017).

Previous work suggests that tinnitus can be the consequence of cortical map plasticity, possibly involving the rearrangement of tonotopic representations contributors to tinnitus development (Engineer et al., 2011). Reduced lateral inhibition after frequency specific deafferentation and a change in frequency tuning can lead to increased synchronous gain (Chawla et al., 1999), which is often considered a functional tinnitus correlate. This may explain why tinnitus may be perceived not directly in the frequency range of the hearing loss but in frequencies at the edges of the affected spectrum. It has been described that noise-induced hearing loss leads to plasticity in adjacent cortical frequency representations relative to the noise (Eggermont & Komiya, 2000), and, in addition that preventing tonotopic reorganisation mitigates tinnitus (Noreña & Eggermont, 2006a). That said, tinnitus can be perceived within the frequency spectrum of the hearing loss (König et al., 2006) and it remains to be shown to what degree tonotopic reorganisation is mediating tinnitus formation (Koops et al., 2020). Animals in this study developed hearing impairments in a broad range of frequencies in and adjacent to the NOE frequency range. Hearing loss was less immediate and less pronounced in some adjacent frequencies, which could warrant an imbalance in cortical tonotopic representation.

Overall, the results in this study cannot ultimately confirm or rule out whether animals developed tinnitus after noise overexposure. Yet, the behavioural changes, especially in

the tendency to detect non-gap sounds as well as the growing uncertainty in the silence detection paradigm are in line with the possibility of latent effects beyond noise-induced hearing loss.

Supplementary figures and tables



Supp. Fig.1: Operant gap-in noise detection: proportion of left responses by gap length for BL and Post NOE conditions. (A) Gap detection performance (left responses) by gap length across time (Baseline, Post NOE 1, Post NOE 2). Left responses are correct responses in gap trials and incorrect responses in non-gap trials. **(B-F)** Behavioural performance as shown in panel A, but separated by stimulus type (1kHz NBN, 4kHz NBN, 8kHz NBN, 16kHz NBN, BBN) during baseline testing (blue markers), Post 1 (red) and Post2 (orange). Individual data points are animal averages. Box plots mark interquartile ranges across all session averages. Horizontal lines in the box plots depict the median across session averages. Note that 8kHz NBN stimuli were assessed for 4 out of the 8 animals.

WaveID	1	ID			TIME		
					1 Mean	2 Mean	3 Mean
	1	ID	1	WaveMAG	.0000005851	.0000000216	.0000003323
			2	WaveMAG	.0000003050	.0000002331	.0000000786
			3	WaveMAG	.0000005086	.0000003642	.0000000320
			4	WaveMAG	.0000001384	.0000001683	.
			5	WaveMAG	.0000003189	.0000001139	.
			6	WaveMAG	.0000000242	.0000001124	.
			7	WaveMAG	.0000003334	.0000002493	.
	2	ID	1	WaveMAG	.0000010176	.0000004645	.0000000097
			2	WaveMAG	.0000010389	.0000000117	.0000001198
			3	WaveMAG	.0000016812	.0000007241	.0000000955
			4	WaveMAG	.0000006554	.0000013899	.
			5	WaveMAG	.0000004775	.0000001778	.
			6	WaveMAG	.0000000853	.0000005863	.
			7	WaveMAG	.0000006809	.0000007140	.
	3	ID	1	WaveMAG	.0000000000	.0000000686	.0000000000
			2	WaveMAG	.0000000031	.0000002139	.0000001764
			3	WaveMAG	.0000000898	.0000000000	.0000004449
			4	WaveMAG	.0000000000	.0000002294	.
			5	WaveMAG	.0000006790	.0000001753	.
			6	WaveMAG	.0000004199	.0000000066	.
			7	WaveMAG	.0000003646	.0000004487	.
	4	ID	1	WaveMAG	.0000007473	.0000000145	.0000003436
			2	WaveMAG	.0000006989	.0000001500	.0000000064
			3	WaveMAG	.0000006602	.0000004640	.0000002416
			4	WaveMAG	.0000001057	.0000003971	.
			5	WaveMAG	.0000006780	.0000003820	.
			6	WaveMAG	.0000000667	.0000000303	.
			7	WaveMAG	.0000000000	.0000000000	.
	5	ID	1	WaveMAG	.0000003065	.0000002613	.0000000000
			2	WaveMAG	.0000000286	.0000003671	.0000000917
			3	WaveMAG	.0000000000	.0000000000	.0000000000
			4	WaveMAG	.0000001329	.0000001292	.
			5	WaveMAG	.0000000000	.0000000000	.
			6	WaveMAG	.0000003515	.0000003296	.
			7	WaveMAG	.0000004141	.0000007008	.

Supp. Table 1: ABR wave magnitudes for click stimuli. ABR wave magnitudes (in volts) for 90dB click stimuli. The y-dimension (WaveID) depicts the ABR wave identity from 1 to 5 (i.e. waves I to V). ID stands for animal identification. The x-dimension (TIME) corresponds to BL (1), Post1 (2) and Post2 (3).

WaveID	1	ID			TIME		
					1 Mean	2 Mean	3 Mean
	1	ID	1	WaveLat_ms	1.16	.80	1.20
			2	WaveLat_ms	1.16	1.28	1.24
			3	WaveLat_ms	1.16	.96	1.28
			4	WaveLat_ms	1.16	1.16	.
			5	WaveLat_ms	1.44	1.24	.
			6	WaveLat_ms	.76	1.00	.
			7	WaveLat_ms	1.24	1.16	.
	2	ID	1	WaveLat_ms	1.92	1.64	2.04
			2	WaveLat_ms	1.92	1.84	2.00
			3	WaveLat_ms	1.84	1.72	1.80
			4	WaveLat_ms	1.92	1.88	.
			5	WaveLat_ms	2.32	2.04	.
			6	WaveLat_ms	1.72	1.88	.
			7	WaveLat_ms	2.16	1.92	.
	3	ID	1	WaveLat_ms	.	2.40	.
			2	WaveLat_ms	2.76	2.64	2.80
			3	WaveLat_ms	2.72	.	2.16
			4	WaveLat_ms	.	2.80	.
			5	WaveLat_ms	2.76	2.52	.
			6	WaveLat_ms	2.44	2.48	.
			7	WaveLat_ms	3.00	2.64	.
	4	ID	1	WaveLat_ms	2.88	2.80	3.08
			2	WaveLat_ms	3.12	3.04	3.12
			3	WaveLat_ms	2.92	3.04	3.28
			4	WaveLat_ms	3.00	3.20	.
			5	WaveLat_ms	3.52	3.56	.
			6	WaveLat_ms	3.08	3.04	.
			7	WaveLat_ms	.	.	.
	5	ID	1	WaveLat_ms	3.76	3.64	.
			2	WaveLat_ms	4.04	4.04	4.12
			3	WaveLat_ms	.	.	.
			4	WaveLat_ms	3.64	3.72	.
			5	WaveLat_ms	.	.	.
			6	WaveLat_ms	3.80	3.60	.
			7	WaveLat_ms	4.00	3.56	.

Supp. Table 2: ABR wave latencies for click stimuli. ABR wave latencies (in ms) for 90dB click stimuli. The y-dimension (WaveID) depicts the ABR wave identity from 1 to 5 (i.e. waves I to V). ID stands for animal identification. The x-dimension (TIME) corresponds to BL (1), Post1 (2) and Post2 (3).

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	WavLT_mod	-.524285714	-.500000000	-.357142857
	2	WavLT_mod	-.477142857	-.260000000	-.800000000
	3	WavLT_mod	-.254285714	-.750000000	-.200000000
	4	WavLT_mod	-.442857143	-.545714286	.
	5	WavLT_mod	-.300000000	.566666667	.
	6	WavLT_mod	-.840000000	-.190000000	.
	7	WavLT_mod	-.180000000	-.217142857	.

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	WavMag_mod	.0000000208	.0000000082	-.000000002
	2	WavMag_mod	.0000000221	-.000000003	.0000000031
	3	WavMag_mod	.0000000349	.0000000121	.0000000021
	4	WavMag_mod	.0000000144	.0000000247	.
	5	WavMag_mod	.0000000126	.0000000025	.
	6	WavMag_mod	.0000000013	.0000000109	.
	7	WavMag_mod	.0000000141	.0000000147	.

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	RMS_mod	.0000000050	.0000000014	.0000000006
	2	RMS_mod	.0000000049	.0000000017	.0000000009
	3	RMS_mod	.0000000072	.0000000021	.0000000008
	4	RMS_mod	.0000000028	.0000000055	.
	5	RMS_mod	.0000000109	.0000000016	.
	6	RMS_mod	.0000000021	.0000000030	.
	7	RMS_mod	.0000000038	.0000000037	.

Supp. Table 3: ABR modulation by stimulus intensities for click stimuli.

Top to bottom; ABR latency modulation, ABR wave II magnitude modulation, ABR RMS modulation. The y-dimension (ID) stands for animal identification. The x-dimension (Time) corresponds to BL (1), Post1 (2) and Post2 (3).

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	WavMag_total	.0000171161	.0000062238	.0000157052
	2	WavMag_total	.0000197984	.0000045692	.0000025568
	3	WavMag_total	.0000307062	.0000058392	.0000013985
	4	WavMag_total	.0000190854	.0000179248	.
	5	WavMag_total	.0000091626	.0000008890	.
	6	WavMag_total	.0000009218	.0000098293	.
	7	WavMag_total	.0000152785	.0000161908	.

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	RMS_total	.0000066831	.0000046223	.0000062289
	2	RMS_total	.0000067181	.0000078762	.0000047894
	3	RMS_total	.0000092211	.0000051073	.0000051488
	4	RMS_total	.0000055717	.0000076095	.
	5	RMS_total	.0000302250	.0000033817	.
	6	RMS_total	.0000041211	.0000033651	.
	7	RMS_total	.0000053577	.0000054424	.

Supp. Table 4: ABR total magnitude across all stimulus intensities for click stimuli. Top to bottom; ABR wave II total magnitude, ABR RMS total magnitude. The y-dimension (ID) stands for animal identification. The x-dimension (Time) corresponds to BL (1), Post1 (2) and Post2 (3).

					TIME		
					1	2	3
					Mean	Mean	Mean
WaveID	1	ID	1	WaveMAG	.0000005886	.0000000026	.0000003138
			2	WaveMAG	.0000000247	.0000001538	.0000000365
			3	WaveMAG	.0000000556	.0000004093	.0000003648
			4	WaveMAG	.0000004000	.0000001285	.
			5	WaveMAG	.0000003688	.00000009611	.
			6	WaveMAG	.0000002719	.0000004316	.
			7	WaveMAG	.0000012203	.0000000000	.
	2	ID	1	WaveMAG	.0000020938	.0000006333	.0000002541
			2	WaveMAG	.0000026966	.0000000922	.0000000325
			3	WaveMAG	.0000017946	.0000012297	.0000014716
			4	WaveMAG	.0000014698	.0000006563	.
			5	WaveMAG	.0000007438	.0000005181	.
			6	WaveMAG	.0000009054	.0000008275	.
			7	WaveMAG	.0000017094	.0000015942	.
	3	ID	1	WaveMAG	.0000000449	.0000000000	.0000006486
			2	WaveMAG	.0000001967	.0000000074	.0000001438
			3	WaveMAG	.0000015703	.0000010591	.0000000000
			4	WaveMAG	.0000000000	.0000009422	.
			5	WaveMAG	.0000011669	.0000005425	.
			6	WaveMAG	.0000007066	.0000000225	.
			7	WaveMAG	.0000015145	.0000007181	.
	4	ID	1	WaveMAG	.0000016793	.0000001171	.0000000000
			2	WaveMAG	.0000017726	.0000003310	.0000001016
			3	WaveMAG	.0000000000	.0000000843	.0000008408
			4	WaveMAG	.0000000077	.0000000000	.
			5	WaveMAG	.0000010707	.0000000000	.
			6	WaveMAG	.0000000000	.0000005034	.
			7	WaveMAG	.0000000000	.0000000000	.
	5	ID	1	WaveMAG	.0000002763	.0000000079	.0000002000
			2	WaveMAG	.0000002663	.0000001958	.0000000000
			3	WaveMAG	.0000000000	.0000000696	.0000000000
			4	WaveMAG	.0000000000	.0000000000	.
			5	WaveMAG	.0000000000	.0000003275	.
			6	WaveMAG	.0000007726	.0000007021	.
			7	WaveMAG	.0000000000	.0000009717	.

Supp. Table 5: ABR wave magnitudes for 8kHz NBN stimuli. ABR wave magnitudes (in volts) for 90dB click stimuli. The y-dimension (WaveID) depicts the ABR wave identity from 1 to 5 (i.e. waves I to V). ID stands for animal identification. The x-dimension (TIME) corresponds to BL (1), Post1 (2) and Post2 (3).

					TIME		
					1	2	3
					Mean	Mean	Mean
WaveID	1	ID	1	WaveLAT_ms	1.12	1.04	1.00
			2	WaveLAT_ms	.80	.80	.96
			3	WaveLAT_ms	.72	.96	1.08
			4	WaveLAT_ms	1.44	1.32	
			5	WaveLAT_ms	1.36	1.60	.
			6	WaveLAT_ms	1.04	.88	.
			7	WaveLAT_ms	1.32	.	.
	2	ID	1	WaveLAT_ms	1.76	1.68	1.60
			2	WaveLAT_ms	1.64	1.76	1.88
			3	WaveLAT_ms	1.76	1.76	1.88
			4	WaveLAT_ms	2.24	2.00	.
			5	WaveLAT_ms	2.28	2.40	.
			6	WaveLAT_ms	1.60	1.60	.
			7	WaveLAT_ms	2.28	1.72	.
	3	ID	1	WaveLAT_ms	2.52	.	2.04
			2	WaveLAT_ms	2.40	2.36	2.52
			3	WaveLAT_ms	2.52	2.56	.
			4	WaveLAT_ms	.	2.80	.
			5	WaveLAT_ms	2.68	3.20	.
			6	WaveLAT_ms	2.48	2.40	.
			7	WaveLAT_ms	3.20	2.60	.
	4	ID	1	WaveLAT_ms	2.72	2.64	.
			2	WaveLAT_ms	2.72	3.04	2.88
			3	WaveLAT_ms	.	3.28	2.88
			4	WaveLAT_ms	3.68	.	.
			5	WaveLAT_ms	3.60	.	.
			6	WaveLAT_ms	.	2.60	.
			7	WaveLAT_ms	.	.	.
	5	ID	1	WaveLAT_ms	3.68	3.80	3.72
			2	WaveLAT_ms	3.68	3.72	.
			3	WaveLAT_ms	.	3.72	.
			4	WaveLAT_ms	.	.	.
			5	WaveLAT_ms	.	4.36	.
			6	WaveLAT_ms	3.36	3.32	.
			7	WaveLAT_ms	.	3.40	.

Supp. Table 6: ABR wave latencies for 8kHz NBN stimuli. ABR wave latencies (in ms) for 90dB click stimuli. The y-dimension (WaveID) depicts the ABR wave identity from 1 to 5 (i.e. waves I to V). ID stands for animal identification. The x-dimension (TIME) corresponds to BL (1), Post1 (2) and Post2 (3).

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	WavLT_mod	-.494285714	-.302857143	.444444444
	2	WavLT_mod	-.577142857	-.405714286	-.510000000
	3	WavLT_mod	-.271428571	-.311428571	-.257142857
	4	WavLT_mod	-.300000000	-.484285714	.
	5	WavLT_mod	-.250000000	-.300000000	.
	6	WavLT_mod	-1.300000000	-.880000000	.
	7	WavLT_mod	-.311428571	-.251428571	.

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	WavMag_mod	.0000000401	.0000000148	.0000000036
	2	WavMag_mod	.0000000540	-.000000001	-.000000001
	3	WavMag_mod	.0000000315	.0000000269	.0000000303
	4	WavMag_mod	.0000000222	.0000000118	.
	5	WavMag_mod	.0000000148	.0000000094	.
	6	WavMag_mod	.0000000222	.0000000149	.
	7	WavMag_mod	.0000000345	.0000000260	.

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	RMS_mod	.0000000117	.0000000027	.0000000048
	2	RMS_mod	.0000000124	-.000000002	.000000000
	3	RMS_mod	.0000000112	.0000000078	.0000000073
	4	RMS_mod	.0000000048	.0000000038	.
	5	RMS_mod	.0000000073	.0000000023	.
	6	RMS_mod	.0000000042	.0000000027	.
	7	RMS_mod	.0000000089	.0000000066	.

Supp. Table 7: ABR modulation by stimulus intensities for 8kHz NBN stimuli. Top to bottom; ABR latency modulation, ABR wave II magnitude modulation, ABR RMS modulation. The y-dimension (ID) stands for animal identification. The x-dimension (Time) corresponds to BL (1), Post1 (2) and Post2 (3).

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	WavMag_total	.0000408559	.0000165763	.0000012704
	2	WavMag_total	.0000467537	.0000056878	.0000041607
	3	WavMag_total	.0000451718	.0000305650	.0000278931
	4	WavMag_total	.0000211618	.0000142232	.
	5	WavMag_total	.0000403804	.0000049547	.
	6	WavMag_total	.0000201465	.0000191158	.
	7	WavMag_total	.0000358219	.0000442845	.

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	RMS_total	.0000146341	.0000071469	.0000131571
	2	RMS_total	.0000153175	.0000068607	.0000049702
	3	RMS_total	.0000152955	.0000107271	.0000103832
	4	RMS_total	.0000103164	.0000066084	.
	5	RMS_total	.0000313407	.0000082982	.
	6	RMS_total	.0000086624	.0000073479	.
	7	RMS_total	.0000135515	.0000141035	.

Supp. Table 8: ABR total magnitude across all stimulus intensities for 8kHz NBN stimuli. Top to bottom; ABR wave II total magnitude, ABR RMS total magnitude. The y-dimension (ID) stands for animal identification. The x-dimension (Time) corresponds to BL (1), Post1 (2) and Post2 (3).

					TIME		
					1	2	3
					Mean	Mean	Mean
WaveID	1	ID	1	WaveMAG	.0000008385	.0000002024	.0000004218
			2	WaveMAG	.0000002425	.0000001679	.0000004952
			3	WaveMAG	.0000007157	.0000006348	.0000002855
			4	WaveMAG	.0000004801	.0000001384	.
			5	WaveMAG	.0000010383	.0000002998	.
			6	WaveMAG	.0000002620	.0000002744	.
			7	WaveMAG	.0000012704	.0000005466	.
	2	ID	1	WaveMAG	.0000013107	.0000007961	.0000001982
			2	WaveMAG	.0000017901	.0000002367	.0000000629
			3	WaveMAG	.0000019518	.0000014636	.0000007754
			4	WaveMAG	.0000007642	.0000000859	.
			5	WaveMAG	.0000005254	.0000003475	.
			6	WaveMAG	.0000006404	.0000006337	.
			7	WaveMAG	.0000009992	.0000009867	.
	3	ID	1	WaveMAG	.0000000000	.0000000682	.0000000000
			2	WaveMAG	.0000001388	.0000000936	.0000000446
			3	WaveMAG	.0000017635	.0000008196	.0000000587
			4	WaveMAG	.0000003296	.0000003326	.
			5	WaveMAG	.0000011150	.0000007041	.
			6	WaveMAG	.0000003609	.0000006808	.
			7	WaveMAG	.0000000000	.0000008226	.
	4	ID	1	WaveMAG	.0000009582	.0000005305	.0000000046
			2	WaveMAG	.0000011766	.0000004001	.0000000417
			3	WaveMAG	.0000001685	.0000004076	.0000009592
			4	WaveMAG	.0000006713	.0000001172	.
			5	WaveMAG	.0000023342	.0000000000	.
			6	WaveMAG	.0000001273	.0000000000	.
			7	WaveMAG	.0000015051	.0000000000	.
	5	ID	1	WaveMAG	.0000003374	.0000004881	.0000006646
			2	WaveMAG	.0000002719	.0000002708	.0000003607
			3	WaveMAG	.0000000681	.0000007385	.0000008817
			4	WaveMAG	.0000002046	.0000000470	.
			5	WaveMAG	.0000000000	.0000001836	.
			6	WaveMAG	.0000006433	.0000000633	.
			7	WaveMAG	.0000000000	.0000000000	.

Supp. Table 9: ABR wave magnitudes for 16kHz NBN stimuli. ABR wave magnitudes (in volts) for 90dB click stimuli. The y-dimension (WaveID) depicts the ABR wave identity from 1 to 5 (i.e. waves I to V). ID stands for animal identification. The x-dimension (TIME) corresponds to BL (1), Post1 (2) and Post2 (3).

WaveID	1	ID			TIME		
					1 Mean	2 Mean	3 Mean
	1	ID	1	WaveLAT_ms	.88	.64	.84
			2	WaveLAT_ms	.60	1.00	1.52
			3	WaveLAT_ms	1.04	1.12	.88
			4	WaveLAT_ms	.80	1.08	.
			5	WaveLAT_ms	1.40	.84	.
			6	WaveLAT_ms	.92	.88	.
			7	WaveLAT_ms	1.08	.72	.
	2	ID	1	WaveLAT_ms	1.64	1.64	1.88
			2	WaveLAT_ms	1.64	1.68	2.24
			3	WaveLAT_ms	1.68	1.64	1.88
			4	WaveLAT_ms	1.76	1.76	.
			5	WaveLAT_ms	2.20	1.64	.
			6	WaveLAT_ms	1.80	1.64	.
			7	WaveLAT_ms	2.16	1.76	.
	3	ID	1	WaveLAT_ms	.	2.40	.
			2	WaveLAT_ms	2.36	2.36	2.76
			3	WaveLAT_ms	2.52	2.52	2.60
			4	WaveLAT_ms	2.56	2.32	.
			5	WaveLAT_ms	2.56	2.52	.
			6	WaveLAT_ms	2.64	2.52	.
			7	WaveLAT_ms	.	2.60	.
	4	ID	1	WaveLAT_ms	2.60	2.64	3.32
			2	WaveLAT_ms	2.76	2.96	3.40
			3	WaveLAT_ms	3.16	2.80	3.04
			4	WaveLAT_ms	2.80	3.04	.
			5	WaveLAT_ms	3.56	.	.
			6	WaveLAT_ms	2.96	.	.
			7	WaveLAT_ms	3.28	.	.
	5	ID	1	WaveLAT_ms	3.48	3.44	3.68
			2	WaveLAT_ms	3.76	3.68	4.16
			3	WaveLAT_ms	3.36	3.28	3.44
			4	WaveLAT_ms	3.52	3.64	.
			5	WaveLAT_ms	.	3.36	.
			6	WaveLAT_ms	3.44	3.64	.
			7	WaveLAT_ms	.	.	.

Supp. Table 10: ABR wave latencies for 16kHz NBN stimuli. ABR wave latencies (in ms) for 90dB click stimuli. The y-dimension (WaveID) depicts the ABR wave identity from 1 to 5 (i.e. waves I to V). ID stands for animal identification. The x-dimension (TIME) corresponds to BL (1), Post1 (2) and Post2 (3).

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	WavLT_mod	-.588571429	-.491428571	-.268571429
	2	WavLT_mod	-.594285714	-.350000000	-.365714286
	3	WavLT_mod	-.614285714	-.417142857	-.314285714
	4	WavLT_mod	-.222857143	-.522857143	.
	5	WavLT_mod	.611111111	-1.05000000	.
	6	WavLT_mod	-1.05000000	-.737142857	.
	7	WavLT_mod	-.168571429	-.614285714	.

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	WavMag_mod	.0000000334	.0000000149	.0000000038
	2	WavMag_mod	.0000000399	.0000000096	.0000000003
	3	WavMag_mod	.0000000455	.0000000196	.0000000169
	4	WavMag_mod	-.000000004	-.000000002	.
	5	WavMag_mod	.0000000075	.0000000059	.
	6	WavMag_mod	.0000000114	.0000000051	.
	7	WavMag_mod	.0000000231	.0000000210	.

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	RMS_mod	.0000000065	.0000000043	.0000000012
	2	RMS_mod	.0000000087	.0000000018	.0000000008
	3	RMS_mod	.0000000112	.0000000065	.0000000051
	4	RMS_mod	.0000000022	.0000000014	.
	5	RMS_mod	-.000000002	.0000000019	.
	6	RMS_mod	.0000000021	.0000000021	.
	7	RMS_mod	.0000000072	.0000000038	.

Supp. Table 11: ABR modulation by stimulus intensities for 16kHz NBN stimuli. Top to bottom; ABR latency modulation, ABR wave II magnitude modulation, ABR RMS modulation. The y-dimension (ID) stands for animal identification. The x-dimension (Time) corresponds to BL (1), Post1 (2) and Post2 (3).

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	WavMag_total	.0000410175	.0000185280	.0000084897
	2	WavMag_total	.0000561540	.0000115850	.0000027799
	3	WavMag_total	.0000560600	.0000189192	.0000160888
	4	WavMag_total	.0000381739	.0000098179	.
	5	WavMag_total	.0000026270	.0000028517	.
	6	WavMag_total	.0000058537	.0000184956	.
	7	WavMag_total	.0000194233	.0000280001	.

			Time		
			1	2	3
			Mean	Mean	Mean
ID	1	RMS_total	.0000130990	.0000082467	.0000068687
	2	RMS_total	.0000163108	.0000087200	.0000069402
	3	RMS_total	.0000190748	.0000102898	.0000087926
	4	RMS_total	.0000132029	.0000051916	.
	5	RMS_total	.0000270780	.0000080666	.
	6	RMS_total	.0000060160	.0000083170	.
	7	RMS_total	.0000152090	.0000117179	.

Supp. Table 12: ABR total magnitude across all stimulus intensities for 16kHz NBN stimuli. Top to bottom; ABR wave II total magnitude, ABR RMS total magnitude. The y-dimension (ID) stands for animal identification. The x-dimension (Time) corresponds to BL (1), Post1 (2) and Post2 (3).

Chapter 3: The effect of noise overexposure on sleep-wake dynamics

Background

Progress has been made in recent years to identify functional brain correlates of tinnitus in both human tinnitus sufferers (Leaver et al., 2011; Schlee et al., 2009; Sedley et al., 2012, 2015) and in animal models of induced tinnitus (Bauer et al., 2008; Berger & Coomber, 2015; Brozoski et al., 2002; Engineer et al., 2011; Kalappa et al., 2014; Kaltenbach et al., 2004; Manzoor et al., 2013; Noreña & Eggermont, 2003, 2006; Rauschecker et al., 2010, 2010; Seki & Eggermont, 2003; Wu et al., 2016) with local changes along the ascending auditory pathway, such as altered spontaneous neural activity and increased synchronous firing as the most prominent correlates.

Yet tinnitus is also associated with changes in global brain activity. Especially in cases of long-lasting tinnitus, global changes in brain connectivity and activity have been described (Leaver et al., 2011; Rauschecker et al., 2010b). Indeed, it has been argued that a wide representation of tinnitus in the brain is necessary for it to become conscious in the first place (De Ridder et al., 2011, 2014). These areas cover non-auditory regions, such as the frontal cortex and the limbic system. For example, aberrant activity in the prefrontal cortex is correlated with subjective tinnitus loudness (Seydell-Greenwald et al., 2012). Furthermore, changes in cortical oscillations in tinnitus patients have been observed in temporal, parietal and limbic cortices (Sedley et al., 2015), as well as within the default mode network, the cerebellum and the insula (Sedley et al., 2012). While the

pathophysiological significance of these changes is not entirely clear, it has been shown that in tinnitus the default activity in auditory regions is negatively correlated with activity in non-auditory regions, possibly suggesting an adaption in brain connectivity to reduce the saliency of tinnitus when attention is directed towards non-auditory events (Burton *et al.*, 2012).

Oscillatory brain activity, especially in the gamma-band frequency range, has long been thought to play a role in binding visual stimuli features into a conscious percept (Singer, 1999). Similarly, gamma-band activity could be associated to the generation of pathological phantom percepts (Llinás *et al.*, 1999) and tinnitus in particular (Llinás *et al.*, 1999; Weisz *et al.*, 2007; De Ridder *et al.*, 2015). In patients with long tinnitus history, oscillatory coupling in the gamma frequency range is most widely distributed across the cortical hemispheres, including the prefrontal cortex, the orbitofrontal cortex and the parieto-occipital region (Schlee *et al.*, 2009). Furthermore, high frequency activity in tinnitus has been described in the auditory cortex (Weisz *et al.*, 2007) and has been related to hyperactivity in the cortical regions close to the deafferented cortical areas. This has been described to occur subsequent to hearing loss due to the loss of inhibition from deafferented areas, a phenomenon described as ‘the edge effect’ (Llinás *et al.*, 2005). The findings of brain-wide oscillatory changes in persistent tinnitus raise the question of how tinnitus interacts with natural dynamics of local and global oscillatory activity.

The arguably most prominent of such dynamics is the alternation of brain states between wakefulness and sleep. In terms of brain activity, non-rapid eye movement (NREM) sleep is characterised by the emergence of synchronized activity spanning vast

areas of the brain and visible in the form of slow (0.5-4Hz) oscillations in the cortical EEG, also called slow-wave activity (SWA). Cortical SWA is an indicator for sleep homeostasis as its amplitude correlates with sleep pressure accumulated during preceding wakefulness (Brunner et al., 1993; Guillaumin et al., 2018; Vyazovskiy et al., 2011). And while stimuli disrupting SWA also disrupt the global sleep state, the artificial enhancement of SWA can improve sleep function, such as memory consolidation (Moreira et al., 2021). Slow-wave activity is therefore both a hallmark of sleep and a possible mediator for the function of sleep. It might, at the same time, be sensitive to interruption via aberrant spontaneous brain activity.

Indeed, while the spatial expression of SWA depends on preceding sleep-wake history (Vyazovskiy et al., 2011) as well as on waking experience (Milinski et al., 2021a), there are areas that typically express pronounced sleep-dependent SWA. In fact, many of the brain areas that are hubs of SWA expression (Finelli et al., 2001; Murphy et al., 2009; Nir et al., 2011) show lasting changes in tinnitus on both functional and structural levels (Elgoyhen et al., 2015b). If the increased spontaneous activity observed in tinnitus during the waking state and anaesthesia (Bauer et al., 2008; Berger & Coomber, 2015; Brozoski et al., 2002; Engineer et al., 2011; Kalappa et al., 2014; Kaltenbach et al., 2004; Leaver et al., 2011; Manzoor et al., 2013; Noreña & Eggermont, 2003, 2006; Rauschecker et al., 2010, 2010; Schlee et al., 2009; Sedley et al., 2012, 2015; Seki & Eggermont, 2003; Wu et al., 2016) persists during sleep, it is likely to interfere with coordinated activity such as globally synchronized SWA, and possibly causing impaired sleep maintenance and changes in sleep architecture. This could be at the basis of many of tinnitus' comorbidities, such as depression, stress and anxiety.

Changes in sleep are indeed one of the most common side-effects of tinnitus. Altered sleep architecture (Attanasio et al., 2013; Burgos et al., 2005; Hébert et al., 2011a) and insomnia (Asnis et al., 2018) are commonly found in tinnitus patients. Hébert and colleagues (Hébert et al., 2011a) found a correlation between subjectively reported sleep problems and reduced average EEG spectral power in the delta frequency band in tinnitus patients. Other studies identified a bias in tinnitus patients towards longer sleep latency (Burgos et al., 2005) and lighter sleep (Attanasio et al., 2013; Burgos et al., 2005), correlated with increased subjective tinnitus loudness (Attanasio et al., 2013). Thus, sleep seems to be shallower and less stable in tinnitus patients than in control individuals, although tinnitus participants still entered all sleep stages, including slow-wave and REM sleep (Attanasio et al., 2013; Burgos et al., 2005; Hébert et al., 2011a). The magnitude of changes in sleep might be related to tinnitus loudness experienced during wakefulness: studies based on patient ratings demonstrate a correlation between subjective tinnitus loudness and symptoms of changed sleep, such as sleep disruptions (Folmer & Griest, 2000; Hallam, 1996; Izuhara et al., 2013a; Koning, 2019a; Schecklmann et al., 2015) and the ability to initiate sleep (Koning, 2019a).

It is important to acknowledge that the association between tinnitus-related brain activity and altered sleep architecture, while robust, is so far only based on correlational studies. And while there is a clear association between tinnitus and alterations in sleep architecture it remains unclear how these changes are related to the emergence of tinnitus. How does brain activity during sleep change after a triggering event and how do possible changes correlate with behavioural signs of tinnitus during wakefulness?

To address these questions, the animal model of tinnitus introduced in Chapters 1 and 2 was combined with chronic recordings of brain activity across vigilance states. Specifically, to link behavioural signs of emerging tinnitus with changes in sleep architecture, neural recordings were obtained before and up to six months after the initial tinnitus trigger.

Method summary

The experimental timeline

Adult female ferrets (n=3) underwent behavioural testing before and after noise overexposure as described in Chapter 2. In addition, each animal underwent periods of chronic electrophysiological recordings to assess sleep-wake architecture. To this end, animals were implanted with frontal and occipital EEG screw electrodes over the cortical hemisphere ipsilateral to the noise exposed ear, a multi-channel laminar (Neuronexus) probe in the primary auditory cortex in the hemisphere contralateral to the noise exposed ear and with neck muscle electrodes. Implantation took place prior to noise exposure but subsequent to completed baseline behavioural testing (Fig.1, see also Methods: Surgery).

During electrophysiological recording sessions animals were housed individually in a custom-made chamber (Fig.2A,B) with food and water available *ad libitum* and in a set 15/9 light-dark cycle identical to standard housing conditions. During the recording sessions, which lasted for 2-3 consecutive days on each occasion, the animals were

continuously monitored using a surveillance camera with night vision (infrared) capability.

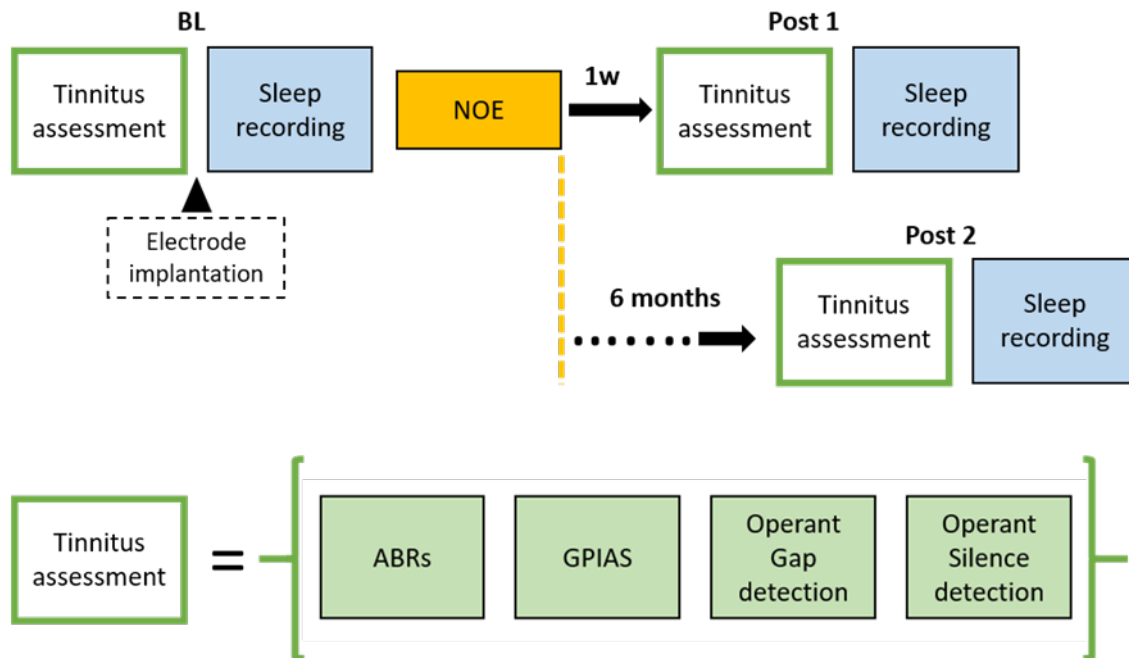


Fig. 1: Experimental timeline. Ferrets were assessed for tinnitus, hearing loss, EEG brain activity and sleep-wake behaviour before and after noise overexposure (NOE). Assessments of all these metrics took place once under baseline conditions and on two occasions after NOE, the first assessment (Post1) commencing 1 week after NOE and the second (Post2) approximately 6 months after NOE. In addition to the ‘tinnitus assessment’ (see chapter 2 for details of the used paradigms), brain activity was recorded (based on frontal and occipital EEG) in the freely behaving animals for approximately 48 hours in each condition (BL, Post1 and Post2).

The first recording session took place before noise overexposure as part of the baseline assessment of each animal (baseline recordings, BL), and two further sessions after NOE, one approximately 2 weeks post NOE (Post1) and the other approximately 6 months post NOE (Post2). See Fig.1 for a timeline of the experiment. Vigilance state scoring was performed offline and manually, prior to spectral analysis and assessment of sleep architecture. Manual scoring was based on filtered EEG and EMG signals and on the

video recordings (see Methods: EEG signal processing and vigilance state scoring). This chapter focuses on the analysis of the EEG to evaluate changes in sleep-wake architecture subsequent to NOE.

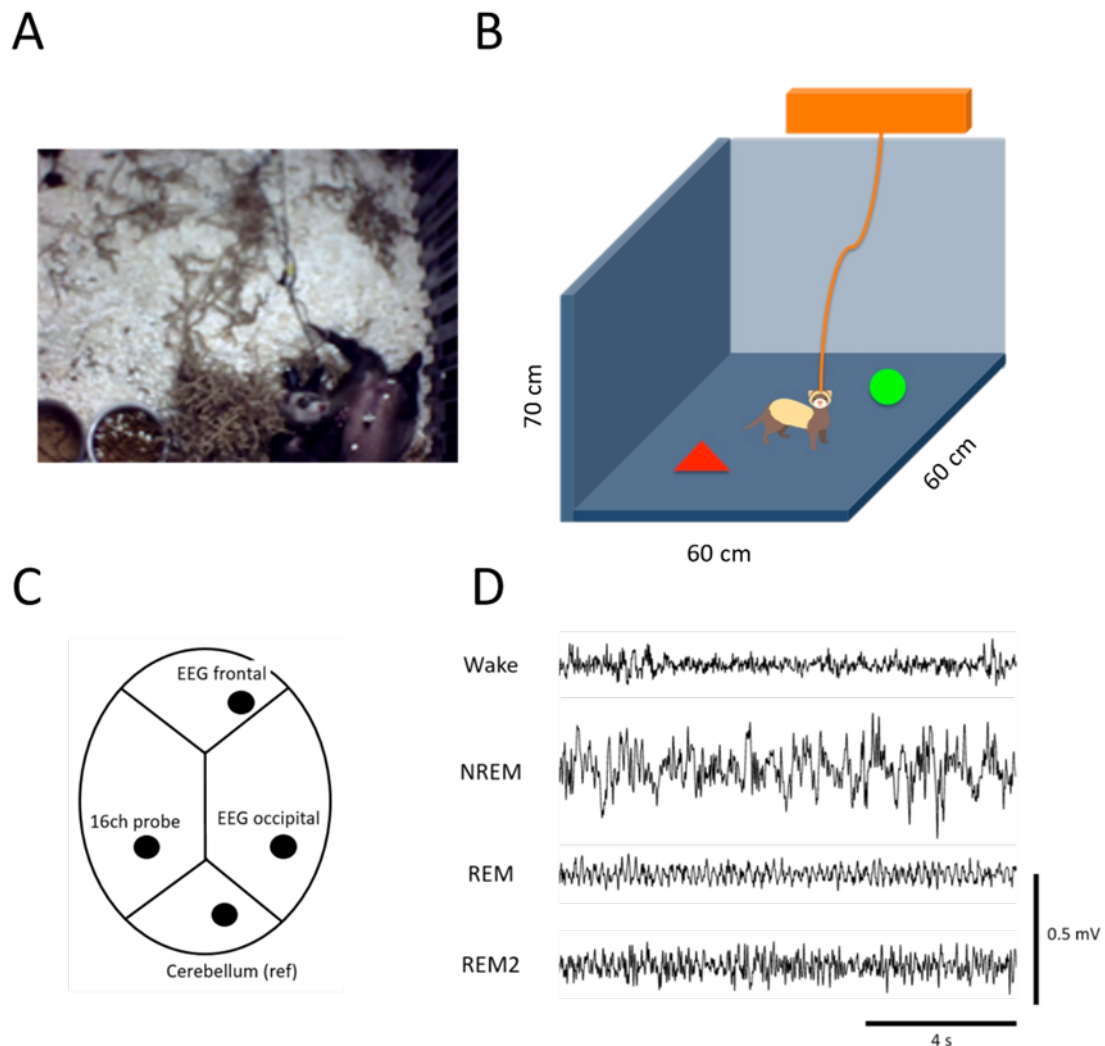


Fig. 2: Chronic EEG recordings in the ferret: setup and vigilance states. (A) Snapshot of a ferret during a recording session, acquired from the continuous video recording obtained during recording sessions. (B) Schematic depiction of the recording chamber pictured in A, with dimensions. (C) Locations of implanted electrodes: EEG screw electrodes over frontal and occipital cortices ipsilateral relative to the noise exposed ear, a reference screw electrode over the cerebellum and a 16-channel laminar probe in the primary auditory cortex contralateral relative to the exposed ear. (D) Example EEG traces during wakefulness (Wake), non-rapid eye movement sleep (NREM), rapid eye movement sleep (REM) and REM2 sleep (REM2). EEG signals displayed in this panel are band-pass filtered (0.5-30Hz) and were obtained under baseline conditions (before NOE).

Data analysis

Statistical analysis was performed using custom written MATLAB (Simulink, USA) scripts and SPSS (IBM). Data were graphically assessed for normality by using histograms and by estimating the linearity of q-q-plots (Chambers, 2018).

Spectral power analysis was performed based on the EEG. Analysis was based on 4-sec epochs of the raw signal (spanning ~48hrs of continuous recording per condition, i.e. BL, Post1 and Post2), computed over the 0.25-20Hz frequency range using frequency bins spanning 0.25Hz. This frequency range was chosen since the primary objective of the EEG analysis was to identify changes in sleep pattern and composition and since the spatiotemporal brain activity central to vigilance state dynamics in the ferret is in the range of 0.5-4Hz (EEG delta band), 4.5-8Hz (EEG theta band) and 8.5-20Hz (EEG alpha and low beta bands). Epochs confounded by artefacts – as identified during the manual scoring procedure – were excluded from the analysis. Artefacts in specific frequency bins that had remained unnoticed during manual data scoring were excluded offline (see Methods: EEG signal processing and vigilance state scoring). As EEG data are not-normally distributed, analysis was performed on log-transformed data. Specifically, the transformation was applied to the power per 0.25Hz frequency bin, spanning 0.25 to 20Hz. Statistically significant effects were estimated using a two-way repeated measures ANOVA (factors frequency bin and condition) analysis with Tukey's multiple comparisons for individual differences.

For the analysis of the time course of SWA (delta range, 0.5-4Hz) for each vigilance state, SWA power was calculated per 15-min interval, before resulting values were merged

into 1h intervals, resulting in 4 datapoints per 1h bin. As two consecutive 24h-recordings constituted the recording session in each condition (BL, Post1, Post2) each 1h interval contained 8 values per condition. Importantly, not every 15min interval contained all vigilance states, which resulted in some 1h intervals having less than 8 SWA values. To equalize the number of values per 1h interval for statistical comparison between conditions, missing values were treated as values equal to the median of the values present in the respective 15min interval. 1h intervals with less than 3 original values were marked with a green square in Figures 15, 16, 26, 27, 37 and 38. Statistical analysis was performed with two-way ANOVAs (factors time interval and condition) and Tukey's multiple comparisons for individual differences.

Statistical analysis of wake episode lengths between animals (to compare animal differences in BL conditions) were performed using a one-way ANOVA and Tukey's multiple comparisons. The effect of condition in individual animals (BL vs Post1 and BL vs Post2) was estimated via Wilcoxon rank-sum tests.

Total vigilance state durations across conditions and total wake episode numbers across conditions were not statistically assessed for individual animals as variability of each respective measure could not be estimated based on a single sample per animal.

Definition of an index for tinnitus, for changes in ABRs and sleep amount

As a summary index for behavioural evidence for tinnitus and hearing loss a behavioural tinnitus index (TI, see Eq(4) below) was defined. These metrics were defined to obtain representative indicators for behavioural changes that are corroborating or

contradicting tinnitus and to then construct a tinnitus index incorporating all metrics. Since the direction of behavioural changes after NOE that indicate tinnitus differed between paradigms and aspects of behaviour included, the mathematical definition for each metric was adapted accordingly. For example, tinnitus is corroborated by a decrease in silence detection ability after NOE and by an increase in non-gap detection ability. To construct the TI, a normalised metric for performance changes in the first and second assessment after NOE (Post1 and Post2) was calculated for each paradigm.

The metric for operant silence detection Eq(1) refers to the change in silence detection ability (percent correct in silence trials) after NOE.

$$\text{Eq(1):} \quad M(\text{silence}) = 1 - \frac{\text{Post \% correct}}{\text{BL \% correct}}$$

Thus, positive values describe the magnitude of a decrease in silence detection ability (and therefore evidence for tinnitus) and negative values describe the magnitude of an increase in silence detection ability. For example, a value of $M(\text{silence}) = 0.2$ would indicate a decrease in performance by 20% relative to baseline performance.

The metric for operant gap detection performance refers to the change of ability in detecting the continuous sound (non-gap) across all tested stimulus frequencies (Eq(2)) and to the change in gap-detection threshold across all stimuli (Eq(3)).

$$\text{Eq(2):} \quad M(\text{cont}) = \frac{\text{Post \% correct}}{\text{BL \% correct}} - 1$$

The metric for a change in gap detection threshold (Eq(3)) was based on a normalised performance value instead of thresholds corresponding to gap lengths. To establish a normalised threshold, each gap length (3, 5, 10, 20, 50, 100, 270ms) was assigned a

performance value based on the assumption that a threshold of 3ms corresponds to 100% performance and the remaining gap lengths correspond to evenly spaced decrements in threshold (85.71%, 71.43%, 57.14%, 42.86%, 14.29%). This approach of defining threshold changes is a compromise to obtain a metric reflecting the direction of threshold change and its magnitude on a scale between 0 and 1, the same scale as in the other defined metrics of the tinnitus index (Eq(1-3)).

$$\text{Eq(3): } M(thresh) = 1 - \frac{Post\ threshold(normalised)}{BL\ threshold(normalised)}$$

For $M(\text{Silence})$, $M(\text{cont})$, and $M(\text{thresh})$ positive values describe changes in line with tinnitus development (an impairment in silence detection, an improvement in continuous sound detection, an increase in gap detection threshold, respectively).

The behavioural tinnitus index (TI, see Eq(4)) is the sum of these metrics (Eq (1-3)) with the aim to parametrize the level of tinnitus and hearing loss experienced by each animal after of NOE.

$$\text{Eq(4): } TI = M(silence) + M(cont) + M(thresh) + M(GPIAS)$$

Index for changes in ABRs

Changes in ABRs were summarised in form of two metrics: first, based on changes in ABR thresholds relative to baseline (BL) and second, based on changes in ABR total magnitude relative to BL.

The metric for thresholds refers to the average change in thresholds across all tested stimuli (in dB SPL), defined as differential between BL and Post NOE values:

Eq(5):

$$M(Thresholds) = (avg. threshold BL - avg. threshold Post)$$

The metric for the ABR magnitude is defined as follows: first, to consider the entire ABR response the magnitude of the ABR was defined as the root-mean square of the signal after stimulus presentation in a predefined response window (1.6-4ms, with a shift of 0.16ms for every step in decreasing sound level). The total magnitude was then calculated for each animal and each stimulus by measuring the area under the level-response graph, as shown in Fig. 2.2B. The input 'ABRmag' used for the metric below is the average over total ABR magnitudes for all stimuli (1, 2, 4, 8, 16 kHz NBN and BBN) per animal. The metric for each animal is defined as

$$Eq(6): \quad M(ABR \text{ magnitude}) = \left(\frac{Post \ ABRmag}{BL \ ABRmag} - 1 \right) \times 100$$

Positive values indicate the magnitude of a response increase (in %) whereas negative values indicate a reduced response (evidence for hearing loss).

Index for changes in sleep amount

The metric here refers to the change in sleep amount (percent of recording time spend in NREM, REM and REM2 sleep over ~48 hours) after as compared to before NOE. The metric for each sleep state is defined as

$$M(\text{sleep dur. change}) = \left(\frac{\text{Post \% time (in vigilance state)}}{\text{BL \% time (in vigilance state)}} - 1 \right) \times 100$$

Thus, positive values describe the magnitude of increase in sleep duration (in %) whereas negative values describe the magnitude of a decrease.

Results

After the habituation period (3-5 sessions of 30 min and longer) ferrets fell asleep while in the recording chamber as evaluated through video observation (Fig.2A). Vigilance states were readily distinguishable based on the EEG signal (Fig.2D), in combination with the EMG and video recordings. EEG spectra computed on the recorded signal during the different vigilance states (Wake, NREM sleep, REM and REM2 sleep) exhibited marked state-specific characteristics: wakefulness was characterised by an active EMG and a relatively even distribution of spectral power across frequencies, especially in the frontal derivation (Fig.3A). Non-rapid eye movement sleep or NREM was characterised by behavioural quiescence and pronounced activity in the EEG delta band (0.5-4Hz)(Fig.3) and rapid eye movement sleep or REM by pronounced activity in the EEG theta range (4.5-8Hz)(Fig.3). In line with previous work in ferrets (Jha et al., 2006) an additional REM-like sleep state, classified as REM2, was identified, which showed predominant EEG theta activity as well as higher frequency components in the alpha and beta frequency bands (8.5-20Hz)(Fig.3).

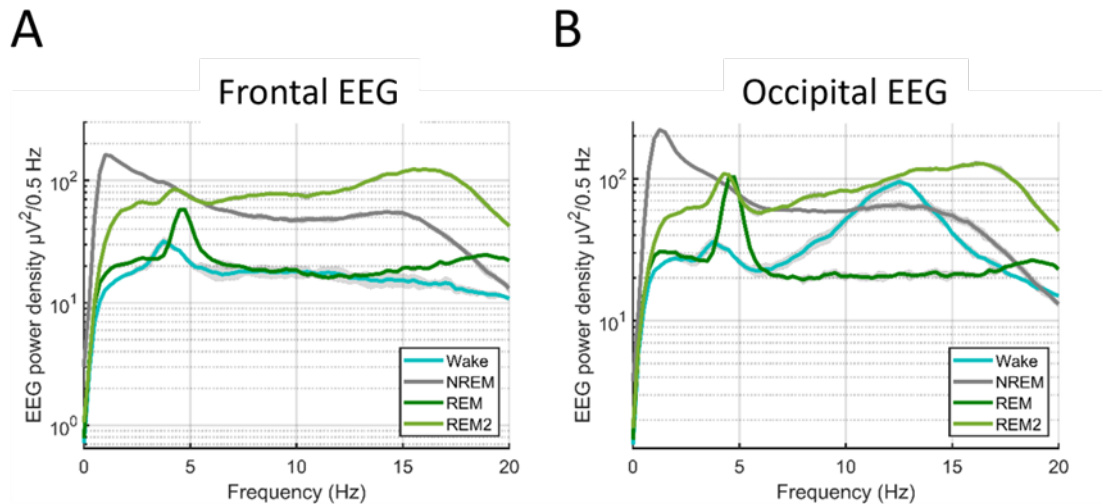


Fig. 3: EEG vigilance state spectra in the ferret (example based on Case2). Data are means across EEG spectra of **(A)** the frontal and **(B)** the occipital derivation calculated for two consecutive 24-hour recordings, based on 0.25 Hz frequency bins. Shading depicts the standard error. Vigilance states (Wake, NREM, REM and REM2) are colour coded (see inlet figure legend).

In recordings under baseline conditions (~48 hours of continuous recordings) all animals (n=3) spent most of the time asleep (85.6%, 70.6% and 71.5% of recording time, respectively), similar to previously reported sleep durations in the ferret ($70.34 \pm 1.69\%$, Jha, Coleman and Frank, 2006). Sleep was dominated by NREM sleep across all animals (51.1%, 39.9% and 41.4% of recording time, Fig.4A-C) with approximately a quarter of recording time spent in REM and REM2 sleep (30.9%, 24.3% and 26.7%, respectively). Two out of the three animals spent more time in REM than in REM2 (Case1 and Case3, Fig.4A,C). Only Case2 spent more time in REM2 (16.2%) than in REM (8.1%)(Fig.4B).

Ferrets slept during both the light and the dark phase, yet all animals showed a marked increase in time awake after light onset (see ZT0 in Fig.4D-F). In two cases, the following 4-5 hours were dominated by wakefulness (Fig.4E,F), whereas Case1 – the animal that slept most overall – showed only a brief (~2h) increase in wakefulness (Fig.4D). During most of the remaining time in the light phase and in the dark phase (marked by the black

horizontal bar in Fig.4D-F) animals were asleep. Note that the spike in time awake at ZT10 (Case1 and Case3) coincided with the manual start and restart of the recording software every 24hours (at ZT10), which required the presence of the experimenter in the room.

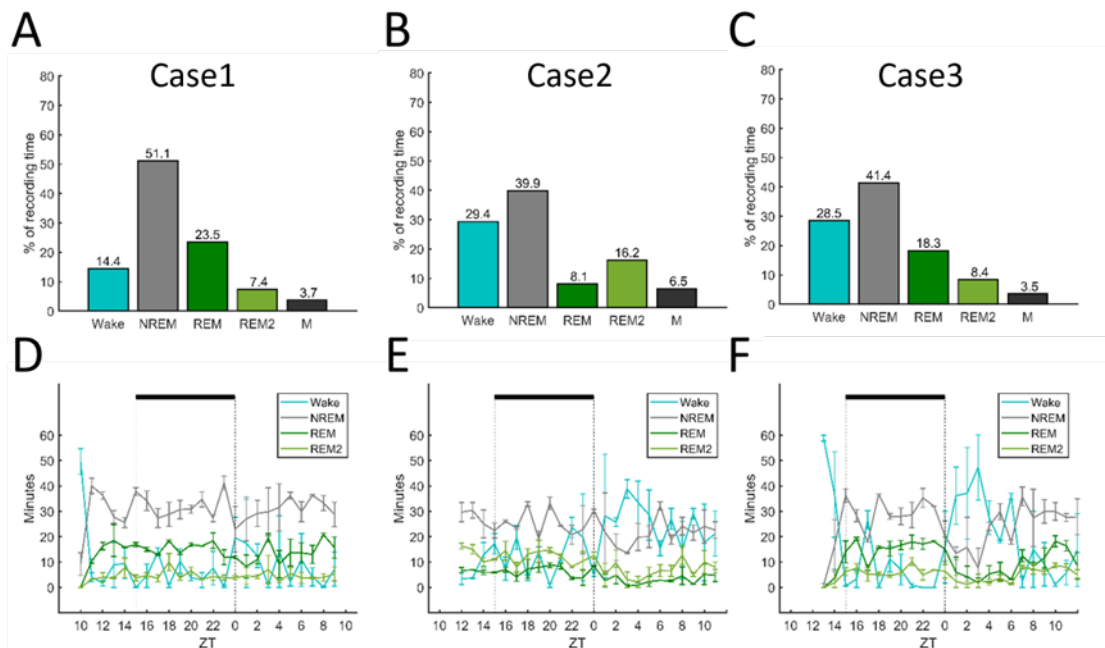


Fig. 4 Vigilance state distributions under baseline conditions. (A-C) Amount of time spent in Wake, NREM, REM, REM2 and M (movement artefacts within sleep episodes) as % of total recording time. Each panel (A-C) represents a different animal (Case1, Case2, Case3). **(D-F)** Time course of Wake, NREM, REM and REM2 states across 24 hours for Cases 1-3. Durations spent in each state are depicted in minutes per 1h-bin. Data are averages over 2 consecutive 24h recording sessions (i.e. based on 2 datapoints per bin). Error bars are standard deviations. ZT is the Zeitgeber time, the black horizontal bar depicts the dark phase (light onset at ZT0).

The animal with the largest amount of sleep during baseline recordings also showed the lowest number of wake episodes, i.e. sleep was least fragmented in this animal (Fig.5A). Both other animals showed more wake episodes, indicating that their sleep was more often interrupted, either due to external triggers or spontaneous awakening. Wake episodes were in general rather brief ($9.9 \pm 9.93 \text{ min}$, $5.83 \pm 7.37 \text{ min}$, $11.73 \pm 17.39 \text{ min}$ for Cases 1-3, Fig.5B) but differed between animals ($F_{(2,231)} = 6.28$, $p = 0.002$, ANOVA) with

Case2 showing significantly shorter episodes than Case3 ($p=0.002$, *Tukey post-hoc*).

Cases 2 and 1 were similar ($p=1.3$), as were Cases 3 and 1 ($p=0.71$).

Therefore, all ferrets spent the majority of time asleep, including during much of the light phase. And while animals showed little wakefulness throughout the recording, they did show a common increase in propensity for wakefulness after the end of the dark phase, suggesting an arousing effect of light onset or an intrinsic induction of more wakefulness after ZT0, possibly due to circadian rhythmicity. Indeed, previous work suggest that ferrets have only a modest diurnal organization of sleep-wake amounts (Jha et al., 2006), in line with this study. Despite the commonalities in sleep-wake architecture between Cases1-3, animals showed varying sleep duration and sleep fragmentation as well as different proportions of sleep stages, especially regarding REM and REM2.

As reported in Chapter2, NOE was associated with significant changes in behaviour and auditory processing. Alongside that, the three animals that were part of the chronic experiment (Cases1-3) showed varying changes in sleep architecture and spectral composition after NOE, as is described below. To evaluate these dynamics in the context of the individual changes in behaviour and in ABR paradigms, the three cases are in the following discussed individually. Results of Case1 are summarized in Figures 6-16, Case2 in Figures 17-27 and Case3 in figures 28-38, with a common summary in Fig. 39.

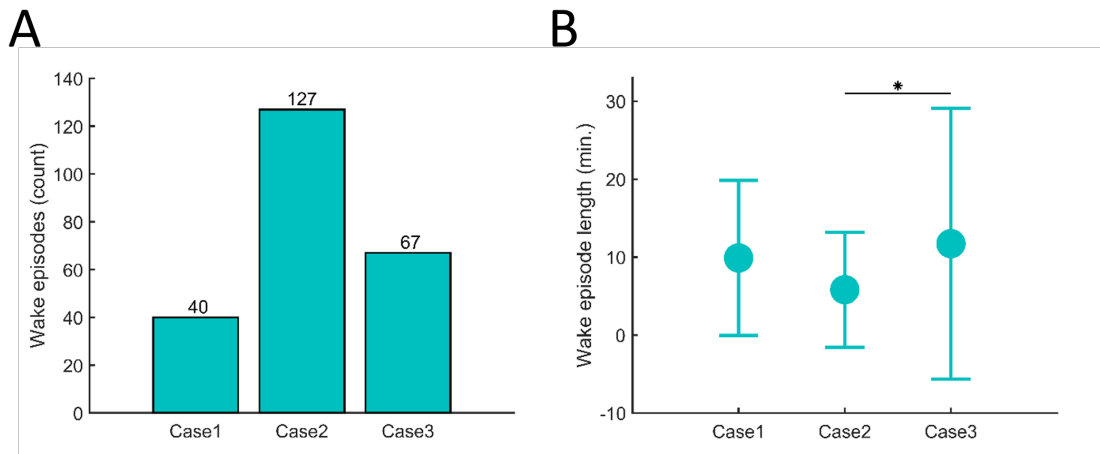


Fig. 5: Sleep fragmentation under baseline conditions. (A) Number of wake episodes (>1min) for Cases 1-3 during ~48 hours of baseline recording. The y-axis and the number displayed above bars depicts the count of wake episodes. **(B)** Duration of wake episodes for Cases 1-3. Data are animal means and standard deviations. ANOVA (factor animal), Tukey's multiple comparisons; horizontal marker with * signifies significant difference ($p<0.01$).

Case 1

Sleep-wake pattern: increased wakefulness and fragmented sleep after NOE

Initially after noise exposure Case1 showed little difference in time awake between BL and Post1, yet time awake was 6% higher in Post2 (*BL 14.4%, Post1 12.7%, Post2 21.4%*, Fig.6A). This was not due to a decrease in NREM sleep, as the amount in NREM was nearly identical to BL conditions in Post2 (*BL 51.1%, Post1 55.4%, Post2 51%*, Fig.6B). However, REM sleep decreased progressively after NOE, over 7% lower than in BL 6 months after NOE (*BL 23.5%, Post1 19.3%, Post2 15.9%*, Fig.6C). REM2 showed an opposite trend, in that durations spent in this state were longer after NOE, although the proportion of REM2 remained overall low with no more than 10% of recording time spent in REM2 sleep.

In parallel to spending more time awake, the number of wake episodes (>1min of uninterrupted wakefulness) increased nearly two-fold in the Post2 condition relative to Baseline (*BL vs Post2: 70 vs 136 wake episodes*, Fig.7A). The duration of wake episodes remained stable throughout the experiment (*BL vs Post1, 9.9±9.93min vs 11.04±11.09min, p=0.94, BL vs Post2, 9.9±9.93min vs 7.82±9.3min, p=0.17, Wilcoxon rank-sum test*, Fig.7B) further corroborating the possibility that the animal's sleep was reduced due to more wake episodes and not because the animal maintained continuous wakefulness for longer. This raises the possibility that 6 months after NOE the animal's sleep episodes might not have been long enough to facilitate the transition from NREM to REM sleep, leading to the observed decrease in REM throughout the experiment. The effect on REM2 might have been less obvious due to its overall low propensity.

The possible interaction between increased wakefulness and loss of REM sleep is further illustrated by the time course of vigilance states: 6 months after NOE, the animal showed prominent bouts in time awake not only briefly after light onset as during BL conditions, but throughout the entire light phase (Fig.8B) and wakefulness amount even superseded the amount of NREM sleep occasionally (Fig.8D). Also, in the first 10 hours of the light period, the animal accumulated the most time awake (see 'Post2' in Fig.9A). During the same time interval, the animal accumulated hardly any time of REM sleep (see 'Post2' in Fig.9C), corroborating the possibility that increased wakefulness after NOE led to loss of REM sleep.

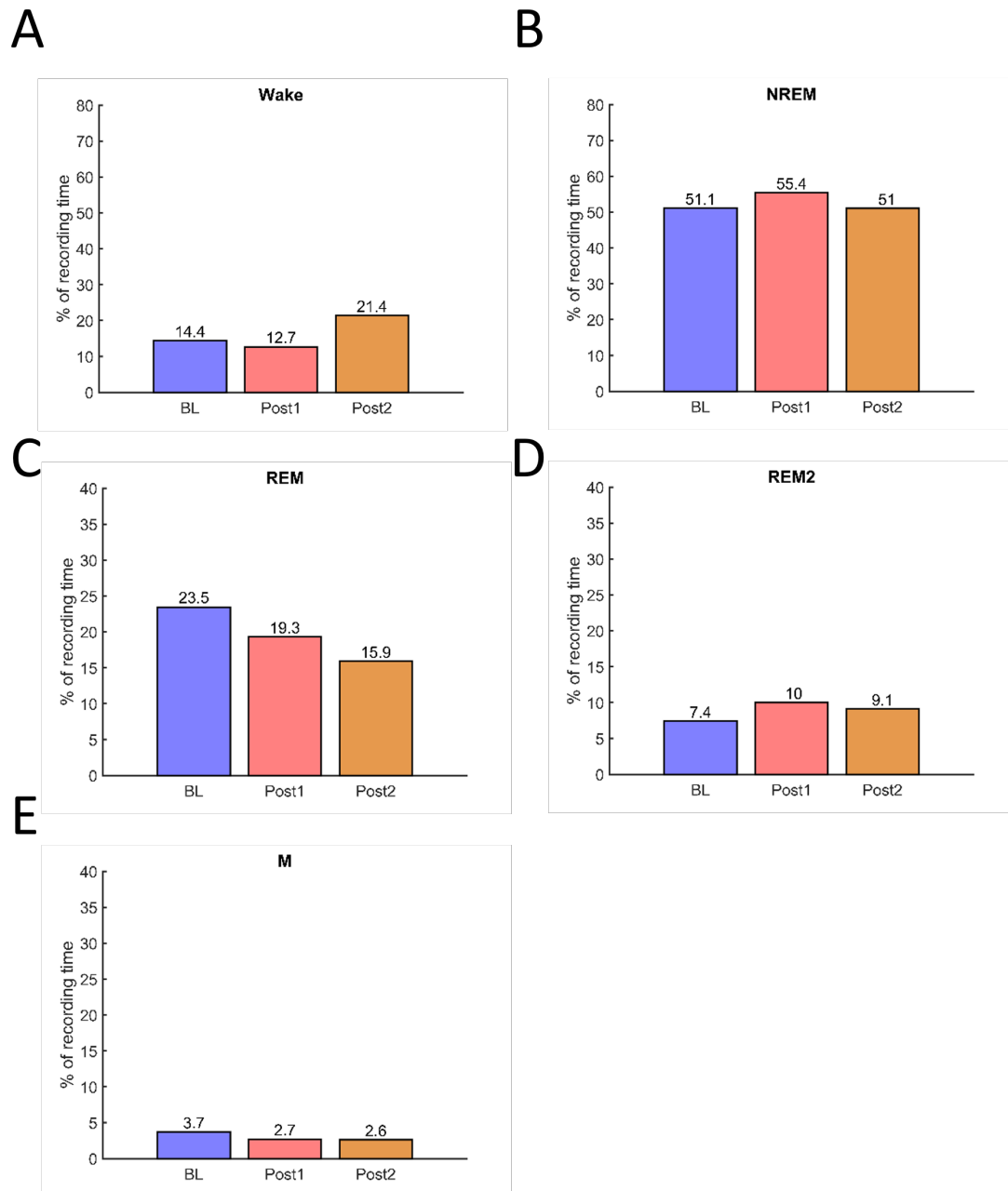


Fig. 6: Total vigilance state duration before and after NOE (Case1). Amount of time spent in Wake, NREM, REM, REM2 and M (movement artefacts within sleep episodes) as % of total recording time. Panels (A-E) represent Wake, NREM, REM, REM2 and M, respectively.

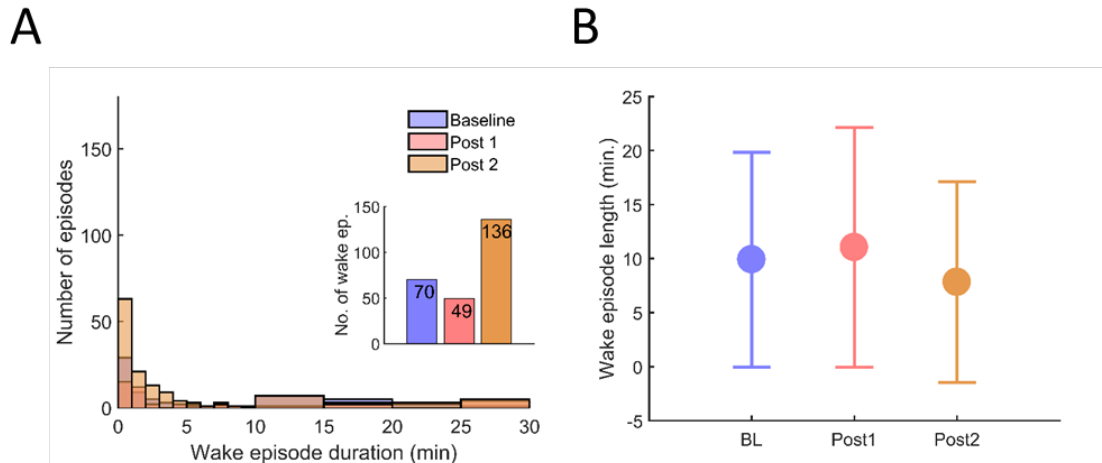


Fig.7: Sleep fragmentation before and after NOE (Case1). (A) Number of wake episodes during ~48 hours of recording (BL, Post1, Post2). The distribution of wake episodes by episode duration (ranging from 0-1min to 25-30 min. durations) is shown in the larger plot. The small inlet plot show the total numbers of all wake episodes during Baseline, Post1 and Post2 conditions. The y-axis and the number displayed above bars depicts the count of wake episodes. (B) Duration of wake episodes. Data are means $\pm$ standard deviations.

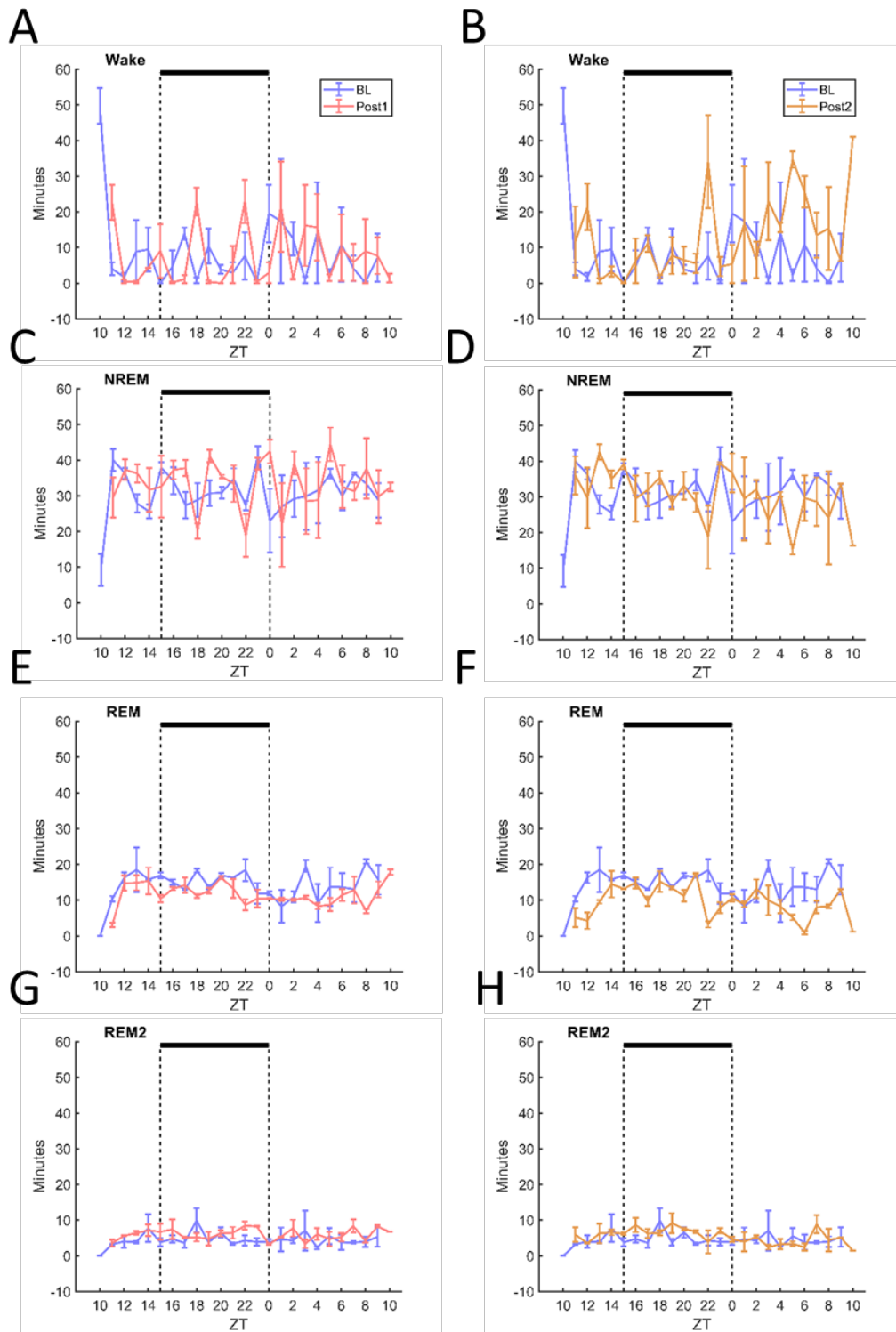


Fig.8: Time course of vigilance states before and after NOE (Case1). Time course of Wake, NREM, REM and REM2 states across 24 hours. Durations spent in each state are depicted in minutes per 1h-bin. Data are averages over 2 consecutive 24h recording sessions (i.e. based on 2 datapoints per bin) for the BL condition (blue markers), Post1 (red) and Post2 (brown). Error bars are standard deviations. ZT is the Zeitgeber time, the black horizontal bar depicts the dark phase (light onset at ZT0). Note that recording sessions started at varying times between ZT10 and ZT13 across BL, Post1 and Post2 sessions. The first datapoint of each plot is matched to the respective start time.

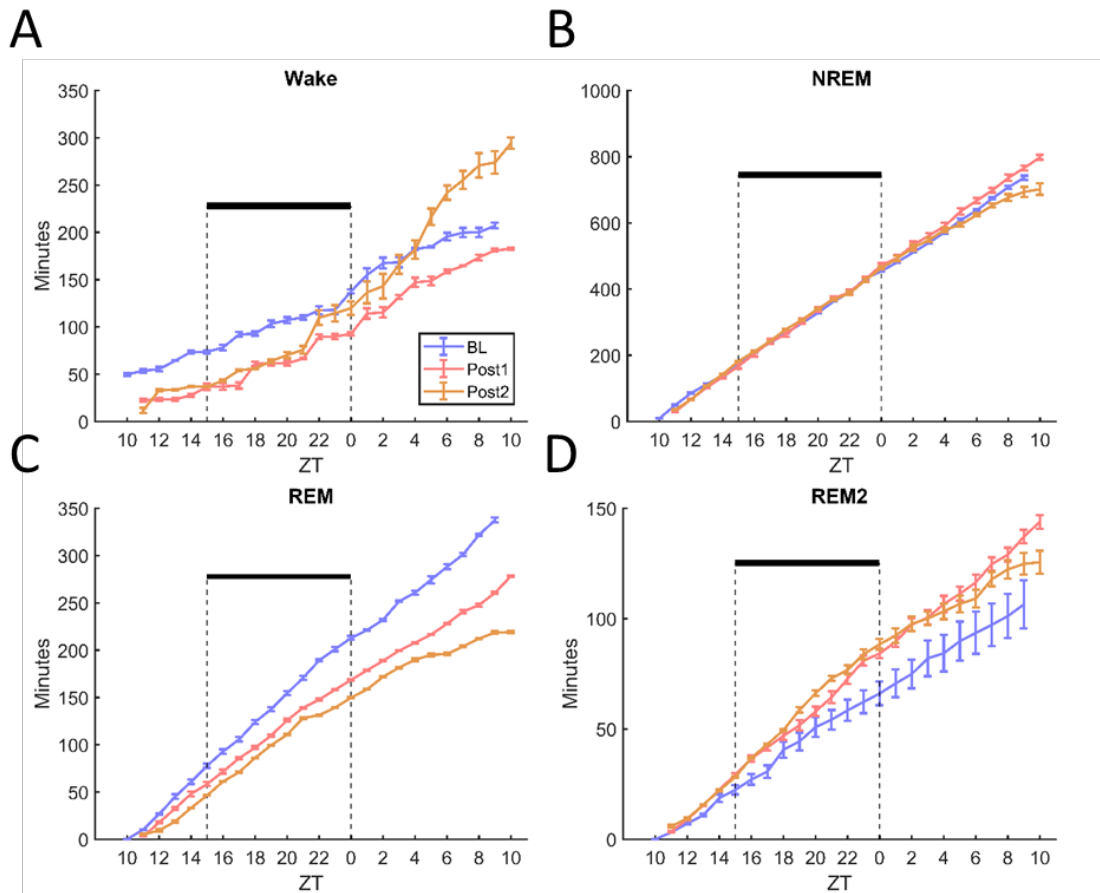


Fig.9 : Cumulative amount per vigilance state before and after NOE (Case1). Increment in state durations for **(A)** Wake, **(B)** NREM, **(C)** REM and **(D)** REM2 across the 24h cycle are depicted in steps of 1h bins. Data are averages over 2 consecutive 24h recording sessions (i.e. based on 2 datapoints per bin) for the BL condition (blue markers), Post1 (red) and Post2 (brown). Error bars are standard errors. ZT is the Zeitgeber time, the black horizontal bar depicts the dark phase (light onset at ZT0). Note that recording sessions started at varying times between ZT10 and ZT13 across BL, Post1 and Post2 sessions. The first datapoint of each plot is matched to the respective start time.

EEG spectral composition: widespread increase in frontal cortical EEG power, accompanied by opposite dynamics in occipital EEG

Initially after NOE, wake EEG spectra remained largely similar to BL conditions in the frontal EEG, although theta activity and activity in the beta range were elevated

(Fig.10A). 6 months after NOE, however, spectral power was elevated across wide frequency ranges (Fig.10B). Occipitally, spectral power broadly decreased initially after NOE (Fig.10C) but this change was not maintained long-term (Fig.10D).

During NREM sleep, the broad elevation in frontal EEG spectral power evident during wakefulness 6 months after NOE was already evident much earlier, in Post1 conditions (Fig.11A) and remained long-term (Fig.11B), whereas occipitally, a decrease in power across the entire spectrum (0.5 – 20Hz) became evident 6 months after NOE (Fig.11D).

During REM and REM2 sleep a similar widespread decrease in occipital spectral power was evident after NOE (Figs.12C,D and 13C,D), but a frontal increase in power was only temporary and restricted to mostly the delta and theta ranges for REM sleep (Fig.12A,B), while for REM2 this was also evident in higher frequency ranges and remained 6 months after NOE, similar to NREM sleep and wakefulness.

Thus, the frontal increase in spectral power and the occipital decrease in the right cortical hemisphere was visible in all states although most clearly in NREM sleep, the most abundant vigilance state (see Fig.14 for summary). As the effect was present across the majority of the analysed spectrum and visible in all states, the elevation in spectral power may be an indicator for increased cortical excitability possibly related to consequences of noise exposure.

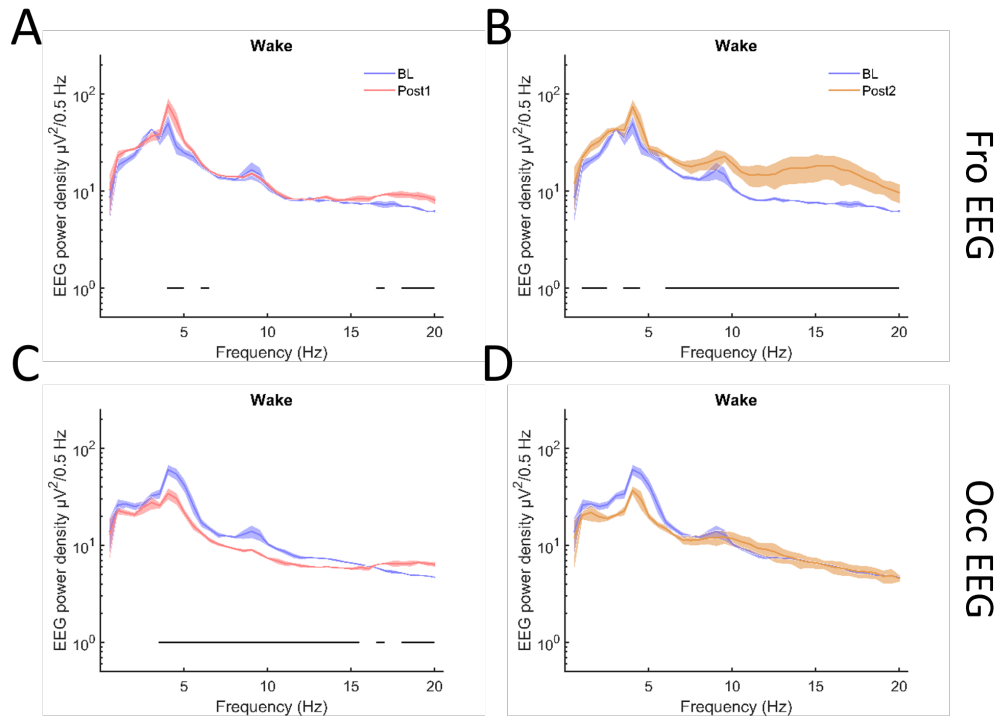


Fig.10: Wake EEG power spectra before and after NOE (Case1). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

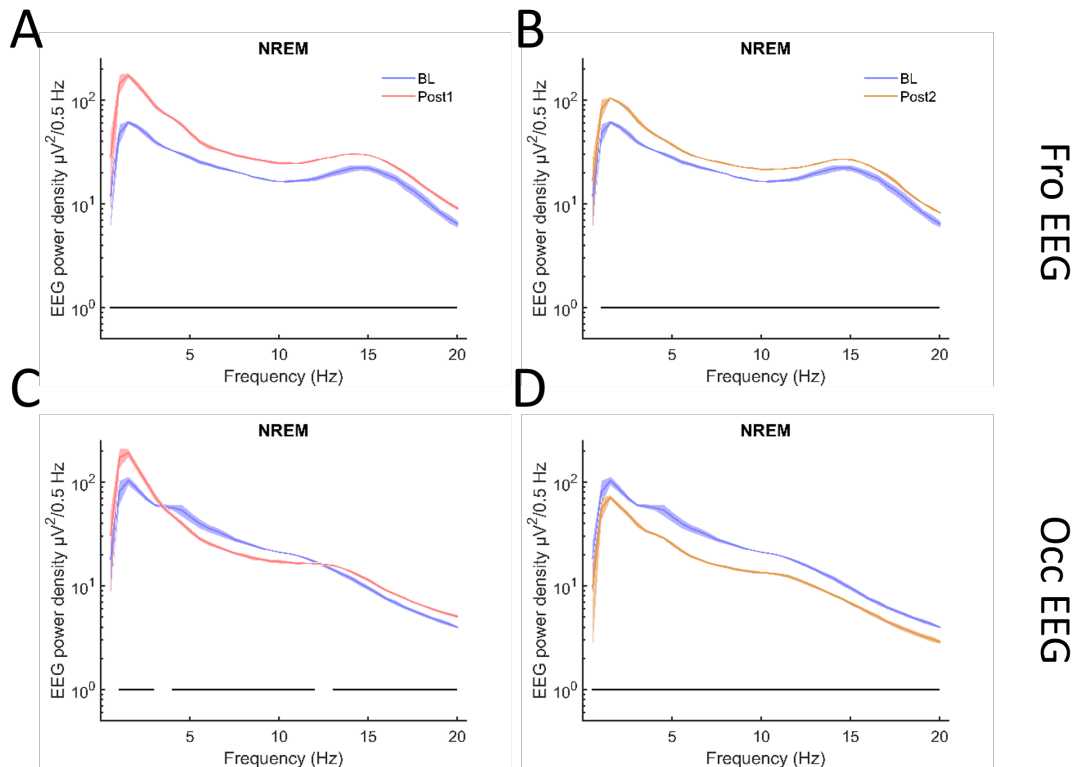


Fig.11: NREM EEG power spectra before and after NOE (Case1). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

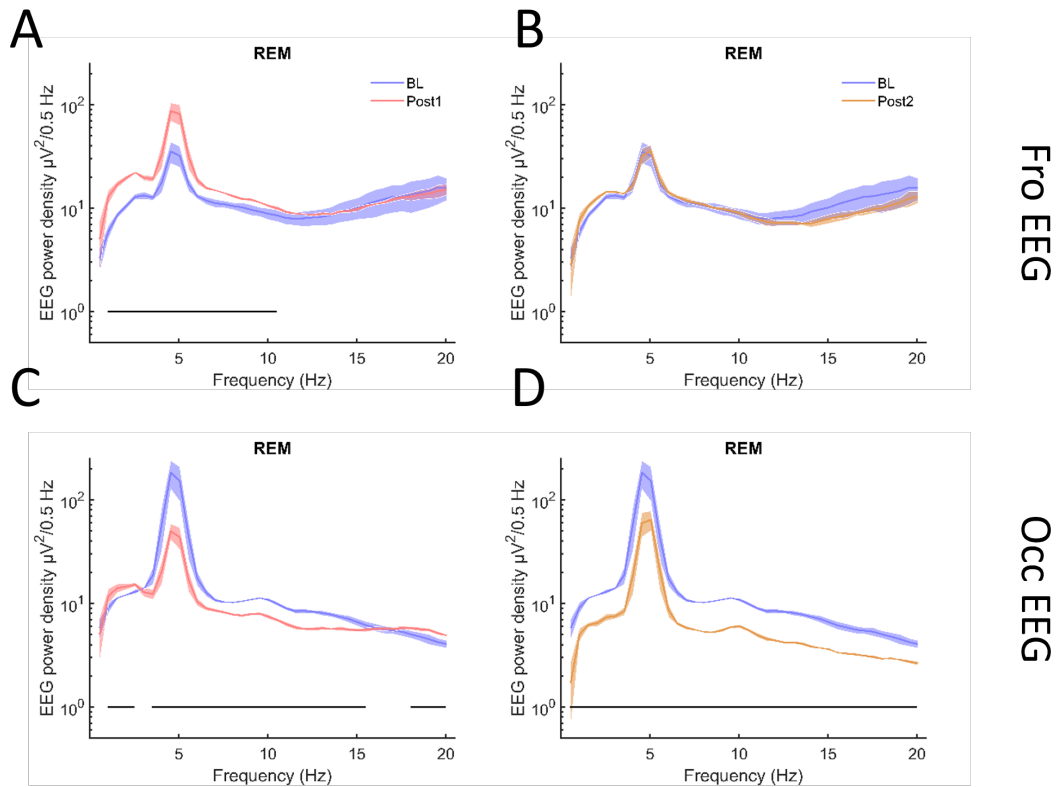


Fig.12: REM EEG power spectra before and after NOE (Case1). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

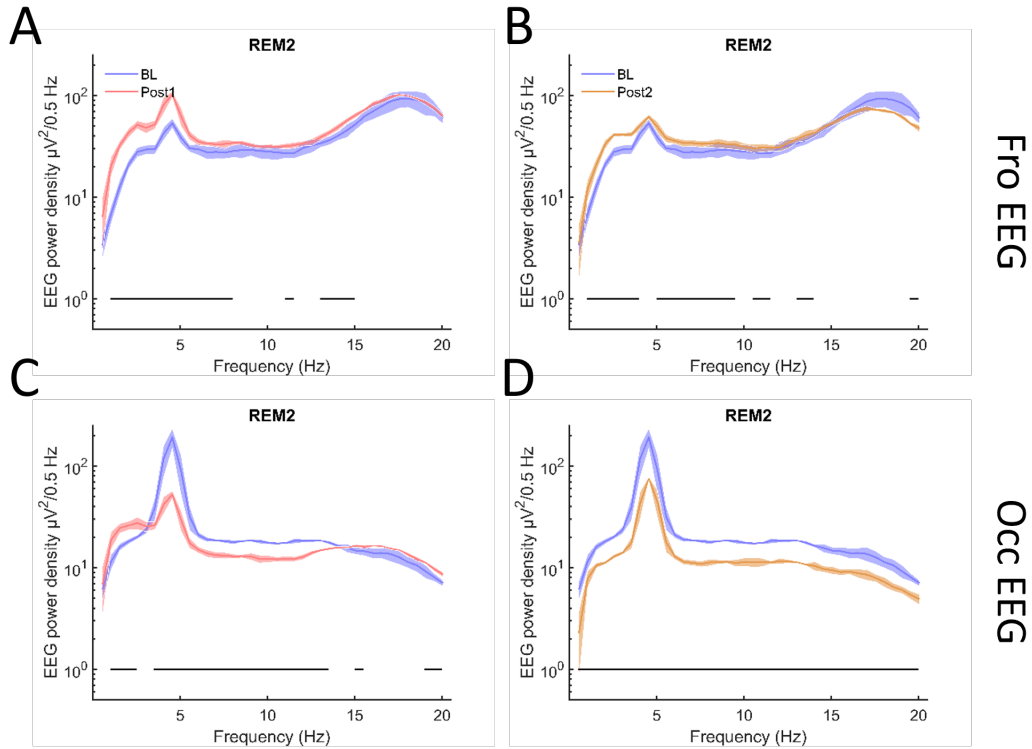


Fig.13: REM2 EEG power spectra before and after NOE (Case1). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

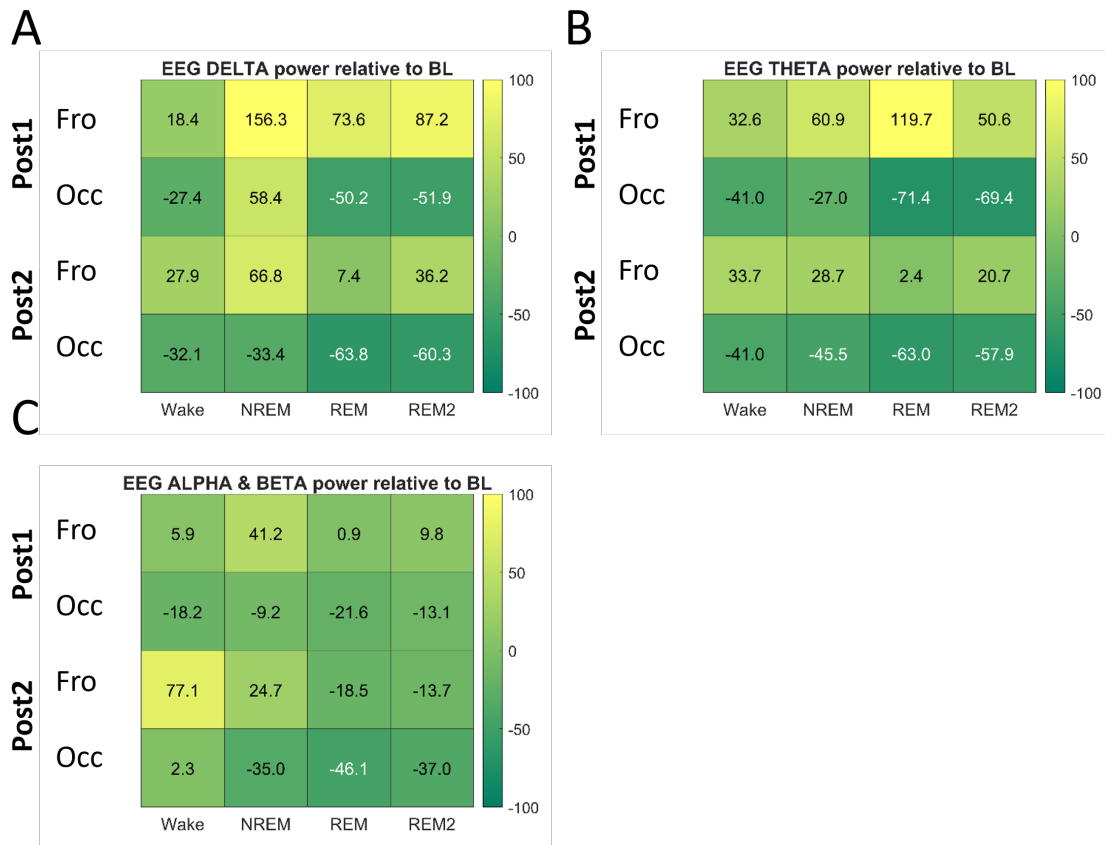


Fig.14: Change in EEG spectral power in all vigilance states after NOE relative to BL (in %)(Case1). (A) EEG Delta power (0.5-4Hz), (B) EEG Theta power (4.5-8Hz), (C) EEG Alpha and low Beta power (8.5 – 20Hz). Values depict changes in average spectral power density in percent relative to baseline based on recordings over approx. 48 hours on two separate occasions after NOE (Post1 and Post2) . Data are separated by vigilance state (Wake, NREM, REM and REM2) and by EEG derivation (frontal (FRO) and occipital (OCC)).

Evidence for elevated homeostatic sleep need after NOE

A notable dynamic that differed from the general frontal increase and occipital decrease in EEG spectral power after NOE was EEG delta power during NREM sleep.

This increased in both frontal and occipital derivations early after NOE (Fig.11A,C). This elevated frontal delta activity was maintained throughout the 24h cycle (Fig.15C), yet the increase in occipital delta activity was mainly present after light onset and less so during the dark period (Fig.16C). Interestingly, an increase in delta was evident in REM

and REM2 sleep as well and also most pronounced in the first half of the light period (Fig.15E). Therefore, the increase in delta activity was mainly evident during the time of day where the animal tended to show more wakefulness, even though in Post1 conditions sleep was rather evenly distributed throughout the 24h cycle (Fig.8). That said, in the Post1 condition the animal also slept more overall and sleep was less fragmented than during BL (Fig.5, Fig.6A), which, in combination with the globally elevated EEG delta activity (frontally and occipitally) points towards elevated homeostatic sleep need. It is well described that delta activity – often referred to as slow-wave activity – is elevated after wakefulness and decreases during subsequent sleep, representing recovery from elevated homeostatic sleep need (Bernardi et al., 2015; Hung et al., 2013; Tobler & Borbély, 1986; Vyazovskiy et al., 2011). The elevated sleep SWA in both frontal and occipital derivations could be a sign of increased sleep intensity in response to high sleep pressure.

This, however, was only evident temporarily as 6 months after NOE (Post2) NREM SWA elevation was less pronounced in the frontal derivation (Fig.15D,F) and disappeared entirely in the occipital EEG, where EEG spectral power broadly decreased (Fig.16D). As the animal spent more time in wakefulness in the Post2 condition (Fig.7A) and sleep was also more disrupted at that point (Fig.8A) it seems likely that the initial increase in sleep need after NOE was unrelated to time spent awake and rather due to other factors such as waking experience (Fisher et al., 2016; Milinski et al., 2021a), which could be impacted by drastically changing sensory experience after noise overexposure.

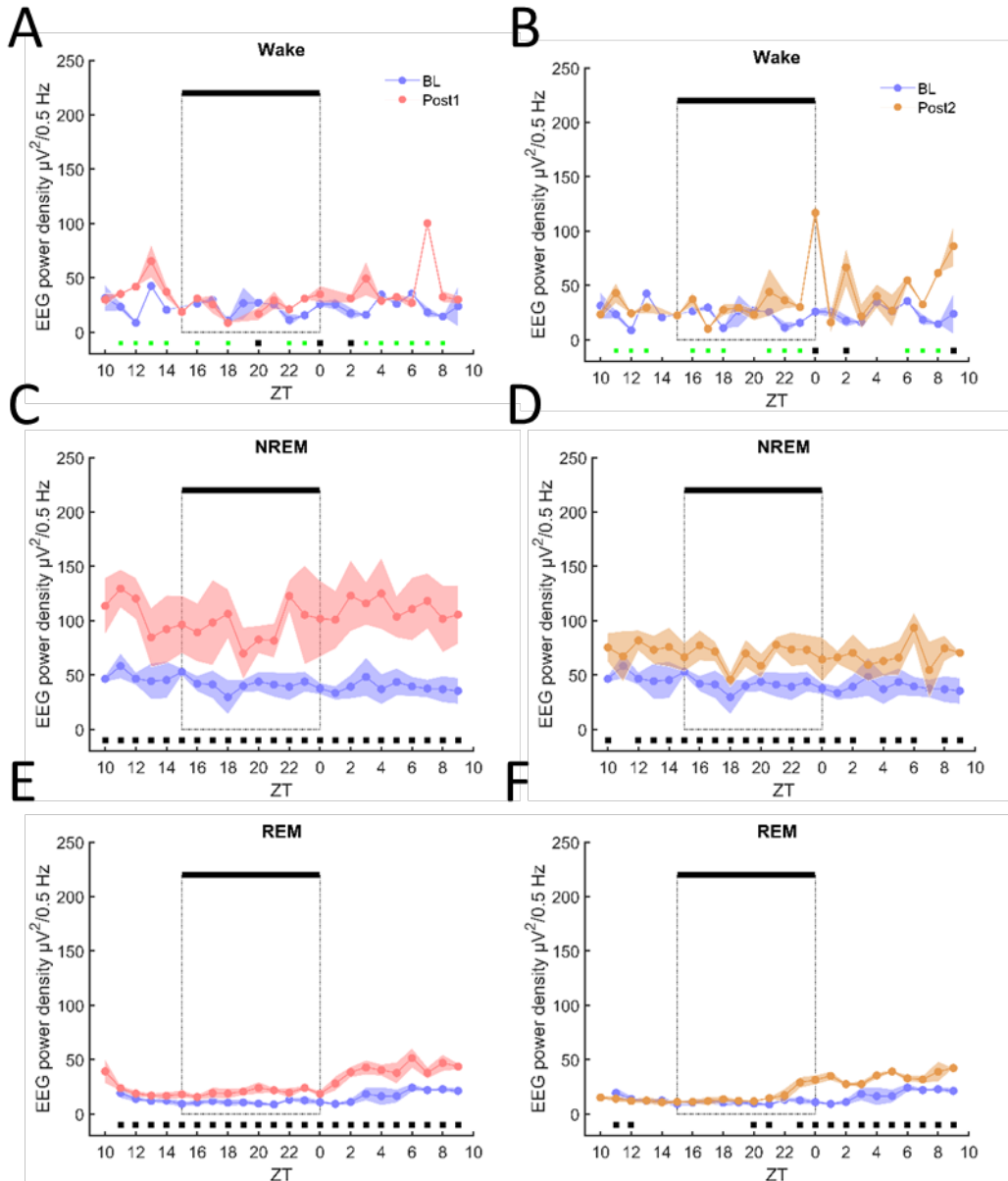


Fig.15: Time course of frontal EEG SWA across vigilance states (Case1). (A,B) Time course of Wake SWA BL vs Post1 and for BL vs Post2. (C,D) Time course of Wake SWA BL vs Post1 and for BL vs Post2. (E,F) Time course of REM SWA (REM and REM2 are merged in this analysis) for BL vs Post1 and for BL vs Post2. Initially, SWA across 48 hours of recording for each vigilance state was calculated per 15-min interval, before being merged into 1h intervals. Data of corresponding 1h-intervals in the two consecutive 24h cycles of 48 hours of continuous recording were then merged for analysis, resulting in 8 datapoints per 1h interval per condition. Shaded error bars are standard deviations. Two-way ANOVA (factors condition and time interval), Tukey's multiple comparisons; black square markers = $p < 0.05$. Green squares mark time intervals with less than 3 datapoints in either condition.

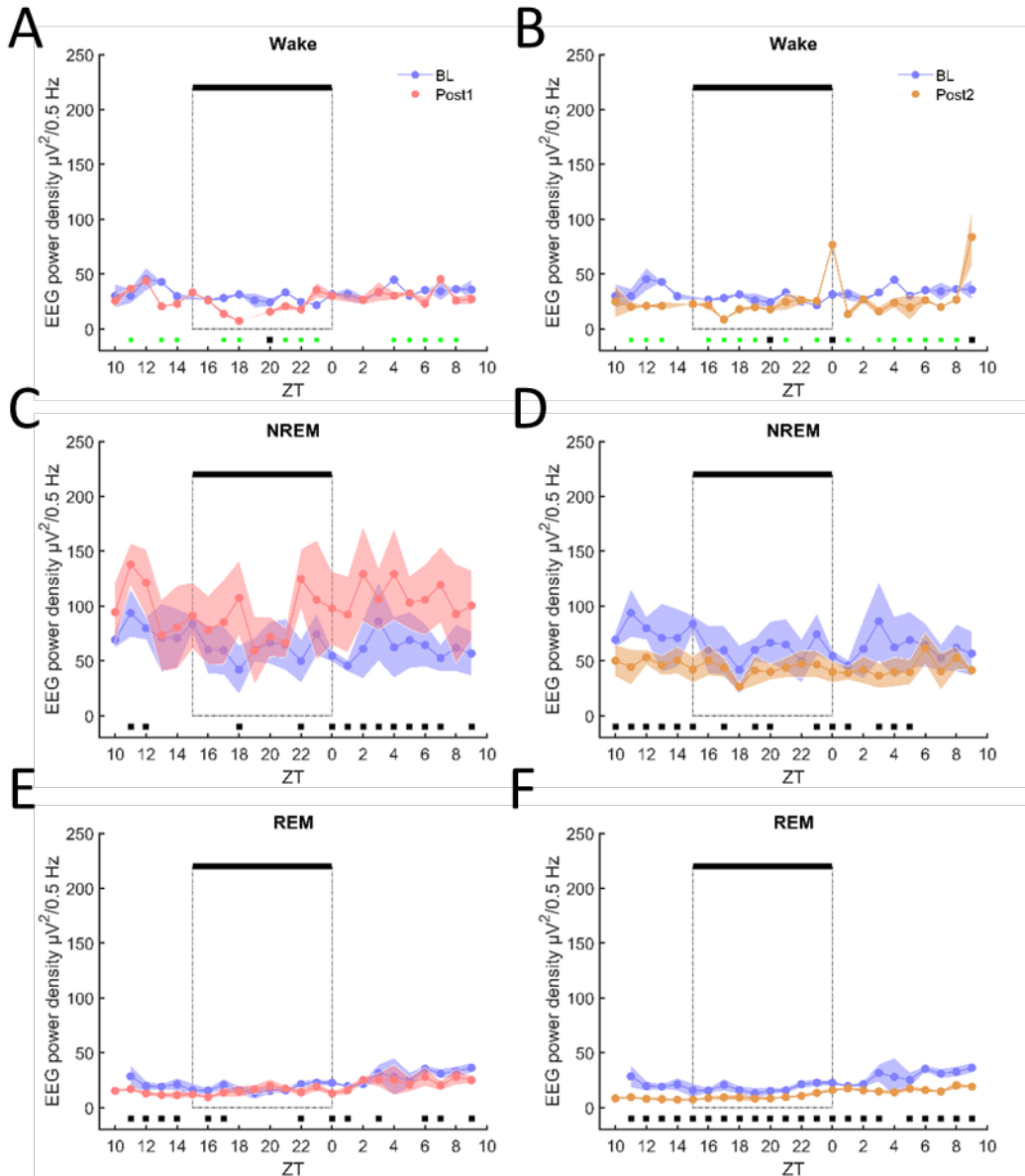


Fig.16: Time course of occipital EEG SWA across vigilance states (Case1). Time course of Wake SWA BL vs Post1 and for BL vs Post2. **(C,D)** Time course of Wake SWA BL vs Post1 and for BL vs Post2. **(E,F)** Time course of REM SWA (REM and REM2 are merged in this analysis) for BL vs Post1 and for BL vs Post2. Initially, SWA across 48 hours of recording for each vigilance state was calculated per 15-min interval, before being merged into 1h intervals. Data of corresponding 1h-intervals in the two consecutive 24h cycles of 48 hours of continuous recording were then merged for analysis, resulting in 8 datapoints per 1h interval per condition. Shaded error bars are standard deviations. Two-way ANOVA (factors condition and time interval), Tukey's multiple comparisons; black square markers = $p < 0.05$. Green squares mark time intervals with less than 3 datapoints in either condition.

Summary of Case1:

Evidence for tinnitus in behavioural performance, disturbed sleep and changes in cortical excitability

Case1 showed a positive Tinnitus Index after NOE (TI Post1: 0.5, Post2: 0.5), representing behavioural evidence for tinnitus (see 'TI' for Case1 in Figs.39,40) in parallel with a substantial decrease in ABR magnitude by over 30% compared to BL and a threshold elevation of 13.3 dB SPL (Figs.39,40). In the context of the evidence for elevated sleep pressure at this time (increased NREM and REM SWA, and reduced sleep fragmentation) the question remains of whether temporary dynamics in brain plasticity or in sensory experience after NOE affected sleep homeostasis.

Notably, 6 months after NOE, thresholds elevated further (by 25 dB SPL relative to BL) while ABR magnitude recovered partially (from Post1: -31.2% decrease to Post2: -7.7% decrease), raising the possibility of a long-term compensation through central or peripheral gain elevation. Tinnitus in this animal remained stable six months after NOE. The increased sleep fragmentation at the same time (Fig.7A, 'Post2') suggests that the animal might have become more sensitive to external stimuli (hyperacusis) or to internal triggers for arousal (possibly tinnitus). Indeed, increased gain in the auditory brainstem or possibly on cortical level after NOE could play a role in amplifying external sensory stimulation or spontaneously generated activity and therefore making sleep less stable. In line with this, the animal spent more time awake in the Post2 assessment.

Case 2

Sleep-wake pattern: increased sleep and less sleep fragmentation after noise overexposure

Case2 showed – opposite to Case1 – a continuous decrease in wakefulness after noise exposure (Fig.17A). In turn, NREM and REM sleep amount increased (*NREM BL 39.9%, Post1 48%, Post2 46.2%; REM BL 8.1%, Post1 7.8%, Post2 10%*, Fig.17B,C). Only REM2 sleep, while initially after NOE nearly identical to BL conditions, was lowest in the Post2 condition (REM BL 16.2%, Post1 16.1%, Post2 9.2%, Fig.17D).

Even though the animal spent more time asleep after NOE, the ferret's predominant waking time remained during the light phase, starting with a surge in wakefulness at light onset (Fig.19A,B). Interestingly, while during BL conditions the surge in wakefulness at ZT0 was mostly at the cost of REM and REM2 sleep ('BL' in Fig.20C,D), this dynamic was increasingly dampened after NOE, with both REM and REM2 accumulation following an approximately linear function in the Post2 condition ('Post2' in Fig.20C,D). The distribution of NREM sleep across the 24h cycle was largely maintained after NOE (Fig.19C,D, Fig.20B), even though NREM amount overall was increased.

In line with the continuously increasing sleep amount after NOE, the animal's sleep became progressively more stable: the number of wake episodes decreased after NOE after NOE (*BL vs Post1 vs Post2, 297 vs 203 vs 154 episodes*, Fig.18A). Wake episode lengths remained stable (*BL vs Post1, 5.83±7.37min vs 6.52±7.3min, p=0.72, BL vs Post2, 5.83±7.37 vs 6.72±7.5, p=0.61, Wilcoxon rank-sum test*, Fig.18B). This suggests that the animal did not have impaired ability to sustain wakefulness but rather maintained sleep for longer once in that state.

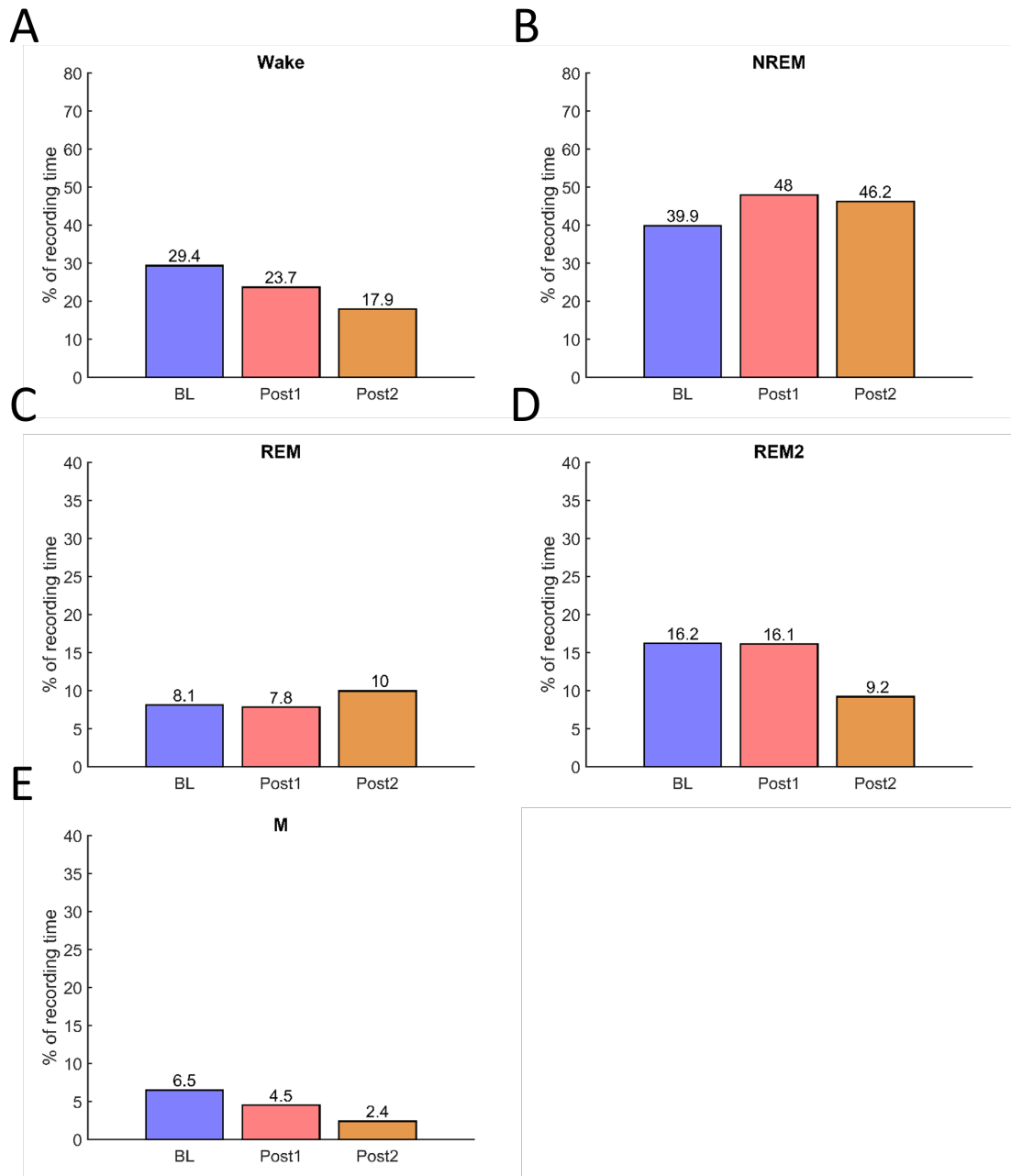


Fig.17: Total vigilance state duration before and after NOE (Case2). Amount of time spent in Wake, NREM, REM, REM2 and M (movement artefacts within sleep episodes) as % of total recording time. Panels (A-E) represent Wake, NREM, REM, REM2 and M , respectively.

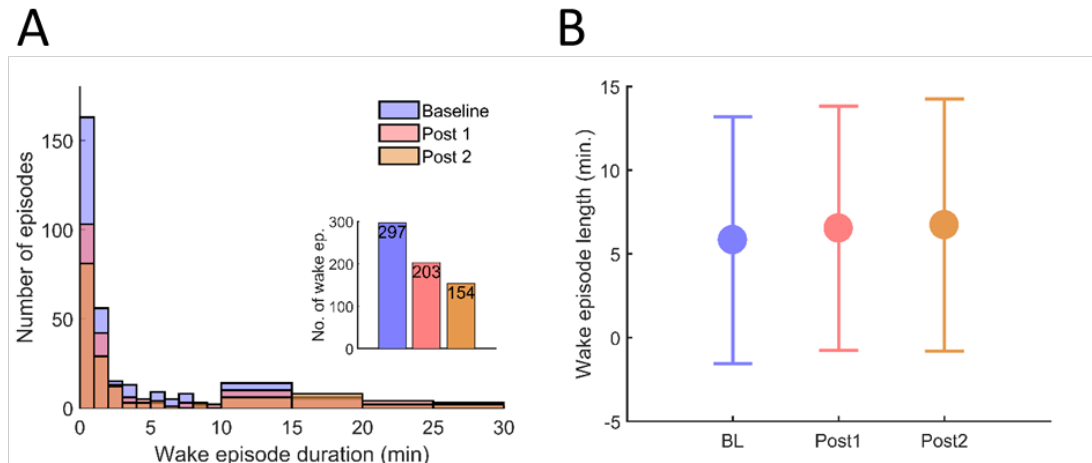


Fig.18: Sleep fragmentation before and after NOE (Case2). (A) Number of wake episodes during ~48 hours of recording (BL, Post1, Post2). The distribution of wake episodes by episode duration (ranging from 0-1min to 25-30 min. durations) is shown in the larger plot. The small inlet plot show the total numbers of all wake episodes during Baseline, Post1 and Post2 conditions. The y-axis and the number displayed above bars depicts the count of wake episodes. (B) Duration of wake episodes. Data are means $\pm$ standard deviations.

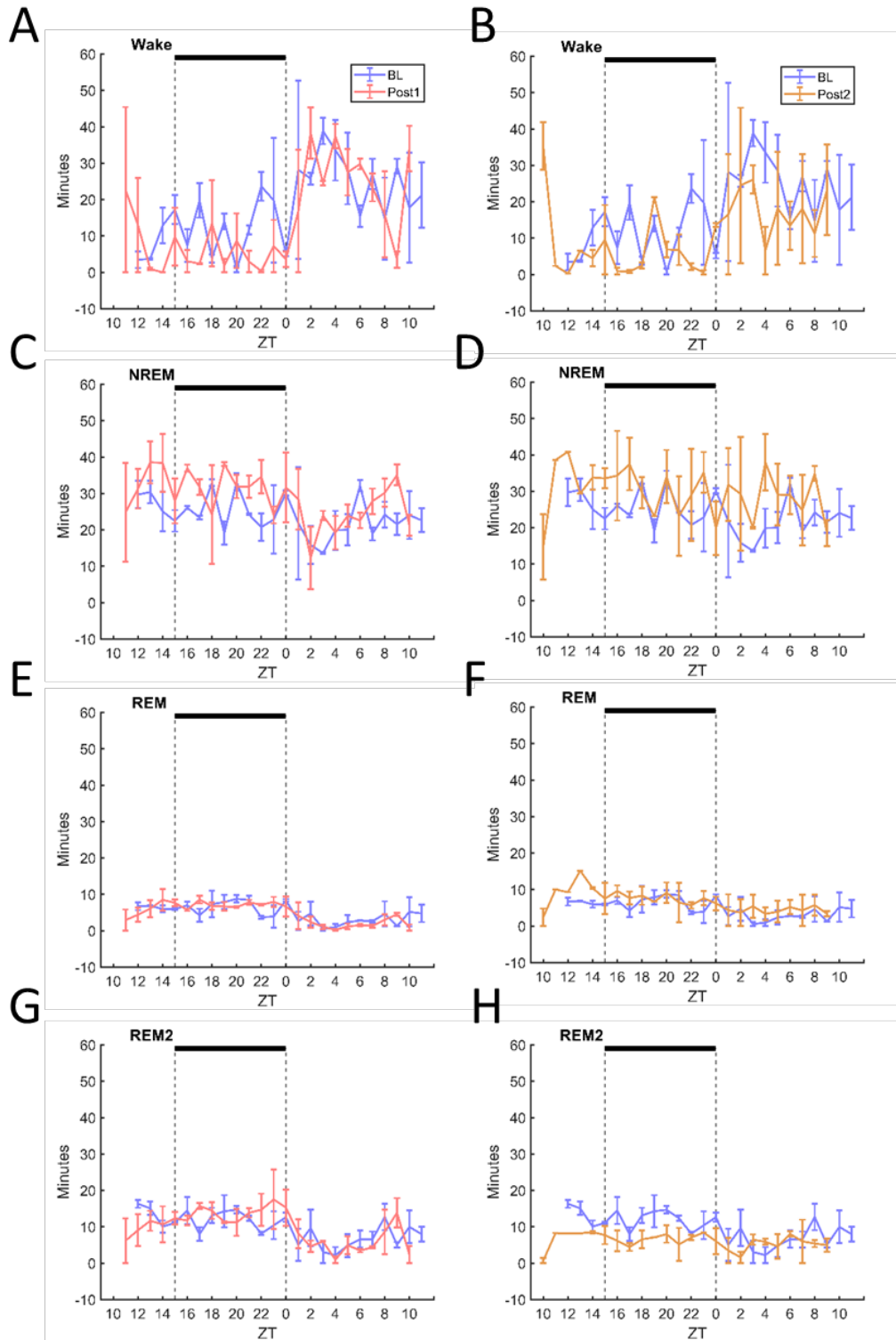


Fig.19 : Time course of vigilance states before and after NOE (Case2). Time course of Wake, NREM, REM and REM2 states across 24 hours. Durations spent in each state are depicted in minutes per 1h-bin. Data are averages over 2 consecutive 24h recording sessions (i.e. based on 2 datapoints per bin) for the BL condition (blue markers), Post1 (red) and Post2 (brown). Error bars are standard deviations. ZT is the Zeitgeber time, the black horizontal bar depicts the dark phase (light onset at ZT0). Note that recording sessions started at varying times between ZT10 and ZT13 across BL, Post1 and Post2 sessions. The first datapoint of each plot is matched to the respective start time.

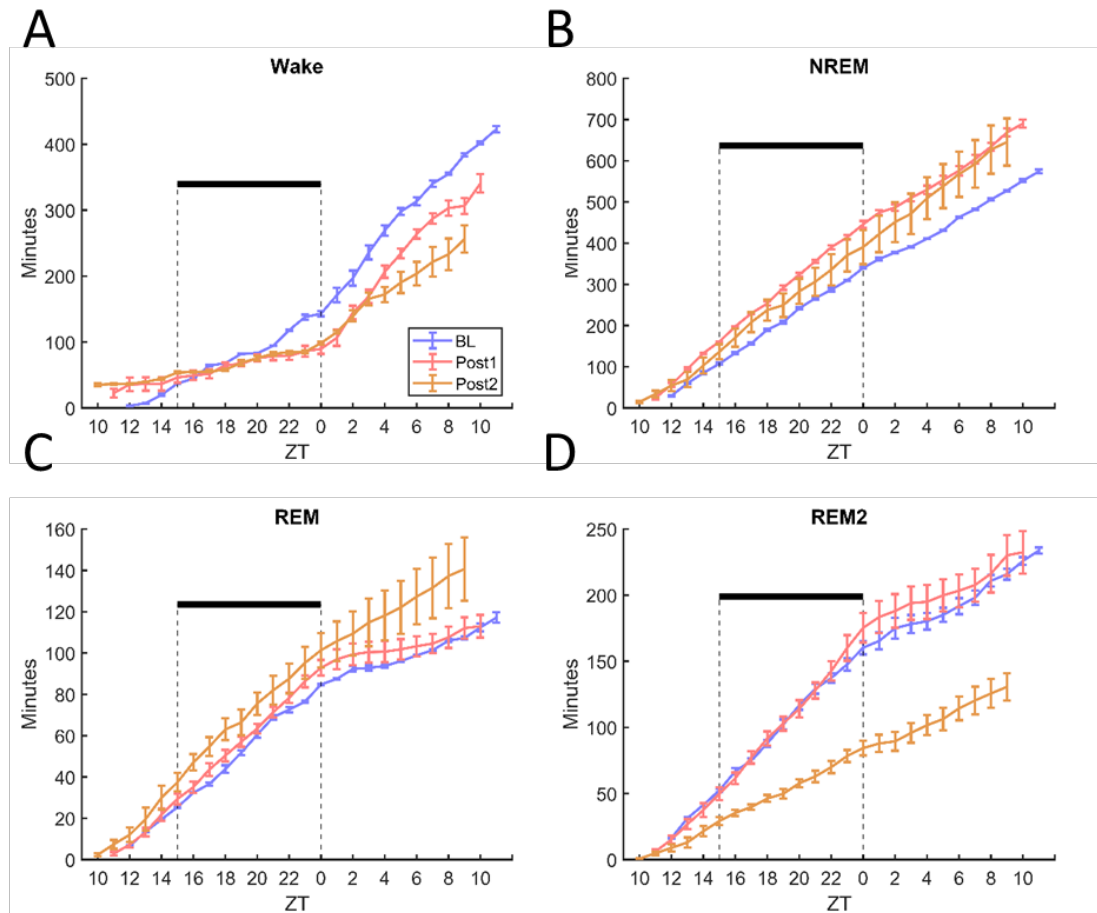


Fig.20 : Cumulative amount per vigilance state before and after NOE (Case2). Increment in state durations for (A) Wake, (B) NREM, (C) REM and (D) REM2 across the 24h cycle are depicted in steps of 1h bins. Data are averages over 2 consecutive 24h recording sessions (i.e. based on 2 datapoints per bin) for the BL condition (blue markers), Post1 (red) and Post2 (brown). Error bars are standard errors. ZT is the Zeitgeber time, the black horizontal bar depicts the dark phase (light onset at ZT0). Note that recording sessions started at varying times between ZT10 and ZT13 across BL, Post1 and Post2 sessions. The first datapoint of each plot is matched to the respective start time.

EEG spectral composition: a widespread decrease in spectral power after NOE during all vigilance states

In this animal EEG spectral power tended to decrease after NOE across wide frequency ranges: during wakefulness, the EEG composition shifted after NOE towards less pronounced delta, theta and low beta frequency ranges in both frontal and occipital EEG derivations (Fig.21). This was not evident anymore in the Post2 condition, when the animal spent little time awake (Fig.17A). During NREM sleep the EEG power decrease was even more widespread across frequencies <20Hz (Fig.22A,B,). In the occipital EEG this effect was less pronounced, with no post NOE power decrease evident in the low beta range during the Post1 condition (Fig.22C) and, in the Post2 condition, the occipital EEG power spectrum was similar to BL in the low delta, alpha and low beta range (Fig.22D). Similarly, during REM and REM2 sleep a widespread EEG power decrease was evident and more so in the frontal derivation for both REM sleep (Fig.23A,B) and REM2 sleep (Fig.24A,B) and less so for the occipital derivation (Fig.23c,D, Fig.24C,D). Interestingly, activity in the delta range was consistently decreased in both derivations in both REM and REM2 sleep.

Overall, it is of note that a predominant decrease in EEG power after NOE was evident in all assessed vigilance states (Fig.25). The fact that power did not decrease across all frequency bands (note especially wakefulness EEG spectra in Post2, Fig.21B,D) suggests that the widespread decrease in spectral power was not driven by a change in overall signal quality.

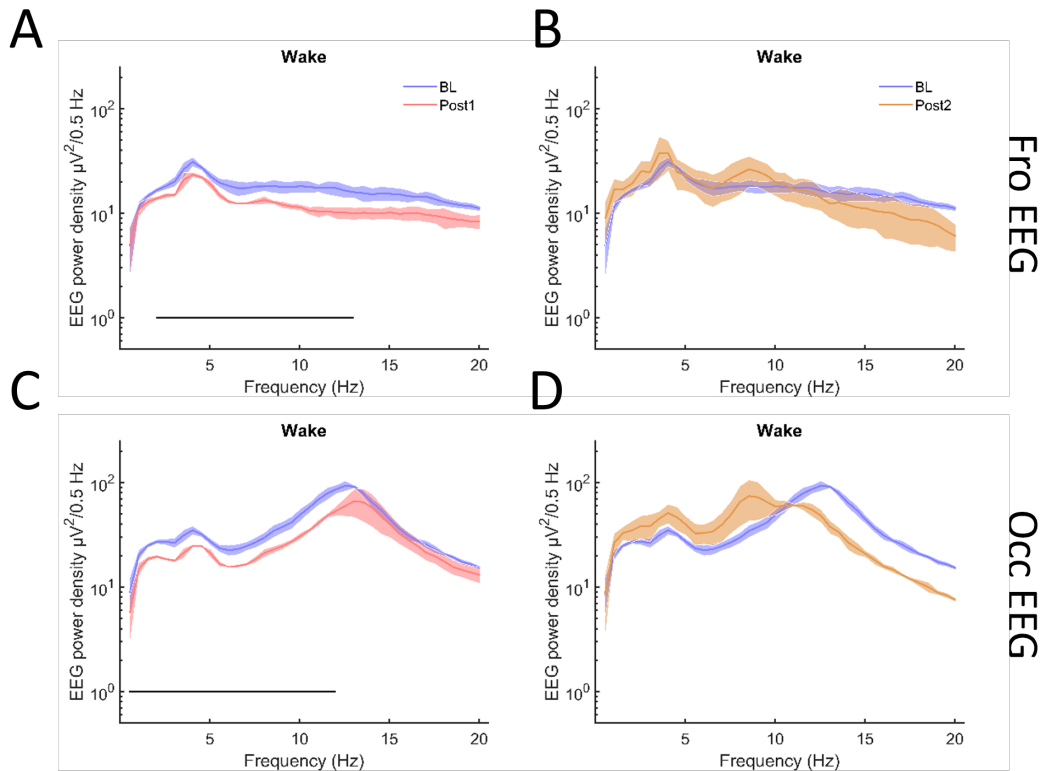


Fig.21: Wake EEG power spectra before and after NOE (Case2). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

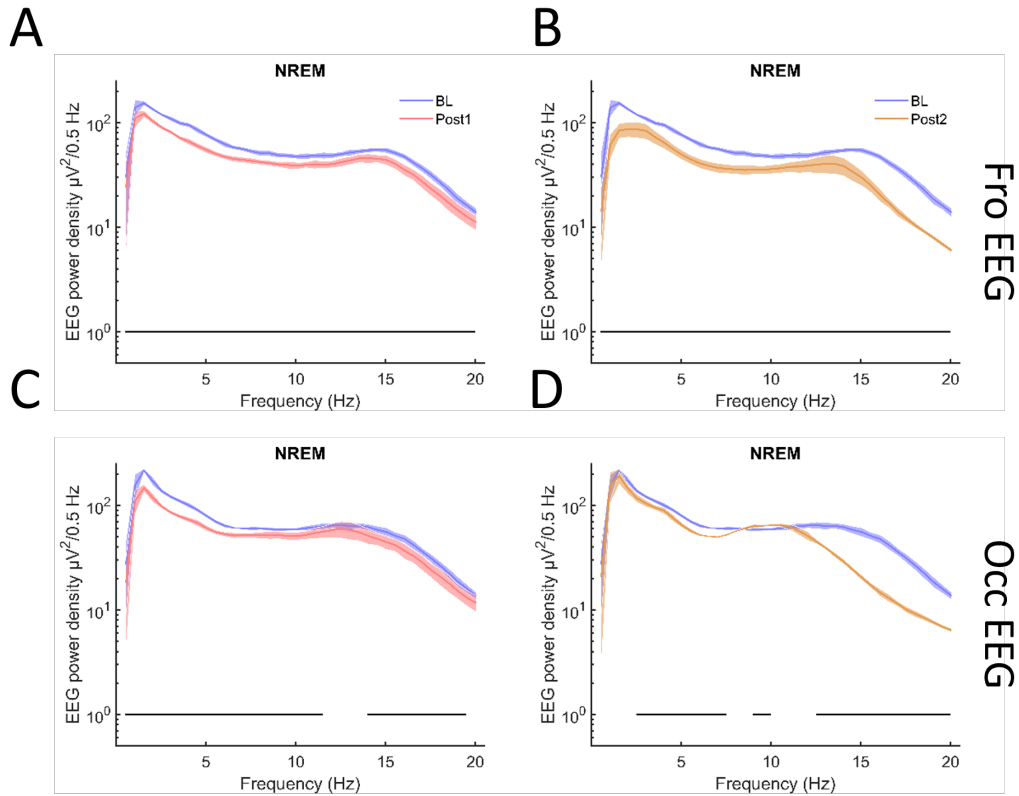


Fig.22: NREM EEG power spectra before and after NOE (Case2). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

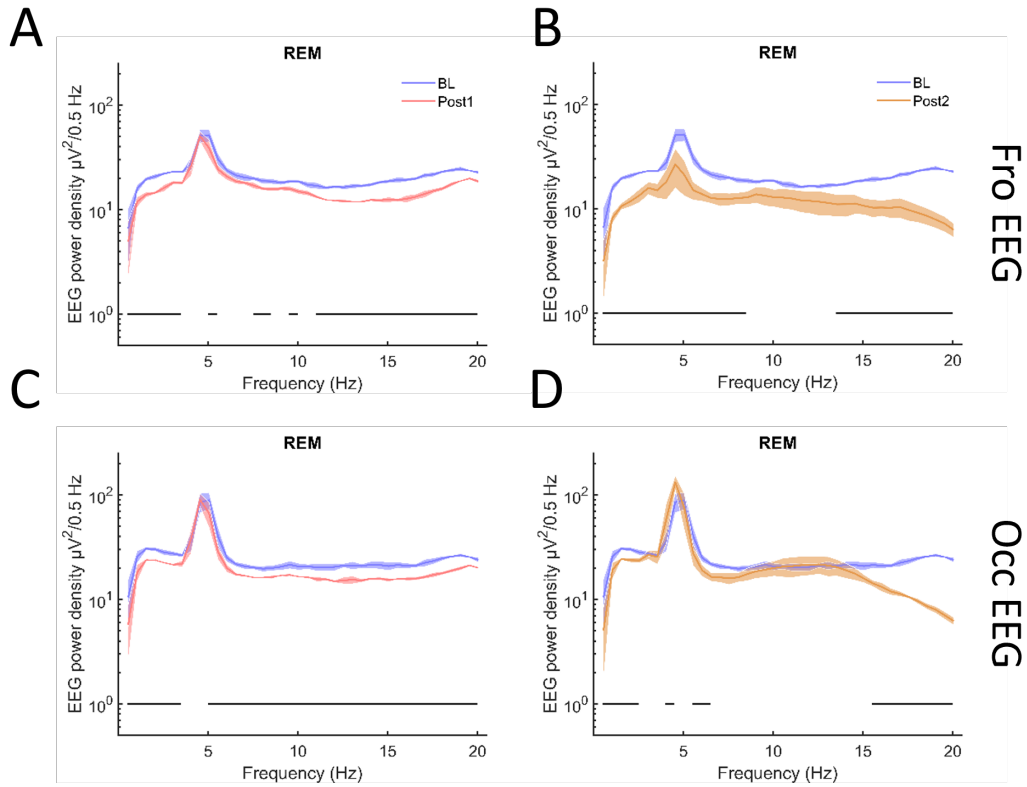


Fig.23: REM EEG power spectra before and after NOE (Case2). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

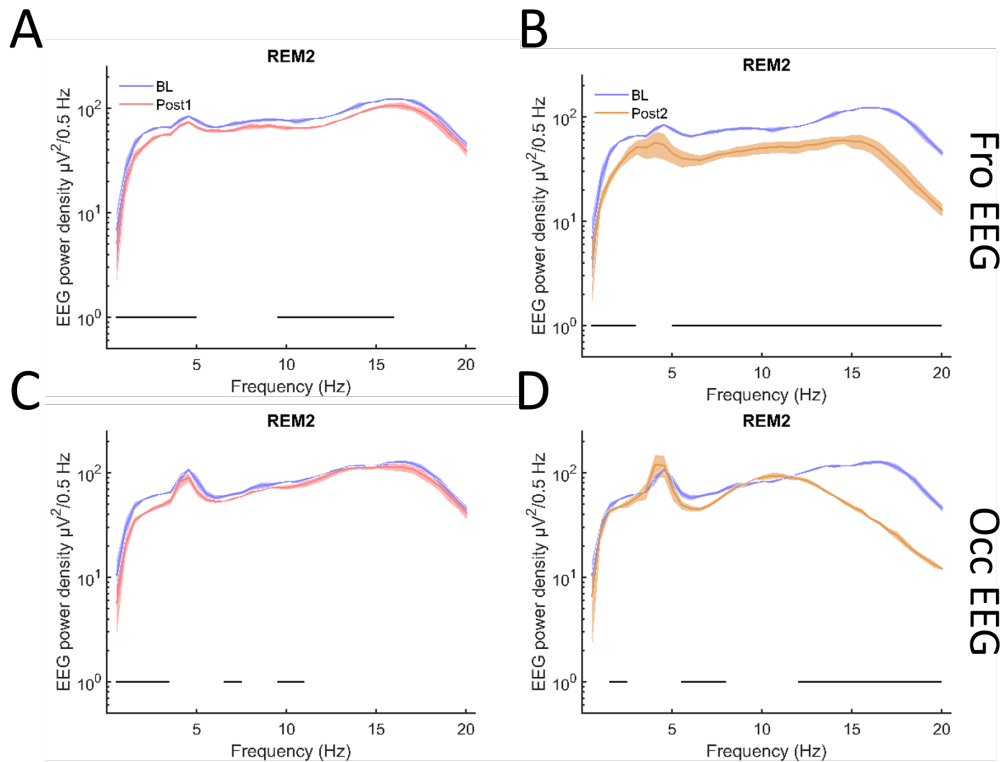


Fig.24: REM2 EEG power spectra before and after NOE (Case2). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

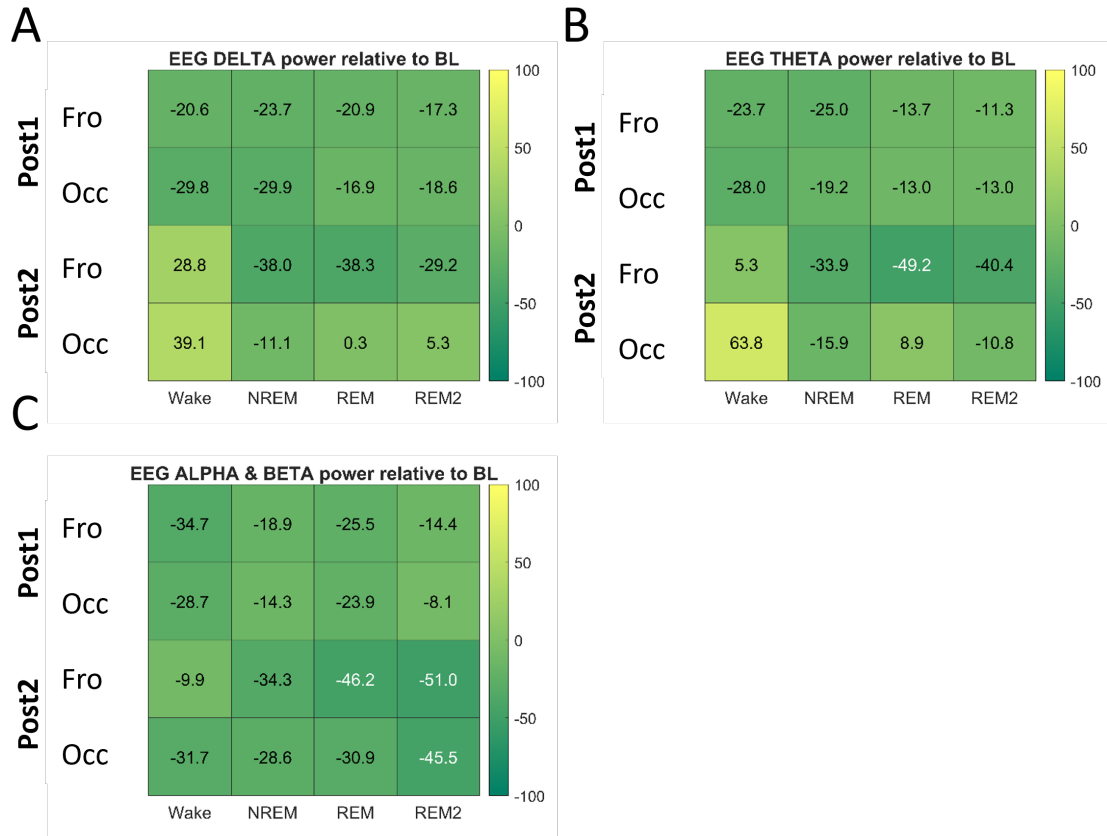


Fig.25: Change in EEG spectral power in all vigilance states after NOE relative to BL (in %)(Case2). (A) EEG Delta power (0.5-4Hz), (B) EEG Theta power (4.5-8Hz), (C) EEG Alpha and low Beta power (8.5 – 20Hz). Values depict changes in average spectral power density in percent relative to baseline based on recordings over approx. 48 hours on two separate occasions after NOE (Post1 and Post2) . Data are separated by vigilance state (Wake, NREM, REM and REM2) and by EEG derivation (frontal (FRO) and occipital (OCC)).

Evidence for reduced sleep intensity after NOE

The post NOE decrease in the frontal SWA range was in NREM sleep pronounced mostly during the dark period and the early light period (Fig.26,27C,D) and mostly during the dark period for REM and REM2 sleep (Fig.26,27E,F). Notably, this animal tended to sleep most in the dark period (Fig.19). The fact that SWA decreased for both NREM and REM during this time but also during the subsequent light phase suggests that sleep might have been 'less intense' in homeostatic terms. This could be related to the increased amount of sleep overall (Fig.17) and therefore less need for deep sleep with pronounced SWA. On the other hand, the increased amount of NREM and REM sleep duration after NOE (Fig.17B,C) could be a compensation in response an impaired ability of entering deep or 'intense' sleep.

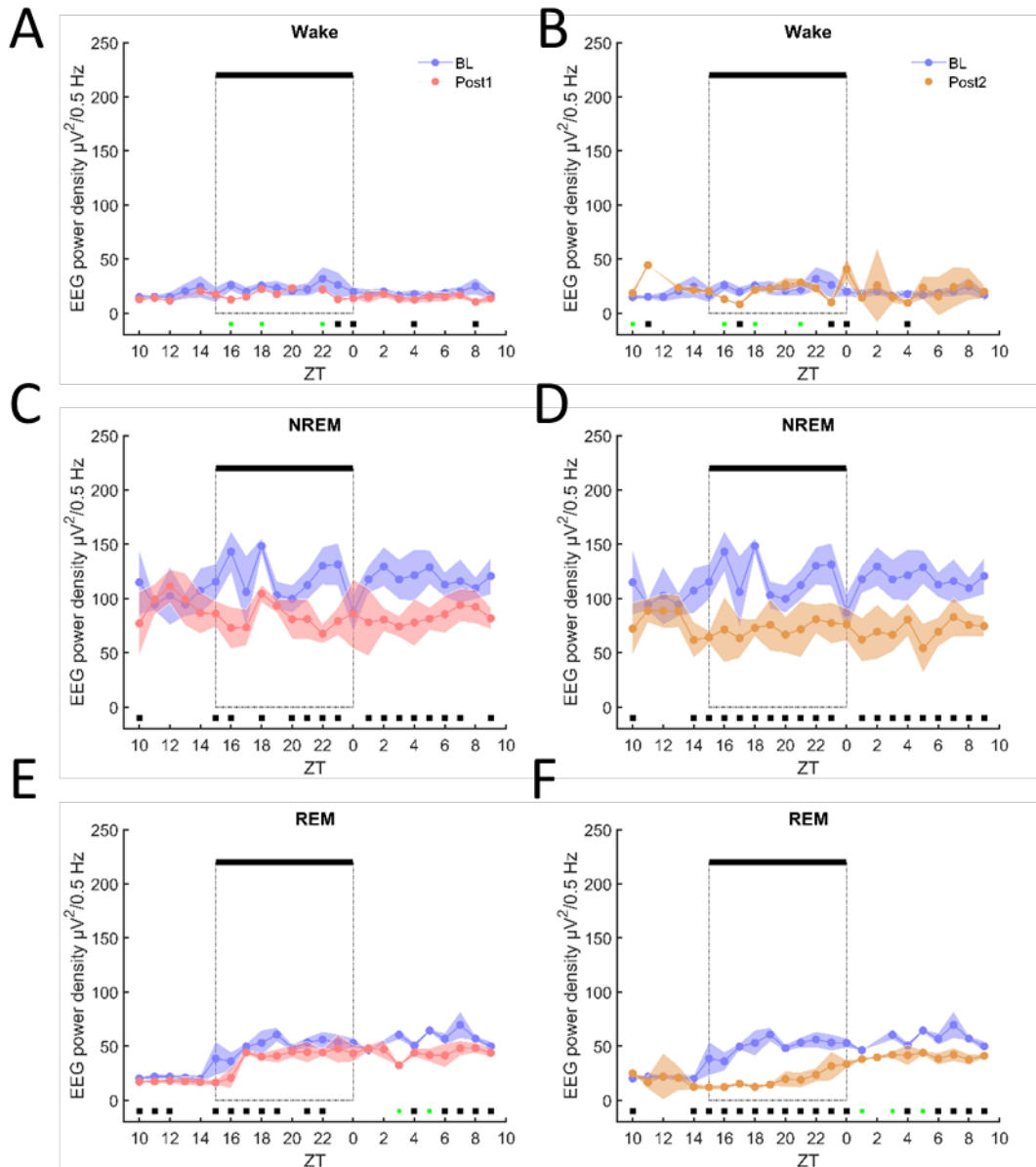


Fig.26: Time course of frontal EEG SWA across vigilance states (Case2). (A,B) Time course of Wake SWA BL vs Post1 and for BL vs Post2. (C,D) Time course of Wake SWA BL vs Post1 and for BL vs Post2. (E,F) Time course of REM SWA (REM and REM2 are merged in this analysis) for BL vs Post1 and for BL vs Post2. Initially, SWA across 48 hours of recording for each vigilance state was calculated per 15-min interval, before being merged into 1h intervals. Data of corresponding 1h-intervals in the two consecutive 24h cycles of 48 hours of continuous recording were then merged for analysis, resulting in 8 datapoints per 1h interval per condition. Shaded error bars are standard deviations. Two-way ANOVA (factors condition and time interval), Tukey's multiple comparisons; black square markers = $p < 0.05$. Green squares mark time intervals with less than 3 datapoints in either condition.

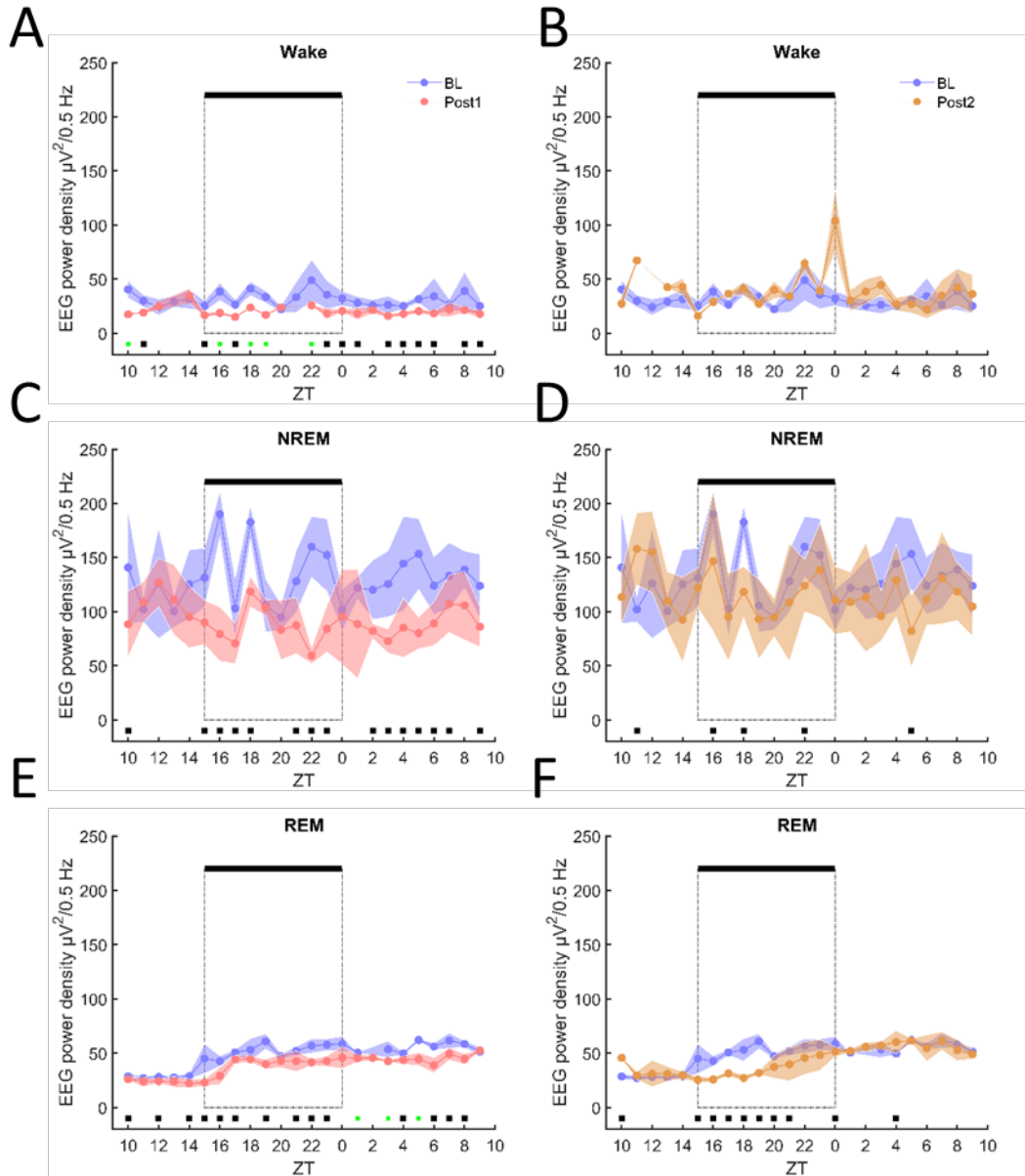


Fig.27: Time course of occipital EEG SWA across vigilance states (Case2). (A,B) Time course of Wake SWA BL vs Post1 and for BL vs Post2. (C,D) Time course of Wake SWA BL vs Post1 and for BL vs Post2. (E,F) Time course of REM SWA (REM and REM2 are merged in this analysis) for BL vs Post1 and for BL vs Post2. Initially, SWA across 48 hours of recording for each vigilance state was calculated per 15-min interval, before being merged into 1h intervals. Data of corresponding 1h-intervals in the two consecutive 24h cycles of 48 hours of continuous recording were then merged for analysis, resulting in 8 datapoints per 1h interval per condition. Shaded error bars are standard deviations. Two-way ANOVA (factors condition and time interval), Tukey's multiple comparisons; black square markers = $p < 0.05$. Green squares mark time intervals with less than 3 datapoints in either condition.

Summary of Case 2:

Prolonged sleep in connection with pronounced hearing loss

Animal 2 showed only mild evidence for tinnitus after NOE (TI, Post1: 0.2, Post2: 0.4 in Figs.39,40), which was the weakest indication for tinnitus across the three cases. Hearing ability on the other hand was markedly affected: thresholds increased by over 30 dB SPL and ABR response magnitude was markedly decreased in the Post1 assessment (by 44.7%), and even more so, by 52.9% relative to BL in the Post2 assessment. This raises the possibility that the impaired behavioural performance after NOE could be partially explained by hearing loss. In fact, the increased sleep amount after NOE could be connected to a higher auditory arousal threshold allowing the animal to sustain sleep even in the face of environmental stimuli that might have otherwise triggered arousal. Therefore, the increased sleep amount after NOE is unlikely to be a compensatory response to 'less efficient' sleep, but, in contrary, the lighter sleep after NOE could be a consequence of the animal's ability to maintain consolidated sleep and accumulate a high amount of sleep overall and therefore never reaching high levels of homeostatic sleep pressure.

Case 3

Sleep-wake pattern: temporarily increased REM sleep, REM2 sleep and sleep fragmentation after noise overexposure

This animal showed a temporary change in sleep amount after NOE. Initially after NOE (Post1), the ferret showed less wakefulness (BL vs Post1, 28.5% vs 23.6%, Fig.28A) and

more REM (*BL vs Post1*, 18.3% vs 20.2%, Fig.28C) and REM2 sleep (*BL vs Post1* 8.4% vs 10.7%, Fig.28D). At the same time sleep was more fragmented, as wake episodes were more numerous in the Post1 condition (*BL vs Post1*, 128 vs 137 episodes, Fig.29A) and wake episode length decreased, although not significantly so (*BL vs Post1*, $11.73 \pm 17.39 \text{ min}$ vs $7.65 \pm 8.6 \text{ min}$, $p=0.96$, Fig.29B). Both measures largely reversed towards Post2 (Wake episodes: *BL vs Post2*, 128 vs 63 episodes, Fig.29A, wake episode length, *BL vs Post2*, 11.73 ± 17.39 vs 15.25 ± 19.89 , $p=0.08$, Fig.29B) as did the amount of sleep (Fig.28).

Sleep wake distribution across the 24h cycle remained largely constant throughout the experiment. The animal showed most wakefulness during the light phase (Fig.30A,B, Fig.31A) and NREM, REM and REM2 sleep phases occurred predominantly in the dark phase (Fig.30C-H) although sleep accumulated slower during the light phase in the Post2 condition (Fig.31B-D).

Thus, the animal showed temporary symptoms of impaired sleep after NOE, with sleep amount largely returning to the BL pattern 6 months after NOE.

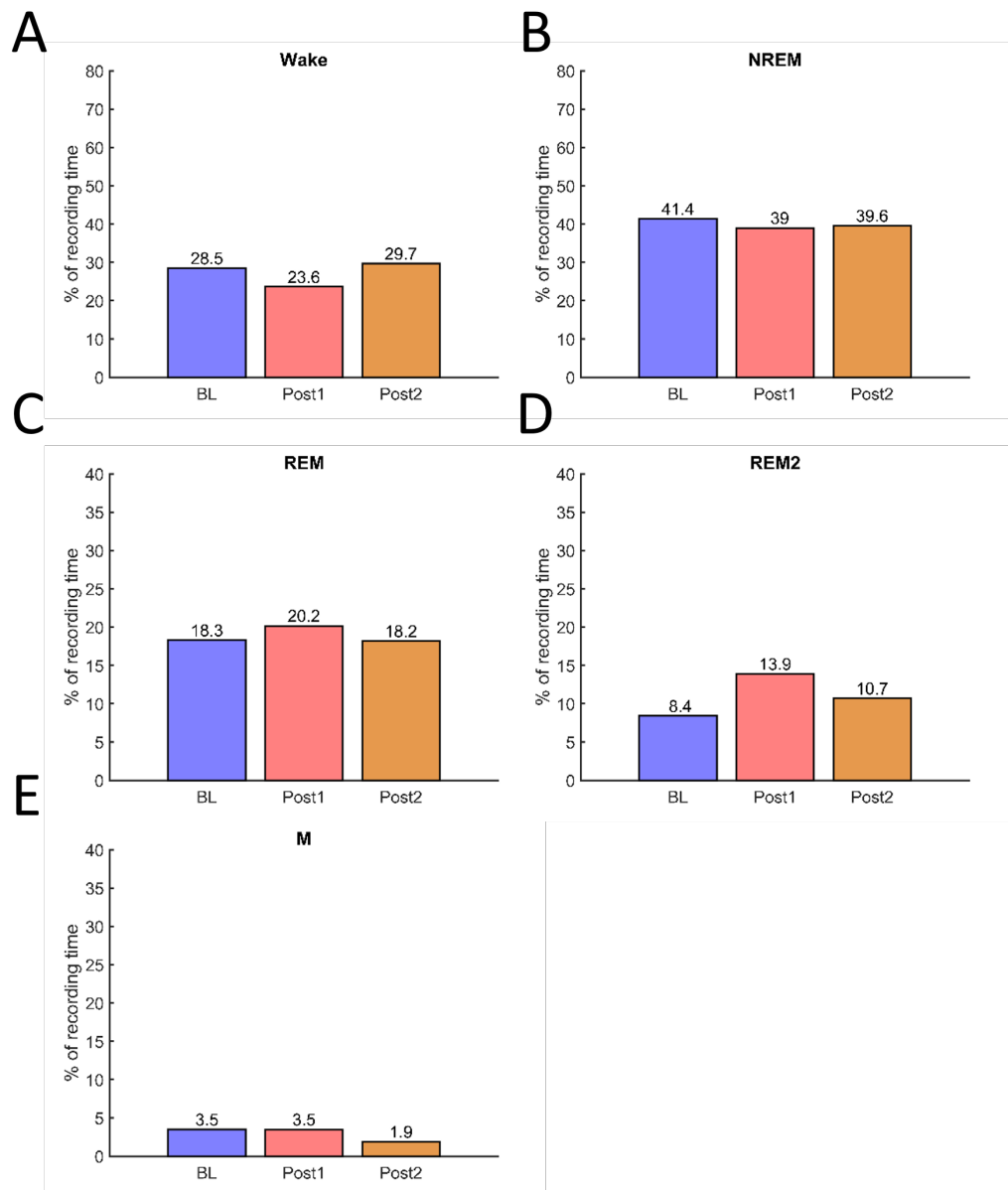


Fig. 28: Total vigilance state duration before and after NOE (Case3). Amount of time spent in Wake, NREM, REM, REM2 and M (movement artefacts within sleep episodes) as % of total recording time. Panels (A-E) represent Wake, NREM, REM, REM2 and M , respectively.

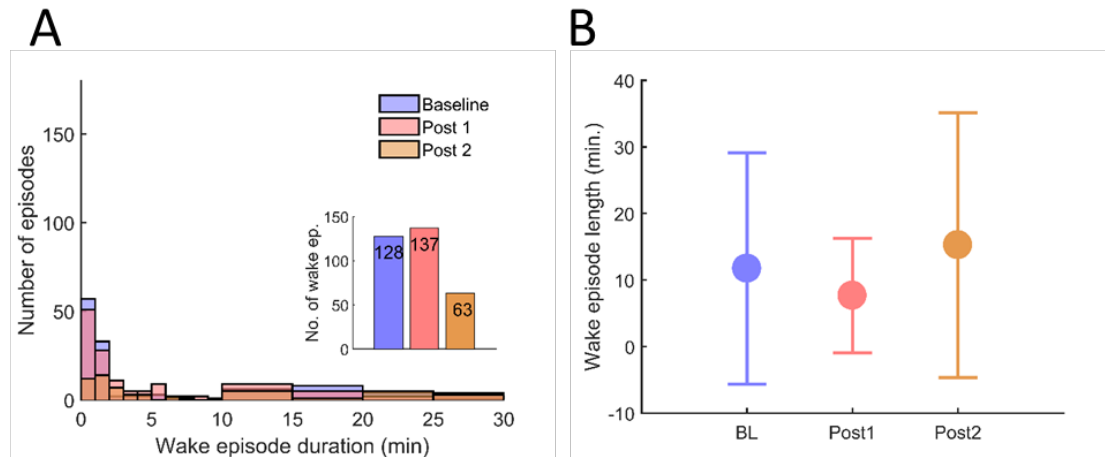


Fig.29: Sleep fragmentation before and after NOE (Case3). (A) Number of wake episodes during ~48 hours of recording (BL, Post1, Post2). The distribution of wake episodes by episode duration (ranging from 0-1min to 25-30 min. durations) is shown in the larger plot. The small inlet plot show the total numbers of all wake episodes during Baseline, Post1 and Post2 conditions. The y-axis and the number displayed above bars depicts the count of wake episodes. (B) Duration of wake episodes. Data are means $\pm$ standard deviations.

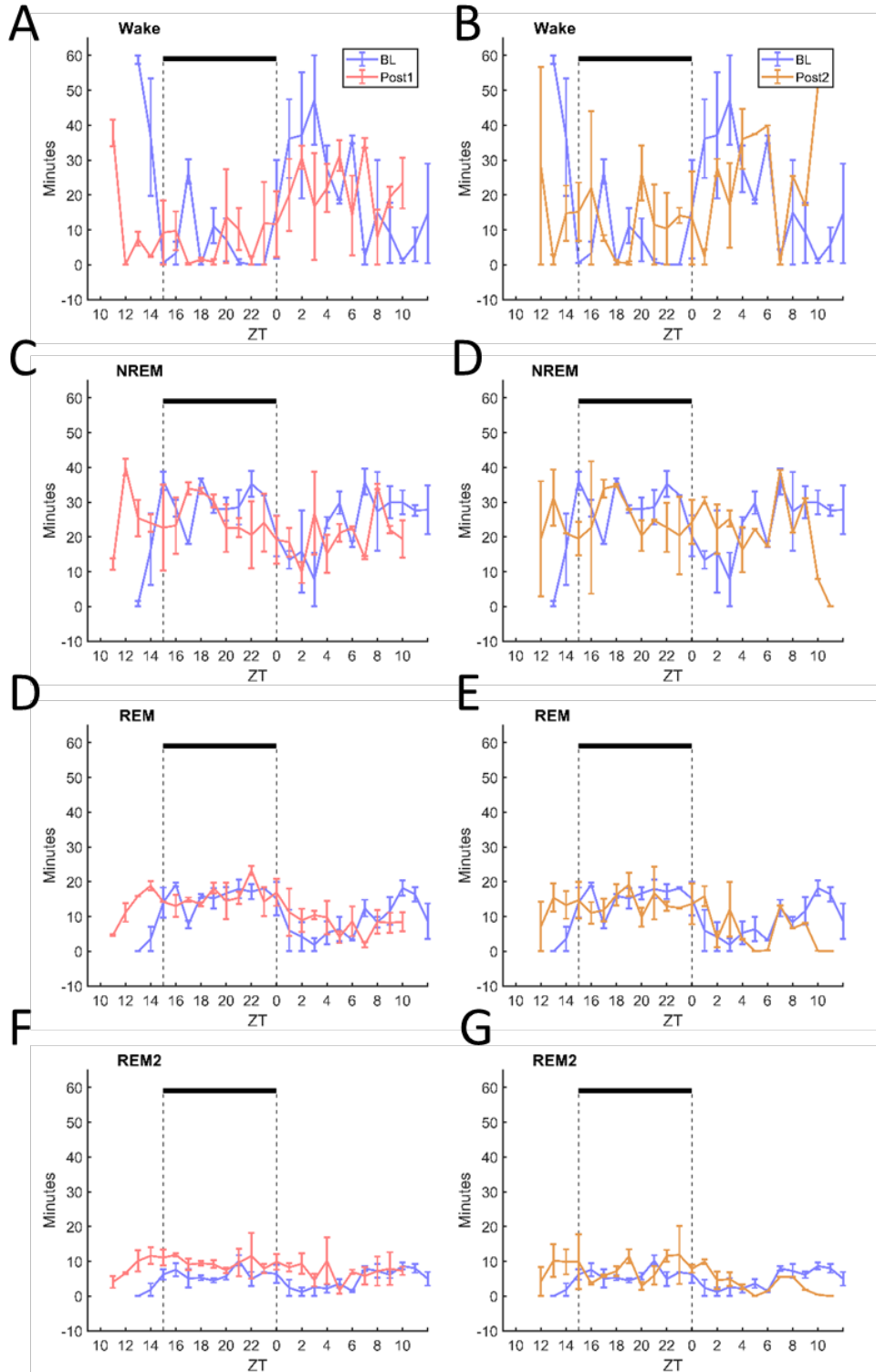


Fig.30 : Time course of vigilance states before and after NOE (Case3). Time course of Wake, NREM, REM and REM2 states across 24 hours. Durations spent in each state are depicted in minutes per 1h-bin. Data are averages over 2 consecutive 24h recording sessions (i.e. based on 2 datapoints per bin) for the BL condition (blue markers), Post1 (red) and Post2 (brown). Error bars are standard deviations. ZT is the Zeitgeber time, the black horizontal bar depicts the dark phase (light onset at ZT0). Note that recording sessions started at varying times between ZT10 and ZT13 across BL, Post1 and Post2 sessions. The first datapoint of each plot is matched to the respective start time.

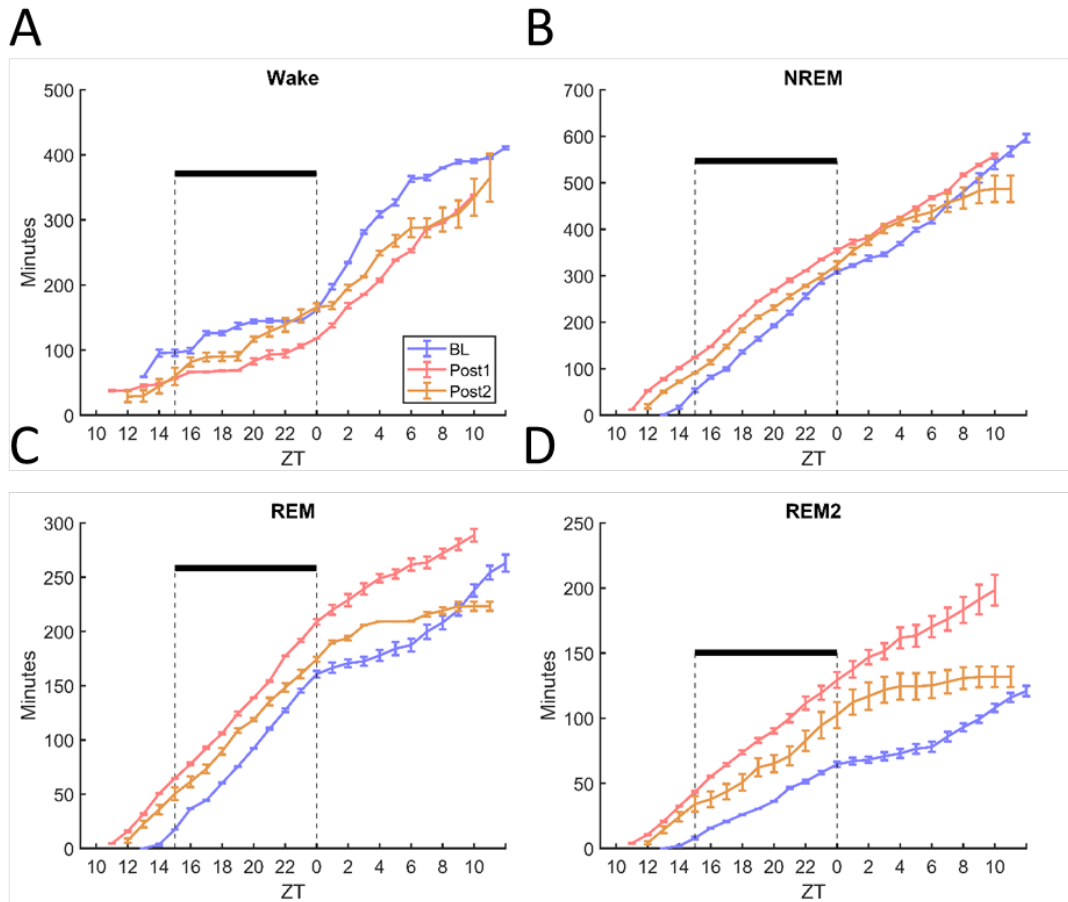


Fig.31 : Cumulative amount per vigilance state before and after NOE (Case3). Increment in state durations for (A) Wake, (B) NREM, (C) REM and (D) REM2 across the 24h cycle are depicted in steps of 1h bins. Data are averages over 2 consecutive 24h recording sessions (i.e. based on 2 datapoints per bin) for the BL condition (blue markers), Post1 (red) and Post2 (brown). Error bars are standard errors. ZT is the Zeitgeber time, the black horizontal bar depicts the dark phase (light onset at ZT0). Note that recording sessions started at varying times between ZT10 and ZT13 across BL, Post1 and Post2 sessions. The first datapoint of each plot is matched to the respective start time.

EEG spectral composition: a temporary increase in spectral power after NOE across vigilance states.

EEG spectral power initially increased after NOE across wide frequency ranges in all vigilance states but decreased to BL or even lower in the late (Post2) assessment, although the signal was confounded by more noise 6 months after NOE.

During the waking state, frontal EEG delta, theta and low beta activity were elevated in the first assessment after NOE (Fig.32A), but not in the late assessment (Fig.32B). A similar pattern was visible for the occipital EEG, with only delta activity remaining stable throughout the experiment.

Similarly, during NREM sleep frontal delta, theta and beta activity were upregulated in the first assessment after NOE and returned to BL levels in the late assessment (Fig. 33A,B). In the occipital EEG, a temporary increase in power was evident only in the theta, alpha and part of the beta range (~17-20Hz, Fig.33C,D).

In REM and REM2 sleep higher frequency EEG bands were elevated in power after NOE in both frontal and occipital derivations (Fig.34,35A,C) and this was reversed in the Post2 condition (Fig.34,35B,D). In REM sleep, occipital delta and theta power surrounding the characteristic frequency peak for REM sleep was now even lower than in BL (Fig.34D).

It is interesting to note that the temporary increase in EEG spectral power (see summary Fig. 36) was present in parallel to signs of disrupted sleep.

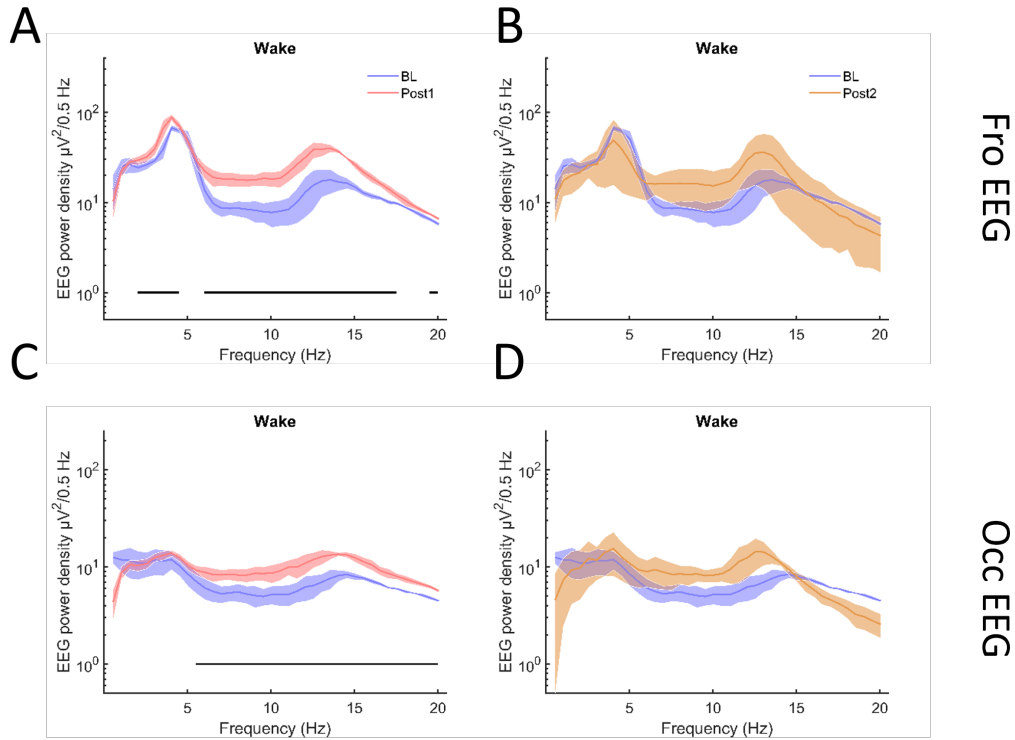


Fig.32: Wake EEG power spectra before and after NOE (Case3). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

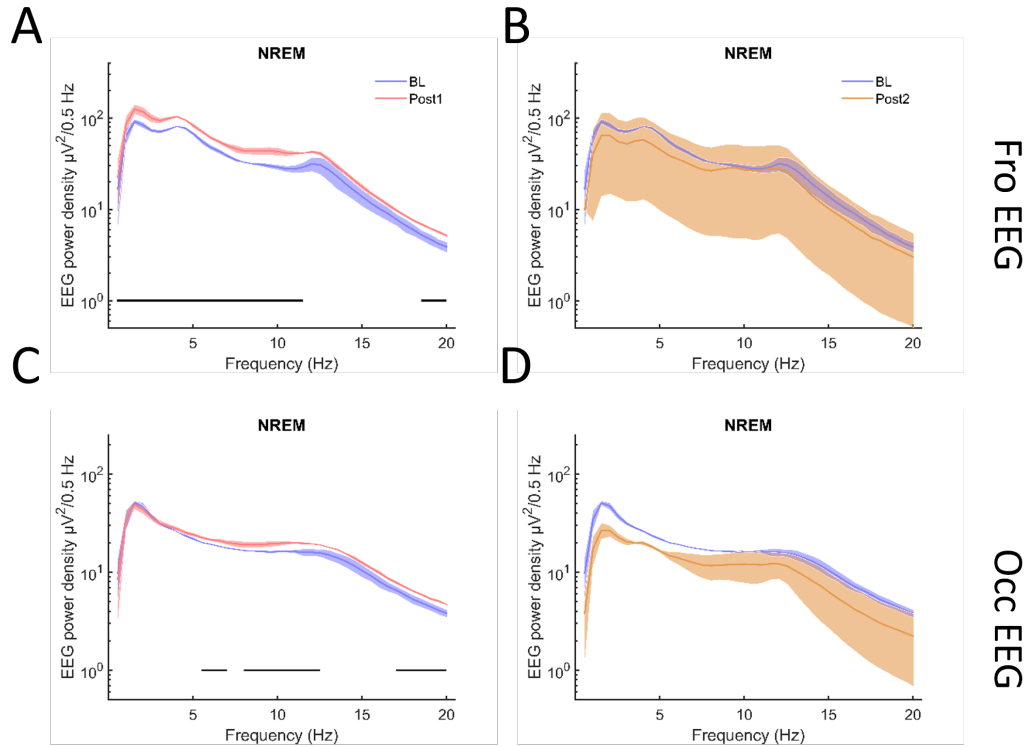


Fig.33: NREM EEG power spectra before and after NOE (Case3). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

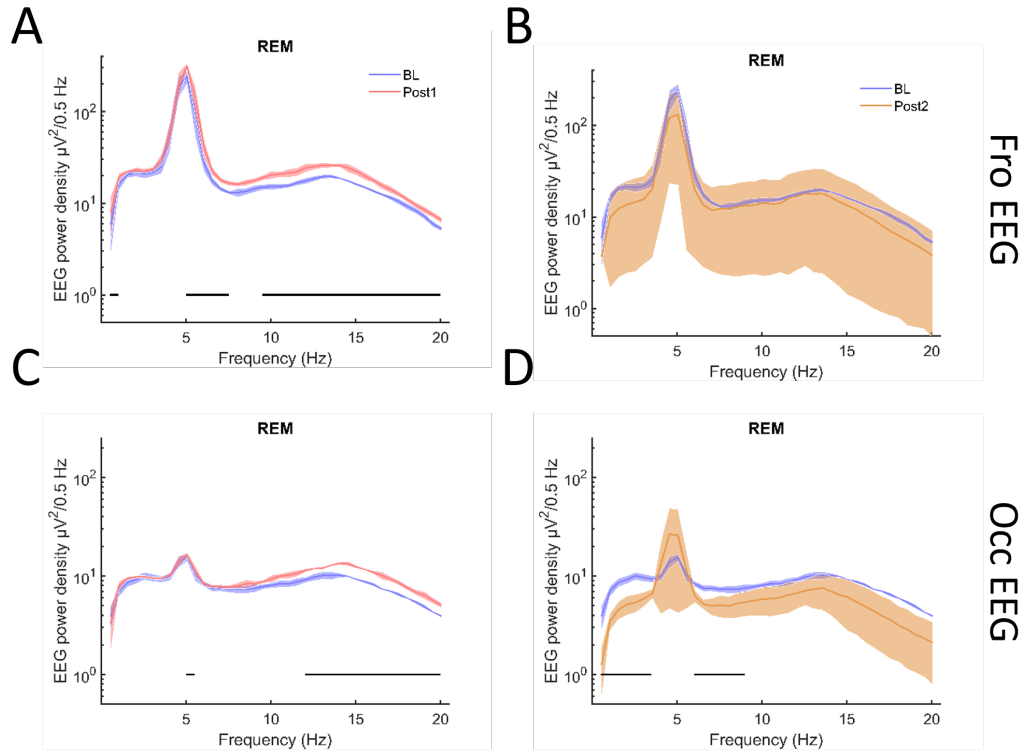


Fig.34: REM EEG power spectra before and after NOE (Case3). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

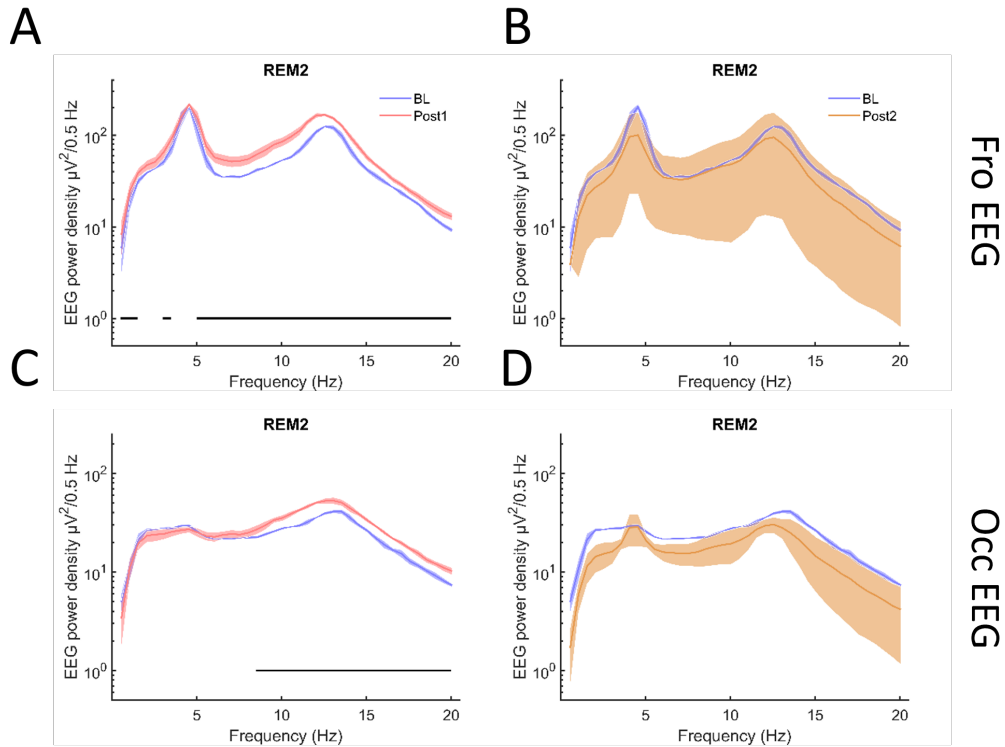


Fig.35: REM2 EEG power spectra before and after NOE (Case3). (A) Baseline (BL) and post NOE 1 (Post1) spectra for the frontal EEG derivation, (B) BL and post NOE 2 (Post2) spectra for the frontal EEG derivation. (C) and (D) show the corresponding spectra based on the occipital EEG derivation. Spectra are means and standard deviations based on recordings over ~48hrs per condition and displayed in bins spanning 0.5Hz. Originally, average 24h EEG spectra were produced with a resolution of 0.25Hz. For comparison between conditions, data of each 0.25Hz bin were merged into 0.5Hz bins, producing 4 datapoints per 0.5 Hz bin per condition. Two-way ANOVA (factors condition and frequency bin), Tukey's multiple comparisons; solid black line = $p < 0.05$.

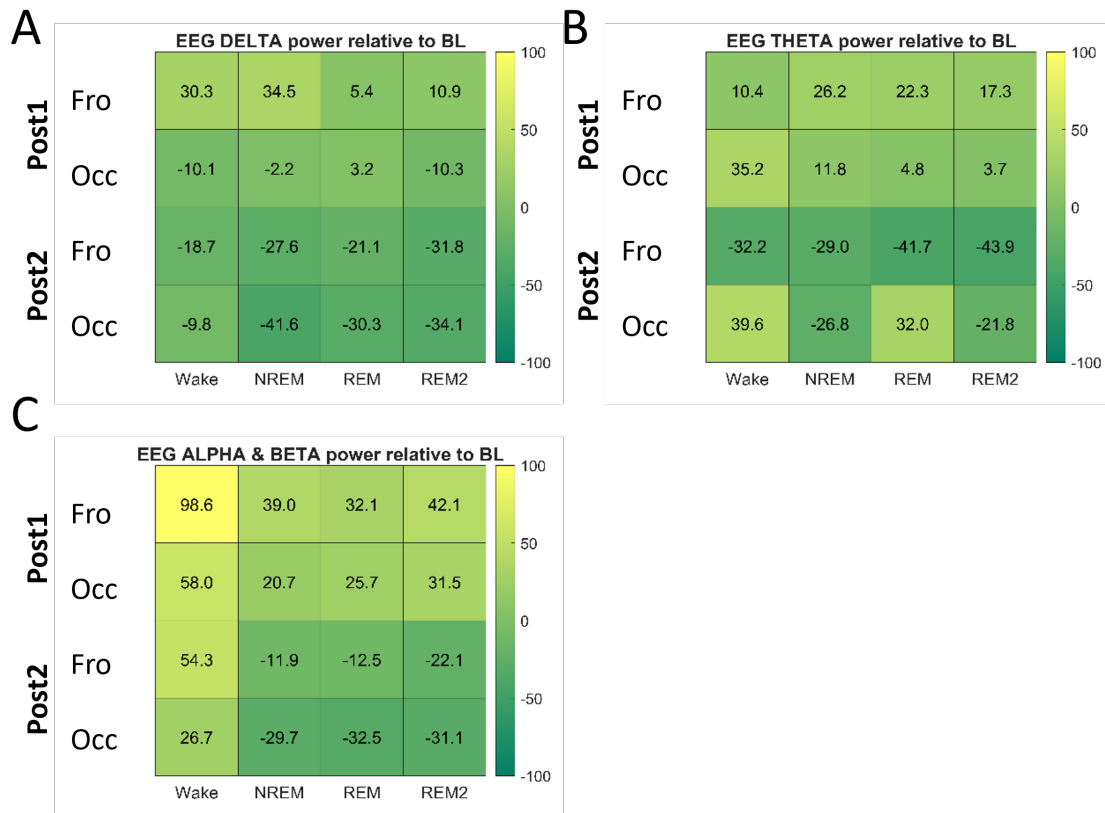


Fig.36: Change in EEG spectral power in all vigilance states after NOE relative to BL (in %)(Case3). (A) EEG Delta power (0.5-4Hz), (B) EEG Theta power (4.5-8Hz), (C) EEG Alpha and low Beta power (8.5 – 20Hz). Values depict changes in average spectral power density in percent relative to baseline based on recordings over approx. 48 hours on two separate occasions after NOE (Post1 and Post2) . Data are separated by vigilance state (Wake, NREM, REM and REM2) and by EEG derivation (frontal (FRO) and occipital (OCC)).

Evidence for transiently elevated sleep pressure and long-term reduced sleep intensity after NOE

EEG SWA across the 24-hour cycle widely mirrored the temporary EEG power increase evident soon after NOE (Post1). Similarly, in the long term (Post2) EEG SWA decreased during certain times within the 24-hour cycle.

Initially after NOE, NREM frontal SWA was elevated mainly during the dark phase (Fig.37C), the predominant sleeping phase of the animal (Fig.30A,B). During REM and

REM2 sleep SWA was elevated mostly in the early light phase, incidentally the time where the amounts of REM and REM2 were increased in the Post1 condition (Fig.28C,D). This suggests a more intense NREM sleep (indicated by increased NREM SWA), and possibly more intrusions of local SWA during REM sleep (Funk et al., 2016) – likely signs of increased sleep pressure.

In the late assessment (Post2), frontal NREM, REM and REM2 SWA was similar to BL during the dark phase but decreased in the early light phase (Fig.37D,F). In the occipital EEG, SWA was decreased during most of the 24h cycle for all sleep states (Fig.38D,F). Sleep amount was similar to BL conditions (Fig.28) but less fragmented than during BL (Fig.29A), which could indicate less sleep interruptions caused by external triggers (such as environmental noise) or an increased sleep need. Since the latter is usually accompanied by increased SWA (Bernardi et al., 2015; Hung et al., 2013; Tobler & Borbély, 1986; Vyazovskiy et al., 2011) this might not have been the case in this animal. Rather, the time course of SWA suggests a decrease in sleep intensity long-term after NOE, possibly due to better sleep maintenance as observed in Case2.

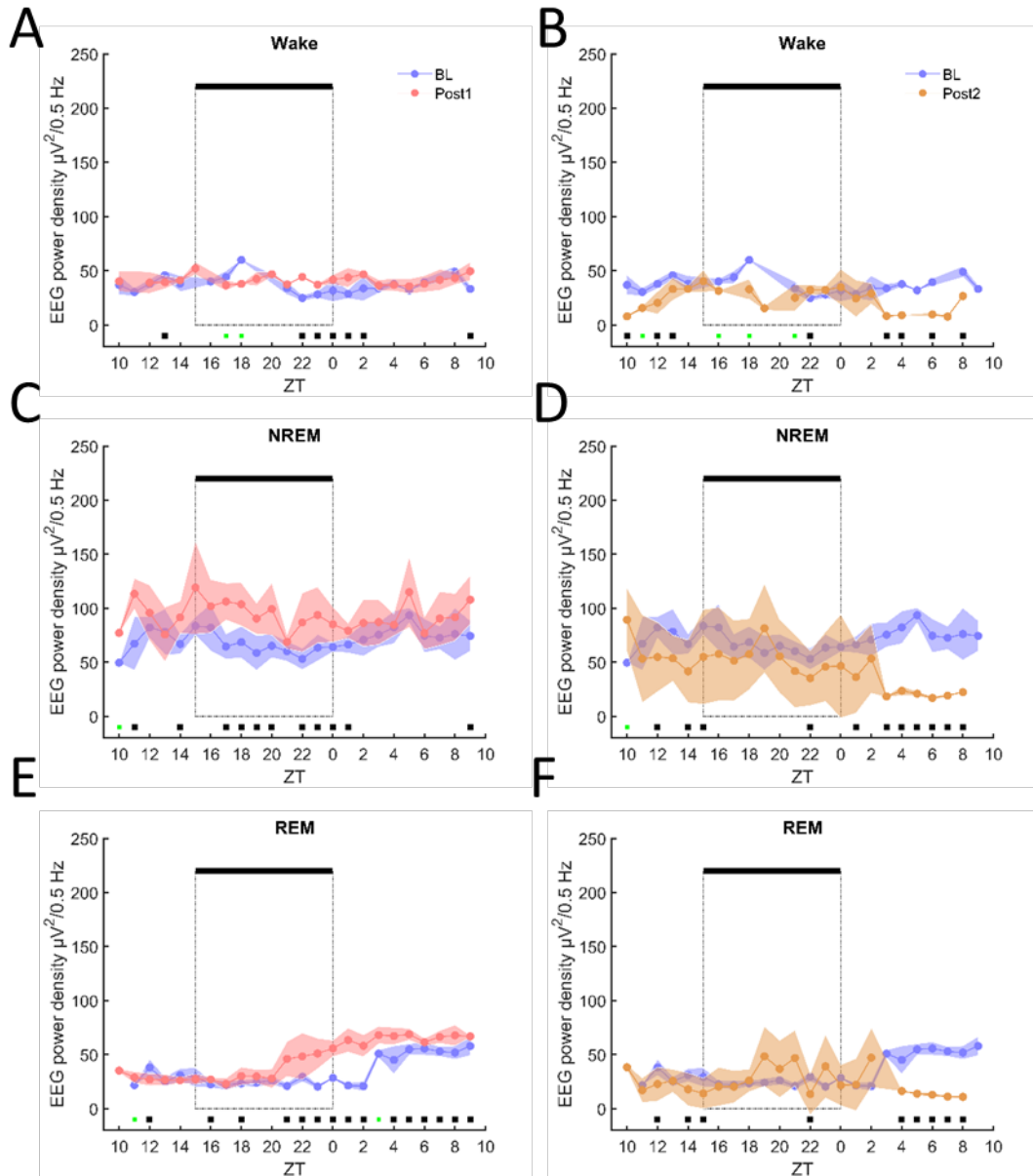


Fig.37: Time course of frontal EEG SWA across vigilance states (Case3). (A,B) Time course of Wake SWA BL vs Post1 and for BL vs Post2. (C,D) Time course of Wake SWA BL vs Post1 and for BL vs Post2. (E,F) Time course of REM SWA (REM and REM2 are merged in this analysis) for BL vs Post1 and for BL vs Post2. Initially, SWA across 48 hours of recording for each vigilance state was calculated per 15-min interval, before being merged into 1h intervals. Data of corresponding 1h-intervals in the two consecutive 24h cycles of 48 hours of continuous recording were then merged for analysis, resulting in 8 datapoints per 1h interval per condition. Shaded error bars are standard deviations. Two-way ANOVA (factors condition and time interval), Tukey's multiple comparisons; black square markers = $p < 0.05$. Green squares mark time intervals with less than 3 datapoints in either condition.

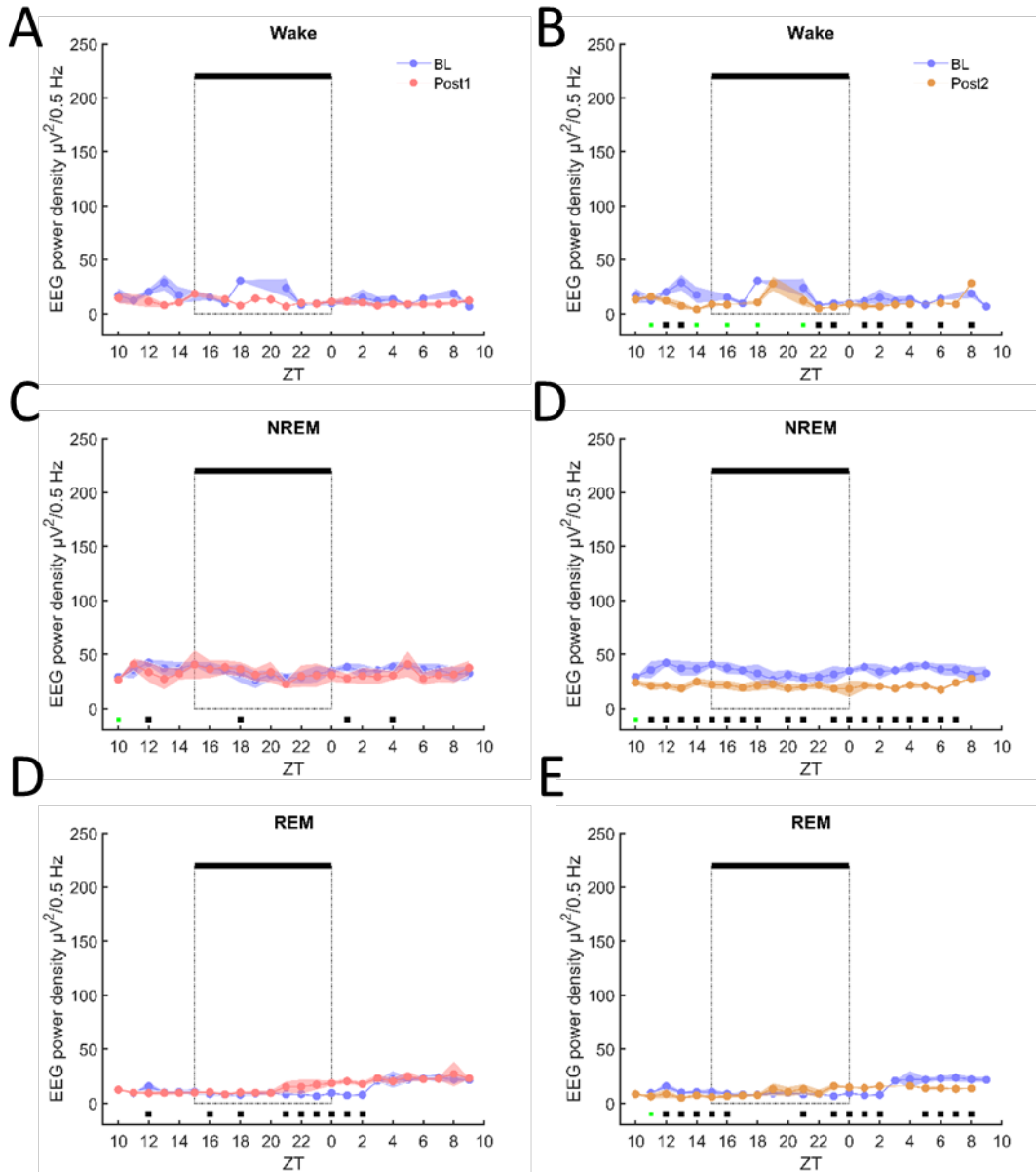


Fig.38: Time course of occipital EEG SWA across vigilance states (Case3). (A,B) Time course of Wake SWA BL vs Post1 and for BL vs Post2. (C,D) Time course of Wake SWA BL vs Post1 and for BL vs Post2. (E,F) Time course of REM SWA (REM and REM2 are merged in this analysis) for BL vs Post1 and for BL vs Post2. Initially, SWA across 48 hours of recording for each vigilance state was calculated per 15-min interval, before being merged into 1h intervals. Data of corresponding 1h-intervals in the two consecutive 24h cycles of 48 hours of continuous recording were then merged for analysis, resulting in 8 datapoints per 1h interval per condition. Shaded error bars are standard deviations. Two-way ANOVA (factors condition and time interval), Tukey's multiple comparisons; black square markers = $p < 0.05$. Green squares mark time intervals with less than 3 datapoints in either condition.

Summary of Case3:

Evidence for progressive tinnitus or hyperacusis and long-term hearing impairment correlated with sleep disruption and facilitation, respectively.

In animal 3 evidence for tinnitus increased progressively after NOE (TI Post1: 0.5) and reached the highest tinnitus index across all animals 6 months after NOE (TI Post2: 0.9, Figs.39,40). In parallel, the animal showed relatively mild signs of hearing impairment based on both thresholds and ABR magnitude (Figs.39,40). The increase in sleep fragmentation in the Post1 assessment (Fig.30) may suggest increased sleep disturbance, possibly due to increased sensitivity to environmental stimuli or due to the presence of tinnitus. Notably, the animal showed temporary elevation in EEG spectral power across vigilance states, which may point towards changes in excitability at the basis of temporary hyperacusis or tinnitus. In the long term, 6 months after NOE, the animal did not show pronounced changes in sleep amount and even more stable sleep than in baseline conditions in combination with decreased sleep delta power. This is unlikely to be due to reduced sensitivity to environmental sleep disruption as consequence of hearing impairment (as seems likely in Case 2) since Case 3 showed only mild hearing impairment. The question remains whether the improved sleep maintenance 6 month after NOE as compared to the initial sleep disruption, despite progressive and marked evidence for tinnitus may be due to state dependent mitigation of tinnitus related aberrant brain activity.

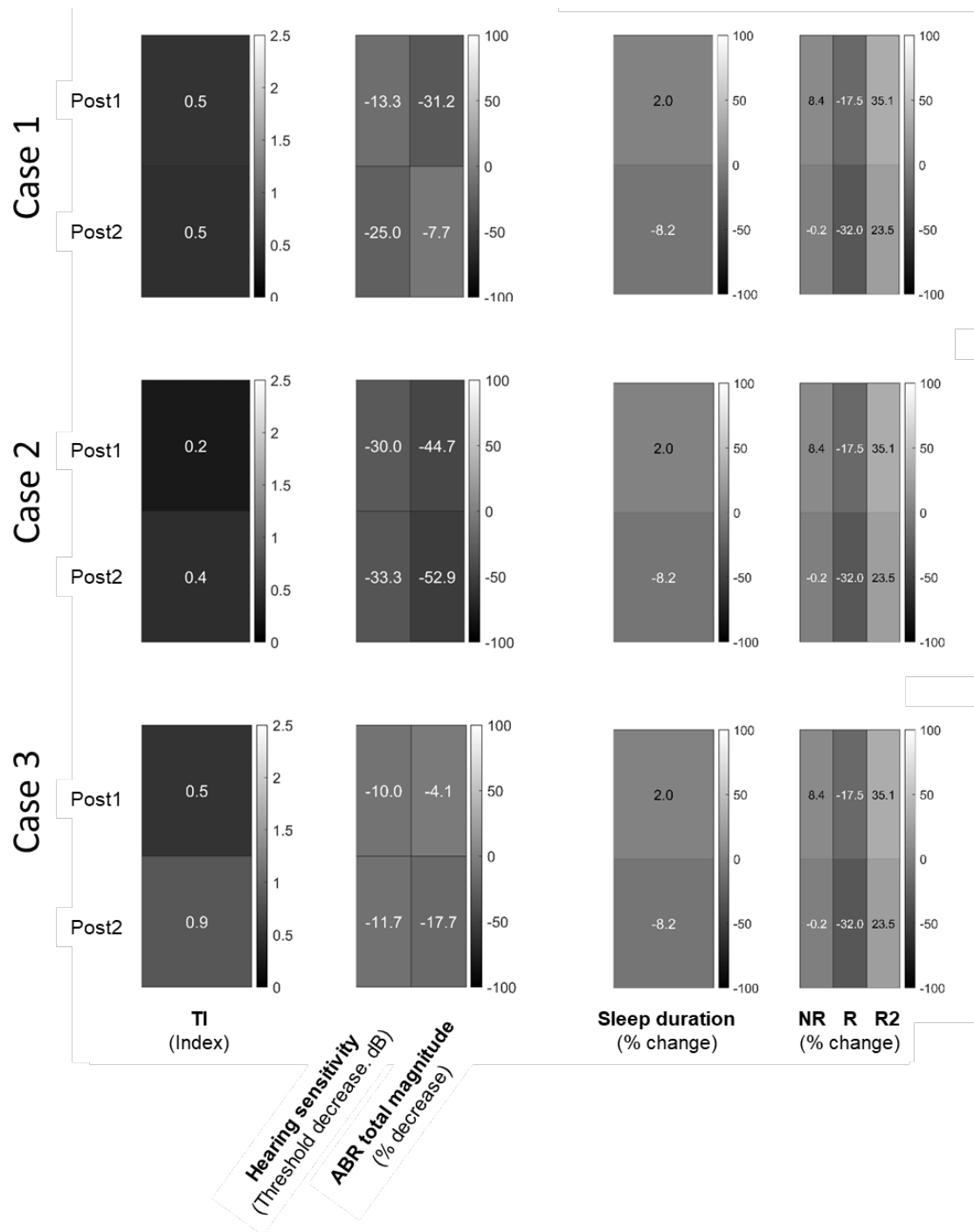


Fig.39: Numerical summary of behavioural performance, hearing ability and sleep-wake amount. Data are shown for Cases1-3, respectively. TI (Tinnitus index) describes the sum of normalised changes in performance across all operant behavioural paradigms (gap and silence detection). Positive values of TI describe changes in line with tinnitus development, negative values signify evidence against tinnitus. Hearing sensitivity is based on ABR threshold changes (depicted in dB) relative to BL. Note that an increase in ABR threshold (e.g. by 20 dB) is here depicted as a decrease in hearing sensitivity (e.g. -20 dB). ABR magnitude shows the change in total ABR response magnitude across sound levels relative to BL (% decrease relative to BL). Sleep duration depicts the change in overall sleep duration (NREM, REM and REM2 sleep) relative to BL. The duration of specific sleep states relative to BL are shown separately for NREM (NR), REM (R) and REM2 (R2). See 'Method Summary' for details on each measure displayed in this figure.

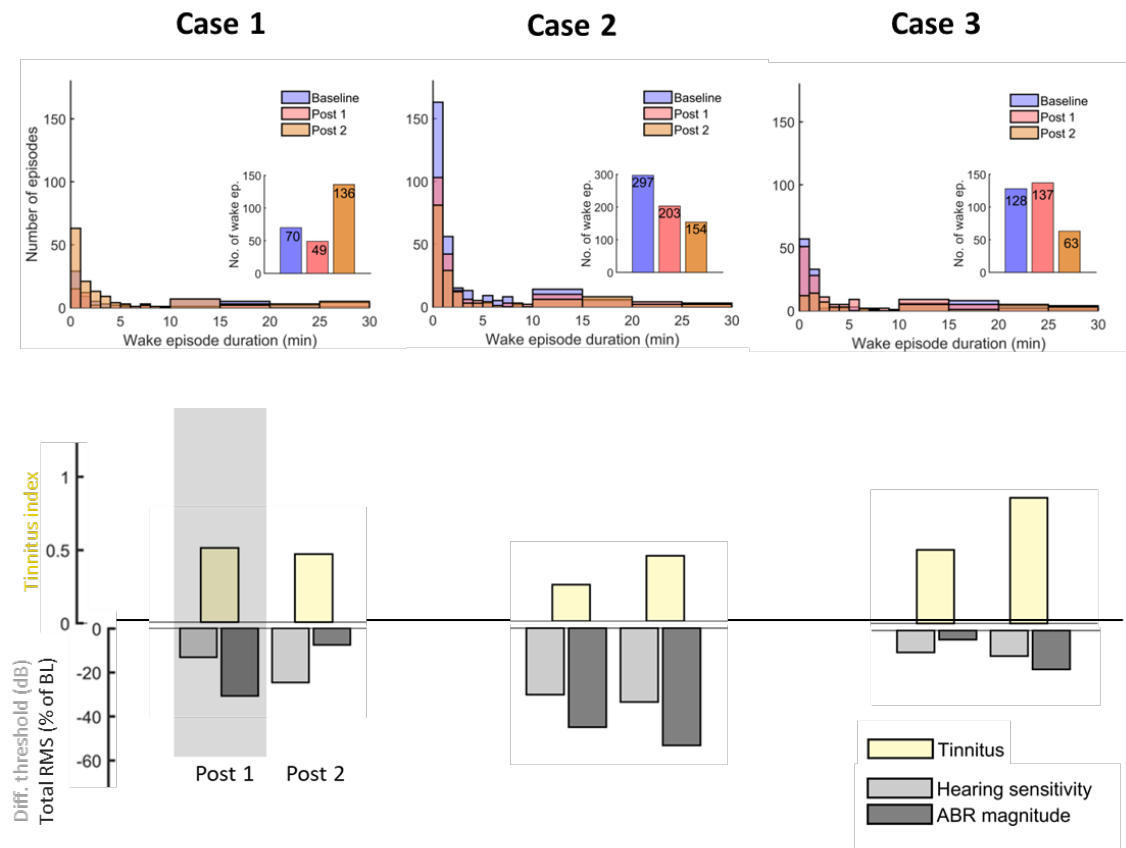


Fig.40: Summary of behavioural performance, hearing ability and sleep-wake amount. Data are shown for Cases1-3 (columns), respectively. The top row shows the number of wake episodes (a measure for sleep disruption) across conditions. The distribution of wake episodes by episode duration (ranging from 0-1min to 25-30 min. durations) is shown in the larger plots. The small inlet plots show total numbers of wake episodes during Baseline, Post1 and Post2 conditions. Bottom row: TI (Tinnitus index) describes the sum of normalised changes in performance across operant behavioural paradigms (gap and silence detection). Positive TI values describe changes in line with tinnitus development, negative values signify evidence against tinnitus. Hearing sensitivity (light grey bars) is based on ABR threshold changes (depicted in dB). Note that an increase in ABR threshold (e.g. by 20 dB) is here depicted as a decrease in hearing sensitivity (e.g. -20 dB). ABR magnitude (dark grey bars) shows the change in total ABR response magnitude across sound levels relative to BL (% of BL).

Discussion

While all animals developed signs of tinnitus after NOE as well as indications for reduced hearing ability, the magnitude of these measures varied between individuals. Indeed, it is reported in rodent studies that the effect of noise overexposure can produce vastly

different effects dependent on individuals, which sometimes can show inherent resistance to tinnitus development (S. Shore & Wu, 2019). Therefore, animals were treated as individual cases and changes in sleep architecture and EEG spectral composition were analysed in this context.

Case1 showed strong indications for an impairment of behavioural performance in line with tinnitus development, in addition to progressive ABR threshold elevations – although ABR response magnitude largely recovered long-term after an initial decrease. It is likely, therefore, that the animal developed a combination of hearing impairment as well as tinnitus. Initially after NOE, the animal showed increased sleep fragmentation and spent more time awake. The elevated SWA during NREM sleep – a correlate of homeostatic sleep pressure – indicates that this increment in waking was not due to reduced sleep need. Instead, the elevated SWA could be a consequence of the increased time awake, possibly due to impaired ability to enter or maintain consolidated sleep. This is in line with the presence of external or internal triggers for arousal that impair sleep stability. Although sleep disruption and reduced amount of sleep could lead to daytime sleepiness and therefore explain part of the impaired behavioural performance evident in this animal, it is of note that reduced sleep maintenance is a common symptom observed in human tinnitus patients (Folmer & Griest, 2000; Hallam, 1996; Izuohara et al., 2013a; Koning, 2019a; Schecklmann et al., 2015).

Case2 showed strong indications for immediate and progressively worsening hearing loss (leading to a decrease of ABR magnitude by over 50% after NOE and a threshold elevation by 33.3 dB SPL), correlated with mild, but progressively increasing tinnitus index over the course of assessments after NOE. It is likely therefore, that a change in

hearing ability played a role in determining the animal's behavioural performance. In line with this, the ferret spend markedly more time asleep, likely not due to increased sleep need (as indicated by overall lower sleep intensity) but possibly connected to a higher waking threshold due to pronounced hearing impairment. It has previously been reported that cochlear lesions in guinea-pigs lead to significant reduction in wakefulness and increased sleep (Pedemonte et al., 1997), corroborating the possibility that auditory sensory deprivation can indeed facilitate sleep.

Case3 showed relatively mild hearing impairment throughout the experiment but strong and progressively increasing evidence for tinnitus. Interestingly, like Case1, which showed similar evidence for tinnitus in the early assessment, Case3 did initially after NOE show signs of sleep disruption and increased sleep intensity, in line with tinnitus-induced sleep disturbance. In the long term, the animal behaved more like Case2 as ABR impairment intensified further, although still mild overall, and the animal's sleep became lighter and less disrupted. It may be, therefore, that the animal suffered from tinnitus without marked hearing loss after NOE but, in the long term, the decrease in total ABR magnitude as well as a mild threshold elevation could signify the degeneration of auditory nerve fibres (ANFs) over time (Kujawa & Liberman, 2009) leading to hearing impairment while possibly fostering tinnitus at the same time.

Importantly, the occurrence of increased spectral power across wide EEG frequency ranges (in Case1, frontal EEG and in Case3, frontal and occipital EEG) co-occurred with signs of disrupted sleep in both cases (increased sleep fragmentation for Case1 and Case3, reduced sleep amount for Case1). This may point towards increased cortical excitability at the same time as animals showed typical signs of sleep disruption seen in

tinnitus patients. While changes in EEG spectral power without the assessment of evoked responses are only an indirect indicator for changes in excitability, elevated excitability in the auditory pathway has been suggested to be a direct correlate of tinnitus (Kalappa et al., 2014; Kaltenbach et al., 2004) and, in recent years, it has become clear that distressing tinnitus is also correlated with changes in non-auditory brain regions such as in the prefrontal cortex, hippocampus and the amygdala (Elgoyhen et al., 2015b). The observed elevations in spectral power may therefore represent aspects of a neural tinnitus correlate.

Overall, this study produced three cases exhibiting common signs of hearing impairment and tinnitus after NOE, yet with unique dynamics of these conditions in each case. We show that noise-induced hearing loss may facilitate sleep and, in turn, keep sleep pressure consistently low. This suggests not only that sensory deprivation facilitates sleep (Pedemonte et al., 1997), but that it may specifically promote rather light sleep. Interestingly, sleep in hearing-impaired human subjects with cochlear implants also gets biased towards more light sleep when the implant is switched off at night, and subjects report less restful sleep as compared to when the implant is switched on (Velluti et al., 2010). It is unclear whether subjects would normally switch their implant off at night and whether sudden sensory deprivation may have a different effect on subsequent sleep than chronic or life-long deafness. Nevertheless, it is possible that prolonged light sleep as observed in our study points towards persistent low-quality sleep, in turn promoting an increase in sleep time. If animals with auditory sensory deprivation have indeed an impaired ability to meet their sleep demand, then SWA build-up during wakefulness would be expected to be at equal or even increased levels as compared to conditions of normal hearing. Yet in the case with the most severe hearing impairment

in combination with better sleep maintenance, SWA was reduced in all states, including wakefulness, suggesting that improved sleep maintenance was not due to increased demand for sleep but indeed due to a more direct sleep facilitation through improved sensory disconnection.

Further, our findings suggest a temporal link between the development of tinnitus in healthy animals, the appearance of sleep disturbance and signs for shifts in cortical excitability. To our knowledge, this is the first observation of developing symptoms of induced tinnitus and long-term changes in sleep-wake pattern and brain activity in parallel. While further work will be needed to clarify the exact correlation and causal connections between symptoms of tinnitus, hearing loss and sleep our findings provide evidence that altered and disturbed sleep so commonly observed in human tinnitus patients may indeed be functionally linked to the condition.

Finally, it is acknowledged that the effect of tinnitus and chronic hearing loss on sleep can be difficult to study in human subjects as hearing loss is commonly associated with increased psychological distress (Bigelow et al., 2020), often linked to feelings of social exclusion, loneliness and failure of coping strategies (Bennett et al., 2022), factors which can itself interfere with sleep (E.-J. Kim & Dimsdale, 2007). Our results demonstrate an approach to study the long-term effects of changed hearing ability and tinnitus that allows for both long-term chronic sleep recordings as well as for the close control of such factors that could interfere with sleep beyond the direct intervention.

Chapter 4: The effect of noise overexposure on auditory evoked activity across sleep and wakefulness

Background

Sleep is affected in many neurological and psychiatric conditions, most of which include changes in the spatiotemporal dynamics of brain activity during sleep (Krystal, 2012). And while the importance of sleep for health and wellbeing is widely acknowledged (Kamdar et al., 2012; Krause et al., 2017; Palchykova et al., 2006), it remains unclear whether natural dynamics in brain state could affect or be affected by conditions that are driven by persistent aberrant brain activity.

Tinnitus offers a unique opportunity to investigate the potential of naturally occurring changes in brain state to modulate the associated pathological brain activity: First, the spatial overlap between NREM slow-wave activity and spontaneous aberrant activity in tinnitus (see introduction of Chapter 2 & Milinski *et al.*, 2022) suggests that natural brain dynamics are well-positioned to affect the functional representation of tinnitus in the brain. Second, the relatively well-defined phantom percept that is tinnitus may allow to test for a sleep-dependent modulation of the condition using paradigms that assess the presence of a specific phantom sound.

While the relationship between sleep and tinnitus has not been tested directly, there is evidence to suggest that the drive to express sleep-dependent brain activity even in the

face of local disruptions, such as under very high sleep pressure, might act as a mitigating factor to the effect of tinnitus on spatiotemporal brain activity. For example, tinnitus patients who show disrupted sleep, possibly due to aberrant tinnitus activity, still express deep NREM sleep (sleep stages 3 and 4, and SWS) (Attanasio et al., 2013; Burgos et al., 2005; Hébert et al., 2011a). In addition, tinnitus patients have reported sleep as a state of reduced tinnitus (De Ridder et al., 2014; Stouffer et al., 1991). It remains an open question however, whether sleep – in particular NREM sleep – could temporarily interfere with aberrant tinnitus-related brain activity (e.g. Burton *et al.*, 2012; Sedley *et al.*, 2012, 2015; Seydell-Greenwald *et al.*, 2012) and act as a mitigating factor on tinnitus saliency.

The arguably most likely mechanism for an interference of sleep with local deviations in brain activity is the entrainment of local aberrant dynamics with global slow-wave activity and its effect on cortical signal propagation. Entrainment of local cell populations with cortical SWA is thought to be based on both intrinsic and extrinsic factors: evidence suggests that elevated spiking and synaptic activity, typical of the awake state (Thomas et al., 2020), result in an increased metabolic load and accumulated intrinsic need for cellular maintenance (Vyazovskiy & Harris, 2013). To offset wake-dependent changes and restore cellular homeostasis, neurons may enter periods of silence, so called off periods, which are known to increase after sleep deprivation (McKillop et al., 2018; Thomas et al., 2020; Vyazovskiy et al., 2009). High lateral connectivity in cortex then further promotes global synchronization of SWA and can drive more cells towards an on-off firing pattern – an extrinsic drive for individual neurons to entrain with global SWA. Both the intrinsic and extrinsic drives to engage in this stereotypic firing pattern during sleep may be in direct competition with drives to maintain markedly different,

aberrant firing patterns in the same neural populations. In addition, the off period occurrence may contribute to sensory disconnection and interrupt signal propagation across cortical areas (Rosanova *et al.*, 2018) and thus shunt the effect of aberrant activity on widespread cortical networks. An intriguing possibility is that the functional brain state during NREM sleep could disrupt abnormal tinnitus-related neural activity, resulting in a suppression of the phantom perception. Thus, we hypothesize that NREM sleep under physiologically elevated sleep pressure, associated with high global SWA, might ‘override’ aberrant cortical activity associated with tinnitus, or suppress its effect on wider brain networks.

Arguably, the ideal way to address this possibility is to assess an objective correlate of tinnitus across vigilance states. While structural and functional changes in tinnitus have been described in a variety of brain areas (Elgoyhen *et al.*, 2015a; Leaver *et al.*, 2011; Rauschecker *et al.*, 2010b), a particularly prominent correlate of tinnitus is elevated activity and excitability along the auditory pathway including the auditory cortex (Engineer *et al.*, 2011; Leaver *et al.*, 2011; R. Salvi *et al.*, 2000). It has been suggested that increased gain in the ascending auditory pathway is a direct consequence of reduced afferent input. Neurons adjacent to the deafferented neurons in the tonotopic organisation are relieved from lateral inhibition and show hyperactivity (Eggermont & Komiya, 2000; Noreña & Eggermont, 2003a). This may ultimately increase the neural tone and elevate the excitability for stimuli these cells are sensitive to, which has been argued to produce phantom precepts in adjacent frequency ranges to the deafferented frequency range (Eggermont & Roberts, 2004c). Importantly, this is not always the case in tinnitus sufferers (König *et al.*, 2006) and it remains uncertain whether increased evoked activity after noise overexposure is ultimately linked to tinnitus and not merely

a homeostatic compensation in response to reduced afferent input, such as on level of the auditory brainstem (Schaette & McAlpine, 2011). Yet increased spontaneous activity has been shown in tinnitus models on multiple levels of the ascending auditory pathway, perhaps most prominently in the cochlear nucleus (Brozoski et al., 2002; Kaltenbach et al., 2004; Wu et al., 2016) and on cortical level (chinchillas: Salvi, Wang and Ding, 2000, cats: Noreña, Tomita and Eggermont, 2003, guinea pigs: Syka, Rybalko and Popelář, 1994; Coomber *et al.*, 2014 and rats: Syka and Rybalko, 2000). Once such aberrant activity affects cortical processing, it has potential to interfere with vigilance state dynamics requiring the modulation of global cortical activity (Vyazovskiy & Harris, 2013) and, in turn, might be affected by such dynamics itself.

Thus, the assessment of auditory evoked responses may provide a valuable tool to measure a putative tinnitus correlate in passive subjects, irrespective of their vigilance state. Indeed, while the brain state can affect the magnitude of evoked cortical activity (Sela et al., 2020), evoked potentials are largely preserved across sleep states (Nir et al., 2015; Sela et al., 2016). Evoked brain activity could therefore be an indicator for the interference of sleep with functional changes underlying increased excitability in tinnitus (Fig.1A). Here, stimulus-specific evoked activity was assessed across different vigilance states in freely-moving ferrets with behavioural symptoms of tinnitus.

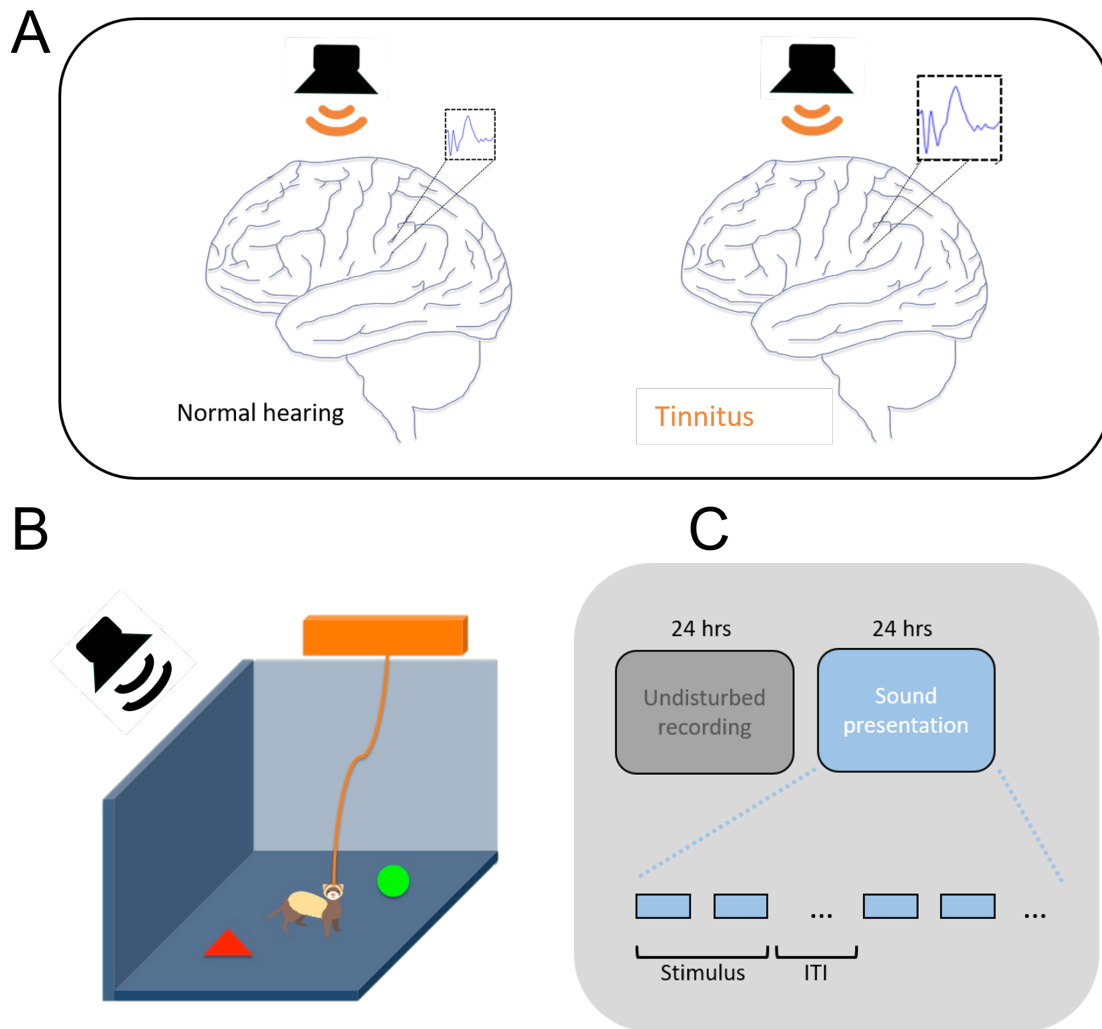


Fig. 1: Setup and experimental paradigm. (A) Hyperexcitability in the auditory pathway may increase auditory evoked activity in tinnitus. In this experiment evoked activity is tested before and after NOE, and the influence of the vigilance state on evoked activity is evaluated. **(B)** Schematic depiction of the recording setup (identical to the recording setup described in Result Chapter 3), including a free-field speaker installed close to the cage ceiling. **(C)** Summary of the paradigm and sound presentation. Animals are recorded for two continuous days (48 hrs), whereas they are left undisturbed in the first 24 hours. Subsequently, narrow-band sounds with central frequencies of 1, 4, 8 and 16 kHz, stimulus duration 820ms, (including a silent gap of 38ms at the centre of the stimulus) are presented at 40, 50, 60 and 65 dB SPL with a randomized inter trial interval (ITI) of 10-42 seconds. Each sound stimulus-intensity combination is presented 200 times over the course of 24 hours.

Method summary

Animals

The data reported in this chapter were obtained from the same ferrets as described in Chapter 3.

Sound presentation during sleep and wakefulness

Auditory evoked responses were assessed on three occasions: initially, under baseline conditions before NOE; then approximately 1 week after NOE (Post1) and approximately 6 months after NOE (Post2) (see Chapter3, Fig.1). Ferrets were kept in identical conditions as during chronic electrophysiological recordings (see Chapter 3): animals were housed individually in a custom-made recording chamber in a 15/9h light-dark cycle. Frontal and occipital EEG and neck-muscle EMG recordings were obtained in the freely-moving animal over ~48 hours per recording session. Signals from the laminar probe in the contralateral ACx relative to the EEG electrodes were not included in the analysis due to insufficient signal quality. In the first 24 hours per recording session the animal was left undisturbed (Fig.1C). Over the course of the second 24 hours of the session auditory stimuli were presented via a free-field speaker installed over the recording cage (Fig.1B,C). Presented sounds were narrow-band stimuli of similar frequency ranges as the stimuli used in the operant gap detection paradigm described in Chapter 1 with centre frequencies of 1, 4, 8 and 16 kHz. Stimuli had a duration of 820ms and had a silent gap of 38ms at the centre of the stimulus. Stimuli were presented at 40, 50, 60 and 65 dB SPL (as measured at floor level in the centre of the recording cage). Inter-stimuli intervals (ITIs) were of a random duration between 10 and 42s and

each stimulus-intensity combination was presented 200 times. Stimuli were generated via MATLAB and produced using an RP2.1 Enhanced Real-Time Processor (TDT) and an Alesis RA150 Amplifier. Stimulus presentation was controlled via a custom written MATLAB script.

Analysis of EEG evoked responses

Vigilance states (Wake, NREM, REM, REM2, see Chapter 3) were manually assigned for each 4-s epoch of the recording, based on visual inspection of the filtered signals and the video recordings of the animal (See Methods: Vigilance state scoring). For analysis of EEG evoked potentials, raw data were transformed into a MATLAB compatible format (.mat) using the *tdtbin2mat* MATLAB function (provided by TDT). Evoked EEG responses were analysed within a time window set between -0.5s and +5s relative to sound stimulus onset. Each stimulus presentation is referred hereafter as a trial. Trials that fell into an epoch that had been scored as artefact in the manual vigilance scoring procedure were excluded from further analysis. To aid computing efficiency the signal was down sampled by a factor of 2 (from an original sampling rate of 3051.7578Hz). Trials were then sorted into groups based on stimulus frequency, intensity, and vigilance state. Due to marked noise in the EEG signal of individual trials, which was not possible to exclude by conventional filtering, it was not possible to define peaks and troughs of auditory evoked potentials (AEPs) reliably. To reduce the impact of noise on AEP detection 20 bootstrapped means were drawn to serve as a representative signal sample for subsequent analysis, allowing for detection of peaks and troughs with minimal bias of noise while still reflecting the variability in the original dataset. Bootstrapped means were drawn from each group of trials (all trials for the same condition, vigilance state,

stimulus and stimulus intensity), respectively. Peaks and troughs of evoked potentials were detected after smoothing the signal using a moving average of ~8 points (implemented by the `smooth` MATLAB function). As the shape of the evoked potential was not uniform across animals (Fig.5), criteria for identification of peaks and troughs were defined based on the individual animal and derivation. Peaks and troughs were automatically detected in a time window after stimulus onset that was predefined using a custom written MATLAB script and the *findpeaks* MATLAB function. Time windows for early (R1), mid (R2) and late (R3) response components were defined based on the average latency of peaks and troughs in the respective derivation (see Fig.5 for illustration). Note that for Case1 R1, R2 and R3 could be identified, for Case2 R1 and R2, and for Case3 only one response component, R1.

Statistical analysis

Statistical analysis of evoked responses was performed in SPSS. Analysis of main effects and group interactions were estimated by fitting a generalised linear mixed model (GLMM) with the repeated measures condition, stimulus, vigilance state, trial and response component (R1-R3). Note that analysis was performed on populations of bootstrapped averages (see 'Analysis of evoked responses' above), which may have led to the observed small standard errors of within-group-variability. Statistical analysis of sleep episode lengths and numbers of brief awakenings were performed in MATLAB using Wilcoxon rank sum tests.

Results

Sound evoked responses were observed in all vigilance states without modifying the sleep-wake cycle

Prior to noise overexposure all animals showed sound evoked responses in both frontal and occipital EEG derivations across all vigilance states (Figs.2,4,6). Note that variability in EEG electrode positions likely accounts for differences in latencies and shapes of evoked responses between animals, as well as in the presence or prominence of secondary or tertiary AEPs, such as responses to the offset of the sound stimulus. Analysis focused on onset responses only, as this only AEPs present in all derivations and animals. EEG evoked responses were associated with mild EMG responses in Case2 and Case3, yet these were unlikely to be pronounced startle responses as EMG responses were low in amplitude and, in Case3, only present in one derivation (Figs. 3,5,7).

Despite evident sound evoked EEG responses, ferrets spent the majority of time asleep while sounds were presented (Fig.8A,B,C). In fact, the amount of sleep was either similar to baseline recordings without sound presentation (**Case1**, No sound vs Sound, 84.9% vs 84.2% of recording time, Fig.8A) or even increased in recordings with sound presentation (**Case2**, No sound vs Sound, 71.8% vs 84%, **Case3**, No sound vs Sound, 70.9% vs 73.6%, Fig.8B,C), suggesting that sound presentation did not prevent ferrets from accumulating similar sleep amounts as in undisturbed conditions. Further, sleep was not more disrupted during sound presentation, as sleep episode lengths were similar between undisturbed recordings and sound presentation and showed high variability in both conditions (**Case1**, No sound vs Sound, $42.15 \pm 44.48 \text{ min}$ vs $50.44 \pm 47.97 \text{ min}$, $p=0.22$, **Case2**, No sound vs Sound, $12.03 \pm 10.41 \text{ min}$ vs 33.9 ± 88.78 , $p=0.05$, **Case3**, No sound vs

Sound, $23.05 \pm 47.67 \text{ min}$ vs $23.84 \pm 42.12 \text{ min}$, $p=0.4$, Wilcoxon rank sum test, Fig.8D,E,F).

The numbers of brief awakenings (waking or movement episodes <16s during sleep) were also similar for Case1 and Case3 (**Case1**, No sound vs Sound, 16.88 ± 7.24 vs 15.08 ± 4.99 , $p=0.63$, **Case3**, No sound vs Sound, 9.96 ± 4.37 vs 9.25 ± 5.61 , $p=0.46$, Fig.8G,I) and were even lower during sound presentation for Case2 (**Case2**, No sound vs Sound, 19.13 ± 6.41 vs 10.21 ± 8.55 , $p<0.001$, Wilcoxon rank sum test, Fig.8H).

These results indicate that the presented sounds were sufficiently salient to trigger evoked EEG responses but did not disrupt sleep or prevent animals from sleeping.

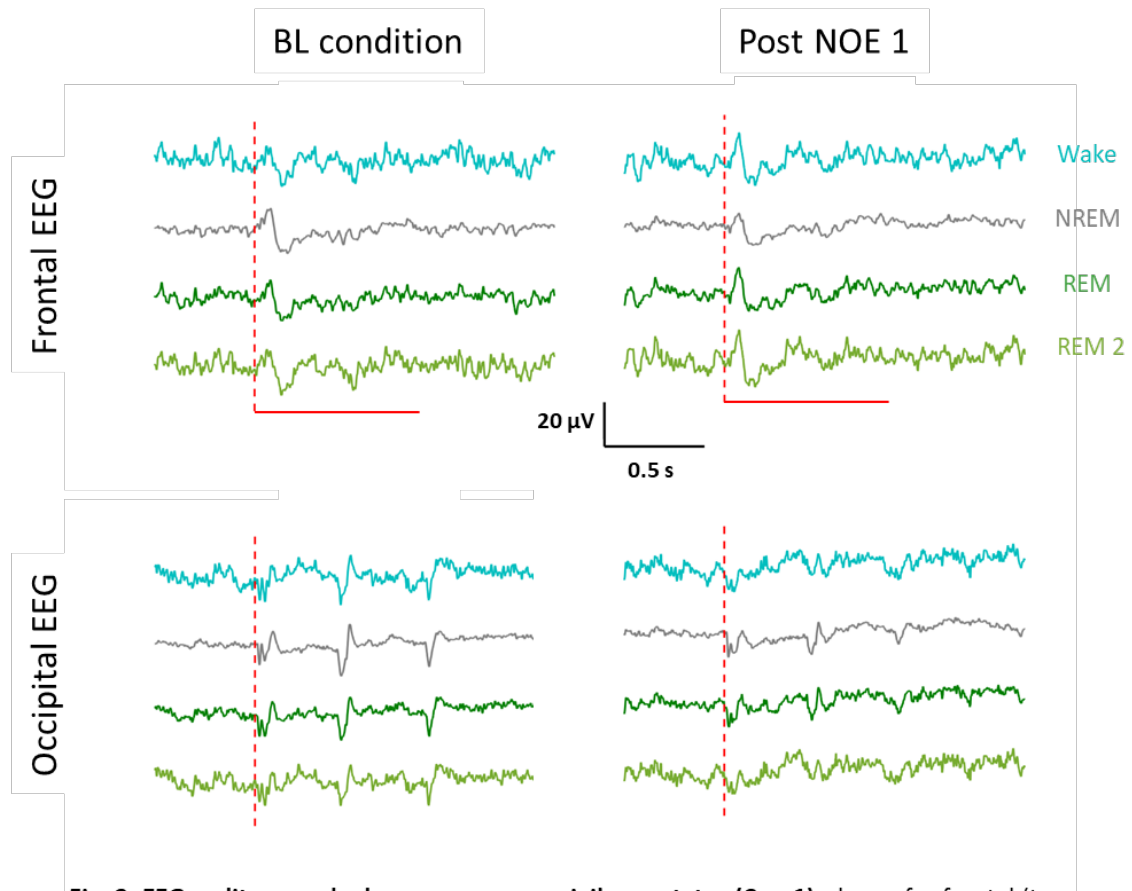


Fig. 2: EEG auditory evoked response across vigilance states (Case1), shown for frontal (top panels) and occipital derivations (bottom panels) during the BL condition and Post1, respectively. The dotted red vertical line in each panel depicts the stimulus onset. The averages displayed in this figure are based on snippets of raw recording data after exclusion of signal artefacts (see Methods: vigilance state scoring) and are averages over signals for all stimulus types (820ms narrow-band sounds centred around 1, 4, 8 and 16 kHz with a 38ms silent gap at the centre of the stimulus,) and intensities (40, 50, 60 and 65 dB SPL). Stimuli were presented via a free-field speaker installed close to the ceiling of the recording chamber (see Method summary).

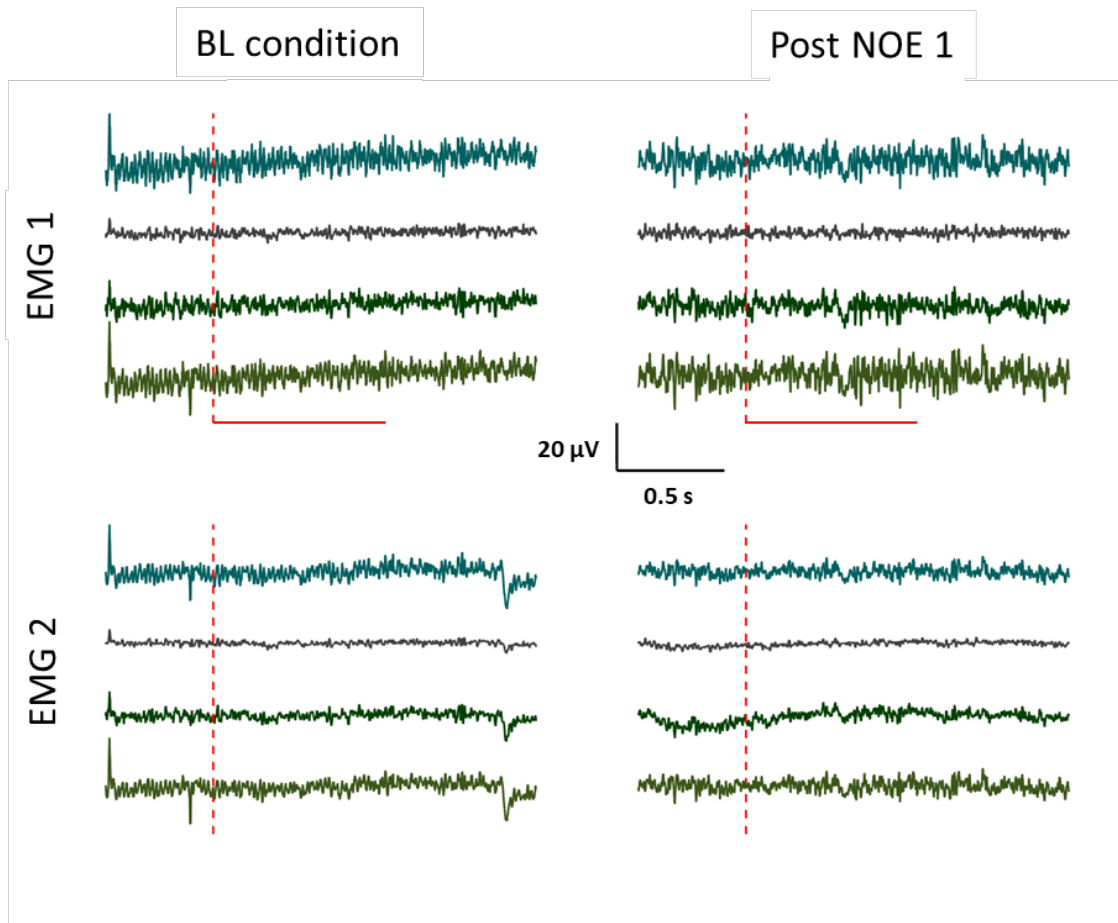


Fig. 3: EMG traces across vigilance states (Case1), shown for both neck muscle EMG derivations (EMG1 (top) and EMG2 (bottom)) during BL conditions and Post1, respectively. The dotted red vertical line in each panel depicts the stimulus onset. The averages displayed in this figure are based on snippets of raw recording data after exclusion of signal artefacts (see Methods: vigilance state scoring) and are averages over signals for all stimulus types (820ms narrow-band sounds centred around 1, 4, 8 and 16 kHz with a 38ms silent gap at the centre of the stimulus,) and intensities (40, 50, 60 and 65 dB SPL). Stimuli were presented via a free-field speaker installed close to the ceiling of the recording chamber (see Method summary).

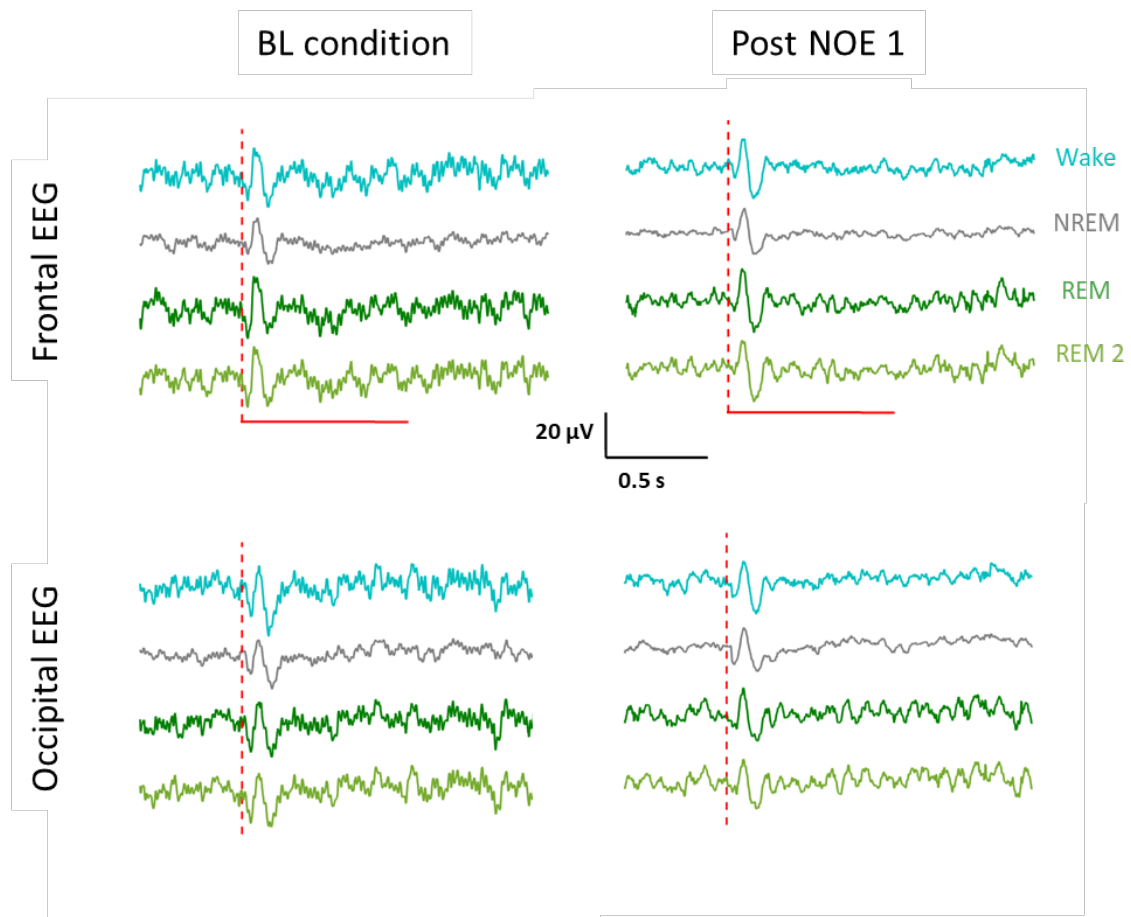


Fig. 4: EEG auditory evoked responses across vigilance states (Case2), shown for frontal (top panels) and occipital derivations (bottom panels) during the BL condition and Post1, respectively. The dotted red vertical line in each panel depicts the stimulus onset. The averages displayed in this figure are based on snippets of raw recording data after exclusion of signal artefacts (see Methods: vigilance state scoring) and are averages over signals for all stimulus types (820ms narrow-band sounds centred around 1, 4, 8 and 16 kHz with a 38ms silent gap at the centre of the stimulus,) and intensities (40, 50, 60 and 65 dB SPL). Stimuli were presented via a free-field speaker installed close to the ceiling of the recording chamber (see Method summary).

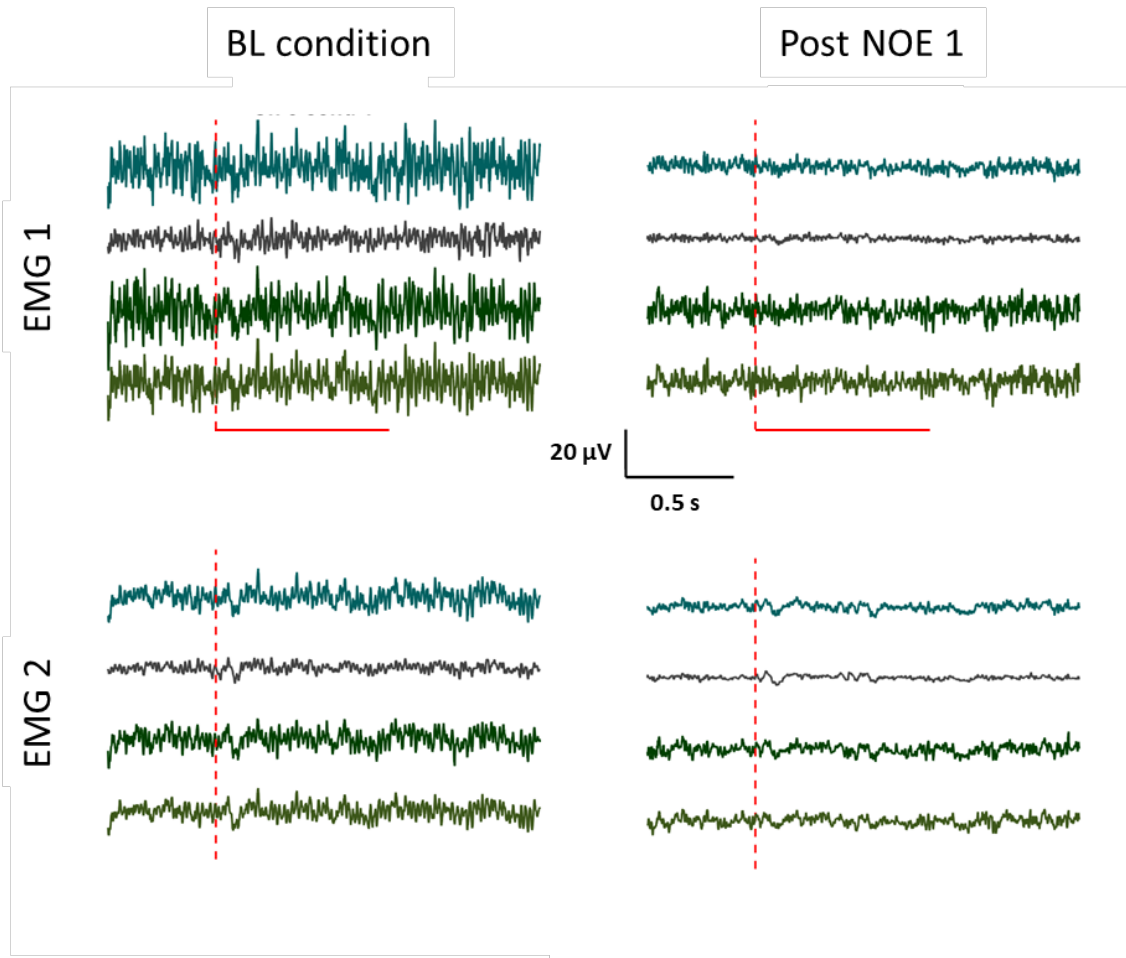


Fig. 5: EMG traces across vigilance states (Case2), shown for both neck muscle EMG derivations (EMG1 (top) and EMG2 (bottom)) during BL conditions and Post1, respectively. The dotted red vertical line in each panel depicts the stimulus onset. The averages displayed in this figure are based on snippets of raw recording data after exclusion of signal artefacts (see Methods: vigilance state scoring) and are averages over signals for all stimulus types (820ms narrow-band sounds centred around 1, 4, 8 and 16 kHz with a 38ms silent gap at the centre of the stimulus,) and intensities (40, 50, 60 and 65 dB SPL). Stimuli were presented via a free-field speaker installed close to the ceiling of the recording chamber (see Method summary).

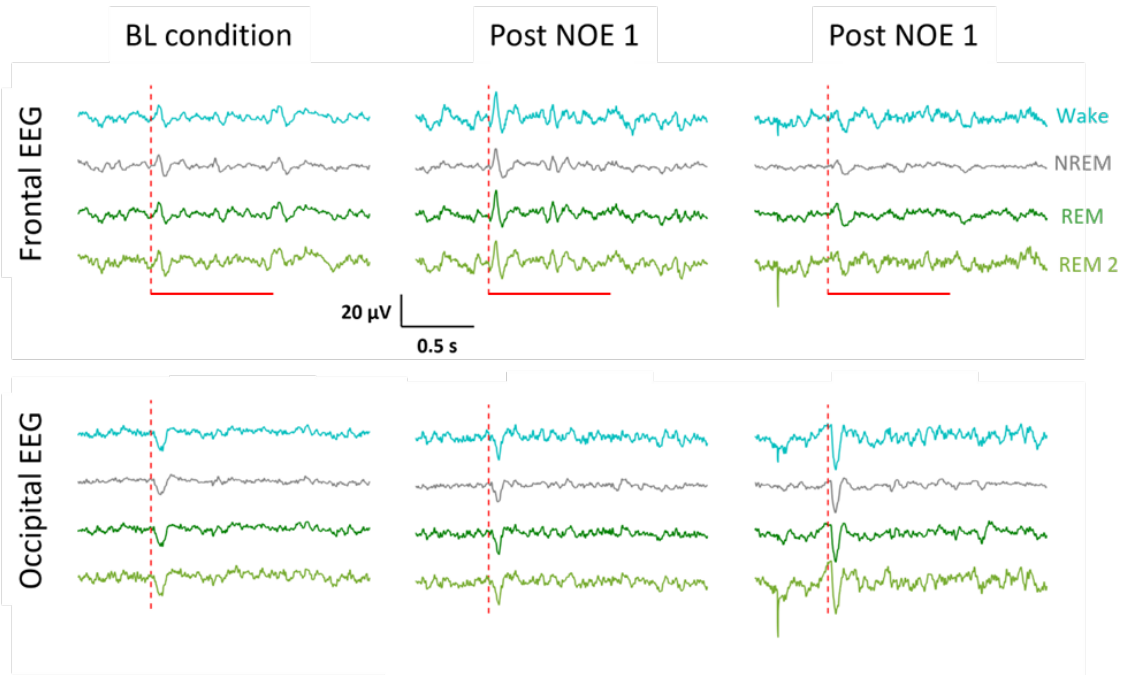


Fig. 6: EEG auditory evoked responses across vigilance states (Case3), shown for frontal (top panels) and occipital derivations (bottom panels) during the BL condition, Post1 and Post2, respectively. The dotted red vertical line in each panel depicts the stimulus onset. The averages displayed in this figure are based on snippets of raw recording data after exclusion of signal artefacts (see Methods: vigilance state scoring) and are averages over signals for all stimulus types (820ms narrow-band sounds centred around 1, 4, 8 and 16 kHz with a 38ms silent gap at the centre of the stimulus,) and intensities (40, 50, 60 and 65 dB SPL). Stimuli were presented via a free-field speaker installed close to the ceiling of the recording chamber (see Method summary).

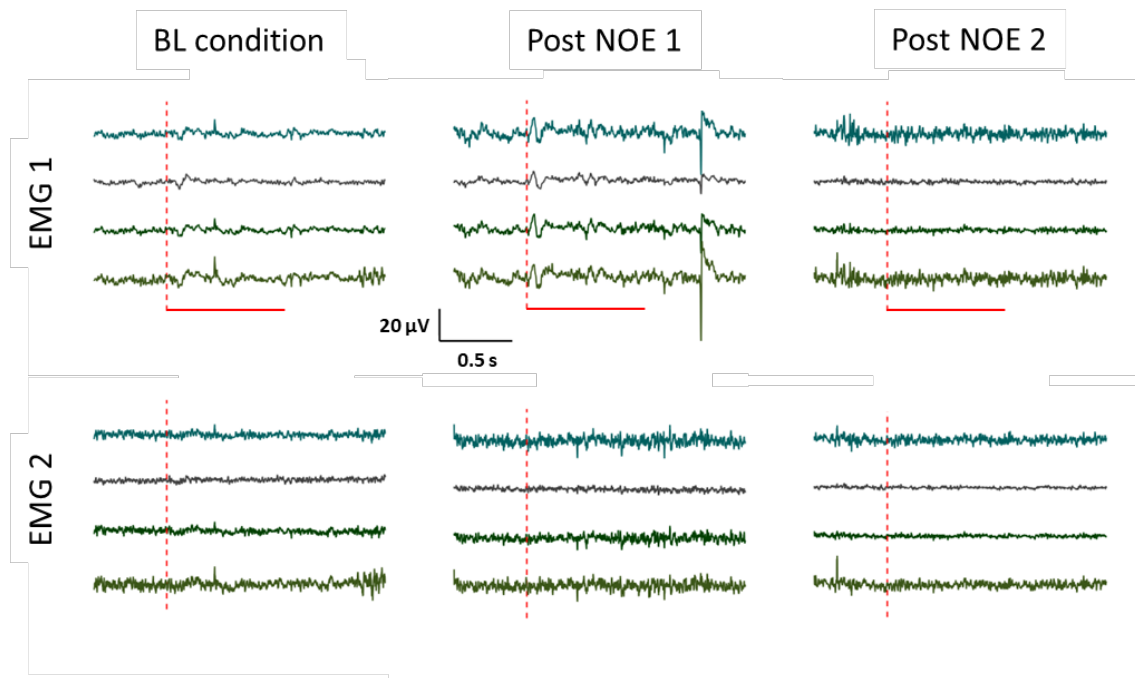


Fig. 7: EMG traces across vigilance states (Case3), shown for both neck muscle EMG derivations (EMG1 (top) and EMG2 (bottom)) during BL conditions, Post1 and Post2, respectively. The dotted red vertical line in each panel depicts the stimulus onset. The averages displayed in this figure are based on snippets of raw recording data after exclusion of signal artefacts (see Methods: vigilance state scoring) and are averages over signals for all stimulus types (820ms narrow-band sounds centred around 1, 4, 8 and 16 kHz with a 38ms silent gap at the centre of the stimulus,) and intensities (40, 50, 60 and 65 dB SPL). Stimuli were presented via a free-field speaker installed close to the ceiling of the recording chamber (see Method summary). Note that the prominent spike towards the end of the recorded snippets in Post NOE 1 (EMG1) is likely an artefact, as a physiological muscular twitch of this magnitude would be evident in the adjacent EMG signal (EMG2).

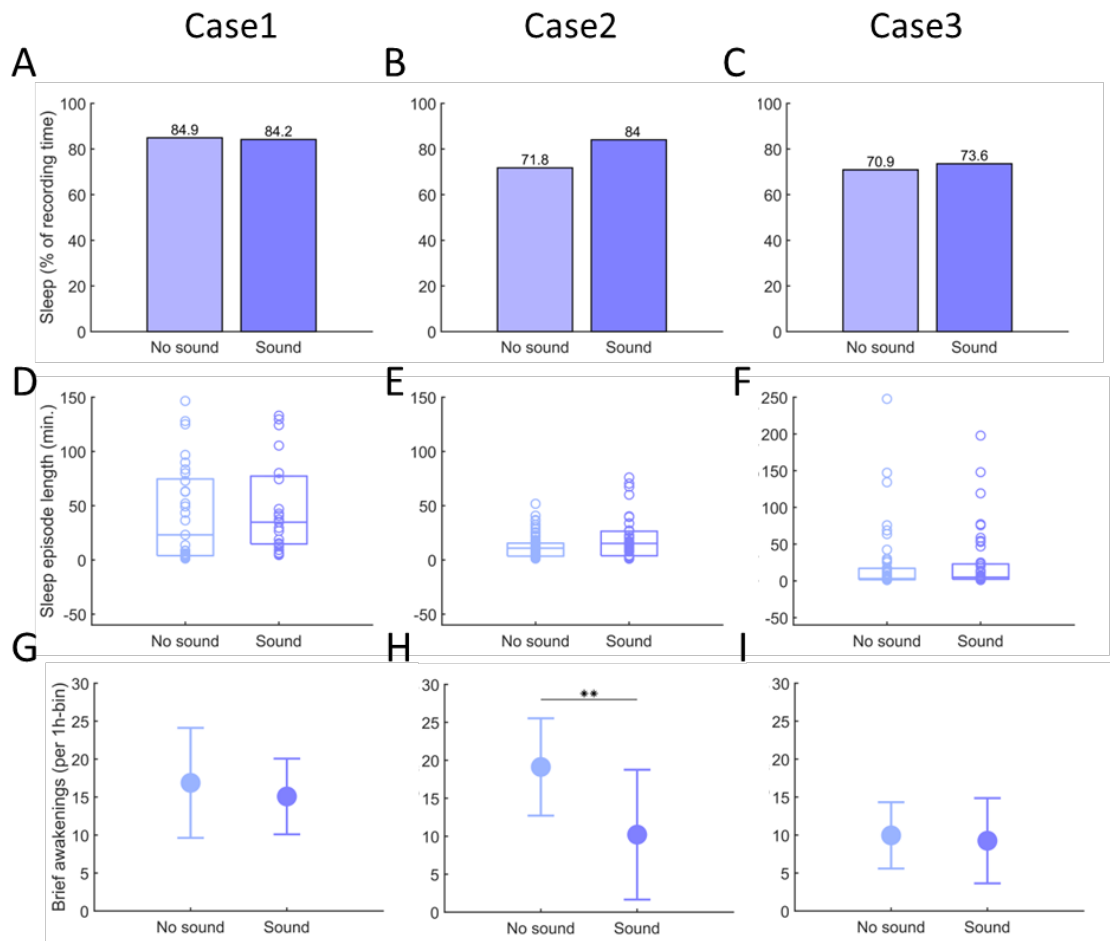


Fig.8: Sleep architecture with and without sound presentation before NOE. (A,B,C) Sleep amount as % of recording time under baseline conditions and either in an undisturbed recording without sound presentation ('No sound') or with sound presentation ('Sound'). Each panel shows data from one animal (Cases1,2,3). (D,E,F) Sleep episode lengths (of sleep episodes > 1 min) in undisturbed recordings and recordings with sound presentation. Boxes show interquartile ranges and the median. Datapoints depict lengths of individual episodes. Each panel shows data from one animal (Cases1,2,3). (G,H,I) Number of brief awakenings per 1h time interval (defined as <16 s of wakefulness or movement preceded and followed by sleep). Data and error bars are means and standard deviations. Each panel shows data from one animal (Cases1,2,3). Horizontal line with ** markers depicts $p < 0.001$, Wilcoxon rank sum test.

NREM sleep is associated with lowest evoked responses across vigilance states

To assess changes in the auditory evoked response (AER) throughout the experiment, the amplitude of the wave in the respective EEG signal after stimulus onset was detected with a custom-made algorithm (see ‘Analysis of evoked potentials’ in the Method summary). Since the shape of evoked responses varied between animals, likely because of variations in exact electrode location, the response windows for early and late components of the response were defined for each animal individually (Fig.9). Response magnitudes for each response component were then defined as the difference between the maximum and subsequent minimum of the signal in the response window for the respective response component (Fig.9B,D,F). Specifically, for Case1, the difference in voltage between the first peak and subsequent trough of the response (R1, Fig. 9B), between second peak and subsequent trough (R2) and between the third peak and subsequent trough (R3) were measured. For Case2 only two response components could be reliably determined (early and late, R1 and R2) and only one component for Case3 (Fig.9D,F).

To gain a picture of how the evoked response is modulated by the vigilance state AER magnitudes of all measurement variables except ‘vigilance state’ were pooled (stimulus, intensity, trial number and response component). All animals showed a significant interaction between the magnitude of the AER and the vigilance state in both the frontal EEG (**Case1**, $F_{(1,1915)}=1.364E+31$, $p<0.001$, **Case2**, $F_{(1,1276)}=1.423E+32$, $p<0.001$, **Case3**, $F_{(1,956)}=1.874E+30$, $p<0.001$, Fig.10A,C,E) and the occipital EEG (**Case1**, $F_{(1,1916)}=7.802E+24$, $p<0.001$, **Case2**, $F_{(1,1276)}=4.892E+29$, $p<0.001$, **Case3**, $F_{(1,956)}=8.092E+30$, $p<0.001$, Fig.10B,D,F). In all animals and derivations, AER magnitudes

were lowest during NREM sleep (**Frontal EEG: Case1, Wake vs NREM vs REM vs REM2**, $27.05 \pm 17.11 \mu V$ vs $14.17 \pm 10.09 \mu V$ vs $21.49 \pm 13.72 \mu V$ vs $28.05 \pm 17.09 \mu V$, **Case2, Wake vs NREM vs REM vs REM2**, $40.52 \pm 16.19 \mu V$ vs $24.71 \pm 10.66 \mu V$ vs $40.57 \pm 16.7 \mu V$ vs $39.89 \pm 17.95 \mu V$, **Case3, Wake vs NREM vs REM vs REM2**, $37.78 \pm 11.31 \mu V$ vs $22.42 \pm 5.66 \mu V$ vs $31.98 \pm 8.21 \mu V$ vs $42.29 \pm 9.98 \mu V$, **Occipital EEG: Case1, Wake vs NREM vs REM vs REM2**, $23.51 \pm 9.08 \mu V$ vs $13.47 \pm 3.49 \mu V$ vs $18.66 \pm 6.23 \mu V$ vs $23.58 \pm 8.16 \mu V$, **Case2, Wake vs NREM vs REM vs REM2**, $40.2 \pm 15.63 \mu V$ vs $25.59 \pm 7.77 \mu V$ vs $40.55 \pm 15.08 \mu V$ vs $39.09 \pm 14.55 \mu V$, **Case3, Wake vs NREM vs REM vs REM2**, $37.58 \pm 13.07 \mu V$ vs $22.52 \pm 4.49 \mu V$ vs $31.54 \pm 7.6 \mu V$ vs $42.75 \pm 16.4 \mu V$) and highest during Wake and REM2 sleep, suggesting that NREM sleep may dampen sound evoked activity. The modulation of AERs by the vigilance state was similar across sound intensities (Supp.Figs.4,5). As stimulus intensity did not consistently affect evoked responses across any vigilance state these results do not indicate whether NREM sleep dampens evoked activity independent of sound intensity or whether it raises the threshold for sound evoked responses.

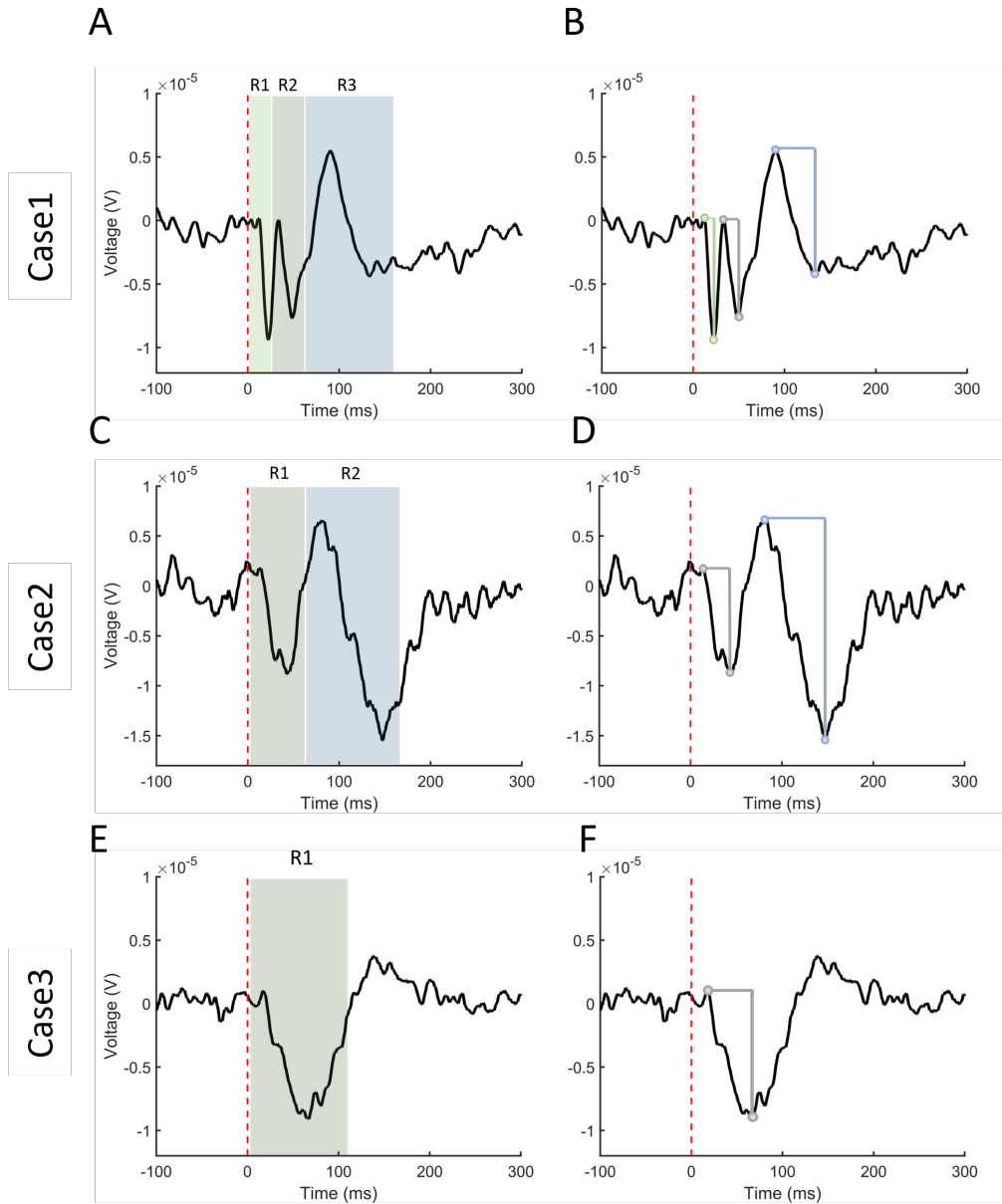


Fig.9: Definitions of early, mid and late components of the AER and AER magnitudes. (A,C,E) Time windows for response components. Average occipital EEG evoked potential during BL with an outline of the time windows the response components (R1,R2,R3) were defined in. Similar windows were applied for the frontal EEG signal. Note that a different number of response windows was defined for different animals. **(B,D,F)** Definition of response magnitudes. An custom-written MATLAB algorithm selected the maximum value within each response window and the subsequent minimum to compute the response magnitude for each response component. The difference between the designated maximum and minimum in each time window defined the response magnitude.

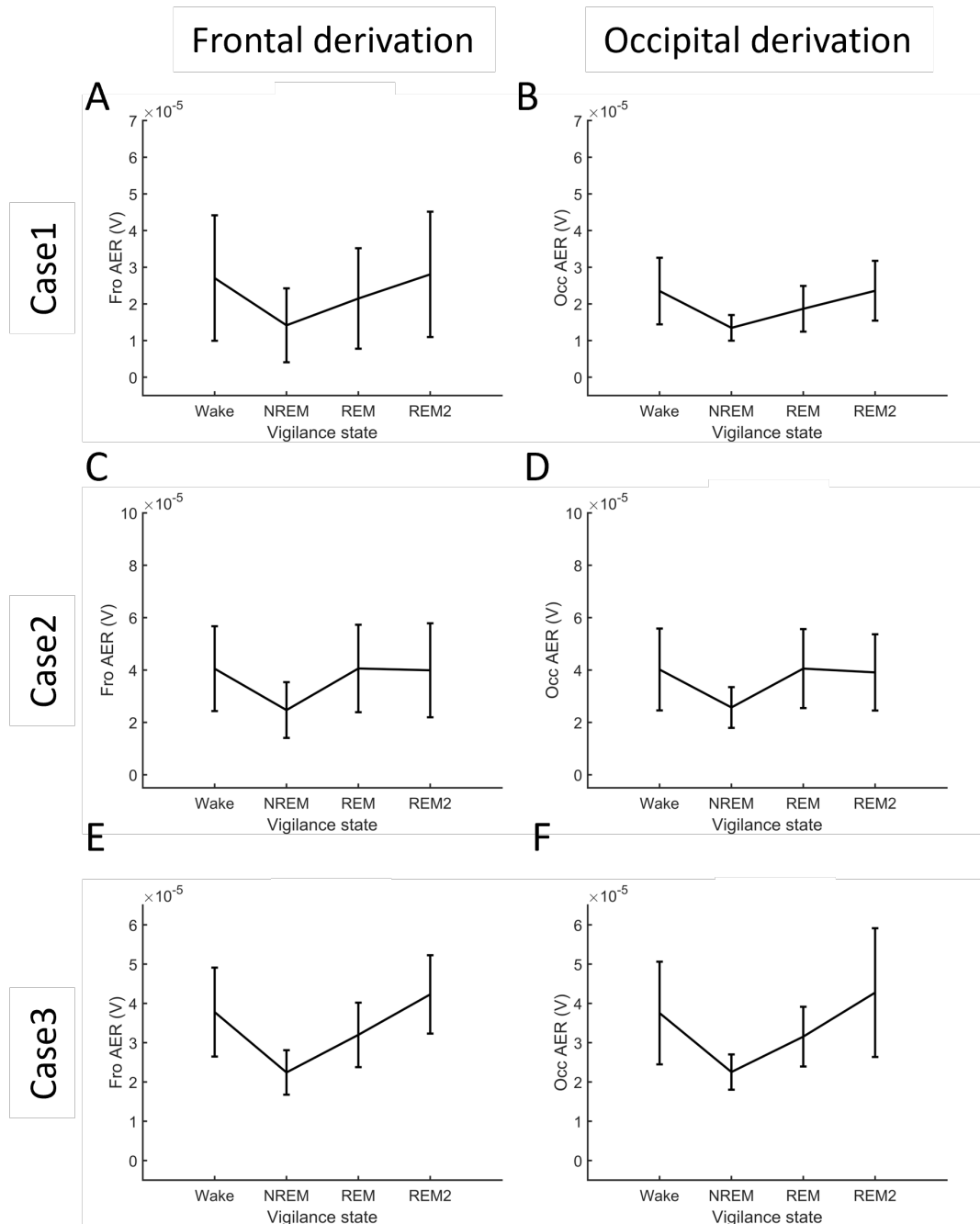


Fig.10: Modulation of EEG evoked responses by vigilance state. Each panel shows the average auditory evoked response (AER) magnitude of one animal and EEG derivation, separated by vigilance state. Averages are calculated across conditions, sound stimuli, stimulus intensities and response windows for each vigilance state (Wake, NREM sleep, REM sleep, REM2 sleep), respectively. Data are means and standard deviations. **(A,B)** Data for Case1 based on the frontal and occipital EEG, respectively. **(C,D)** Data for Case2 and **(E,F)** Case3 are shown in the same manner.

Noise exposure is associated with changes in evoked activity

After noise exposure (Post1) animals showed changes in EEG sound evoked activity, with varying dynamics between animals. Individual differences were consistent with the specific cases of tinnitus and/or changed hearing ability each animal represented (see Fig. 39, Chapter 3). Briefly, Cases 1 and 3, which showed evidence for tinnitus without or with minor hearing impairment showed overall increased sound evoked activity after NOE, whereas the animal with indications for pronounced hearing loss showed decreased evoked activity. Notably, these individual changes were often not as pronounced during sleep as they were during wakefulness. 6 months after NOE, all animals showed a tendency towards increased auditory evoked activity. As in the previous chapter, results are in detail discussed for each individual animal. Since neither animal showed consistent modulation of evoked responses with changing stimulus intensities (see also the chapter's Discussion: Limiting factors), analysis was based on average responses across stimulus intensities.

Case1: Increased evoked activity after NOE, an effect dampened during NREM sleep.

Overall AERs (pooled across all response components) in Case1 were increased already in the first assessment after noise overexposure (Post1) as compared to BL (effect of condition, Fro EEG, $F_{(1,1917)}=7.122E+31$, $p<0.001$, Occ EEG, $F_{(1,1918)}=1.217E+31$, $p<0.001$), in both frontal and occipital signals (Fig. 11C,D). AERs were significantly increased for all sound stimuli in all EEG derivation, in the frontal EEG (*BL vs Post1, 1 kHz NBN, 24.16±17.67µV vs 24.26±15.36, 4 kHz NBN, 21.9±13.9µV vs 23.2±17.84µV, 8 kHz NBN, 20.06±13.3µV vs 23.06±16.04µV, 16 kHz NBN, 20.56±13.4µV vs 24.3±17.46µV*, see Table

1 for statistics) and in the occipital EEG (**1 kHz NBN**, $18.28 \pm 6.53 \mu V$ vs $21.4 \pm 9.1 \mu V$, **4 kHz NBN**, $18.92 \pm 8.33 \mu V$ vs $20.02 \pm 8.75 \mu V$, **8 kHz NBN**, $19.21 \pm 7.85 \mu V$ vs $19.61 \pm 7.25 \mu V$, **16 kHz NBN**, $19.83 \pm 7.76 \mu V$ vs $21.16 \pm 9.31 \mu V$, Table 2).

However, while the increase in evoked activity was rather global (evident in both EEG derivations) this was modulated across vigilance states and in a more local manner: in the occipital EEG signal, the increase in evoked responses after NOE was evident in all vigilance states (*occipital EEG*, BL vs Post1, **Wake**, $23.41 \pm 8.61 \mu V$ vs $23.61 \pm 9.55 \mu V$, **NREM**, $13.18 \pm 3.29 \mu V$ vs $13.75 \pm 3.66 \mu V$, **REM**, $17.36 \pm 19.96 \mu V$, **REM2**, $22.29 \pm 7.11 \mu V$ vs $24.87 \pm 8.91 \mu V$, Table 4, Fig.11B), yet in the frontal EEG, NREM sleep was associated with a reduced evoked response after NOE as compared to baseline (*frontal EEG*, BL vs Post1, **Wake**, $25.99 \pm 16.35 \mu V$ vs $28.12 \pm 17.81 \mu V$, **NREM** $15.14 \pm 10.52 \mu V$ vs $13.18 \pm 9.55 \mu V$, **REM**, $19.74 \pm 13.12 \mu V$ vs $23.22 \pm 14.09 \mu V$, **REM2**, $25.8 \pm 15.47 \mu V$ vs $30.3 \pm 18.34 \mu V$, Table 3, Fig.11A). Notably, the reduced NREM AER in the frontal EEG was present for all sound stimuli (Fig.12A). Thus, NREM sleep might have a dampening effect on frontal cortical overexcitability after NOE.

In addition, there was an interaction between the response component (R1,R2,R3), vigilance state and condition for all tested stimuli in the frontal derivation (Table 5, Fig.13): not all components showed an increase in magnitude in all states, except that the early component (R1) tended to be the most elevated after NOE for most tested stimuli and vigilance states. The depression of post NOE response magnitude in NREM was evident for most response components and consistently most pronounced for the mid component (R2).

Similar to the frontal EEG signal, there was an interaction between the response component (R1,R2,R3), vigilance state and condition in the occipital signal (Table 6, Fig.14). While response magnitudes were elevated in the occipital EGG to varying degrees across response components, it is interesting to note that, as in the frontal derivation, the mid component showed a largely consistent NREM-specific decreased after NOE relative to BL.

6 months after NOE, the animal showed evidence for a further increased auditory evoked responses (Post2, Supp. Fig. 1). Due to decreased signal quality in the Post2 assessment this could not be quantitatively verified, yet it is of note in this regard that even without sound presentation, the animal spent more time awake in the Post2 assessment (BL vs Post2, 14.4% vs 21.4%, Fig.15A, grey bars) and sound presentation amplified this effect even further (time awake, BL vs Post2, 15.8% vs 44%, Fig.15A) at the cost of all sleep states (Fig.15B,C,D). This indicates that the animal may have become more responsive to presented sounds – consistent with increased cortical excitability - and was therefore unable to accumulate the same amount of sleep as during sound presentation before noise exposure.

Thus, Case1 showed indications for increased frontal and occipital sound-evoked excitability after NOE, associated with reduced sleep and in line with tinnitus symptoms described in Chapter 3. During NREM sleep, the increase in evoked activity was initially dampened, possibly explaining why sleep durations were initially little affected after NOE.

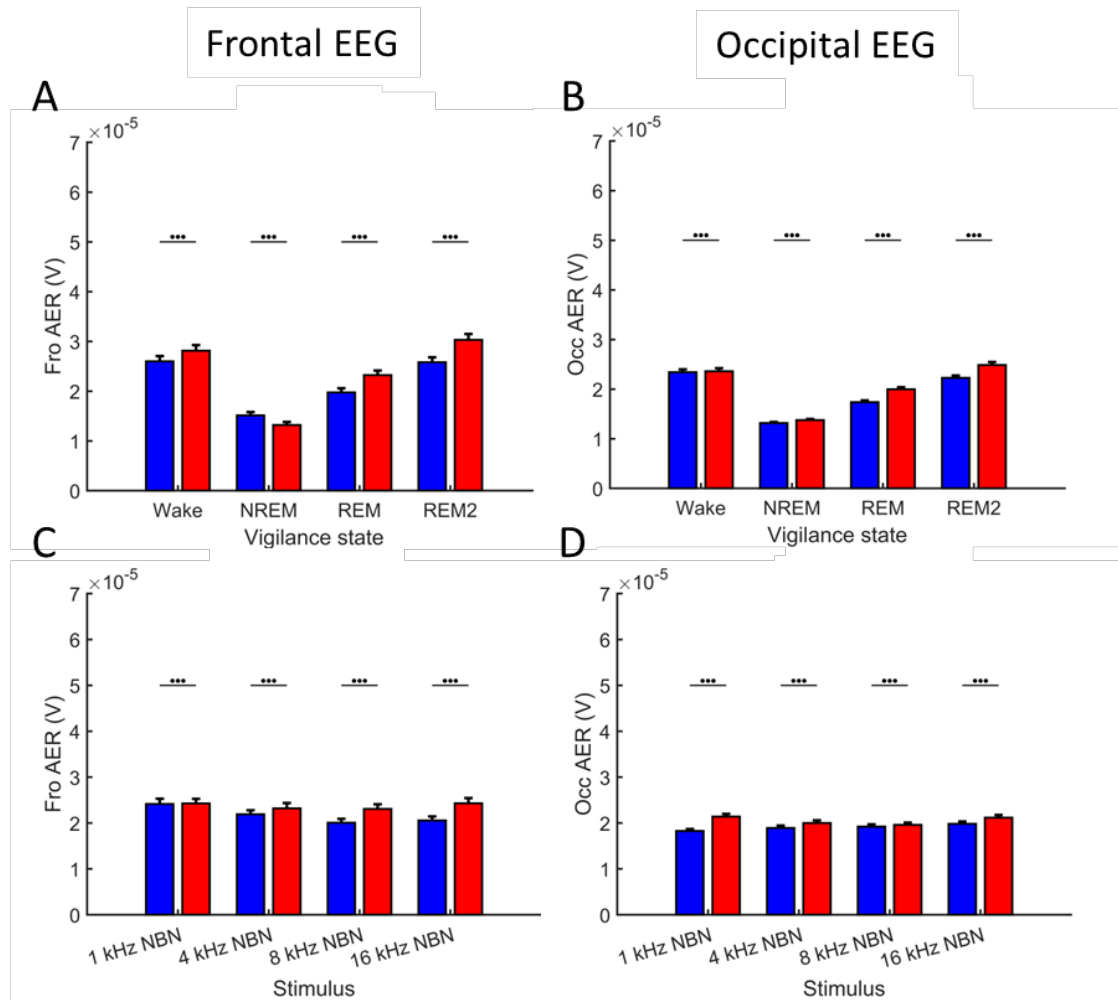


Fig. 11: EEG auditory evoked response magnitude before and after NOE (Case1). (A,B) Average AER magnitude during the baseline condition (blue markers) and Post NOE 1 (red markers) for each vigilance state. Data are averages across stimuli, stimulus intensities and response windows. (C,D) Average AER magnitude for each stimulus. Data are averages across vigilance states, stimulus intensities and response windows. Error bars are standard errors. *** signify significant differences ($p < 0.001$, GLMM).

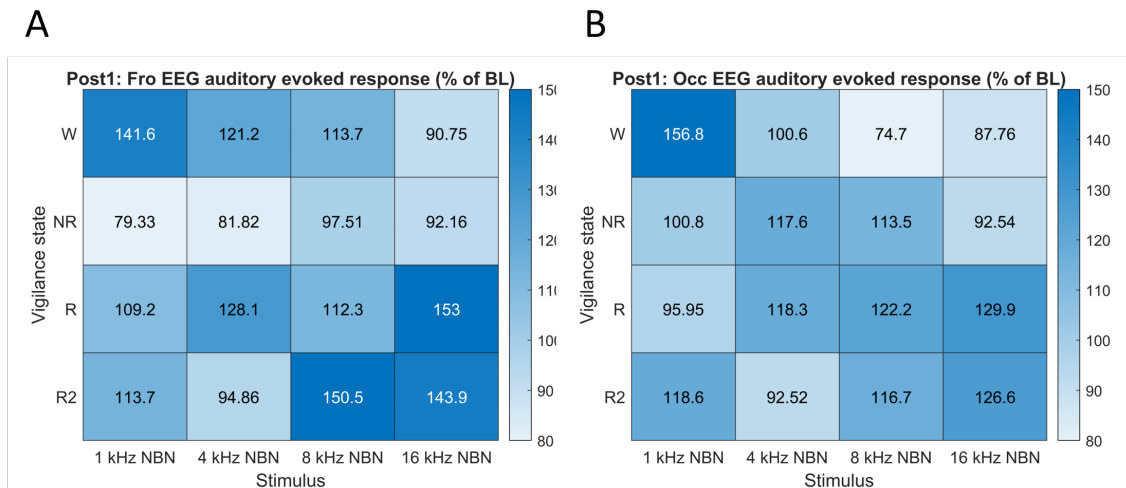


Fig. 12: Change in evoked responses after noise exposure during sleep and wake in Case1. Values are average Post1 evoked responses as % of the baseline condition for the frontal EEG (**A**) and the occipital EEG (**B**). Data are averaged across stimulus intensities and response windows. The x-axis represents stimuli, the y-axis represents the vigilance state (Wake, NREM, REM, REM2).

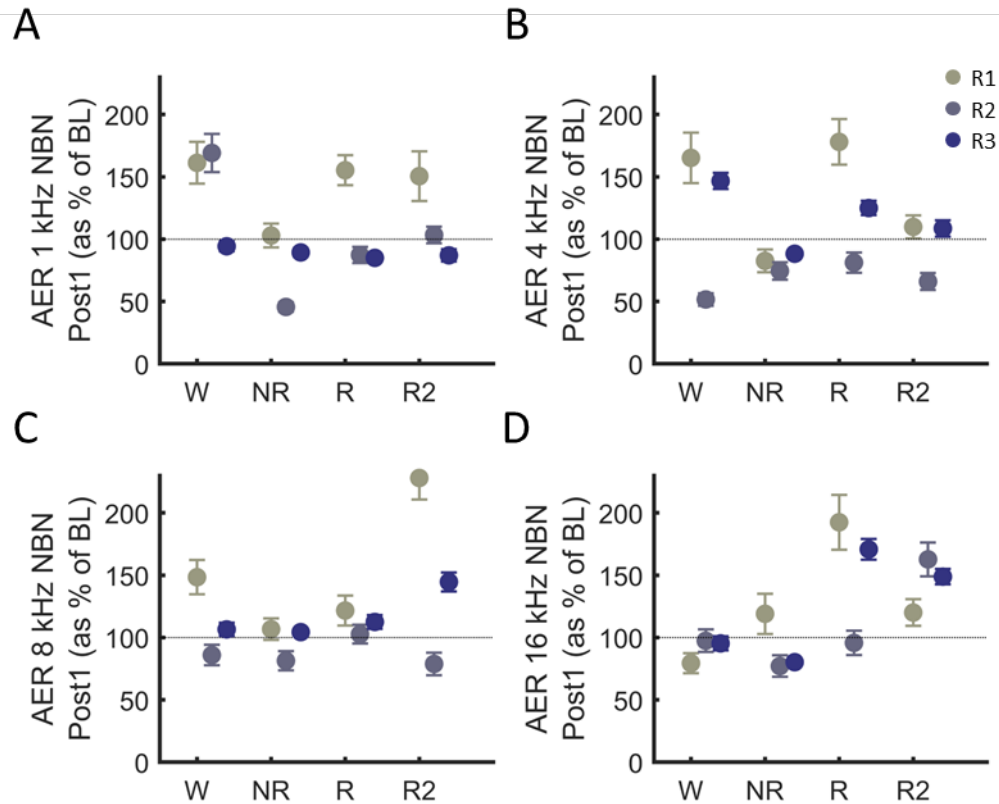


Fig.13: Change in EEG auditory evoked response magnitude after NOE for each response component (Case1, frontal EEG). Each panel shows data separated by vigilance state and AER magnitude measure (early, mid, late response window) for one stimulus type, respectively. Panels **A**, **B**, **C** and **D** show data for 1kHz , 4kHz, 8kHz and 16kHz centred NBN respectively. Data are averages and standard errors as % of the corresponding data in the baseline condition. The dotted horizontal line depicts 'no change' between BL and post NOE (i.e. 100%).

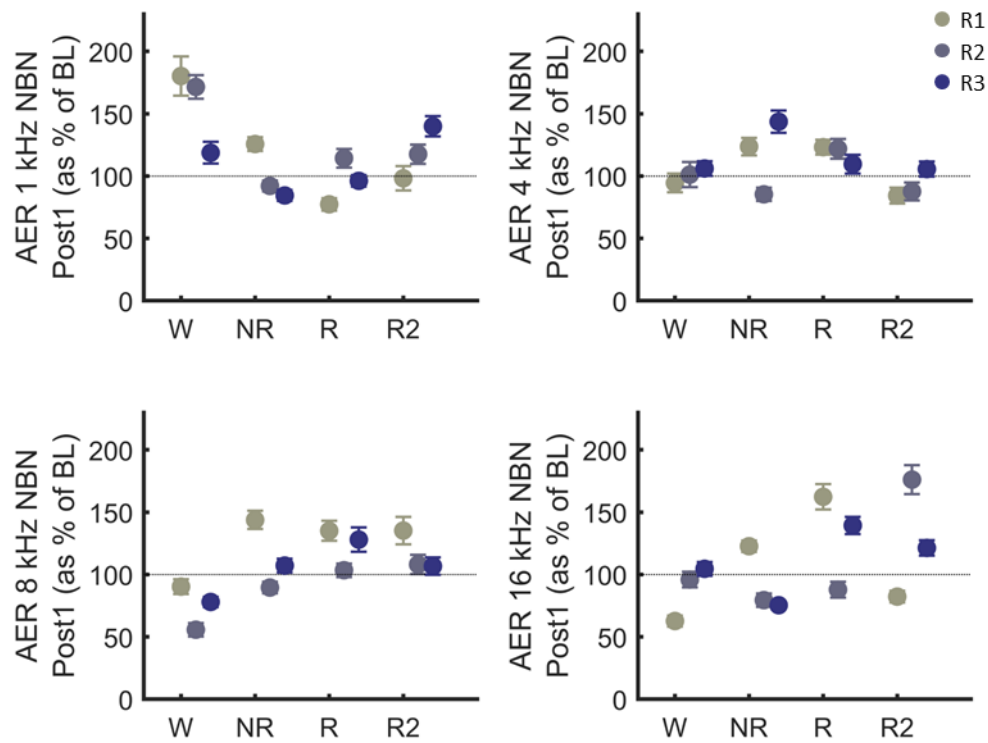


Fig.14: Change in EEG auditory evoked response magnitude after NOE for each response component (Case1, occipital EEG). Each panel shows data separated by vigilance state and AER magnitude measure (early, mid, late response window) for one stimulus type, respectively. Panels **A**, **B**, **C** and **D** show data for 1kHz , 4kHz, 8kHz and 14kHz centred NBN respectively. Data are averages and standard errors as % of the corresponding data in the baseline condition. The dotted horizontal line depicts 'no change' between BL and post NOE (i.e. 100%).

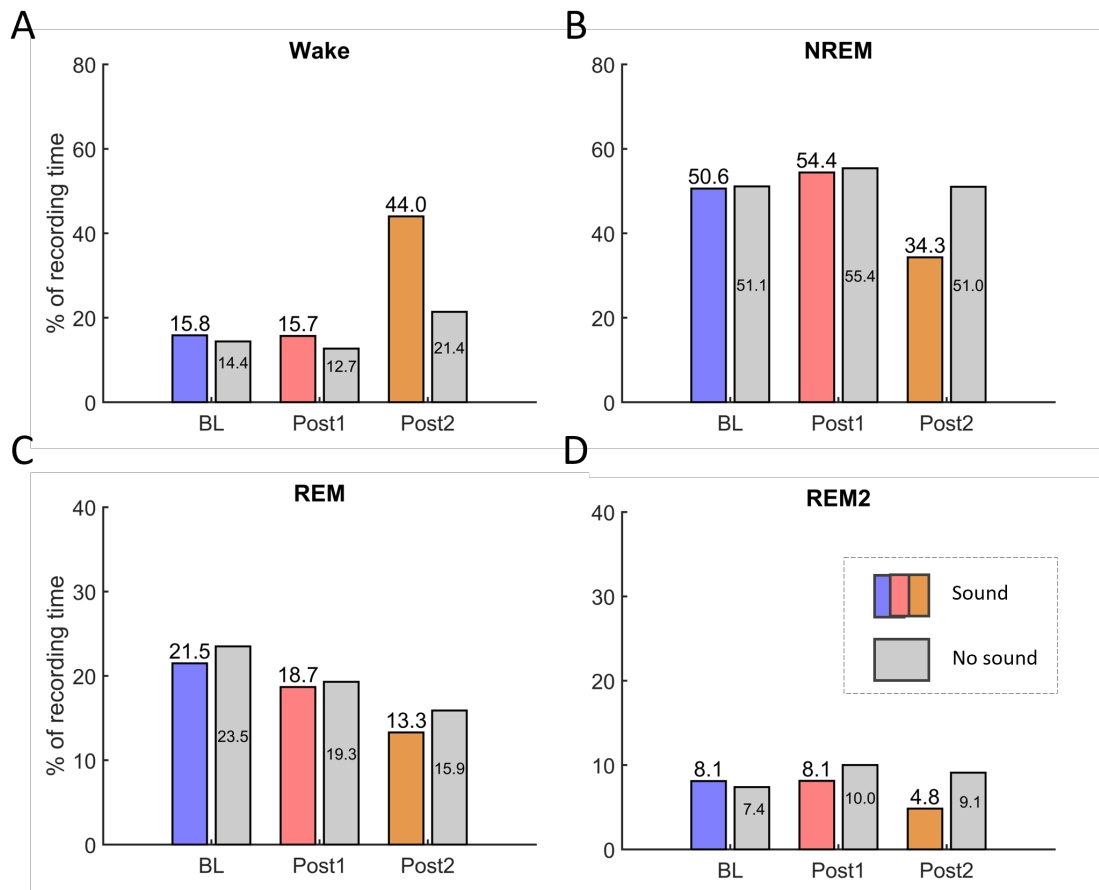


Fig. 15: Vigilance state durations before and after NOE (Case1). (A) Time spent in wakefulness, (B) in NREM, (C) in REM and (D) in REM2 sleep during ~24hrs of recording with sound presentation before NOE (BL), early after NOE (Post1) and late after NOE (Post2). Grey bars show state durations during the same conditions (BL, Post1, Post2) but without sound presentation.

Case2: Decreased evoked activity after NOE, with a trend towards occipital increase in excitability in the long term

Case2 showed reduced evoked activity (pooled across all response components) in the first assessment after NOE (Post1) as compared to BL (*effect of condition, Fro EEG, $F_{(1,1278)}=1.070E+31, p<0.001$, Occ EEG, $F_{(1,1278)}=2.210E+33, p<0.001$, Fig.16C,D*). This applied to all sound stimuli in the frontal EEG signal (*BL vs Post1, 1 kHz NBN, $37.13\pm18.15\mu V$ vs $32.43\pm15.48\mu V$, 4 kHz NBN, $42.47\pm18.84\mu V$ vs $31.24\pm13.63\mu V$, 8 kHz NBN, $41.55\pm18.48\mu V$ vs $34.61\pm17.22\mu V$, 16 kHz NBN, $37.44\pm14.74\mu V$ vs $34.5\pm15.75\mu V$,*

Table 7) and in the occipital signal (BL vs Post1, **1 kHz NBN**, $41.96 \pm 16.66 \mu V$ vs $30.83 \pm 12.03 \mu V$, **4 kHz NBN**, $41.67 \pm 17.54 \mu V$ vs $29.97 \pm 11.0 \mu V$, **8 kHz NBN**, $41.19 \pm 14.37 \mu V$ vs $32.75 \pm 13.62 \mu V$, **16 kHz NBN**, $41.3 \pm 12.88 \mu V$ vs $31.38 \pm 13.22 \mu V$, Table 8). The reduction in AERs was evident during all vigilance states in both the frontal EEG derivation (BL vs Post1, **Wake**, $45.94 \pm 17.55 \mu V$ vs $35.09 \pm 12.59 \mu V$, **NREM**, $26.41 \pm 11.28 \mu V$ vs $23.02 \pm 9.73 \mu V$, **REM**, $43.06 \pm 16.61 \mu V$ vs $38.08 \pm 16.47 \mu V$, **REM2**, $43.18 \pm 17.74 \mu V$ vs $36.6 \pm 17.61 \mu V$, Table 9)(Fig.16A) and in the occipital derivation (BL vs Post1, **Wake**, $48.59 \pm 14.94 \mu V$ vs $31.81 \pm 11.21 \mu V$, **NREM**, $28.07 \pm 8.5 \mu V$ vs $23.32 \pm 6.13 \mu V$, **REM**, $45.01 \pm 13.6 \mu V$ vs $36.08 \pm 15.21 \mu V$, **REM2**, $44.45 \pm 14.99 \mu V$ vs $33.72 \pm 11.94 \mu V$, Table 10, Fig.16B). Only during REM and REM2 sleep there were signs for stable or slightly elevated evoked activity for 8 and 16 kHz stimuli, respectively, yet this was only evident in the frontal EEG (Fig.17A).

Although there was an interaction between response components (R1,R2) in both the frontal EEG (Table 11, Fig.18) and the occipital EEG (Table 12, Fig. 19), the decreased magnitude after NOE was visible for both early (R1) and late (R2) components in most vigilance states for all of the tested sound stimuli.

This changed 6 months after NOE (Post2) where the animal showed indications for an increased AEP in the occipital derivation (Supp. Fig.2). This could not be quantitatively analysed due to reduced signal quality.

While in undisturbed conditions (without sound stimulation) the animal spent progressively less time awake subsequent to NOE (Fig.20A, grey bars) there was no similar trend over time with sound stimulation (Fig.,20A, coloured bars). Here, in the first assessment after NOE, the animal even spent more time awake than during BL

(Fig.20A), with reduced time in all sleep states (Fig.20B,C,D). It may be that the markedly reduced hearing ability in this animal after NOE (see Chapter 3, Fig.39) created an unusually silent environment to the animal, which may have elevated the saliency of some presented stimuli despite overall reduced evoked activity. In the long term, with further degradation in hearing ability, the animal might have become increasingly unresponsive, in line with the high sleep amount evident 6 months after NOE. In fact, locally increased occipital excitability at this point in time may not have been sufficient to govern a disruptive effect of environmental noise on sleep or was possibly less pronounced during sleep.

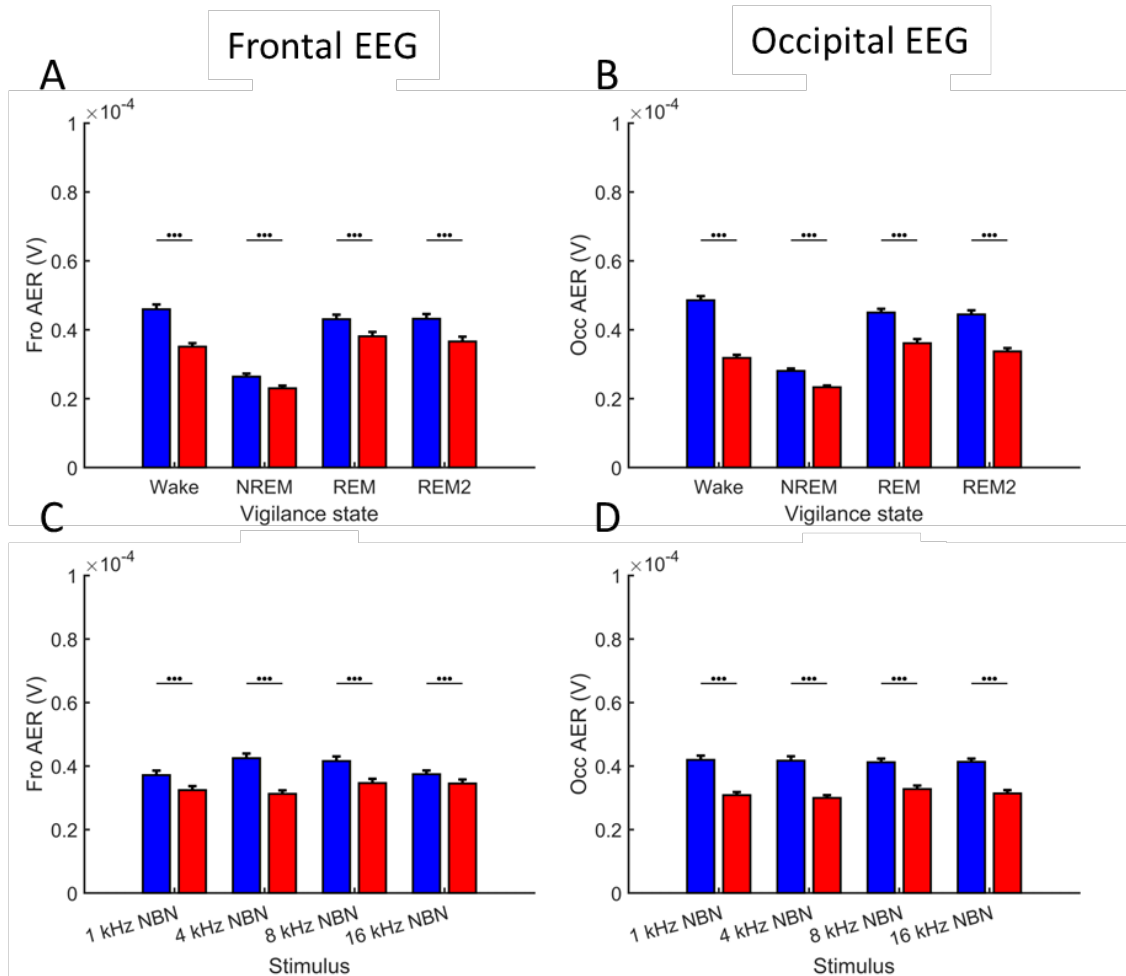


Fig. 16: EEG auditory evoked response magnitude before and after NOE (Case2). (A,B) Average AER magnitude during the baseline condition (blue markers) and Post NOE 1 (red markers) for each vigilance state. Data are averages across stimuli, stimulus intensities and response windows. (C,D) Average AER magnitude for each stimulus. Data are averages across vigilance states, stimulus intensities and response windows. Data are averages across vigilance states, stimulus intensities and response windows. Error bars are standard errors. *** signify significant differences ($p < 0.001$, GLMM).

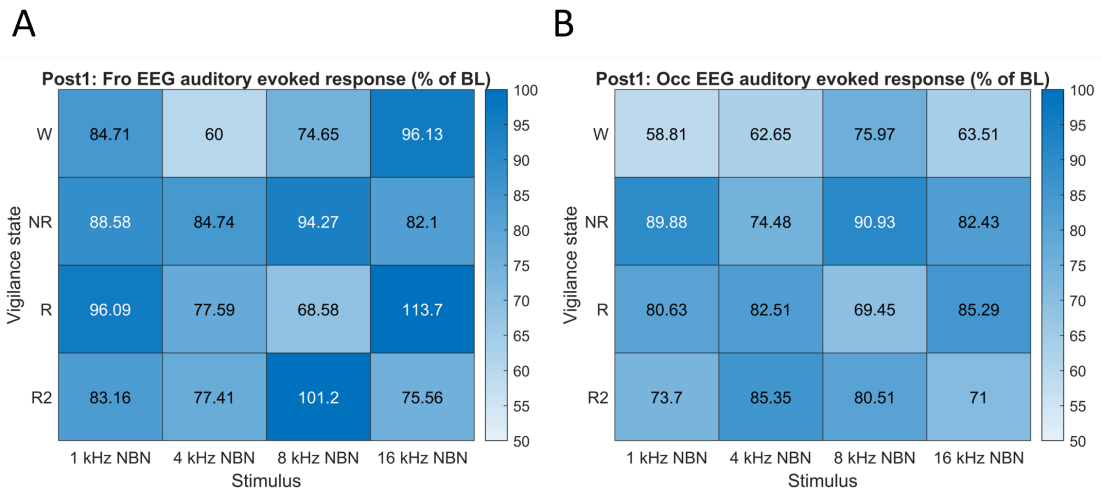


Fig. 17: Change in evoked responses after noise exposure during sleep and wake in Case2. Values are average Post1 evoked responses as % of the baseline condition for the frontal EEG (**A**) and the occipital EEG (**B**). Data are averaged across stimulus intensities and response windows. The x-axis represents stimuli, the y-axis represents the vigilance state (Wake, NREM, REM, REM2).

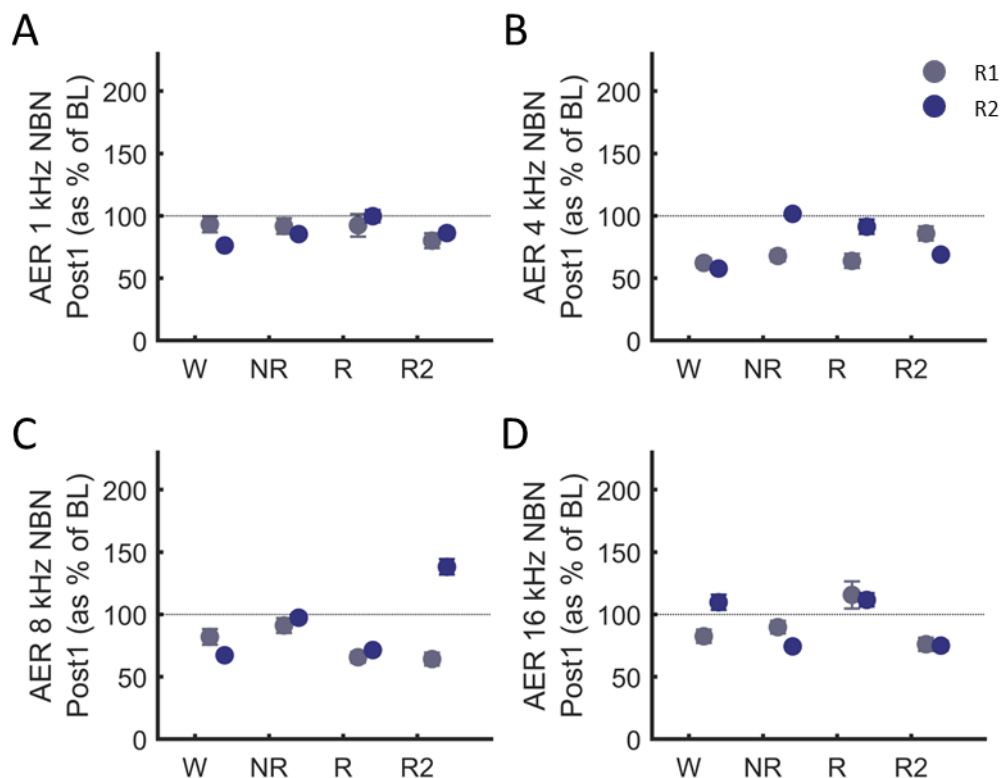


Fig.18: Change in EEG auditory evoked response magnitude after NOE for each response component (Case2, frontal EEG). Each panel shows data separated by vigilance state and AER magnitude measure (early, mid, late response window) for one stimulus type, respectively. Panels **A**, **B**, **C** and **D** show data for 1kHz, 4kHz, 8kHz and 16kHz centred NBN respectively. Data are averages and standard errors as % of the corresponding data in the baseline condition. The dotted horizontal line depicts 'no change' between BL and post NOE (i.e. 100%).

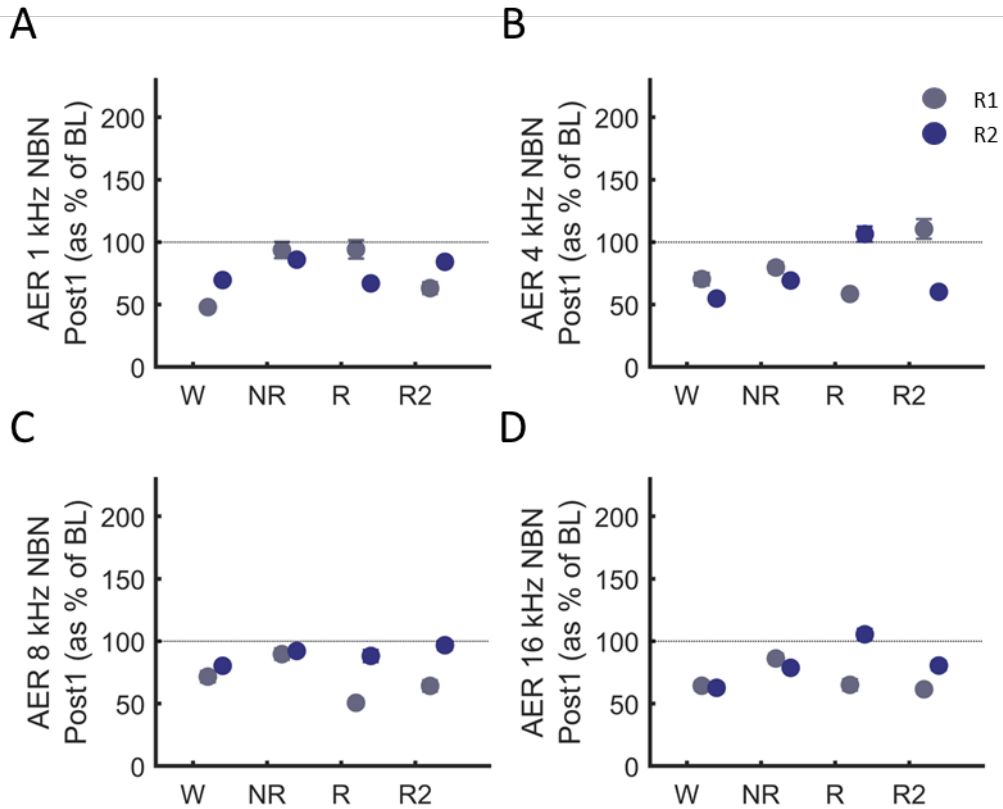


Fig.19: Change in EEG auditory evoked response magnitude after NOE for each response component (Case2, occipital EEG). Each panel shows data separated by vigilance state and AER magnitude measure (early, mid, late response window) for one stimulus type, respectively. Panels **A**, **B**, **C** and **D** show data for 1kHz , 4kHz, 8kHz and 16kHz centred NBN respectively. Data are averages and standard errors as % of the corresponding data in the baseline condition. The dotted horizontal line depicts 'no change' between BL and post NOE (i.e. 100%).

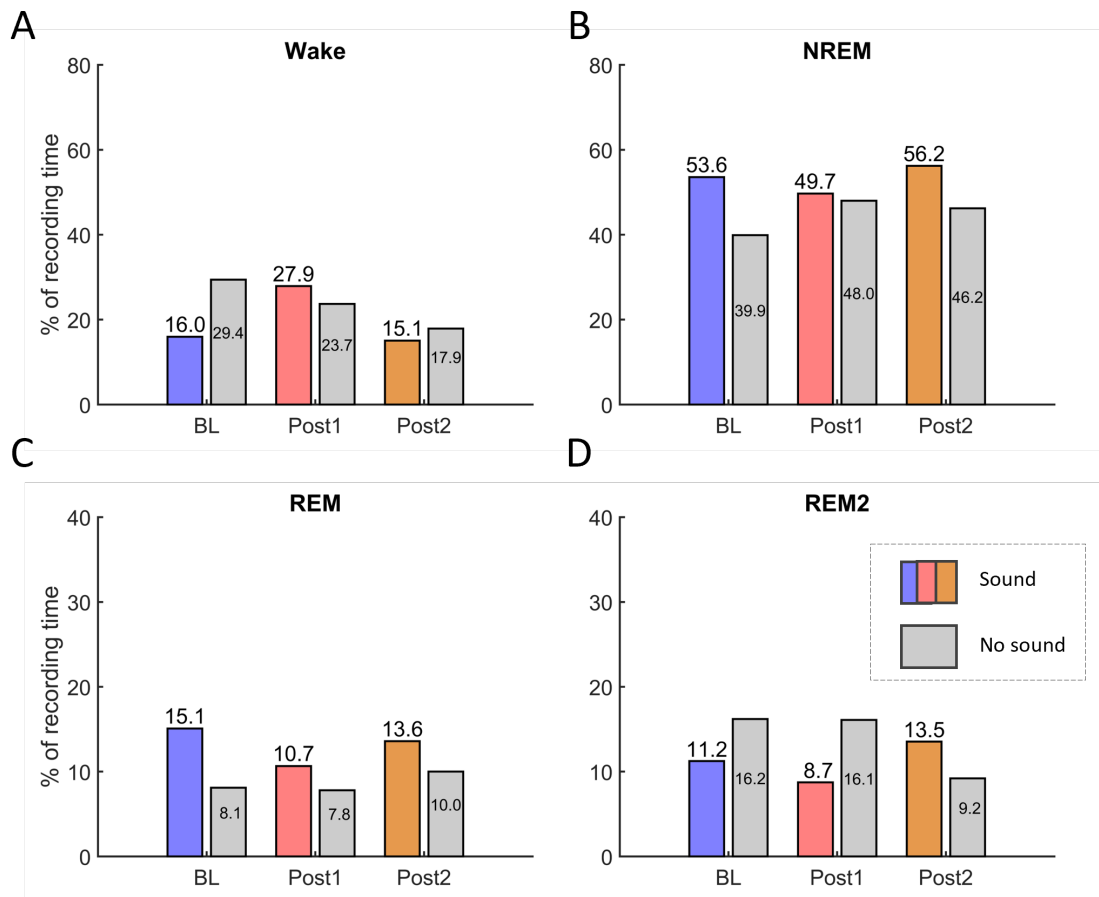


Fig. 20: Vigilance state durations before and after NOE (Case2). (A) Time spent in wakefulness, (B) in NREM, (C) in REM and (D) in REM2 sleep during ~24hrs of recording with sound presentation before NOE (BL), early after NOE (Post1) and late after NOE (Post2). Grey bars show state durations during the same conditions (BL, Post1, Post2) but without sound presentation.

Case3: Widely increased evoked activity transiently after NOE, with a further local increase in the long term.

Case3 showed an initial increase in evoked activity after NOE (Post1) as compared to BL, which was especially pronounced in the frontal EEG derivation: the magnitude of the evoked response (pooled across response components) increased for all stimuli (BL vs Post1, **1 kHz NBN**, $32.6 \pm 10.03 \mu V$ vs $41.64 \pm 10.44 \mu V$, **4 kHz NBN**, $33.71 \pm 8.04 \mu V$ vs $42.23 \pm 12.48 \mu V$, **8 kHz NBN**, $27.18 \pm 5.26 \mu V$ vs $39.02 \pm 9.91 \mu V$, **16 kHz NBN**, $32.92 \pm 9.23 \mu V$

vs $35.67 \pm 10.68 \mu V$, Table 13, Fig.21C). In the occipital EEG derivation this effect was less pronounced (BL vs Post1, **1 kHz NBN**, $27.06 \pm 7.66 \mu V$ vs $28.48 \pm 6.38 \mu V$, **4 kHz NBN**, $30.51 \pm 6.21 \mu V$ vs $29.66 \pm 7.21 \mu V$, **8 kHz NBN**, $26.54 \pm 6.01 \mu V$ vs $38.44 \pm 16.74 \mu V$, **16 kHz NBN**, $27.54 \pm 5.93 \mu V$ vs $27.77 \pm 5.58 \mu V$, Table 14) and not present for 4kHz NBN (Fig.21D). As in Case1, the increase in excitability after NOE dependent on the vigilance state: it was most pronounced during wakefulness in both EEG derivations (*Fro EEG*, BL vs Post1, **Wake**, $31.17 \pm 6.93 \mu V$ vs $47.53 \pm 9.27 \mu V$, Table 15; *Occ EEG*, $28.47 \pm 4.78 \mu V$ vs $33.11 \pm 6.51 \mu V$, Table 16, Fig.21A,B). During sleep states the increase was less pronounced (*Fro EEG*, BL vs Post1, **NREM**, $23.52 \pm 3.11 \mu V$ vs $27.43 \pm 3.83 \mu V$, **REM**, $31.64 \pm 6.65 \mu V$ vs $37.51 \pm 8.17 \mu V$, **REM2**, 40.08 ± 8.0 vs $46.09 \pm 8.69 \mu V$, Table 15, Fig.21A) or, as in the occipital EEG, even absent for REM and REM2 sleep (*Occ EEG*, BL vs Post1, **NREM**, $19.88 \pm 2.39 \mu V$ vs $20.77 \pm 2.3 \mu V$, **REM**, 28.81 ± 4.06 vs 28.36 ± 5.65 , **REM2**, $34.49 \pm 4.78 \mu V$ vs $31.71 \pm 4.52 \mu V$, Table 16, Fig.26B).

In the late assessment, 6 months after NOE (Post2), the change in evoked activity reversed and AERs for all stimuli approached baseline levels or below in the frontal derivation (BL vs Post2, **1 kHz NBN**, $32.6 \pm 10.03 \mu V$ vs $31.34 \pm 12.21 \mu V$, **4 kHz NBN**, $33.71 \pm 8.04 \mu V$ vs $26.05 \pm 5.8 \mu V$, **8 kHz NBN**, $27.18 \pm 5.26 \mu V$ vs $27.27 \pm 12.52 \mu V$, **16 kHz NBN**, $32.92 \pm 9.23 \mu V$ vs $33.78 \pm 15.63 \mu V$, Table 13, Fig.21C), which was due to drastically reduced AERs during NREM and REM sleep (BL vs Post2, **Wake**, $31.18 \pm 6.93 \mu V$ vs $34.66 \pm 10.12 \mu V$, **NREM**, $23.52 \pm 3.11 \mu V$ vs $16.3 \pm 2.86 \mu V$, **REM**, $31.64 \pm 6.65 \mu V$ vs $26.79 \pm 5.9 \mu V$, **REM2**, $40.08 \pm 8.0 \mu V$ vs $40.69 \pm 11.82 \mu V$, Table 15, Fig.21A). In the occipital derivation on the other hand, evoked activity increased further for all stimuli (BL vs Post2, **1 kHz NBN**, $27.06 \pm 7.66 \mu V$ vs $48.59 \pm 19.95 \mu V$, **4 kHz NBN**, $30.51 \pm 6.21 \mu V$ vs $43.38 \pm 10.11 \mu V$, **8 kHz NBN**, $26.54 \pm 6.01 \mu V$ vs $38.44 \pm 16.74 \mu V$, **16 kHz NBN**, $27.54 \pm 5.93 \mu V$ vs $47.07 \pm$

18.25 μ V, Table 14, Fig.21D), an effect that was most pronounced during Wake and REM2 sleep (*BL vs Post2*, **Wake**, 28.47 $\pm$ 4.78 μ V vs 51.06 $\pm$ 12.78 μ V, **NREM**, 19.88 $\pm$ 2.39 μ V vs 26.93 $\pm$ 4.49 μ V, **REM**, 28.81 $\pm$ 4.06 μ V vs 37.45 $\pm$ 8.56 μ V, **REM2**, 34.49 $\pm$ 4.78 μ V vs 62.05 $\pm$ 14.16 μ V, Table 16, Fig.21B).

The frontal, temporary increase in excitability was evident for all stimuli during wakefulness (Fig.22A), and so was the occipital late-onset increase in excitability during wakefulness and REM2 sleep (Fig.22D). Notably, the late onset-occipital increase in AERs mirrors the effect seen in Case2 (which also showed an occipital AER increase in the Post2 assessment) and could be linked to a similar process of lingering degradation of hearing ability and compensatory gain elevation.

The animal showed a similar trend in time spent awake throughout the experiment as in the undisturbed assessments where no sounds were presented: in both types of assessment, with and without sound presentation, Case3 showed a transient decrease in time awake after NOE (Post1) and an increase 6 months after NOE where the wake amount was slightly higher than during baseline (Fig.23A). Only the amount of NREM sleep showed a different trend over time with sound presentation than in the undisturbed assessment as time in NREM sleep progressively increased from BL to Post2. This could be a compensatory response of the animal towards progressively less efficient sleep due to increasingly disruptive evoked or spontaneous brain activity.

In summary, this animal showed a temporary increase in evoked activity alongside strong behavioural evidence for progressively increasing tinnitus and little change in hearing ability, an effect notably least pronounced or absent during sleep. In the long term, the frontal elevation in evoked activity reduced slightly (yet was still increased

compared to BL during wakefulness), while occipital evoked activity increased even further. Interestingly, the pronounced reduction in evoked activity during NREM sleep may explain why this animal showed no sleep disturbance despite strong behavioural evidence for tinnitus during this time.

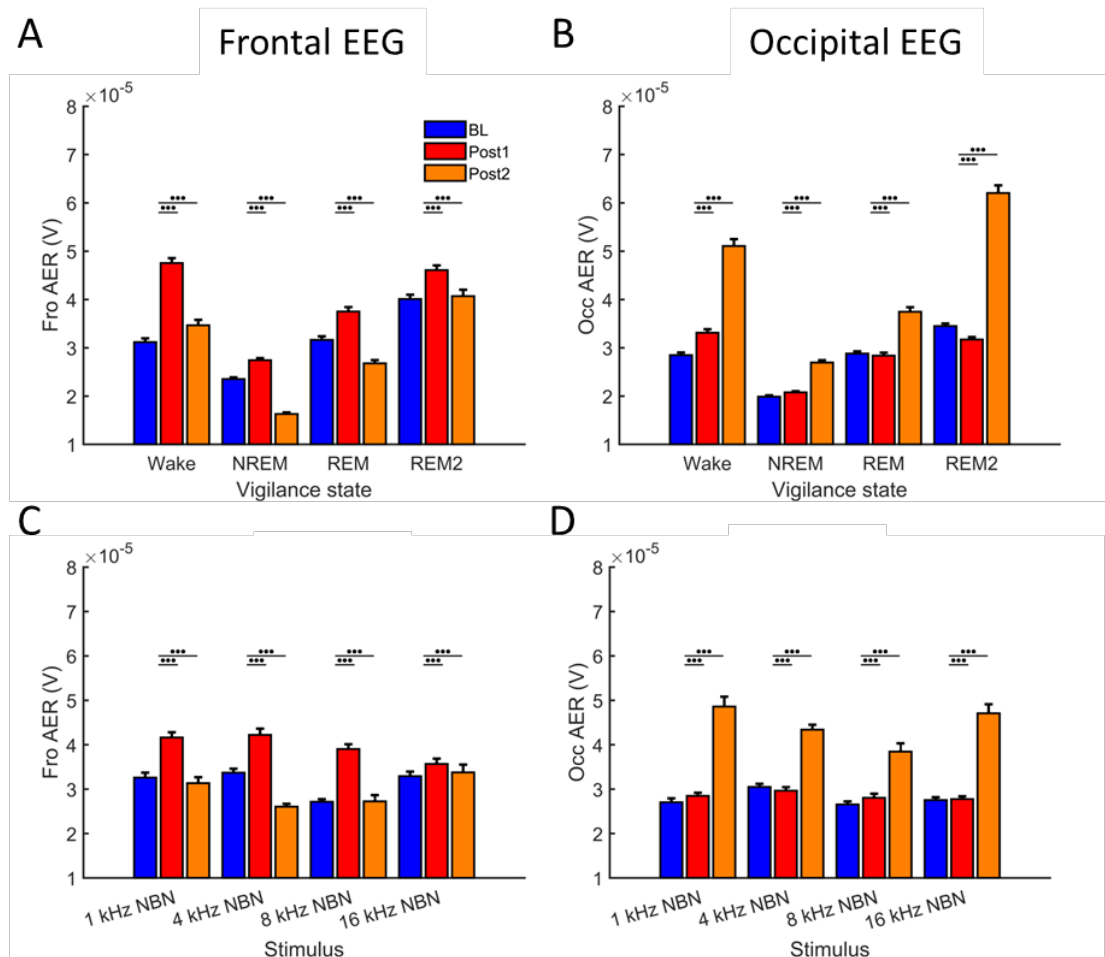


Fig. 21: EEG auditory evoked response magnitude before and after NOE (Case3). (A,B) Average AER magnitude during the baseline condition (blue markers) and Post NOE 1 (red markers) for each vigilance state. Data are averages across stimuli, stimulus intensities and response windows. (C,D) Average AER magnitude for each stimulus. Data are averages across vigilance states, stimulus intensities and response windows. Data are averages across vigilance states, stimulus intensities and response windows. Error bars are standard errors. *** signify significant differences ($p < 0.001$, GLMM).

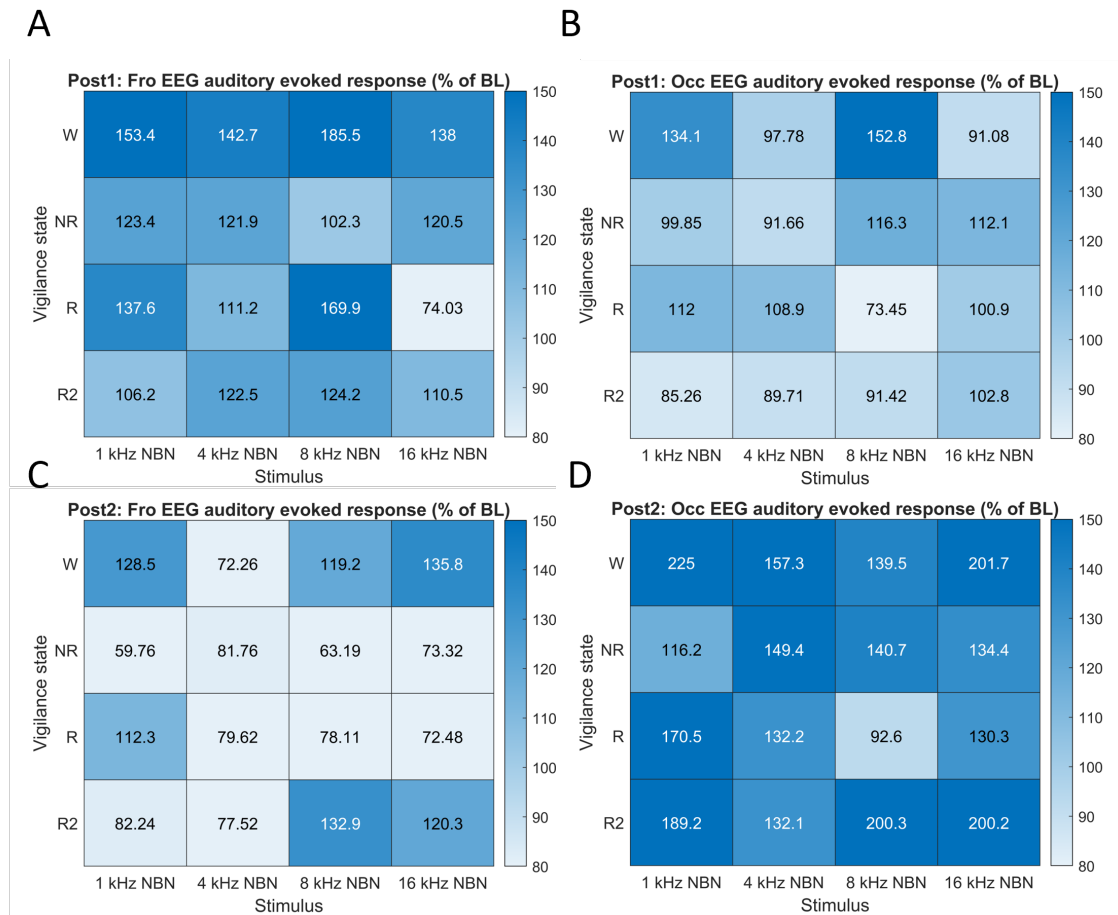


Fig. 22: Change in evoked responses after noise exposure during sleep and wake in Case3.

Values are average Post1 evoked responses as % of the baseline condition for the frontal EEG (**A**) and the occipital EEG (**B**). Data are averaged across stimulus intensities and response windows. The x-axis represents stimuli, the y-axis represents the vigilance state (Wake, NREM, REM, REM2).

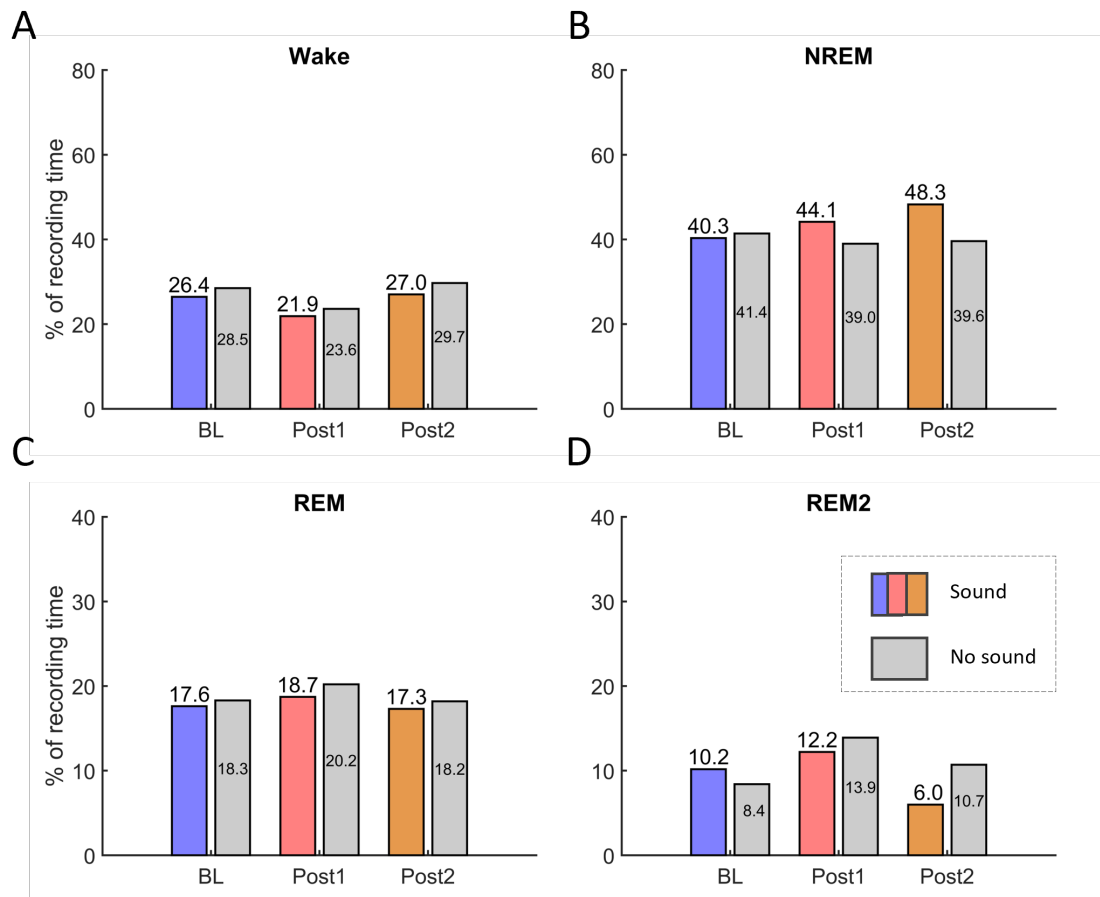


Fig. 23: Vigilance state durations before and after NOE (Case3). (A) Time spent in wakefulness, (B) in NREM, (C) in REM and (D) in REM2 sleep during ~24hrs of recording with sound presentation before NOE (BL), early after NOE (Post1) and late after NOE (Post2). Grey bars show state durations during the same conditions (BL, Post1, Post2) but without sound presentation.

Discussion

Animals showed varying changes in evoked activity after NOE, yet, in the long term, all of them developed signs of increased evoked activity in the occipital EEG derivation. Although this could not be quantitatively assessed in two animals and the increased evoked activity after NOE could not be linked to a distinct cause this still raises the possibility that all animals developed tinnitus eventually. This, however, took place on different timescales and with differing degrees of hearing impairment in parallel. The changes in evoked activity that differed between animals were especially apparent soon after NOE and, notably, were in line with the respective evidence for tinnitus and hearing loss in each Case. These findings may provide important insights into the association between neural correlates of tinnitus and sleep disruptions, as well as into the modulation of tinnitus by the vigilance state.

An association between cortical excitability and sleep disruptions

Case1 was an animal that over time developed behavioural signs for tinnitus without pronounced hearing impairment (see Chapter 3, Figs.39,40). This animal showed a marked increase in excitability in all EEG derivations after NOE, associated with increased sleep disruptions in undisturbed recordings, which were even further amplified by sound presentation. This suggests a strong effect of underlying exaggerated responsiveness to sound stimulation, given that after NOE the animal was already in a state of reduced sleep overall. Further, it suggests that lasting increased excitability after noise exposure can manifest on cortical or thalamic level rather than subcortically. The fact that sound evoked activity was elevated in all EEG derivations after NOE while EEG spectra were generally elevated only in the frontal derivation (see Chapter 3) suggests

that the increased excitability in the occipital EEG may be specific to auditory stimulation, whereas frontally the animal may have developed a general increase in excitatory tone. Importantly, as further described below, a general increase in frontal excitability may signify a neural correlate of tinnitus governing sleep disruptions.

A putative role of frontal excitability in tinnitus is further corroborated by Case3, which also showed strong evidence for tinnitus without pronounced hearing impairment (see Chapter 3, Figs.39, 40). This animal, like Case1, showed increased sound evoked activity after NOE in both EEG derivations, although slightly more pronounced in the frontal EEG. General excitability, based on EEG spectra in undisturbed recordings, was also elevated after NOE and frontally more so than occipitally. In the long term, frontal evoked activity returned partially to baseline levels during wakefulness and, during sleep, even below. Notably, evidence for disrupted sleep was only present while frontal excitability was generally elevated, both in terms of evoked activity and general excitability based on EEG spectra. This is a further indication for an association between frontal cortical overexcitability, tinnitus and sleep disruptions.

Indeed, the animal that never showed signs of increased frontal excitability after NOE (Case2), neither in terms of evoked activity nor based on EEG spectra, developed progressively more stable sleep and spent increasingly more time asleep after NOE. Notably, this was the only animal that showed pronounced hearing impairment immediately after NOE and, while tinnitus cannot be ruled out, the behavioural impairment seen in this animal may be largely explained by hearing impairment alone.

Therefore, increased excitability, in particular in frontal areas, could be a correlate of tinnitus after noise overexposure. Indeed, it has previously been shown that the

prefrontal cortex of tinnitus sufferers shows increased activity in the resting state (Schlee et al., 2009), although there are many changes in other non-auditory brain regions, such as in limbic areas (Leaver et al., 2011; Sedley et al., 2015), which are thought to govern tinnitus persistence (Rauschecker et al., 2010b). The increase in frontal excitability in particular could amplify the disruptive effect of spontaneous brain activity on sleep, since NREM slow-wave activity is usually most pronounced in these areas (Finelli et al., 2001). And although sleep slow-wave activity is regulated locally in the brain (Finelli et al., 2001; Krueger et al., 2019) the extensive interconnectivity of cortical areas predisposes for a widespread effect of locally amplified spontaneous or evoked activity, which could eventually disrupt a global sleep state (Vyazovskiy & Harris, 2013) and may therefore underly the sleep disruption observed in this study and also govern aspects of disturbed sleep observed in tinnitus sufferers (Folmer & Griest, 2000; Hallam, 1996; Izuhara et al., 2013a; Koning, 2019a; Schecklmann et al., 2015).

Sleep as mitigating factor on noise-induced hyperexcitability

In both Cases that showed widespread increase in cortical excitability (Case1 and Case3), this change was least pronounced during sleep, in particular during NREM sleep. In Case1 the increase in frontal evoked activity was dampened during NREM sleep and in Case3, the increased evoked activity was initially less pronounced during all sleep states as compared to wakefulness. Eventually, 6 months after NOE, frontal evoked activity was markedly reduced in NREM and REM sleep, which might explain why this animal did not show disrupted sleep at this time despite having tinnitus.

The state-dependent difference in evoked activity could be related to spontaneous oscillatory activity in the brain, in particular to slow oscillations, which are pronounced

during NREM sleep (Brown et al., 2012) but which can also occur during REM sleep (Funk et al., 2016). It is known that sleep – NREM sleep in particular – is associated with reduced evoked responses on cortical level (Sela et al., 2020), possibly due to impaired cortical signal propagation associated with off-periods (Rosanova et al., 2018). This sleep-dependent sensory disconnection may reduce the effect of persistent hyperactivity on wider cortical networks.

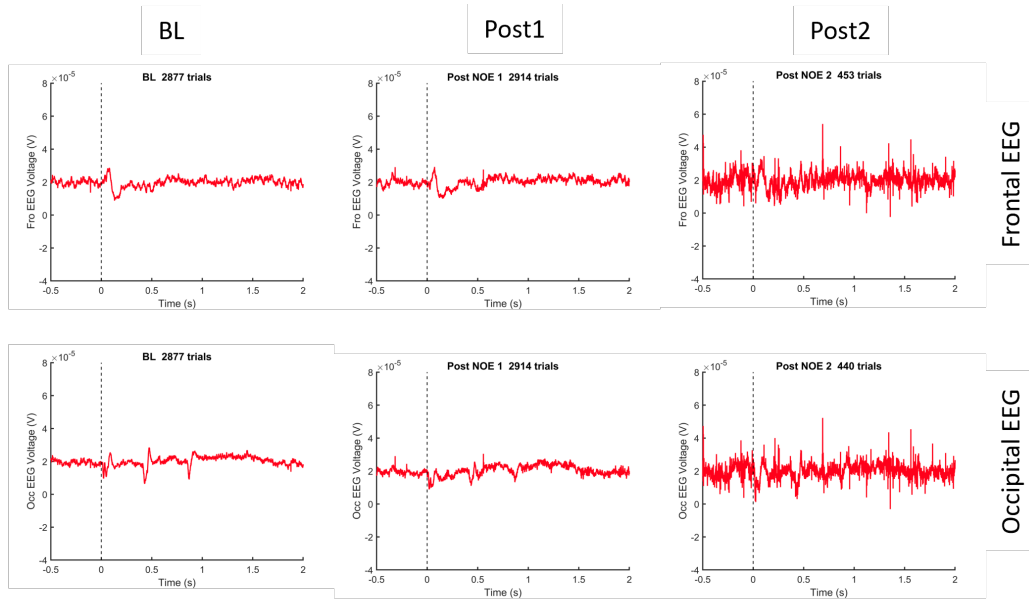
Of course, tinnitus may still interfere with SWA and with sleep in general, as this work and previous studies indicate (Folmer & Griest, 2000; Hallam, 1996; Izuhara et al., 2013a; Koning, 2019a; Schecklmann et al., 2015). Yet the dynamics of natural vigilance states are regulated by homeostatic drives that are expected to facilitate sleep maintenance even in the face of aberrant activity such as in tinnitus. As described in the introduction of this chapter, prolonged waking leads to the accumulation of homeostatic sleep pressure (Thomas et al., 2020; Vyazovskiy et al., 2009), which on the level of the individual cortical neuron is reflected in the increased tendency to engage in a firing pattern that is in accordance with global SWA but incompatible with ongoing hyperactivity (Vyazovskiy & Harris, 2013) and may therefore transiently mitigate tinnitus. This applies especially to brain regions that were particularly active in the preceding waking period (Hung et al., 2013; Vyazovskiy et al., 2011). Such regions in particular show locally intense SWA and highly synchronised on-off firing patterns across neurons (Vyazovskiy et al., 2011). Such locally pronounced sleep may also apply to brain regions that are overactive due to tinnitus. This may explain why the dampening of evoked activity during NREM sleep may be even more pronounced after NOE – when the animals may have developed tinnitus – in line with the results of this study.

In summary, the two animals that showed greater behavioural signs of tinnitus also developed increased neural evoked activity after NOE, whereas the animal with less evidence for tinnitus but severe hearing loss showed reduced auditory evoked activity after NOE. When tinnitus was more severe and frontal evoked activity was elevated sleep was disturbed, but not when occipital evoked activity was elevated, which may have important implications for the notion of frontal or brain-wide tinnitus-representation playing a role in sleep disruption. In both cases with elevated evoked activity after NOE, auditory evoked activity was lowest during sleep, suggesting a role for natural brain state dynamics in modulating putative tinnitus-related activity. Ultimately, this may provide a physiological basis for sleep-induced relief from tinnitus that patients describe (De Ridder et al., 2014; Stouffer et al., 1991).

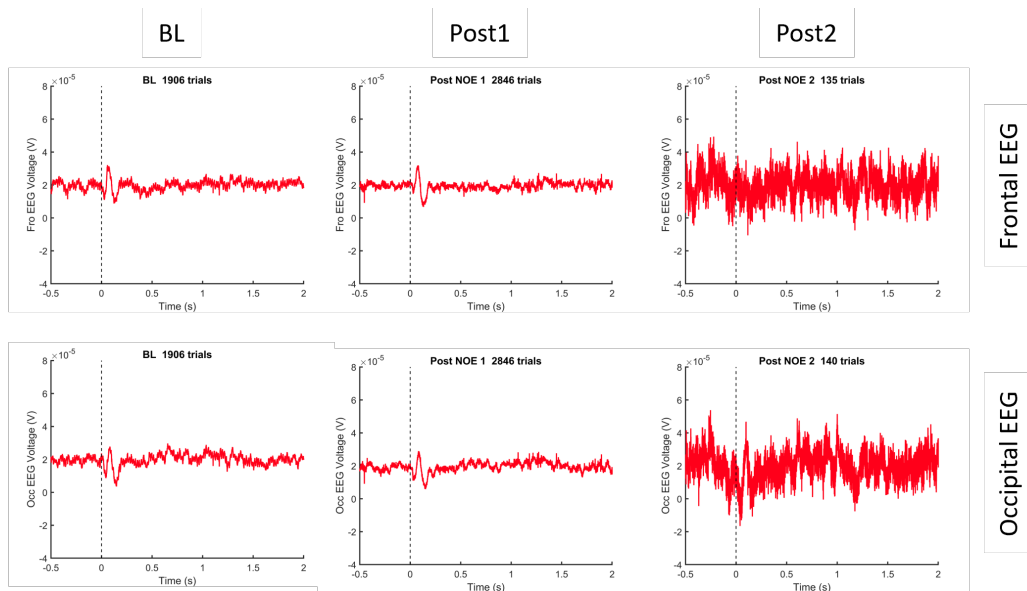
Limiting factors:

The actual intensity of the presented sound stimuli at ear level of the animals could not be ascertained as the animals moved freely in the recording cage and, when resting or sleeping, adopted different postures. Therefore, despite the nesting area being in a fixed position in the recording cage, the animals did not always sleep or rest in the same location. Furthermore, the ferrets included in this study exhibited no single stereotypical sleeping position – they often curled up or rested on their back (Fig.2A, Chapter 3) adopting varying head orientations. This may explain why no consistent modulation of AERs by stimulus intensity was observed.

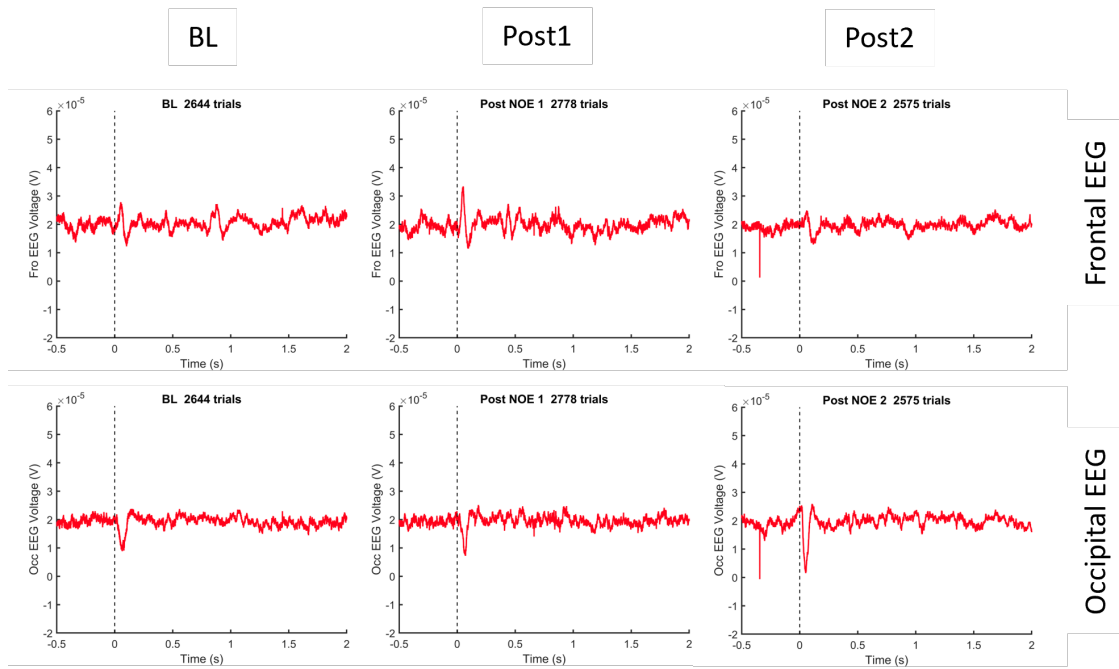
Supplementary figures and tables



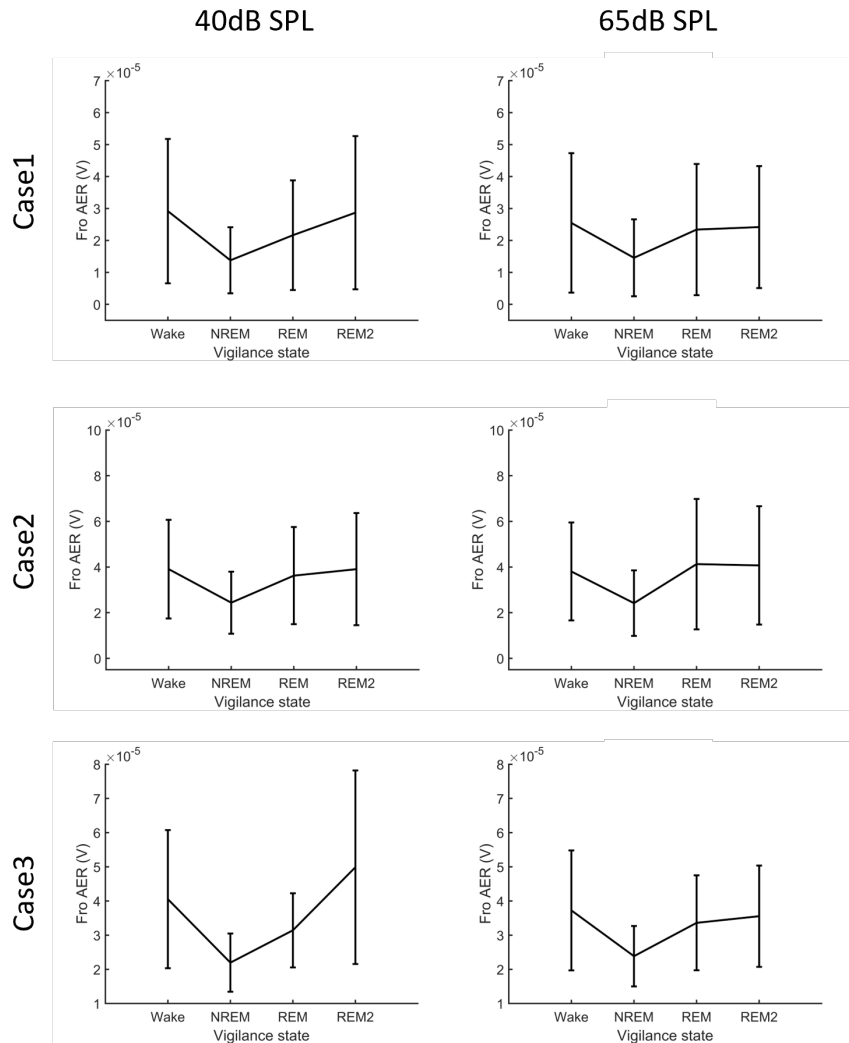
Supp. Fig. 1: Average evoked responses by condition for Case1 and by recording site. Number of trials contributing to the average are indicated above each panel. Note that trials falling into epochs scored as ‘artefact’ during the manual scoring procedure were not included. In addition, signals in the Post3 condition were generally of lower quality, and only trials where the signal’s standard deviation was maximally double the average standard deviation of trials in the ‘post NOE 2’ condition were included.



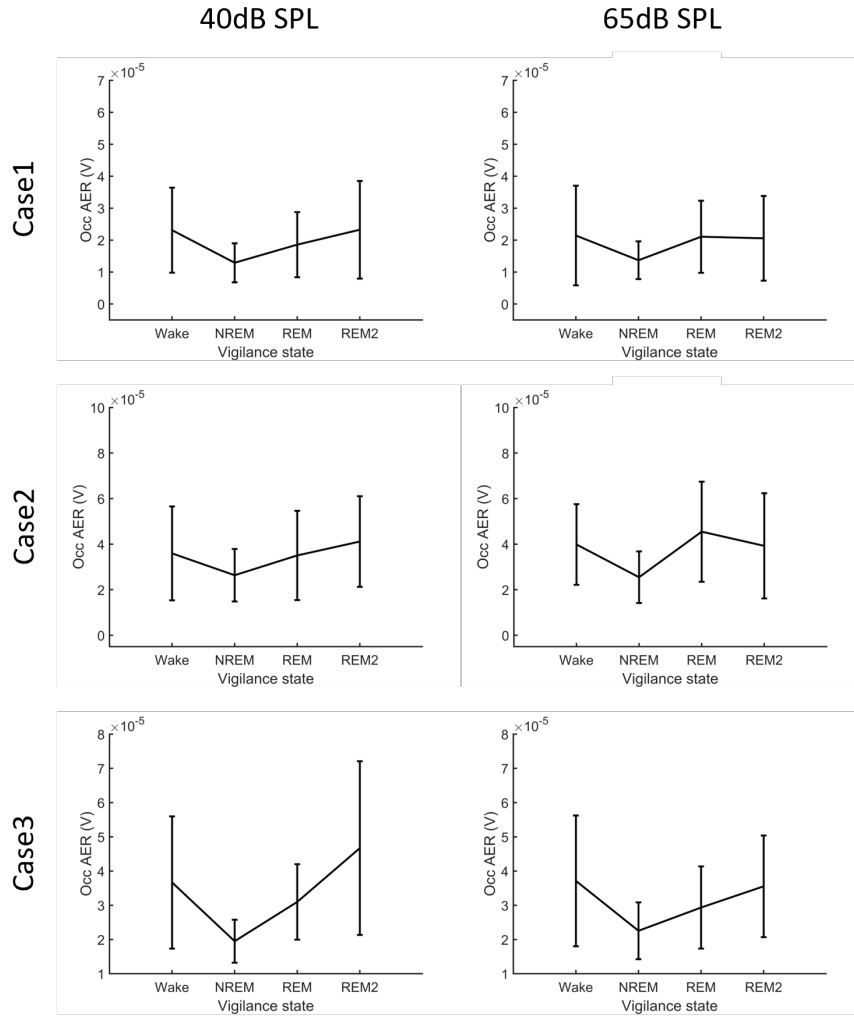
Supp. Fig. 2: Average evoked responses by condition for Case2 and by recording site. Number of trials contributing to the average are indicated above each panel. Note that trials falling into epochs scored as ‘artefact’ during the manual scoring procedure were not included. In addition, signals in the Post3 condition were generally of lower quality, and only trials where the signal’s standard deviation was maximally double the average standard deviation of trials in the ‘post NOE 2’ condition were included.



Supp. Fig. 3: Average evoked responses by condition for Case3 and by recording site. Number of trials contributing to the average are indicated above each panel. Note that trials falling into epochs scored as 'artefact' during the manual scoring procedure were not included. In addition, signals in the Post3 condition were generally of lower quality, and only trials where the signal's standard deviation was maximally double the average standard deviation of trials in the 'post NOE 2' condition were included.



Supp. Fig. 4: Modulation by vigilance state (low and high intensities, frontal EEG). Each panel shows the average auditory evoked response (AER) magnitude of one animal and EEG derivation, separated by vigilance state. Averages are calculated across conditions, sound stimuli, stimulus intensities and response windows for each vigilance state (Wake, NREM sleep, REM sleep, REM2 sleep), respectively. Data are means and standard deviations.



Supp. Fig. 5: Modulation by vigilance state (low and high intensities, occipital EEG). Each panel shows the average auditory evoked response (AER) magnitude of one animal and EEG derivation, separated by vigilance state. Averages are calculated across conditions, sound stimuli, stimulus intensities and response windows for each vigilance state (Wake, NREM sleep, REM sleep, REM2 sleep), respectively. Data are means and standard deviations.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.698E+32	1	1911	.000
Cond * Freq	2.698E+32	1	1911	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_microV_log				

1 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.784E+31	1	478	.000
Cond	1.784E+31	1	478	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_microV_log				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.273	2.0577E-18	6.185E+17	.000	1.273	1.273
Cond=1	-.020	4.7981E-18	-4.224E+15	.000	-.020	-.020
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_microV_log						
b. This coefficient is set to zero because it is redundant.						

4 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.764E+31	1	478	.000
Cond	2.764E+31	1	478	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_microV_log				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.229	3.9896E-20	3.081E+19	.000	1.229	1.229
Cond=1	.010	1.9751E-18	5.258E+15	.000	.010	.010
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_microV_log						
b. This coefficient is set to zero because it is redundant.						

8 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.088E+30	1	478	.000
Cond	1.088E+30	1	478	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_microV				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	22.768	2.4859E-15	9.159E+15	.000	22.768	22.768
Cond=1	-2.593	2.4859E-15	-1.043E+15	.000	-2.593	-2.593
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_microV						
b. This coefficient is set to zero because it is redundant.						

16 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.791E+31	1	477	.000
Cond	2.791E+31	1	477	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_microV_log				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.269	.0000	.	.	1.269	1.269
Cond=1	-.066	1.2468E-17	-5.283E+15	.000	-.066	-.066
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_microV_log						
b. This coefficient is set to zero because it is redundant.						

Table 1: Interaction between condition and stimulus (Case1, Fro EEG). Effect of condition (BL vs Post1) for each stimulus.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.091E+25	1	1912	.000
Cond * Freq	1.091E+25	1	1912	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_MicroV

1 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.312E+25	1	478	.000
Cond	1.312E+25	1	478	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_MicroV

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	20.955	7.1124E-13	2.946E+13	.000	20.955	20.955
Cond=1	-2.579	7.1198E-13	-3.622E+12	.000	-2.579	-2.579
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_MicroV
b. This coefficient is set to zero because it is redundant.

4 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.749E+28	1	478	.000
Cond	1.749E+28	1	478	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_MicroV

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	19.920	7.0477E-15	2.826E+15	.000	19.920	19.920
Cond=1	-.944	7.1407E-15	-1.322E+14	.000	-.944	-.944
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_MicroV
b. This coefficient is set to zero because it is redundant.

8 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	7.258E+30	1	478	.000
Cond	7.258E+30	1	478	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_MicroV

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	19.520	9.3127E-17	2.096E+17	.000	19.520	19.520
Cond=1	-.507	1.8814E-16	-2.694E+15	.000	-.507	-.507
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_MicroV
b. This coefficient is set to zero because it is redundant.

16 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.007E+32	1	478	.000
Cond	2.007E+32	1	478	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_MicroV

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	21.055	1.1638E-17	1.809E+18	.000	21.055	21.055
Cond=1	-1.193	8.4181E-17	-1.417E+16	.000	-1.193	-1.193
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_MicroV
b. This coefficient is set to zero because it is redundant.

Table 2: Interaction between condition and stimulus (Case1, Occ EEG). Effect of condition (BL vs Post1) for each stimulus.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.396E+30	1	1911	.000
Cond * State	2.396E+30	1	1911	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_microV_log

Wake

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.157E+33	1	478	.000
Cond	1.157E+33	1	478	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	1.354	3.4680E-18	3.905E+17	.000	1.354	1.354
Cond=1	-.039	1.1336E-18	-3.402E+16	.000	-.039	-.039
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

NREM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.138E+31	1	477	.000
Cond	2.138E+31	1	477	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	1.000	1.4666E-18	6.817E+17	.000	1.000	1.000
Cond=1	.070	1.5092E-17	4.624E+15	.000	.070	.070
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

REM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	5.422E+32	1	478	.000
Cond	5.422E+32	1	478	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	1.250	8.3891E-18	1.490E+17	.000	1.250	1.250
Cond=1	-.057	2.4434E-18	-2.328E+16	.000	-.057	-.057
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

REM2

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	3.178E+31	1	478	.000
Cond	3.178E+31	1	478	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	1.392	3.1181E-18	4.463E+17	.000	1.392	1.392
Cond=1	-.075	1.3270E-17	-5.637E+15	.000	-.075	-.075
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

Table 3: Interaction between condition and vigilance state (Case1, Fro EEG). Effect of condition (BL vs Post1) for each vigilance state.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.944E+27	1	1912	.000
Cond * State	1.944E+27	1	1912	.000
Probability distribution: Normal Link function: Identity ^a a. Target: EvPot_MicroV				

Wake

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	6.257E+30	1	478	.000
Cond	6.257E+30	1	478	.000
Probability distribution: Normal Link function: Identity ^a a. Target: EvPot_MicroV				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	23.486	1.1665E-17	2.013E+18	.000	23.486	23.486
Cond=1	-.058	2.3306E-17	-2.501E+15	.000	-.058	-.058
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal Link function: Identity ^a a. Target: EvPot_MicroV b. This coefficient is set to zero because it is redundant.						

NREM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.410E+33	1	478	.000
Cond	2.410E+33	1	478	.000
Probability distribution: Normal Link function: Identity ^a a. Target: EvPot_MicroV				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	13.776	1.2646E-17	1.089E+18	.000	13.776	13.776
Cond=1	-.621	1.2646E-17	-4.909E+16	.000	-.621	-.621
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal Link function: Identity ^a a. Target: EvPot_MicroV b. This coefficient is set to zero because it is redundant.						

REM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.094E+32	1	478	.000
Cond	1.094E+32	1	478	.000
Probability distribution: Normal Link function: Identity ^a a. Target: EvPot_MicroV				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	19.920	2.6160E-16	7.615E+16	.000	19.920	19.920
Cond=1	-2.737	2.6160E-16	-1.046E+16	.000	-2.737	-2.737
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal Link function: Identity ^a a. Target: EvPot_MicroV b. This coefficient is set to zero because it is redundant.						

REM2

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	7.651E+31	1	478	.000
Cond	7.651E+31	1	478	.000
Probability distribution: Normal Link function: Identity ^a a. Target: EvPot_MicroV				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	24.776	2.3488E-16	1.055E+17	.000	24.776	24.776
Cond=1	-2.411	2.7559E-16	-8.747E+15	.000	-2.411	-2.411
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal Link function: Identity ^a a. Target: EvPot_MicroV b. This coefficient is set to zero because it is redundant.						

Table 4: Interaction between condition and vigilance state (Case1, Occ EEG). Effect of condition (BL vs Post1) for each vigilance state.

1 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	3.640E+29	1	456	.000
Cond * State * Window	3.640E+29	1	456	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

4 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	6.535E+28	1	456	.000
Cond * State * Window	6.535E+28	1	456	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

8 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	5.798E+27	1	456	.000
Cond * State * Window	5.798E+27	1	456	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

16 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	6.094E+27	1	455	.000
Cond * State * Window	6.094E+27	1	455	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

Table 5: Interaction between condition and vigilance state and response component (Case1, Fro EEG) for each stimulus.

1 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.838E+22	1	456	.000
Cond * State * Window	1.838E+22	1	456	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_MicroV

4 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	6.264E+23	1	456	.000
Cond * State * Window	6.264E+23	1	456	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_MicroV

8 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.927E+19	1	456	.000
Cond * State * Window	1.927E+19	1	456	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_MicroV

16 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.798E+25	1	456	.000
Cond * State * Window	1.798E+25	1	456	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_MicroV

Table 6: Interaction between condition and vigilance state and response component (Case1, Occ EEG) for each stimulus.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.193E+19	1	1272	.000
Cond * Freq	2.193E+19	1	1272	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

1 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.364E+32	1	318	.000
Cond	2.364E+32	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	32.462	2.7370E-16	1.186E+17	.000	32.462	32.462
Cond=1	4.208	2.7370E-16	1.537E+16	.000	4.208	4.208
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

4 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.099E+31	1	318	.000
Cond	1.099E+31	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	31.345	3.0387E-15	1.032E+16	.000	31.345	31.345
Cond=1	10.073	3.0387E-15	3.315E+15	.000	10.073	10.073
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

8 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.991E+31	1	318	.000
Cond	2.991E+31	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	33.121	7.0368E-16	4.707E+16	.000	33.121	33.121
Cond=1	7.923	1.4487E-15	5.469E+15	.000	7.923	7.923
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

16 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	5.388E+32	1	318	.000
Cond	5.388E+32	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	33.002	1.8623E-16	1.772E+17	.000	33.002	33.002
Cond=1	4.347	1.8726E-16	2.321E+16	.000	4.347	4.347
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

Table 7: Interaction between condition and stimulus (Case2, Fro EEG). Effect of condition (BL vs Post1) for each stimulus.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.394E+30	1	1272	.000
Cond * Freq	2.394E+30	1	1272	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_V				

1 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	4.877E+31	1	318	.000
Cond	4.877E+31	1	318	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_V				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	30.711	1.0869E-16	2.826E+17	.000	30.711	30.711
Cond=1	10.840	1.5522E-15	6.984E+15	.000	10.840	10.840
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_V						
b. This coefficient is set to zero because it is redundant.						

4 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.503E+31	1	318	.000
Cond	1.503E+31	1	318	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_V				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	29.809	2.8398E-15	1.050E+16	.000	29.809	29.809
Cond=1	11.009	2.8398E-15	3.877E+15	.000	11.009	11.009
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_V						
b. This coefficient is set to zero because it is redundant.						

8 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	4.315E+32	1	318	.000
Cond	4.315E+32	1	318	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_V				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	30.345	1.4550E-18	2.086E+19	.000	30.345	30.345
Cond=1	10.607	5.1064E-16	2.077E+16	.000	10.607	10.607
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_V						
b. This coefficient is set to zero because it is redundant.						

16 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.302E+32	1	318	.000
Cond	1.302E+32	1	318	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_V				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	29.967	7.5246E-18	3.983E+18	.000	29.967	29.967
Cond=1	11.331	9.9296E-16	1.141E+16	.000	11.331	11.331
Cond=2	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_V						
b. This coefficient is set to zero because it is redundant.						

Table 8: Interaction between condition and stimulus (Case2, Occ EEG). Effect of condition (BL vs Post1) for each stimulus.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	6.868E+20	1	1272	.000
Cond * State	6.868E+20	1	1272	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Wake

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	7.949E+31	1	318	.000
Cond	7.949E+31	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	33.012	1.4481E-15	2.280E+16	.000	33.012	33.012
Cond=1	12.911	1.4481E-15	8.916E+15	.000	12.911	12.911
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

NREM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	6.252E+30	1	318	.000
Cond	6.252E+30	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	25.948	3.7646E-16	6.893E+16	.000	25.948	25.948
Cond=1	.429	1.7138E-16	2.500E+15	.000	.429	.429
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

REM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	3.519E+31	1	318	.000
Cond	3.519E+31	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	39.368	1.9144E-16	2.056E+17	.000	39.368	39.368
Cond=1	3.521	5.9364E-16	5.932E+15	.000	3.521	3.521
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

REM2

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.334E+32	1	318	.000
Cond	1.334E+32	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	36.004	4.9304E-32	7.302E+32	.000	36.004	36.004
Cond=1	6.639	5.7471E-16	1.155E+16	.000	6.639	6.639
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

Table 9: Interaction between condition and vigilance state (Case2, Fro EEG). Effect of condition (BL vs Post1) for each vigilance state.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	6.892E+31	1	1272	.000
Cond * State	6.892E+31	1	1272	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Wake

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	6.821E+31	1	318	.000
Cond	6.821E+31	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	31.834	.0000	.	.	31.834	31.834
Cond=1	16.839	2.0389E-15	8.259E+15	.000	16.839	16.839
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

NREM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	3.907E+31	1	318	.000
Cond	3.907E+31	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	23.139	4.0390E-28	5.729E+28	.000	23.139	23.139
Cond=1	5.178	8.2833E-16	6.251E+15	.000	5.178	5.178
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

REM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.482E+32	1	318	.000
Cond	1.482E+32	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	35.743	7.4505E-16	4.797E+16	.000	35.743	35.743
Cond=1	9.069	7.4505E-16	1.217E+16	.000	9.069	9.069
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

REM2

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.067E+35	1	318	.000
Cond	1.067E+35	1	318	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	33.909	5.2082E-16	6.511E+16	.000	33.909	33.909
Cond=1	9.964	3.0499E-17	3.267E+17	.000	9.964	9.964
Cond=2	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V
b. This coefficient is set to zero because it is redundant.

Table 10: Interaction between condition and vigilance state (Case2, Occ EEG). Effect of condition (BL vs Post1) for each vigilance state.

1 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	4.000E+32	1	304	.000
Cond * State * Window	4.000E+32	1	304	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

4 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	3.839E+27	1	304	.000
Cond * State * Window	3.839E+27	1	304	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

8 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.535E+19	1	304	.000
Cond * State * Window	1.535E+19	1	304	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

16 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.287E+32	1	304	.000
Cond * State * Window	2.287E+32	1	304	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Table 11: Interaction between condition and vigilance state and response component (Case2, Fro EEG) for each stimulus.

1 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.652E+28	1	304	.000
Cond * State * Window	1.652E+28	1	304	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

4 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.806E+25	1	304	.000
Cond * State * Window	2.806E+25	1	304	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

8 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.332E+19	1	304	.000
Cond * State * Window	1.332E+19	1	304	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

16 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	5.470E+17	1	304	.000
Cond * State * Window	5.470E+17	1	304	.000

Probability distribution: Normal
Link function: Identity^a
a. Target: EvPot_V

Table 12: Interaction between condition and vigilance state and response component (Case2, Occ EEG) for each stimulus.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.784E+18	1	948	.000
Cond * Freq	2.784E+18	1	948	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_microV				

1 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.813E+31	1	237	.000
Cond_ref1	1.813E+31	1	237	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_microV				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	31.544	5.7165E-16	5.518E+16	.000	31.544	31.544
Cond_ref1=2	9.977	2.3430E-15	4.258E+15	.000	9.977	9.977
Cond_ref1=3	1.115	9.6321E-16	1.157E+15	.000	1.115	1.115
Cond_ref1=11	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_microV						
b. This coefficient is set to zero because it is redundant.						

4 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	5.217E+36	1	237	.000
Cond_ref1	5.217E+36	1	237	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_microV				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	33.821	3.3930E-18	9.968E+18	.000	33.821	33.821
Cond_ref1=2	7.750	3.3930E-18	2.284E+18	.000	7.750	7.750
Cond_ref1=3	-7.797	1.1707E-15	-6.660E+15	.000	-7.797	-7.797
Cond_ref1=11	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_microV						
b. This coefficient is set to zero because it is redundant.						

8 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.288E+32	1	237	.000
Cond_ref1	2.288E+32	1	237	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_microV				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	27.191	8.1481E-16	3.337E+16	.000	27.191	27.191
Cond_ref1=2	12.324	8.1481E-16	1.513E+16	.000	12.324	12.324
Cond_ref1=3	-.764	8.1481E-16	-9.381E+14	.000	-.764	-.764
Cond_ref1=11	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_microV						
b. This coefficient is set to zero because it is redundant.						

16 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	3.962E+31	1	237	.000
Cond_ref1	3.962E+31	1	237	.000
Probability distribution: Normal				
Link function: Identity ^a				
a. Target: EvPot_microV				

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
Intercept	31.921	5.7342E-16	5.567E+16	.000	31.921	31.921
Cond_ref1=2	3.011	4.7844E-16	6.294E+15	.000	3.011	3.011
Cond_ref1=3	2.092	8.4943E-16	2.462E+15	.000	2.092	2.092
Cond_ref1=11	0 ^b	.	.	.	.	.
Probability distribution: Normal						
Link function: Identity ^a						
a. Target: EvPot_microV						
b. This coefficient is set to zero because it is redundant.						

Table 13: Interaction between condition and stimulus (Case3, Fro EEG). Effect of condition (BL vs Post1, BL vs Post2) for each stimulus.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.601E+33	1	948	.000
Cond * Freq	1.601E+33	1	948	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

1 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	3.921E+29	1	237	.000
Cond_ref1	3.921E+29	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.420	3.6667E-17	3.873E+16	.000	1.420	1.420
Cond_ref1=2	.018	2.8159E-17	6.262E+14	.000	.018	.018
Cond_ref1=3	.226	1.8773E-17	1.203E+16	.000	.226	.226
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

4 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	6.926E+29	1	237	.000
Cond_ref1	6.926E+29	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.474	.0000	.	.	1.474	1.474
Cond_ref1=2	-.005	5.8031E-18	-8.322E+14	.000	-.005	-.005
Cond_ref1=3	.161	.0000	.	.	.161	.161
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

8 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	6.741E+29	1	237	.000
Cond_ref1	6.741E+29	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.410	1.3962E-17	1.010E+17	.000	1.410	1.410
Cond_ref1=2	.011	1.3962E-17	8.211E+14	.000	.011	.011
Cond_ref1=3	.101	1.9586E-17	5.154E+15	.000	.101	.101
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

16 kHz NBN

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	3.692E+31	1	237	.000
Cond_ref1	3.692E+31	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.426	1.3643E-17	1.046E+17	.000	1.426	1.426
Cond_ref1=2	.020	3.3205E-18	6.076E+15	.000	.020	.020
Cond_ref1=3	.238	1.3643E-17	1.743E+16	.000	.238	.238
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

Table 14: Interaction between condition and stimulus (Case3, Occ EEG). Effect of condition (BL vs Post1, BL vs Post2) for each stimulus.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.282E+29	1	948	.000
Cond * State	2.282E+29	1	948	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV

Wake

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	9.491E+31	1	237	.000
Cond_ref1	9.491E+31	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	31.001	1.6232E-15	1.910E+16	.000	31.001	31.001
Cond_ref1=2	15.814	1.6232E-15	9.742E+15	.000	15.814	15.814
Cond_ref1=3	3.012	2.0569E-15	1.465E+15	.000	3.012	3.012
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV
b. This coefficient is set to zero because it is redundant.

NREM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	8.916E+31	1	237	.000
Cond_ref1	8.916E+31	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	23.421	4.1946E-16	5.584E+16	.000	23.421	23.421
Cond_ref1=2	3.961	4.1946E-16	9.443E+15	.000	3.961	3.961
Cond_ref1=3	-7.063	4.1946E-16	-1.684E+16	.000	-7.063	-7.063
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV
b. This coefficient is set to zero because it is redundant.

REM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.786E+32	1	237	.000
Cond_ref1	2.786E+32	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	31.706	3.7258E-16	8.510E+16	.000	31.706	31.706
Cond_ref1=2	6.219	3.7258E-16	1.669E+16	.000	6.219	6.219
Cond_ref1=3	-4.279	3.7258E-16	-1.148E+16	.000	-4.279	-4.279
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV
b. This coefficient is set to zero because it is redundant.

REM2

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.766E+33	1	237	.000
Cond_ref1	1.766E+33	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	40.057	7.6754E-16	5.219E+16	.000	40.057	40.057
Cond_ref1=2	6.105	1.4526E-16	4.202E+16	.000	6.105	6.105
Cond_ref1=3	-.700	3.9504E-16	-1.773E+15	.000	-.700	-.700
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV
b. This coefficient is set to zero because it is redundant.

Table 15: Interaction between condition and vigilance state (Case3, Fro EEG). Effect of condition (BL vs Post1, BL vs Post2) for each vigilance state.

Interaction

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.086E+28	1	948	.000
Cond * State	1.086E+28	1	948	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_MicroV_log

Wake

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	2.523E+30	1	237	.000
Cond_ref1	2.523E+30	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.451	2.3141E-17	6.270E+16	.000	1.451	1.451
Cond_ref1=2	.060	3.8048E-17	1.588E+15	.000	.060	.060
Cond_ref1=3	.241	4.1787E-17	5.778E+15	.000	.241	.241
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

NREM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	1.154E+31	1	237	.000
Cond_ref1	1.154E+31	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.295	5.6456E-18	2.294E+17	.000	1.295	1.295
Cond_ref1=2	.019	5.6456E-18	3.397E+15	.000	.019	.019
Cond_ref1=3	.129	4.9504E-18	2.606E+16	.000	.129	.129
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

REM

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	8.886E+28	1	237	.000
Cond_ref1	8.886E+28	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.455	5.5369E-18	2.628E+17	.000	1.455	1.455
Cond_ref1=2	-.004	1.1869E-17	-2.981E+14	.000	-.004	-.004
Cond_ref1=3	.112	2.5839E-17	4.337E+15	.000	.112	.112
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

REM2

Fixed Effects ^a				
Source	F	df1	df2	Sig.
Corrected Model	8.687E+30	1	237	.000
Cond_ref1	8.687E+30	1	237	.000

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log

Fixed Coefficients ^a						
Model Term	Coefficient	Std. Error	t	Sig.	95% Confidence Interval	
					Lower	Upper
Intercept	1.527	1.1348E-17	1.346E+17	.000	1.527	1.527
Cond_ref1=2	-.033	1.1348E-17	-2.947E+15	.000	-.033	-.033
Cond_ref1=3	.258	1.1348E-17	2.272E+16	.000	.258	.258
Cond_ref1=11	0 ^b	.	.	.	.	.

Probability distribution: Normal
Link function: Identity^a

a. Target: EvPot_microV_log
b. This coefficient is set to zero because it is redundant.

Table 16: Interaction between condition and vigilance state (Case3, Occ EEG). Effect of condition (BL vs Post1, BL vs Post2) for each vigilance state.

Chapter 5: The role of sleep in tinnitus development

Background

It is now widely acknowledged that a tinnitus trigger alone, such as noise trauma, is not sufficient for the development of a persistent phantom percept (S. Shore & Wu, 2019).

In fact, tinnitus formation is considered to consist of two stages: an initial triggering event followed by the consolidation of any structural and functional changes in the brain, which are ultimately responsible for the persistent tinnitus symptoms (McFerran et al., 2019). Within the central auditory pathways these changes likely entail enhanced sensory gain to compensate for loss of afferent inputs and maintain firing homeostasis (Schaette & Kempster, 2006; Sedley, 2019), which may lead to elevated firing and bursting activity in various regions across the auditory pathway [Dorsal cochlear nucleus (Brozoski et al., 2002; Kaltenbach et al., 2004; Manzoor et al., 2013; Wu et al., 2016); Inferior colliculus (Bauer et al., 2008; Berger & Coomber, 2015); Medial geniculate body (Kalappa et al., 2014); Auditory cortex (Engineer et al., 2011; Noreña & Eggermont, 2003b; Seki & Eggermont, 2003b)]. Such changes are especially prominent in the dorsal cochlear nucleus (DCN) where increased neuronal synchrony can be observed in animal models of tinnitus even without auditory threshold changes (Marks et al., 2018). This has been related to plasticity in DCN fusiform cells, which are, due to multimodal input, particularly sensitive to spike-time-dependent plasticity, either in form of long-term depression (LTD) or long-term potentiation (LTP). Another prominent consequence of

loss of afferent input due to a tinnitus-trigger-like noise trauma is the rearrangement of tonotopic maps in the auditory cortex (Eggermont & Roberts, 2004c; Engineer et al., 2011; Noreña et al., 2003).

Importantly, neural plasticity in tinnitus is not restricted to the auditory system but involves a change in activity across cortical hemispheres, including prefrontal cortex, orbitofrontal cortex and the parieto-occipital region (Schlee et al., 2009). These brain-wide changes may have important implications for the pathology and pathogenesis of tinnitus. It has been suggested that tinnitus only emerges once brain-wide networks, consisting of frontal and parietal areas, are affected (De Ridder et al., 2011, 2014). For example, malfunctioning of the corticosteroid gating system, involving the nucleus accumbens (NAc) and functional changes in the ventromedial prefrontal cortex have been reported in tinnitus (Rauschecker et al., 2015). Of particular importance are changes in limbic regions, such as neuronal hyperactivity in NAc (Leaver *et al.*, 2011) or a reduction of amygdala activation by unpleasant sounds, possibly due to an ‘internal modification of [the] emotional response’ (Davies et al., 2017). Such limbic involvement in tinnitus has been related to the emotional impact of the phantom sound (De Ridder et al., 2011; Leaver et al., 2016; Rauschecker et al., 2010b) and limbic dysregulation may be a major factor in driving distress associated with tinnitus (De Ridder, Schlee, et al., 2021). In addition, brain-wide and limbic tinnitus representation may indicate tinnitus chronification (De Ridder et al., 2011; Leaver et al., 2016; Rauschecker et al., 2010).

It is likely therefore, that local and global brain plasticity are associated with tinnitus development and may, in addition, be instrumental in mediating generation of the persistent phantom percept. Indeed, electrical stimulation of the left vagus nerve paired

with auditory stimulation can reverse perceptual impairments associated with noise-induced tinnitus in an rat model (Engineer et al., 2011). In humans, vagus nerve stimulation can lead to small but clinically significant improvements in tinnitus loudness and distress (De Ridder, Langguth, et al., 2021), suggesting that targeted plasticity could develop into a tinnitus treatment. Yet, the possibilities to exploit this process are restricted by a lack in understanding of the scope and timescale of its dynamics. To date, little is known about the timescale and possible ‘critical periods’ of plasticity in brain regions as they become involved in tinnitus.

A natural window of brain plasticity

An intriguing possibility to address this problem may be to focus on naturally occurring time windows where the brain is susceptible to plastic changes and harness this potential for experimental manipulation and new routes of clinical treatment. Each day, the brain cycles through multiple functional states, with the arguably most prominent dynamic being the alternation between wakefulness and sleep (Brown et al., 2012). The temporal structure of this dynamic is determined by a combination of circadian factors (mainly the light-dark cycle) and homeostatic factors (mainly time spent awake)(Borbély, 1982; Borbély et al., 2016). During wakefulness brain activity is determined by a combination of spontaneous and evoked activity whereas in sleep it is determined predominantly by spontaneous activity. While brain plasticity does take place during wakefulness the sleep state is thought to be of particular importance in consolidating these plastic changes (Clawson et al., 2018; Frank et al., 2001; Gais et al., 2007; Káli & Dayan, 2004; O’Neill et al., 2010; Takashima et al., 2009). For example,

performance in sensory association tasks increases when human subjects are allowed to sleep after initial training as compared to participants who stayed awake (Gais et al., 2007; Takashima et al., 2009).

The consolidation of experience dependent plasticity is thought to rely on several sleep-dependent mechanisms, such as synaptic strengthening and weakening (Bellesi & de Vivo, 2020; Chauvette et al., 2012; de Vivo et al., 2017) possibly based on the replay of memory traces (Káli & Dayan, 2004). It was shown that cortical regions highly involved in executing tasks during wakefulness exhibit more pronounced slow-wave activity during subsequent sleep (Huber et al., 2004; Vyazovskiy et al., 2011). In turn, if slow waves are suppressed or disrupted, this can interfere with the benefits sleep otherwise provided in terms of memory formation. Slow-waves during NREM sleep have been proposed to be instrumental in sleep dependent downscaling of synaptic strength, a process thought to be essential to sustain brain plasticity (Tononi & Cirelli, 2006). Further, during NREM sleep the depolarisation of a local cell population can increase the chance of concerted neural firing and, in turn, promote plasticity (Klinzing et al., 2019).

An example of this is systems memory consolidation, where global brain plasticity is driven in a sleep-dependent process as consequence of transient changes in hippocampal-cortical information flow. This process is usually based on replay of activity associated with previous waking experience. This is especially prominent in place cells in the hippocampal CA1 region (de Lavilléon et al., 2015), as well as in the re-activation of grid-cells in the entorhinal cortex (Trettel et al., 2019). The coordination of information flow within the hippocampal-entorhinal loop during sharp-wave ripples in NREM sleep may be central to this process of memory reactivation and consolidation

(Mizuseki & Miyawaki, 2017). Also, activity across sensory cortical areas, such as visual cortex (Ji & Wilson, 2007) and auditory cortex (Rothschild et al., 2017) is correlated with hippocampal replay during sleep. For example, Rothschild and colleagues (2017) demonstrated an information-processing loop between auditory cortex and hippocampus that mediates biasing of hippocampal activity during sleep, either through spontaneous cortical activity or via sound stimulation during sleep. This indicates that system memory consolidation may not only be relevant to multiple modalities but also that altered spontaneous activity, such as it has been described in tinnitus, may impact on the cortical-hippocampal processing loop, and possibly affect plasticity on a global scale. It remains open as to how far systems memory consolidation is a mediator in the brain-wide plasticity observed in cases of persistent tinnitus.

Further, it is possible that NREM sleep is an important mediator in the rearrangement of tonotopic maps, subsequent to a tinnitus trigger such as noise overexposure. The state dependency of cortical map plasticity has been demonstrated in the visual system following monocular deprivation (Frank et al., 2001). Here, the amount of NREM sleep correlated with the degree of ocular dominance plasticity, while REM sleep was weakly negatively correlated with plasticity. Further, it has been shown that plasticity in the visual cortex can be promoted by NREM sleep and inhibited by sleep deprivation (Clawson et al., 2018). The possibility remains that plasticity in the auditory system, subsequent to reduction of peripheral input, is also a state-dependent process and that NREM sleep in particular might be a mediator in tinnitus formation.

Ultimately, even though plasticity observed during sleep shows similarities to plasticity thought to underly tinnitus formation, the role of sleep in tinnitus formation remains

unclear. Here, the effect of sleep and sleep-deprivation on tinnitus development was evaluated in a mouse model of noise-induced tinnitus.

Method summary

Experiments were performed in adult male C57/BL6ntac-cdh23h-9 mice (n=14). The mouse model was chosen for this experiment for mainly two reasons: first, it is an established model in sleep and tinnitus research and in *in vivo* recording of brain activity (e.g. Huber et al., 2000; McKillop et al., 2018; Middleton et al., 2011; Turner et al., 2012). It also allows for a larger number of animals to be included as compared to the ferret model. Therefore, the mouse is well suited for both sleep and tinnitus studies, especially when several experimental groups are required. The C57/BL6ntac-cdh23h-9 strain was chosen for this experiment as these animals, due to a corrected *Cdh23*(ahl) allele, do not show early-onset age-related hearing loss present in commonly used inbred mouse strains (Mianné et al., 2016). In the experiments described in this chapter the formation of tinnitus symptoms was compared between animals that were kept awake after a noise overexposure (NOE) paradigm (sleep deprivation group, SD) and a group that was allowed to sleep *ad libitum* after NOE (control group). The aim of this paradigm was to test the importance of sleep immediately after NOE in the development of tinnitus. For a detailed description of the methods and protocols used, in addition to the summary provided below, see the Methods chapter.

Triggering and assessing tinnitus in the mouse

To trigger tinnitus, all mice were subjected to noise overexposure (NOE) as is commonly used in animal models of tinnitus (Bauer et al., 2008; Engineer et al., 2011; Kaltenbach et al., 2004; J. Turner et al., 2012). Here mice were exposed for two hours to 98 dB SPL 8 kHz centred narrowband noise, while under anaesthesia. Noise was presented via a free-field speaker facing the animal while the animal's left ear was plugged. For a more detailed description of the procedure, see Methods: Noise overexposure.

Tinnitus was assessed in a variant of behavioural gap-in-noise detection, gap-induced inhibition of the behavioural startle (GPIAS, see Methods), which is commonly used to assess tinnitus in the mouse model (e.g. Middleton et al., 2011; Turner et al., 2012). The paradigm is largely based on the 'filling in' hypothesis of tinnitus, which posits that a persistent phantom sound will impair the perception of silent gaps in an external noise. See Chapter 1 for more details on the gap detection paradigm and the 'filling in' hypothesis. In brief, animals were placed in a small experimental chamber on top of a motion-sensitive platform (Fig.2A) and presented with low intensity broadband white noise at 60 dB SPL. At random intervals (3-13 s), brief high-intensity startle stimuli (20 ms white noise burst, 105 dB SPL), causing a behavioural startle response (usually a twitch or jump of the animal), were presented over the background noise. Half of the startle stimuli were preceded by a silent gap (50ms gap duration) in the background noise 100ms before. This commonly leads to a reduced startle of the animal relative to startle responses evoked by startle stimuli not preceded by a silent gap. This relative decrease of the startle response is referred to as GPIAS (gap-induced inhibition of the acoustic startle). Impaired gap detection ability is thought to be reflected in reduced

GPIAS (Turner et al., 2012; Turner et al., 2006). Since animals are prone to adaptation in this paradigm (i.e. a reduced startle after repeated exposure to the startle stimulus), each assessment consisted of only 40 trials (20 trials with a 'silent gap' preceding the startle stimulus and 20 trials without; trials were randomly interspersed). In addition, consecutive GPIAS assessments were interleaved by at least 14 days.

To assess noise-induced hearing impairment (Kujawa & Liberman, 2009; Turner et al., 2012), auditory brainstem responses (ABRs) were obtained in sedated animals before and after NOE. Here, the summed potential of the auditory nerve as well as of further ascending processing stages in the central auditory pathway in response to pure tones and click stimuli was recorded via electrodes placed subcutaneously. For a more detailed description of the procedure, see the Methods chapter.

The experimental timeline

Adult male C57/BL6ntac-cdh23h-9 mice (n=14) were housed in a 12/12 light-dark cycle. Food and water was available *ad libitum*. All animals were implanted with frontal and occipital EEG screw electrodes and neck muscle electrodes at the beginning of the experiment (see Methods: electrode implantation). After at least a week of recovery, baseline auditory brainstem responses (ABRs) were assessed in all animals. Subsequently, baseline GPIAS was tested.

Mice were then transferred into recording cages (suitable for obtaining chronic EEG recordings in the freely-moving animals), which also served as home cages. After habituation to the new cage of at least two days, chronic electrophysiological recordings

and video recordings were obtained continuously in the freely behaving animals. After at least two days of continuous baseline recordings, animals were subjected to noise overexposure (see Methods: Noise exposure) administered under anaesthesia for a duration of 2 hours at the end of the dark phase (ZT22-24). Immediately afterwards, at light onset, mice were transferred into a heated recovery chamber for up to 5 minutes until the animals recovered from overt anaesthesia (i.e. started to walk around without provocation). Mice were then transferred back into their home/recording cages. Animals in the sleep deprivation (SD) group (n=8) were now kept awake for a period of six hours. SD was conducted via provision of novel objects, which is an established method for sleep deprivation in mice (e.g. Huber et al., 2000; McKillop et al., 2018) making use of the animals' natural tendency to explore novel objects and therefore remaining engaged. During SD, animals were continuously monitored by the experimenter and novel objects were provided whenever the animal showed signs of drowsiness (behavioural immobility or increasing delta activity in the EEG, which was continuously recorded while the animal was in the home cage). Animals in the control group (n=6) were left undisturbed after having been transferred back to their home/recording cages and could thus sleep *ad libitum* after experiencing NOE. The time of NOE (at the end of the dark period, the animals' active phase) was chosen to ensure that SD would occur during a natural sleep time of the animals (within the first half of the light phase) and thus ensure that the difference in sleep amount between control and SD animals would be maximal.

Chronic electrophysiological recordings were obtained continuously for two days after NOE. Approximately a week after NOE animals were again assessed in the GPIAS

paradigm and ABRs were obtained. GPIAS was assessed for a third time approximately 8 weeks after noise overexposure (see Fig. 1 for a timeline of the experiment).

Statistical analysis

Statistical analysis of data from the GPIAS paradigm was performed using multiple factors (repeated measures): experimental condition (SD and control), time of testing (BL, Post1w, Post8w), order of presentation (time interval within assessments), response window (50ms and 1000ms) and, as random factor, animal ID. Analysis was performed after exclusion of outliers. Outliers were defined as values larger than the mean of the bootstrapped raw data (5000 iterations) + 5 standard deviations. Statistically relevant effects were estimated by applying a generalised linear mixed model (GLMM, distribution: normal, link: identity).

For ABR recordings, statistical analysis on changes in magnitude and latency of the different waves (waves I-V) with stimuli at 90 dB SPL was performed using a generalised linear mixed model (GLMM) on data with wave number (ABR wave I to V) as repeated factor and, as random factor, animal ID. Normality of data was assessed by inspection of QQ plots. Group differences in response modulation and response magnitude across stimulus intensities were assessed using data after exclusion of outliers (values outside the mean $\pm$ 2 standard deviations) as input for a generalised linear mixed model (repeated factors: experimental condition (SD and control), random factors: animal ID). Statistical analysis for ABRS was only performed on normalised data (Post NOE measure as % of BL).

EEG and EMG recordings were visually assessed to ensure efficiency of the sleep deprivation paradigm, but quantitative analysis was not included in the analysis presented in this chapter.

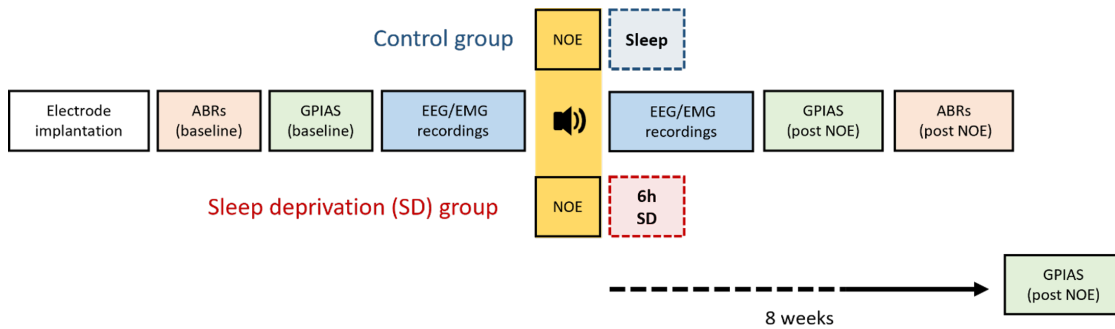


Fig.1: Outline of the experiment. Animals were implanted with frontal and occipital EEG screw electrodes and neck muscle electrodes at the beginning of the experiment. After recovery, auditory brainstem responses (ABRs) were assessed in all animals. Subsequently, baseline gap induced inhibition of the behavioural startle (GPIAS) was tested. Mice were then transferred into recoding cages, which also served as home cages. After a minimum of two days of habituation to the new environment and tethering, chronic electrophysiological recordings and video recordings were obtained continuously in the freely behaving animals. Vigilance state scoring was performed manually based on filtered EEG and EMG signals, and the video recording. After at least two days of continuous baseline recordings, animals were subjected to noise overexposure administered under anaesthesia for a duration of 2 hours at the end of the dark phase (8kHz centred NBN presented at 98dB SPL). Immediately afterwards, at light onset, animals were transferred into a heated recovery chamber for up to 5 minutes until the animals recovered from overt anaesthesia (i.e. started to walk around without provocation). Mice were then transferred back into their home/recording cages. Animals in the SD group (n=8) were kept awake for a period of six hours. Animals in the control group (n=6) were left undisturbed after experiencing NOE. Chronic electrophysiological recordings were obtained continuously for two days after NOE. About 1 week (ca 6 days) after NOE, animals were again assessed in the GPIAS paradigm and subsequently ABR responses were obtained. About 8 weeks after noise overexposure, GPIAS was assessed for a third time.

Results

Sleep deprivation after NOE mitigates gap detection impairment

At the beginning of the experiment, after implantation of chronic electrodes and a recovery period, baseline behaviour in the GPIAS paradigm was measured in all animals. In line with previous studies (e.g. Park et al., 2020; J. Turner et al., 2012), this paradigm served as the main tinnitus measure. GPIAS was assessed by comparing the startle response in gap-trials and non-gap trials, based on the assumption that in animals with normal hearing the startle response in gap trials is inhibited relative to non-gap trials (Fig.2B).

For every trial two parameters were used to quantify the startle response. First, the peak amplitude, defined as maximum - minimum value of the signal within the response window. The second parameter was the average of the signal within the response window. Both measures were assessed in a narrow and a wider response window: one to capture the initial startle response (in the first 50ms after presentation of the startle stimulus), and the other over one second (within the first 1000ms after presentation of the startle stimulus) to include the full startle response. GPIAS was defined as the ratio between the startle response in gap trials and the response in non-gap trials:

$$GPIAS = \frac{startle(gap\ trials)}{startle(non - gap\ trials)}$$

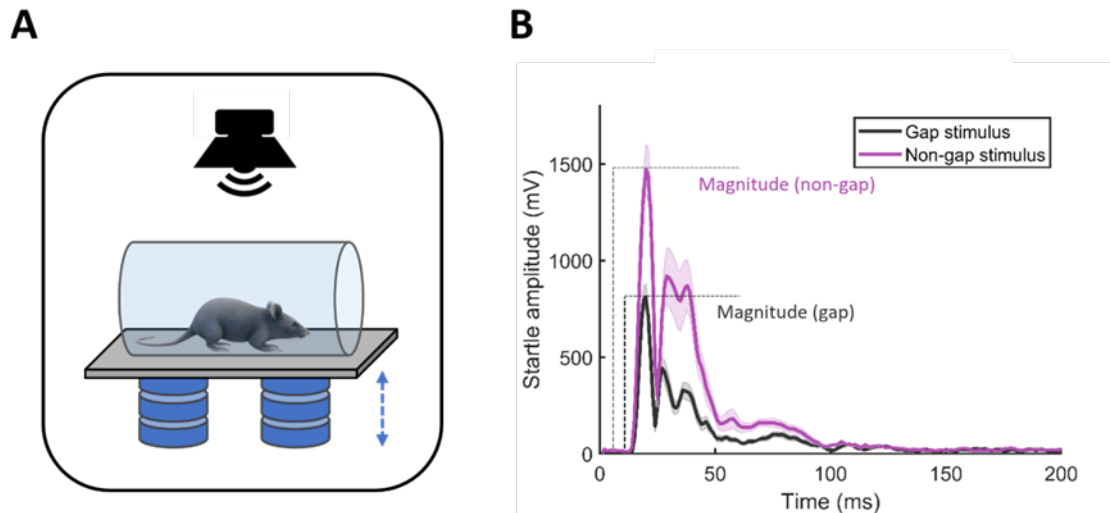


Fig.2: Gap pre-pulse inhibition of the acoustic startle (GPIAS) (A) Schematic depiction of the setup used for the GPIAS paradigm. Animals were placed in an tube-shaped chamber, which was positioned on a motion sensitive platform. Sound stimuli were presented via a speaker located above the chamber. The chamber and speaker were housed in an enclosure (not shown). (B) Example of an average startle signal in gap trials (blue) and non-gap trials (purple). Typically, the startle amplitude is higher in non-gap trials than in trials where the startle sound is preceded by a predictive gap in the ongoing background noise. The startle response was assessed in two ways: first, by measuring the magnitude of only the most pronounced peak in the signal (defined as *maximum - minimum* value of the signal within the response window (see labels in the figure) and, second, by calculating the average of the signal within the response window. These measures were assessed for the onset of the startle response (initial 50ms of the signal), or over the entire recorded response (response window of 1000ms).

Under baseline conditions, before NOE, gap detection ability was evident for both the control group (0.71 ± 0.21 , Fig.3A) and the SD group (0.8 ± 0.21 , Fig.3B), signified by a GPIAS value below 1 in line with previous work in mice (Longenecker et al., 2018; Park et al., 2020). GPIAS at baseline did not differ significantly between groups ($F_{(1,210)}=3.24$, $p=0.07$, *n.s.*). After noise overexposure, both the control and the SD group showed lower gap detection ability (signified by a higher GPIAS value than in BL conditions, Fig.3). And while in a combined analysis, GPIAS developed similarly after NOE between the two groups (*interaction between time and group*, $F_{(5,624)}=1.85$, $p=0.1$, *n.s.*), in a separate analysis of SD and control group, respectively, only mice that were allowed to sleep in the first 6 hours after NOE (control group) developed a significant impairment (**Control**,

$F_{(2,271)}=4.2$, $p=0.02$, BL vs Post1w, 0.71 ± 0.21 vs 0.8 ± 0.21 , $\beta =0.11$, $t_{(273)}=2.64$, $p=0.01$, Fig.3A). Animals in the SD group did not develop a significant impairment (**SD**, $F_{(2,353)}=1.468$, $p=0.23$, BL vs Post1w, 0.8 ± 0.21 vs 0.83 ± 0.23 , $\beta =0.18$, $t_{(355)}=0.36$, $p=0.72$, Fig.3B), suggesting that sleep deprivation may mitigate the effect of NOE on gap detection ability. 8 weeks after noise overexposure, GPIAS performance was similar to BL levels in both groups (**Control**, BL vs Post8w, 0.71 ± 0.21 vs 0.66 ± 0.21 , $\beta =0.02$, $t_{(273)}=0.73$, $p=0.47$, **SD**, BL vs Post8w, 0.8 ± 0.21 vs 0.75 ± 0.12 , $\beta =-0.04$, $t_{(355)}=-1.23$, $p=0.22$, Fig.3), suggesting that impairment was only temporary.

The transient impairment in GPIAS in the control group was evident independent of whether the startle magnitude was defined as the peak response or as the average of the response signal (**Peak**, $F_{(2,132)}=2.38$, $p=0.1$, BL vs Post1w, $\beta =0.1$, $t_{(134)}=2.12$, $p=0.04$, BL vs Post8w, $\beta =0.02$, $t_{(134)}=0.54$, $p=0.59$, **Average**, $F_{(2,136)}=5.75$, $p=0.004$, BL vs Post1w, $\beta =0.12$, $t_{(138)}=2.69$, $p=0.01$, BL vs Post8w, $\beta =-6.677E-6$, $t_{(138)}<0.001$, $p=1$). The transient impairment was also evident when only the long response window was used to define the startle magnitude (**1000ms window**, $F_{(2,134)}=2.46$, $p=0.09$, BL vs Post1w, $\beta =0.1$, $t_{(136)}=2.2$, $p=0.03$, BL vs Post8w, $\beta =-0.27$, $t_{(136)}=-0.99$, $p=0.32$) and showed a trend towards significance for the short response window (**50ms window**, $F_{(2,134)}=2.24$, $p=0.11$, BL vs Post1w, $\beta =0.11$, $t_{(136)}=1.95$, $p=0.05$, BL vs Post8w, $\beta =-0.02$, $t_{(136)}=-0.76$, $p=0.45$).

In the SD group, GPIAS performance remained stable over time for all measures and response windows: (**Peak**, $F_{(2,175)}=2.11$, $p=0.12$, BL vs Post1w, $\beta =0.03$, $t_{(177)}=0.66$, $p=0.51$, BL vs Post8w, $\beta =-0.04$, $t_{(177)}=-1.21$, $p=0.23$, **Average**, $F_{(2,175)}=2.69$, $p=0.07$, BL vs Post1w, $\beta =-0.01$, $t_{(177)}=-0.26$, $p=0.8$, BL vs Post8w, $\beta =-0.06$, $t_{(177)}=-1.56$, $p=0.12$, **50ms window**,

$F_{(2,175)}=3.57$, $p=0.03$, BL vs Post1w, $\beta=0.02$, $t_{(177)}=0.42$, $p=0.67$, BL vs Post8w, $\beta=-0.04$, $t_{(177)}=-1.62$, $p=0.11$, **1000ms window**, $F_{(2,175)}=1.95$, $p=0.15$, BL vs Post1w, $\beta=-0.015$, $t_{(177)}=-0.29$, $p=0.77$, BL vs Post8w, $\beta=-0.06$, $t_{(177)}=-1.65$, $p=0.1$).

Therefore, the mitigating effect of sleep deprivation on transient GPIAS impairment after NOE is unlikely to be related to a specific component of the startle response.

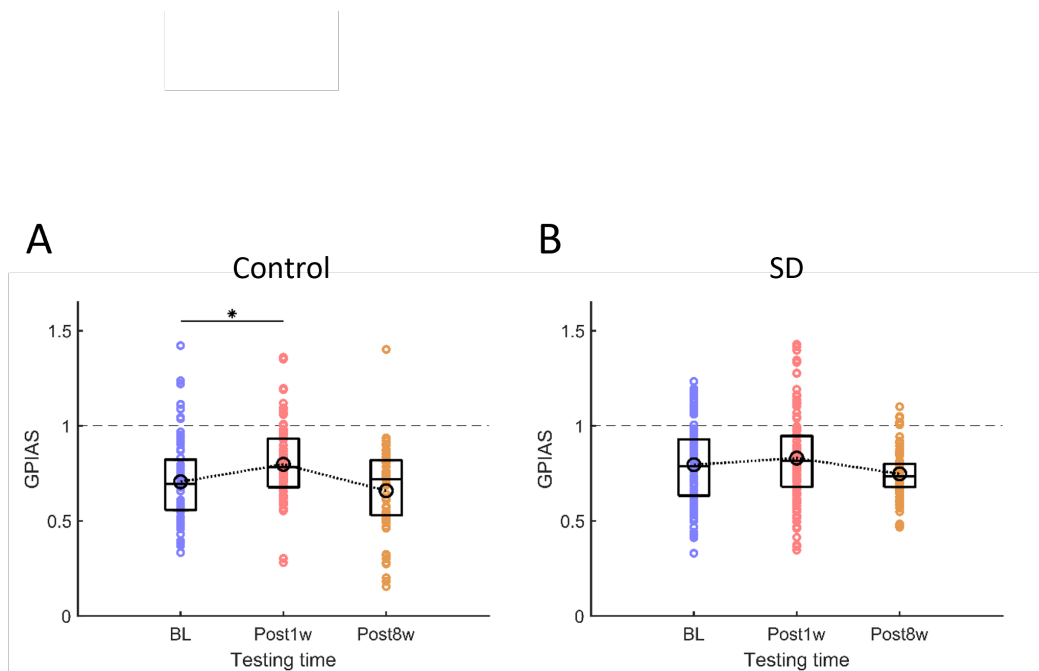


Fig.3: GPIAS: Effects of testing time in Control and SD groups. (A) GPIAS (startle magnitude in gap/startle magnitude in non-gap trials) separated by testing time. Data points depict values of individual animals of the control group, for both measures (peak and average response) and window (50ms and 1000ms). Black circles on top of the box plots depict mean values, connected by a dotted line across groups. Box plots mark interquartile ranges, horizontal lines within boxplots depict the median. The horizontal dotted line depicts GPIAS=1, i.e. no gap induced startle inhibition. **(B)** GPIAS depicted as in Panel A but data are only from the SD group (6 hours of novel object sleep deprivation after NOE). Horizontal solid lines indicate significant group differences with * indicating $p<0.01$, GLMM.

Sleep deprivation after NOE affects intra-session habituation in the GPIAS paradigm

Next to the modulation of GPIAS performance over time, there was a modulation within individual assessments (*Effect of Interval*, $F_{(3,626)}=10.49$, $p<0.001$, Fig.4A), with a steady increase of GPIAS from beginning to end of the assessment (*Early1*, 0.68 ± 0.17 , *Early2*, 0.75 ± 0.18 , *Late1*, 0.79 ± 0.21 , *Late2*, 0.84 ± 0.24). This is in line with habituation of the animal to the startle stimulus, i.e. a steady decrease in startle amplitude and thus also less contrast between startle in gap and non-gap trials.

Intra-session modulation of performance did not remain stable over time (*interaction between Interval and Testing time*, $F_{(11,618)}=66.69$, $p<0.001$). Especially in the late assessment 8 weeks after NOE, GPIAS was more stable throughout the session than in earlier assessments and less of a habituation effect was visible (Fig.4B). Notably, this was most apparent in the SD group (Fig.4C), suggesting that sleep deprivation after NOE may have long-term effects on the ability of animals to adapt to startling sounds.

In summary, these results suggest that NOE led to transiently impaired gap detection ability. Novel object sleep deprivation immediately after NOE mitigated this effect and even reduced animal's habituation within sessions, suggesting that both gap detection ability and sensitivity to startling stimuli may have been maintained or become heightened in these animals.

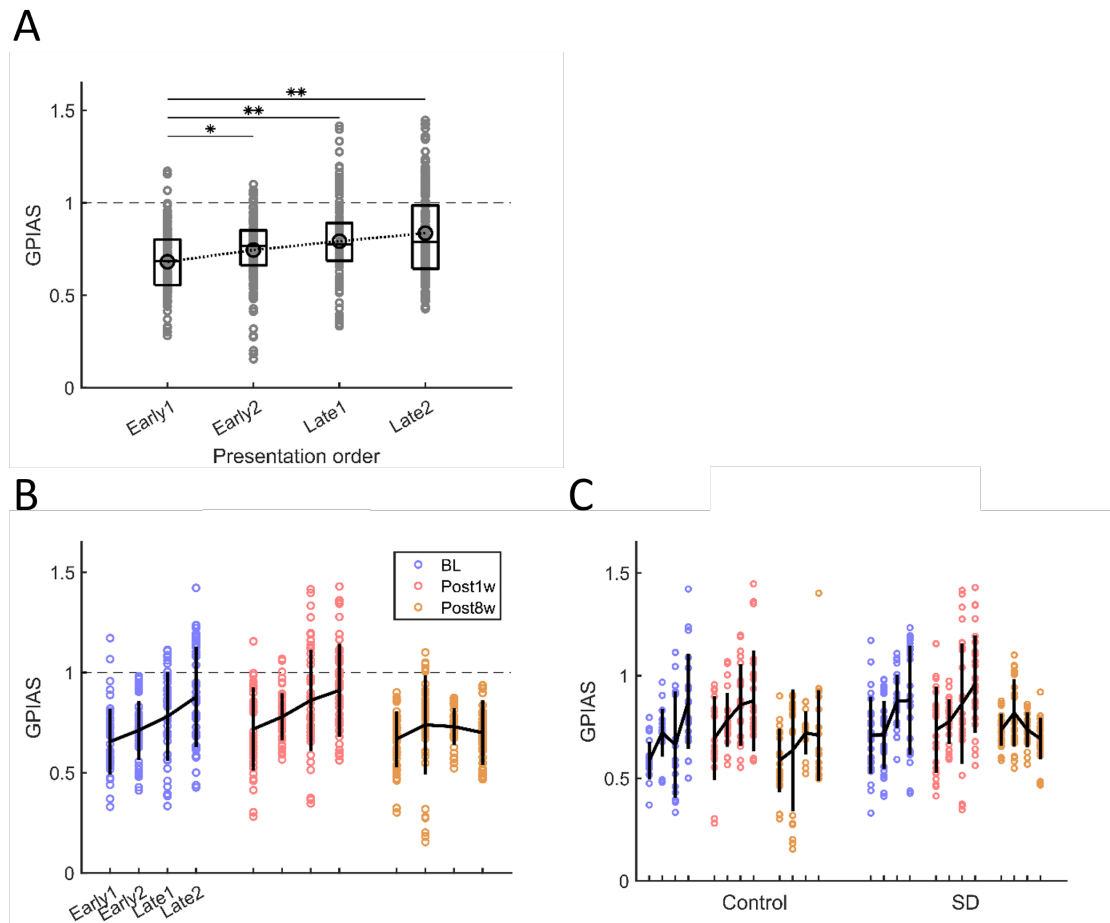


Fig.4: GPIAS: Intra-session dynamics in Control and SD groups. (A) GPIAS (= *startle magnitude in gap trials / startle magnitude in non-gap trials*) depicted across 4 consecutive intervals within the GPIAS assessment (presentation order): ‘Early1’, ‘Early2’, ‘Late1’ and ‘Late2’. For each interval, GPIAS was calculated based on 5 gap and 5 non-gap trials (‘Early1’: trials 1-5, ‘Early2’: trials 6-10, ‘Late1’: trials 11-15, ‘Late2’: trials 16-20 for gap and non-gap trials, respectively). Data points depict values of individual animals of both control and SD groups, all testing times, measures (peak and average response) and windows (50ms and 1000ms). Black circles on top of the box plots depict mean values, connected by a dotted line across groups. Box plots mark interquartile ranges, horizontal lines within boxplots depict the median. The horizontal dotted line depicts GPIAS=1, i.e. no gap induced startle inhibition. Horizontal solid lines indicate significant group differences with * indicating $p < 0.01$ and ** $p < 0.001$, GLMM. **(B)** GPIAS across 4 consecutive intervals (Early1, Early2, Late1, Late2) per assessment depicted as in panel A but separated by testing time (BL, Post1w, Post8w). Error bars are standard deviations, black connecting lines connect group means. **(C)** Depiction as in panel A but data are separated by testing time (BL, Post1w, Post8w) and experimental group (Control and SD). Error bars are standard deviations, black connecting lines connect group means.

The effect of sleep deprivation on noise-induced changes in central brainstem activity

The state of the central auditory brainstem before and after NOE was assessed by obtaining auditory brainstem responses (ABRs) in baseline conditions and approximately 1 week after noise overexposure. Thresholds were assessed through visual assessment of the data under randomised and blinded conditions. In addition, evoked responses were assessed offline via a custom-made algorithm (see Methods) that was applied to obtain amplitudes and latencies of individual response peaks in the ABR signal (Fig. 6A). Subsequently, the analysis focused on two aspects of the ABR response, as previously described in Chapter 2: (1) the magnitude of individual components (ABR waves I to V) of the evoked response to 90 dB stimuli and (2) the total magnitude of responses across stimulus intensities.

Throughout the experiment, animals showed the highest thresholds in low frequency ranges (Fig.5A,B) and sensitivity increased with increasing sound frequency (*effect of sound frequency*, Control: $F_{(4,60)}=1715.47$, $p<0.001$, SD: $F_{(5,84)}=543.64$, $p<0.001$). In addition, there was an interaction between stimulus frequency and time in both the control and the SD group, suggesting that threshold changes after NOE depended on the tested stimulus frequency (*interaction between time and sound stimulus*, control: $F_{(5,60)}=6.0$, $p<0.001$, SD: $F_{(5,84)}=3.64$, $p<0.01$). Threshold elevation for specific stimuli was, however, only evident in the control group, in particular for 8 kHz (*the NOE stimulus*, BL vs Post1, 56.67 ± 3.33 vs 6.0 ± 2.24 dB, $F_{(1,10)}=3.66$, $p=0.09$, *n.s.*) and even more so for 4 kHz stimuli (BL vs Post1, 80 ± 3.65 vs 90 ± 0 dB, $F_{(1,10)}=9.0$, $p<0.05$)(Fig.5C). In the sleep deprived group thresholds did not significantly change for any tested stimulus (Fig.5D).

This suggests that novel object sleep deprivation after NOE might have had a protective effect on hearing sensitivity.

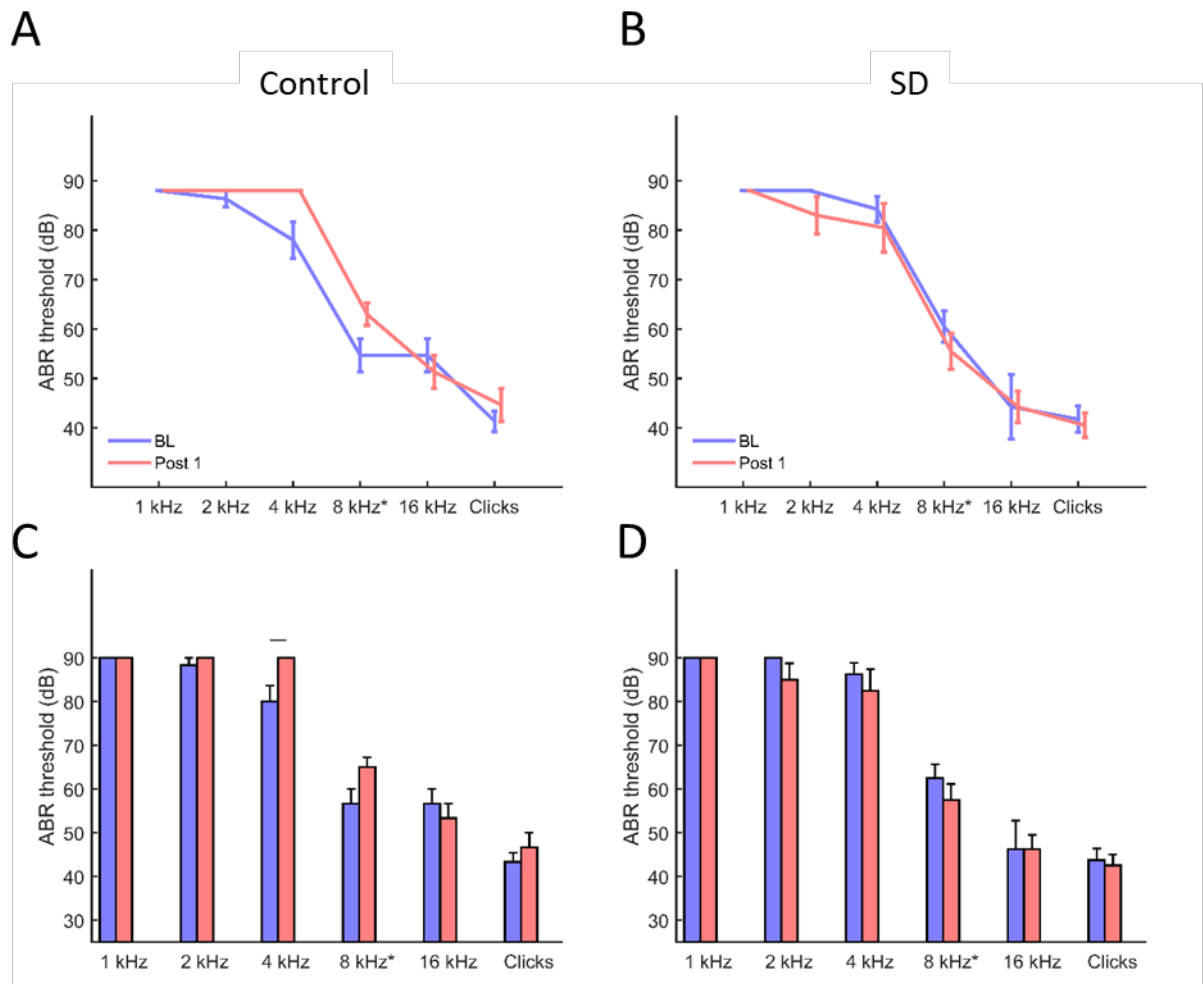


Fig.5: Auditory brainstem responses (ABRs): Thresholds. (A,B) Thresholds for control (A) and SD mice (B) before and after NOE. Thresholds were assessed through visual inspection of raw data by an experienced experimenter under randomised and blind conditions. Data are means and standard errors depicted for all measured sound stimuli. (C,D) Alternative depiction of data displayed in panels A and B. The horizontal bar depicts statistical significant group differences of $p < 0.05$.

To assess in more detail how noise overexposure might have impacted ABRs, response magnitude and latencies were measured. The first tested stimuli were clicks, which, due

to their broad frequency range are most likely to elicit pronounced ABR responses. Generally, animals showed a considerable variability in wave magnitude across ABR waves I to V in response to 90 dB click stimuli (Fig.7A, Table 1). To assess the direction and magnitude of changes in individual ABR waves after NOE for control and SD animals, the change in each respective measure was defined as:

$$\Delta(\text{measure}) = \text{value}(\text{BL}) - \text{value}(\text{post NOE})$$

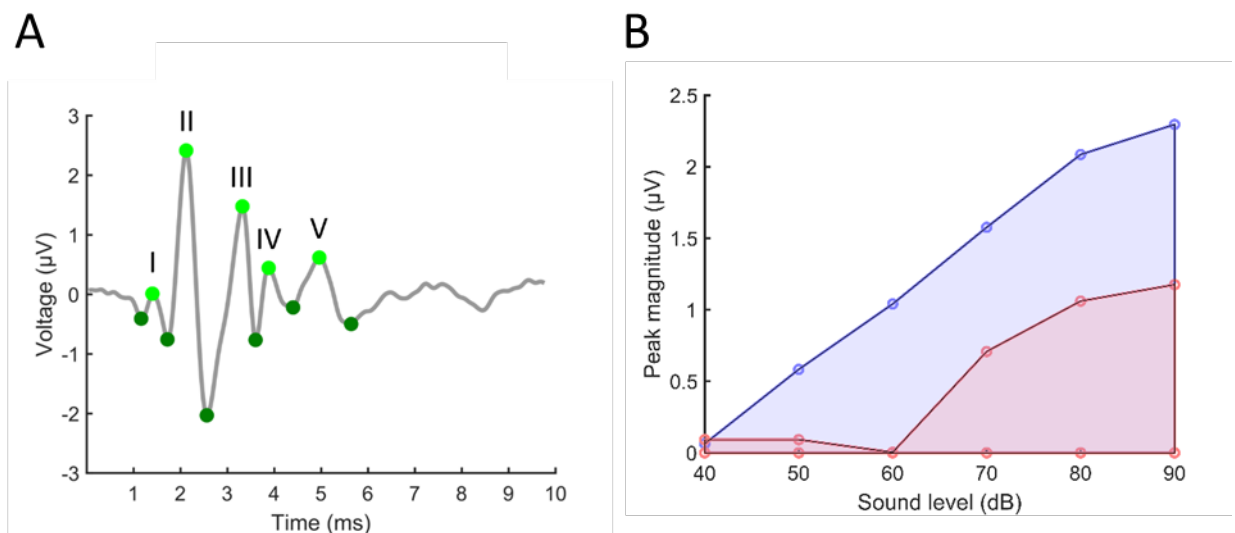


Fig.6: Auditory brainstem responses (ABRs): Methodology and analysis. (A) Example of an ABR signal to 90dB SPL click stimuli (average over 700 trials). Green markers depict detected peaks and troughs of waves (I to V) as detected with a custom-written MATLAB algorithm. **(B)** The magnitude of the ABR response across all sound intensities was estimated by calculating the area under the level-response graph (here the area enclosed by the blue 'BL' and red 'post NOE' graph, respectively).

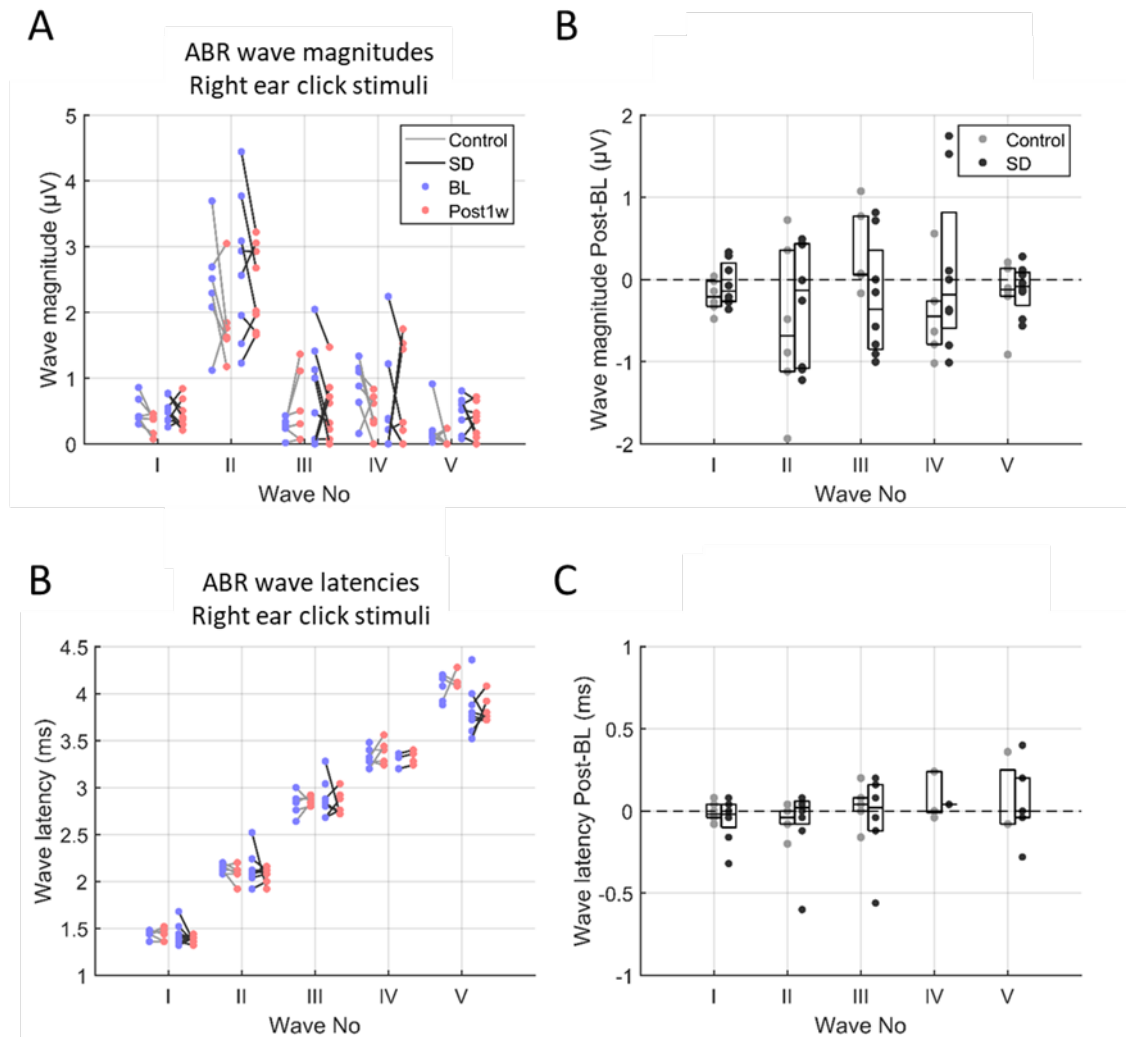


Fig.7: Auditory brainstem responses (ABRs) for 90 dB click stimuli: individual waves. (A) Magnitude of individual ABR waves (I-V) before and after NOE for both Control and SD groups (see figure legend). Datapoints are animal averages. Lines connect animal averages obtained during the BL and Post1w assessments. **(B)** Difference in wave magnitude (Post – BL values) calculated for each animal. Data points represent individual animals. Box plots mark interquartile ranges, horizontal lines within boxplots depict the median. Values below 0 (dotted line) depict a decrease in wave magnitude after NOE relative to BL. **(C)** Wave latencies, depicted as in A. **(D)** Wave latency changes from BL to Post1w, depicted as in B.

Sleep deprivation after NOE mitigates the impairment in click-evoked ABRs

Animals tended to show reduced response magnitudes especially in ABR wave I (*Controls*, $-0.2 \pm 0.19 \mu V$, *SD*, $-0.06 \pm 0.27 \mu V$) and wave II (*Controls*, $-0.56 \pm 0.98 \mu V$, *SD*, $-0.29 \pm 0.74 \mu V$) after NOE (Fig.7B). Control animals did further show an elevation of wave

III magnitude after NOE (by $0.31 \pm 0.49 \mu V$ relative to BL), which was not apparent in SD animals ($-0.24 \pm 0.71 \mu V$), yet there was no significant difference in changes after NOE between control and SD animals for any ABR wave (*interaction ABR wave identity and condition*, $F_{(9,60)}=1.33$, $p=0.24$, Fig.7B). With regards to response latencies (Fig.6C, Table 2) there was an interaction between wave identity and condition ($F_{(9,48)}=3.99$, $p<0.001$) yet latencies did not show significant difference between conditions for any wave identity (**Wave I latency**, Control vs SD, -0.01 ± 0.06 vs -0.05 ± 0.13 ms, $F_{(1,12)}=0.76$, $p=0.4$, **Wave II latency**, Control vs SD, -0.05 ± 0.09 vs -0.07 ± 0.23 ms, $F_{(1,12)}=2.36$, $p=0.15$, **Wave III latency**, 0.03 ± 0.12 vs -0.05 ± 0.28 ms, $F_{(1,10)}=0.08$, $p=0.79$, Control vs SD, **Wave IV latency**, Control vs SD, 0.09 ± 0.14 vs 0.04 ± 0.0 ms, $F_{(1,6)}=2.36$, $p=0.18$, **Wave V latency**, Control vs SD, 0.07 ± 0.25 vs 0.06 ± 0.22 , $F_{(1,8)}=1.47$, $p=0.26$, Fig.7D). This suggests that sleep deprivation did not change the effect of NOE on high-intensity click-evoked responses, in line with thresholds observed for click stimuli.

To assess whether sleep deprivation influenced the effect of NOE on the total ABR magnitude, calculated across responses to all assessed stimulus levels (40, 50, 60, 70, 80, 90 dB SPL clicks), the area under the level-response graph was calculated for each animal and assessment (Fig.6B). This measure was based on the magnitude of ABR wave II and on the RMS of the signal encompassing ABR waves I to V.

Irrespective of the experimental group, animals showed lower total ABR magnitudes after NOE as compared to BL for ABR wave II (74.01 ± 41.64 % of BL and 94.88 ± 27.89 % of BL for control and SD animals, respectively) and the RMS (80.22 ± 31.86 % of BL and 81.53 ± 10.99 % of BL for control and SD animals, respectively), suggesting that both groups were affected by NOE (Fig.8A). The decrease in total magnitude of wave II tended

to be more pronounced in control animals relative to the SD group (**Wave II total magnitude**, Control vs SD, 74.01 ± 41.64 vs $94.88 \pm 27.89\%$ of BL, $F_{(1,12)}=4.58$, $p=0.05$, Fig.8A), indicating that, measured across stimulus intensities, the ABR response was more impaired after NOE when animals were allowed to sleep in the subsequent 6 hours. The decrease in total response magnitude across all ABR waves was similar between control and SD animals (**RMS total magnitude**, Control vs SD, 80.22 ± 31.86 vs $81.53 \pm 10.99\%$ of BL, $F_{(1,11)}=0.02$, $p=0.9$, Fig.8A).

Thus, while click-evoked auditory brainstem responses tended to decrease after NOE in all animals, sleep deprivation was associated with a mitigation of this impairment.

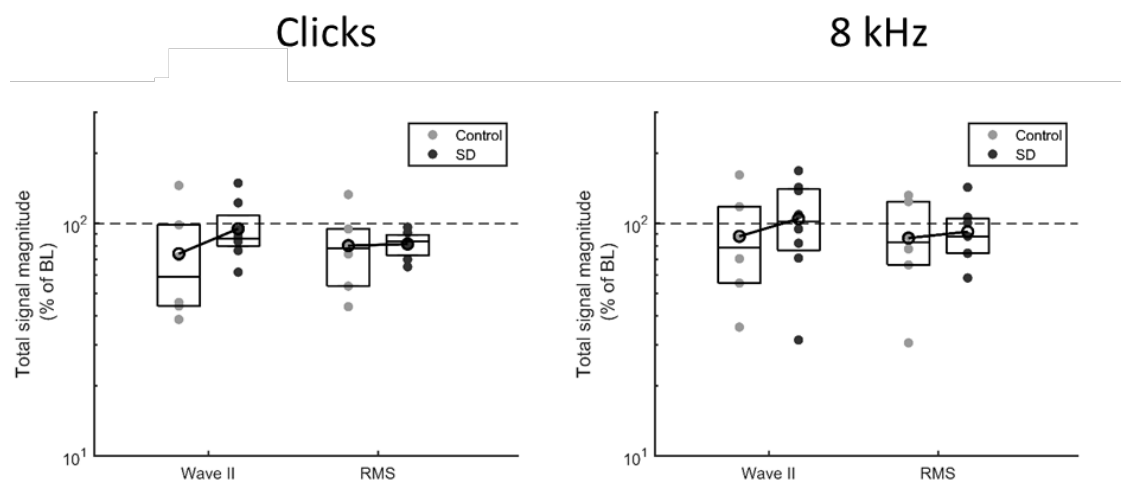


Fig.8: Auditory brainstem responses (ABRs) for click and 8 kHz stimuli: changes after NOE in total magnitude in Control and SD groups. ABR total magnitude (area under the level-response graph, as % of BL), shown for ABR wave II and the RMS of the signal for click stimuli (A) and 8 kHz stimuli (B). Box plots mark interquartile ranges, horizontal lines within boxplots depict the median. Black circles on top of the box plots depict mean values, connected by a dotted line across groups. Values above 1 signify an increase in the respective measure, values below 1 (dotted) line signify a decrease relative to the BL assessment.

Sleep deprivation mitigates impairment in 8 kHz pure tone-evoked ABRs and reduces gain elevation

Since noise overexposure was conducted using an 8 kHz centred NBN stimulus, hearing impairments were expected to be most prominent for stimuli in a similar frequency range, as also suggested by the observed shifts in ABR thresholds after NOE (Fig.5). Therefore, ABRs for 8 kHz pure tones were assessed in more detail, based on response magnitudes and latencies.

Responses to 90 dB tone stimuli had overall lower magnitudes than click stimuli (compare Fig.7A & Fig.9A), but, again, wave II was the most pronounced component of the ABR (Fig.9A). Also, animals showed still high variability in response magnitude (Fig.9A, Table 3).

After NOE, magnitudes of waves I and II tended to be lower than in the BL assessment in both experimental groups (**Wave I magnitude**, Controls, $-0.04 \pm 0.9 \mu V$, SD, $-0.05 \pm 0.9 \mu V$, **Wave II magnitude**, Controls, $-0.24 \pm 0.46 \mu V$, SD, $-0.15 \pm 0.21 \mu V$, Fig.9B), whereas wave III was enhanced (**Wave III magnitude**, Controls, $0.27 \pm 0.18 \mu V$, SD, $0.13 \pm 0.23 \mu V$, Fig.9B). Unlike to click stimulation, differences between control and SD animals were evident in ABR wave specific changes in magnitude, as indicated by a strong interaction between ABR wave identity and experimental condition ($F_{(9,16)}=9.7$, $p<0.001$): in particular the change in magnitude of ABR wave III was more pronounced in control than in SD animals (**Wave III magnitude**, Control vs SD, 0.27 ± 0.18 vs $0.13 \pm 0.23 \mu V$, $F_{(1,12)}=7.85$, $p=0.02$, Fig.9B). This indicates that NOE may have led to reduced evoked activity in early processing stages (auditory nerve and cochlear nucleus) and to a compensatory gain increase in upstream regions associated with ABR wave III, such as in the superior olivary

nucleus (Henry, 1979), which was especially pronounced in control animals. Note that a similar trend was visible in control animals for click stimulation (Fig.7B). In other tone evoked ABR waves the changes after NOE were similar between control and SD groups (**Wave I magnitude**, Control vs SD, -0.04 ± 0.09 vs $-0.05 \pm 0.09 \mu V$, $F_{(1,12)}=0.19$, $p=0.67$, **Wave II magnitude**, Control vs SD, -0.24 ± 0.46 vs $-0.15 \pm 0.21 \mu V$, $F_{(1,12)}=0.52$, $P=0.49$, **Wave IV magnitude**, Control vs SD, -0.03 ± 0.7 vs $-0.09 \pm 0.37 \mu V$, $F_{(1,12)}=0.08$, $p=0.78$, **Wave V magnitude**, Control vs SD, -0.12 ± 0.23 vs $-0.09 \pm 0.17 \mu V$, $F_{(1,12)}=1.56$, $p=0.24$).

Although there was an interaction between latency changes for 90 dB stimuli and group ($F_{(9,39)}=38.85$, $p<0.001$) this was not evident for any particular ABR wave (Fig.8C,D).

The total ABR signal magnitude (encompassing responses to all stimulus intensities) was similarly affected in control and SD animals, even though total magnitude of wave II tended to be more impaired in control animals (**Wave II total magnitude**, Control vs SD, 87.99 ± 45.62 vs $104.72 \pm 44.34\%$ of BL, $F_{(1,12)}=3.77$, $p=0.08$, Fig.8B). As with click evoked ABRs, this was not evident for total magnitude of the RMS encompassing ABR waves I to V (**RMS total magnitude**, Control vs SD, 86.48 ± 37.7 vs $92.13 \pm 27.82\%$ of BL, $F_{(1,11)}=0.29$, $P=0.6$, Fig.8B).

Therefore, 8 kHz pure tone evoked ABRs tended to show larger NOE-induced impairment in control than in SD animals, although this was no significant effect, similar to the results based on click stimuli. In addition control animals showed pronounced elevation of wave III, reminiscent of hyperexcitability, which was not as pronounced in the SD animals further supporting the notion of sleep deprivation as a mitigating factor for the consequences of NOE.

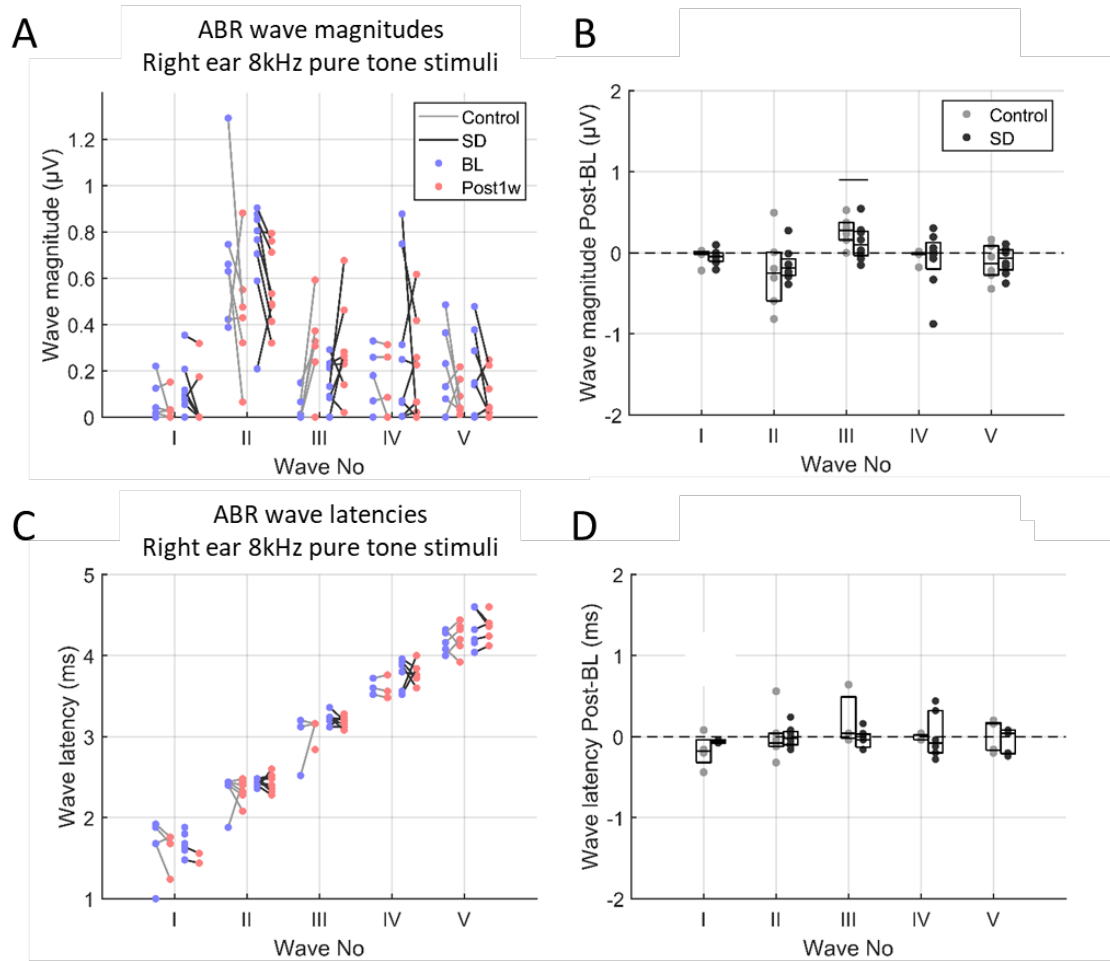


Fig.9: Auditory brainstem responses (ABRs) for 90 dB 8kHz pure tone stimuli: individual waves. (A) Magnitude of individual ABR waves (I-V) before and after NOE for both Control and SD groups (see figure legend). Datapoints are animal averages. Lines connect animal averages obtained during the BL and Post1w assessments. (B) Difference in wave magnitude (Post – BL values) calculated for each animal. Data points represent individual animals. Box plots mark interquartile ranges, horizontal lines within boxplots depict the median. Values below 0 (dotted line) depict a decrease in wave magnitude after NOE relative to BL. (C) Wave latencies, depicted as in A. (D) Wave latency changes from BL to Post1w, depicted as in B.

Discussion

The aim of this experiment was to assess whether sleep after noise overexposure plays a role in the development of tinnitus. To address this, a group of mice ($n=8$) was kept awake for 6 hours immediately after noise exposure. Evidence for tinnitus and hearing loss was assessed and compared to a group of mice ($n=6$) that was left undisturbed after NOE.

Novel object sleep deprivation mitigates effects of noise trauma

After NOE, all animals showed transiently lower gap detection ability, yet a significant impairment was only visible in the control group. This indicates that novel object sleep deprivation after NOE mitigated noise-induced impaired gap detection ability, likely due to reduced hearing loss or tinnitus compared to animals allowed to sleep after NOE. Further to the maintained gap detection ability after NOE, SD animals showed in the long term less habituation within sessions than control animals, suggesting that SD mice remained sensitive to the startle stimulus throughout the assessment. This indicates that SD animals did not only maintain gap detection ability after NOE, but that they may eventually have developed increased sensitivity to startle sounds, decreasing the rate of habituation after repeated exposure within assessments. This could be connected to a latent development of hyperacuity, which is thought to develop over time due to deafferentation after NOE (Kujawa & Liberman, 2009).

To assess whether animals did indeed develop a hearing impairment based on damage to the peripheral auditory pathway, ABRs were acquired for all animals before and after NOE. Here, signs of reduced responses were visible for all animals after NOE, for both

click stimuli and 8 kHz pure tones. However, only control animals showed elevated thresholds and tended to show more pronounced impairment in ABR response magnitudes, to both click stimuli and 8 kHz pure tones. This hearing impairment – with or without tinnitus – could have contributed to the impairment in gap detection performance observed in these animals.

Control animals also showed signs of elevated activity after NOE in ABR wave III (corresponding to activity in the superior olivary nucleus (Henry, 1979)), especially for pure tone stimulation using the centre frequency of the NOE stimulus (8 kHz). The elevation of wave III was significantly more pronounced in the control animals as compared to the SD group, and may indicate increased synchrony or hyperexcitability, which is often described as a functional correlate of noise-induced tinnitus (Bauer et al., 2008; Coomber et al., 2014; Middleton et al., 2011; Noreña & Eggermont, 2006a; Seki & Eggermont, 2003b; Wu et al., 2016). Such activity changes have been described in various stages of the ascending auditory pathway such as in the cochlear nucleus (Brozoski et al., 2002; Kaltenbach et al., 2004; Middleton et al., 2011) and in the inferior colliculus (Bauer et al., 2008; Berger & Coomber, 2015). The superior olivary nucleus has been shown to mediate hyperresponsiveness in tinnitus sufferers (Knudson et al., 2014), suggesting that the elevated ABR wave III in control animals in this study might be connected to tinnitus. Development of hyperexcitability after a noise trauma is usually ascribed to an increase of gain to compensate for loss of afferent input (Auerbach et al., 2014; Sedley, 2019). And while hyperactivity as result of increased gain is considered part of the basis of tinnitus, it also explains why hearing loss and tinnitus are often correlated. In fact, the animals in this experiment showed markedly reduced ABR waves I and II after NOE in response to pure tones in parallel to the increased wave III in control

animals, which is evidence for hearing loss and tinnitus, respectively. ABR magnitude impairment and wave III enhancement was both more pronounced in control as compared to SD animals.

This suggests that novel object sleep deprivation mitigated symptoms of tinnitus and hearing loss, both behaviourally and physiologically, and may therefore have a protective effect against consequences of noise exposure.

Design and characteristics of the sleep deprivation paradigm

To ascertain what aspects of the experimental manipulation may underly the observed group differences, it is important to clarify the rationale behind the timing and design of the used sleep deprivation paradigm.

Acute sleep deprivation rather than chronic sleep restriction was chosen for this experiment as the former is associated with only brief impairments in attention and cognitive abilities, which return to baseline levels after only one night of recovery sleep in human subjects (Philip et al., 2012). Acute sleep deprivation also limits the amount of potentially stressful experience that may be predisposing for tinnitus (Ciminelli et al., 2018b) and therefore minimises confounding effects of the procedure. Further, sleep-dependent plasticity triggered by waking experience is known to already take place in the first night of sleep after learning a task (Gais et al., 2007). Ocular dominance plasticity after monocular occlusion has also been shown to occur already in the first six hours of the subsequent sleep period (Clawson et al., 2018; Frank et al., 2001) suggesting that sleep-dependent plasticity can occur or start within a short timeframe after waking experience and is sensitive to acute sleep deprivation within that time. Sleep-dependent

plasticity involved in tinnitus development might therefore also be affected by acute sleep deprivation soon after noise trauma.

Using novel objects to keep animals engaged and awake was chosen as this method is following an established protocol (e.g. Huber et al., 2000; McKillop et al., 2018) that is making use of the animal's tendency to explore changes in its environment when novel objects are introduced into the cage instead of keeping the animal wake by more intrusive means such as handling. This is thought to minimise procedure related stress. Importantly, any stress that the sleep deprivation paradigm might have induced is unlikely to explain the effects of SD in this study, as stress is considered a predisposing factor for tinnitus development (Ciminelli et al., 2018b).

That said, novel object sleep deprivation introduces other additional variables next to the mere extension of time spent awake. In particular, the animal is engaging in exploratory behaviour and may therefore experiences and acoustic environment not present for the control mice. This may be of relevance in this study, as certain acoustic stimulation after noise overexposure can mitigate tinnitus or hearing loss (Noreña & Eggermont, 2005, 2006a). Importantly, care has been taken to minimise environmental noise related to the SD paradigm: control and SD mice were kept in the same room to keep equipment noise constant between groups. The SD paradigm involved only the placement and removal of small objects (such as wooden cubes or sticks) into or from the animal's cage by a single experimenter. No acoustic stimulation was used to keep animals awake. Cages had an open top, allowing for addition or removal of objects without opening a lid or enclosure. Nevertheless, the animals' explorative behaviour, which occasionally involved the manipulation of novel objects by the mouse, may have

provided a level of acoustic enrichment not experienced by the control animals in the first 6 hours after NOE. However, in previous studies where acoustic enrichment reduced noise trauma (Noreña & Eggermont, 2005, 2006a) animals were exposed to the enriched acoustic environment for several weeks after NOE. Further, the acoustic enrichment consisted of multitone complex stimuli spectrally matched to the expected range of hearing loss. Since any acoustic enrichment the novel object exploration might have provided hardly meets these criteria it is unlikely that it played a substantial role in mitigating the effects of noise exposure.

Therefore, the amount of sleep after NOE is likely the most relevant difference between control and SD animals. During sleep deprivation, animals in the SD group and their EEG and EMG signals were constantly observed for signs of drowsiness. Novel objects effectively engaged animals throughout the 6-hour period. In the control group, animals were left undisturbed and the timing of NOE during the last two hours of the animals' active period (dark phase) within the 24h cycle (ZT 22-24) meant that the time immediately afterwards covered the period where sleep propensity was highest in all mice, ensuring a maximal difference in sleep amount between the two experimental groups. Subsequent sampling of video and EEG recordings corroborated that control animals slept during this period as expected.

Multiple attributes of sleep may alleviate consequences of noise trauma

The fact that 6 hours of acute sleep deprivation after NOE were associated with mitigated impairment of hearing ability and tinnitus symptoms suggests that sleep may mediate or amplify such impairments.

For example, it may be that sensory disconnection during sleep (Neckelmann & Ursin, 1993) plays a role in shielding the animal from an acoustic environment that could otherwise mitigate development of hearing impairment. It has previously been shown that acoustic enrichment after noise trauma mitigates symptoms of hearing loss and tinnitus (Noreña & Eggermont, 2005, 2006a). It is of note, however, that auditory evoked activity is largely maintained during sleep and there are indications that sensory disconnection takes place only on cortical level (Nir et al., 2015). Thus, sleep does not fully prevent the acoustic environment of the animal to still shape brain activity, even though it may be different to the waking state (Rosanova et al., 2018). It remains to be investigated in how far sensory disconnection during sleep may affect the recovery from noise damage.

Moreover, as described in the introduction of this chapter, sleep is involved in mediating and consolidating a range of plastic processes that may be involved in tinnitus development (Clawson et al., 2018; Frank et al., 2001; Gais et al., 2007; Káli & Dayan, 2004; O'Neill et al., 2010; Takashima et al., 2009), such as cortical map plasticity (Aton et al., 2009; Frank et al., 2001) and systems level plasticity (Gais et al., 2007). For example, it is possible that sleep mediates the reorganisation of tonotopic maps after a lasting change in cochlear input, such as through noise-induced damage to the cochlea or loss of auditory nerve fibres. Tonotopic map plasticity is thought to underly tinnitus development (Eggermont & Roberts, 2004b; Engineer et al., 2011) and to be associated with hearing loss (Koops et al., 2020). But also changes in non-auditory brain regions, possibly mediated by sleep-dependent systems level plasticity, are thought to underly the establishment of a brain-wide and limbic representation of tinnitus (Leaver et al., 2011; Rauschecker et al., 2010b). Yet the scope of sleep-dependent plasticity that may

underly the formation of the circuitry ultimately responsible for persistent tinnitus does likely go beyond these examples. For instance, the scaling of synapses (de Vivo et al., 2017) or spatially restricted synapse plasticity (Bellesi & de Vivo, 2020) during sleep might add to the number of processes involved in tinnitus formation.

While future studies will be needed to ascertain which aspect of sleep can affect the formation of noise-induced hearing impairments or tinnitus, the results of this study suggest that natural brain state dynamics provide fruitful ground for investigating and treating the consequences of noise overexposure.

Supplementary tables

		CONDITION			
		1 TIME		2 TIME	
		1 WaveMag Mean	2 WaveMag Mean	1 WaveMag Mean	2 WaveMag Mean
WaveID 1	AnimalID	1	.3927813000	.3738780000	.
		2	.6795055900	.4044358000	.
		3	.3018103000	.1585261000	.
		4	.8580529000	.3799220000	.
		5	.3997880000	.0718769000	.
		6	.4193642000	.4577056000	.
		7	.	.	.5017410000
		8	.	.	.3532300000
		9	.	.	.3709630000
		10	.	.	.7614991000
		11	.	.	.2565746000
		12	.	.	.5507595000
		13	.	.	.7685677000
		14	.	.	.4828520000
2	AnimalID	1	2.691970000	3.048190000	.
		2	2.081623000	1.598522000	.
		3	1.119458000	1.843030000	.
		4	3.696030000	1.760351000	.
		5	2.295490000	1.175480000	.
		6	2.514130000	1.624728000	.
		7	.	.	1.229374000
		8	.	.	2.935000000
		9	.	.	1.525330000
		10	.	.	3.087740000
		11	.	.	1.954114000
		12	.	.	3.771340000
		13	.	.	4.445330000
		14	.	.	2.563560000
3	AnimalID	1	.2495210000	.3058238000	.
		2	.2915630000	1.366411000	.
		3	.0164210000	.0706460000	.
		4	.2355300000	.0687230000	.
		5	.3379853000	1.110424000	.
		6	.4274260000	.5013110000	.
		7	.	.	.0700690000
		8	.	.	.4729429000
		9	.	.	1.411445000
		10	.	.	.0429236000
		11	.	.	1.127006000
		12	.	.	1.002485000
		13	.	.	.0000000000
		14	.	.	2.045254000
4	AnimalID	1	1.155475000	.3665860000	.
		2	.8802500000	.6174520000	.
		3	.1578228000	.7162150000	.
		4	1.095428000	.8318190000	.
		5	.6329210000	.0000000000	.
		6	1.336031000	.3171780000	.
		7	.	.	.3668670000
		8	.	.	1.218010000
		9	.	.	.0000000000
		10	.	.	.3869404000
		11	.	.	.2176440000
		12	.	.	.0000000000
		13	.	.	2.241891000
		14	.	.	.0000000000
5	AnimalID	1	.0254970000	.2371281000	.
		2	.1045960000	.0000000000	.
		3	.1012428000	.2377705000	.
		4	.9123850000	.0000000000	.
		5	.1395907000	.0006570000	.
		6	.2022557000	.0000000000	.
		7	.	.	.5157286000
		8	.	.	.0778550000
		9	.	.	.1202640000
		10	.	.	.5993980000
		11	.	.	.3636536000
		12	.	.	.8066080000
		13	.	.	.6629390000
		14	.	.	.6479370000

Table 1: ABR wave magnitudes (in μ V) before and after NOE for click stimuli, in Control (labelled as condition 1) and SD groups (labelled as condition 2).

			CONDITION				
			1		2		
			TIME		TIME		
			1	2	1	2	
WaveID	1	AnimalID	WaveLat Mean	WaveLat Mean	WaveLat Mean	WaveLat Mean	
	1	AnimalID	1	1.44	1.48	.	.
		2	1.36	1.36	.	.	
		3	1.44	1.52	.	.	
		4	1.44	1.36	.	.	
		5	1.48	1.44	.	.	
		6	1.48	1.44	.	.	
		7	.	.	1.36	1.32	
		8	.	.	1.36	1.40	
		9	.	.	1.68	1.36	
		10	.	.	1.32	1.40	
		11	.	.	1.52	1.36	
		12	.	.	1.40	1.40	
		13	.	.	1.40	1.44	
		14	.	.	1.44	1.40	
	2	AnimalID	1	2.16	2.20	.	.
		2	2.20	2.12	.	.	
		3	2.12	2.12	.	.	
		4	2.08	2.08	.	.	
		5	2.12	1.92	.	.	
		6	2.16	2.08	.	.	
		7	.	.	2.24	2.12	
		8	.	.	2.08	2.12	
		9	.	.	2.52	1.92	
		10	.	.	2.08	2.16	
		11	.	.	2.04	2.08	
		12	.	.	2.12	2.12	
		13	.	.	2.12	2.08	
		14	.	.	1.92	2.00	
	3	AnimalID	1	2.88	2.88	.	.
		2	2.76	2.80	.	.	
		3	2.84	2.92	.	.	
		4	2.64	2.84	.	.	
		5	3.00	2.84	.	.	
		6	2.76	2.80	.	.	
		7	.	.	2.68	2.88	
		8	.	.	2.80	2.76	
		9	.	.	3.28	2.72	
		10	.	.	2.88	3.04	
		11	.	.	2.68	2.76	
		12	.	.	3.04	.	
		13	.	.	.	2.92	
		14	.	.	2.84	2.72	
	4	AnimalID	1	3.40	3.40	.	.
		2	3.32	3.56	.	.	
		3	3.20	3.44	.	.	
		4	3.28	3.24	.	.	
		5	3.48	.	.	.	
		6	3.28	3.28	.	.	
		7	.	.	3.20	.	
		8	.	.	3.36	3.40	
		9	.	.	.	3.36	
		10	.	.	3.20	.	
		11	.	.	3.20	3.24	
		12	.	.	.	.	
		13	.	.	3.32	3.36	
		14	.	.	.	3.28	
	5	AnimalID	1	4.16	4.08	.	.
		2	4.08	.	.	.	
		3	3.92	4.28	.	.	
		4	3.88	.	.	.	
		5	4.20	4.12	.	.	
		6	4.08	.	.	.	
		7	.	.	3.80	3.76	
		8	.	.	4.00	3.72	
		9	.	.	4.36	.	
		10	.	.	3.72	3.72	
		11	.	.	3.60	3.80	
		12	.	.	3.76	3.72	
		13	.	.	3.88	4.08	
		14	.	.	3.52	3.92	

Table 2: ABR wave latencies (in ms) before and after NOE for click stimuli, in Control (labelled as condition 1) and SD groups (labelled as condition 2).

			CONDITION				
			1 TIME		2 TIME		
			1 WaveMag Mean	2 WaveMag Mean	1 WaveMag Mean	2 WaveMag Mean	
WaveID 1	1	AnimalID	1	.1245322000	.1512705000	.	.
			2	.0180870000	.0000000000	.	.
			3	.0180208000	.0323620000	.	.
			4	.0000000000	.0000000000	.	.
			5	.2201127000	.0014129000	.	.
			6	.0407720000	.0264673700	.	.
			7	.	.	.0000000000	.0000000000
			8	.	.	.1174391000	.0000000000
			9	.	.	.0000000000	.0000000000
			10	.	.	.3534714000	.3183496100
			11	.	.	.0545868200	.0000000000
			12	.	.	.0961858000	.0000000000
			13	.	.	.0764198000	.1739525000
			14	.	.	.2073110000	.0000000000
2	AnimalID	1	.7458700000	.5501680000	.	.	
		2	.4221570000	.4291460000	.	.	
		3	.6600970000	.0651338460	.	.	
		4	1.291207000	.4747956000	.	.	
		5	.6294010000	.3210530000	.	.	
		6	.3876050000	.8814590000	.	.	
		7	.	.	.5881910000	.3200950000	
		8	.	.	.8774320000	.4889130000	
		9	.	.	.2082184000	.4823500000	
		10	.	.	.7053580000	.4130120000	
		11	.	.	.7659060000	.5328850000	
		12	.	.	.8532800000	.7121230000	
		13	.	.	.9037750000	.7600600000	
		14	.	.	.8043360000	.7936160000	
3	AnimalID	1	.0000000000	.0000000000	.	.	
		2	.0122010000	.3273965000	.	.	
		3	.0000000000	.2385320000	.	.	
		4	.0000000000	.3723630000	.	.	
		5	.1484132000	.3069270000	.	.	
		6	.0658308000	.5920310000	.	.	
		7	.	.	.0907964000	.0200266000	
		8	.	.	.0839670000	.2450858000	
		9	.	.	.2121630000	.4622630000	
		10	.	.	.2272908000	.2295683000	
		11	.	.	.0000000000	.2815023000	
		12	.	.	.2315261000	.2646659000	
		13	.	.	.1327423000	.6763040000	
		14	.	.	.2912091000	.1394940000	
4	AnimalID	1	.3288980000	.3127755000	.	.	
		2	.0000000000	.0000000000	.	.	
		3	.2585175000	.2592036000	.	.	
		4	.1795670000	.0000000000	.	.	
		5	.0701740000	.0855418000	.	.	
		6	.0000000000	.0000000000	.	.	
		7	.	.	.0037030000	.0212722000	
		8	.	.	.3131270000	.6165770000	
		9	.	.	.0623396000	.2575481000	
		10	.	.	.2491505000	.2254208000	
		11	.	.	.8775700000	.0000000000	
		12	.	.	.0042486000	.0652469000	
		13	.	.	.0702118000	.0000000000	
		14	.	.	.7478850000	.4169927000	
5	AnimalID	1	.1315318000	.2173769000	.	.	
		2	.0000000000	.1634042000	.	.	
		3	.0789267400	.0314351000	.	.	
		4	.2309940000	.0108030000	.	.	
		5	.3640120000	.0903225000	.	.	
		6	.4850070000	.0405910000	.	.	
		7	.	.	.1391714000	.2472768000	
		8	.	.	.0000000000	.0436172100	
		9	.	.	.1488439000	.0186673000	
		10	.	.	.3770740000	.0000000000	
		11	.	.	.0000000000	.0000000000	
		12	.	.	.2864202000	.1218374000	
		13	.	.	.4779695000	.2236486000	
		14	.	.	.0059240000	.0371550000	

Table 3: ABR wave magnitudes (in μV) before and after NOE for NBN 8kHz stimuli, in Control (labelled as condition 1) and SD groups (labelled as condition 2).

			CONDITION			
			1		2	
			TIME		TIME	
			1	2	1	2
			WaveLat	WaveLat	WaveLat	WaveLat
			Mean	Mean	Mean	Mean
WaveID 1	AnimalID	1	1.88	1.68	.	.
		2	1.00	.	.	.
		3	1.68	1.24	.	.
		4	.	.	.	.
		5	1.68	1.76	.	.
		6	1.92	1.76	.	.
		7	.	.	.	.
		8	.	.	1.68	.
		9	.	.	.	.
		10	.	.	1.48	1.44
		11	.	.	1.88	.
		12	.	.	1.80	.
		13	.	.	1.64	1.56
		14	.	.	1.60	.
2	AnimalID	1	2.44	2.48	.	.
		2	1.88	2.44	.	.
		3	2.40	2.08	.	.
		4	2.44	2.40	.	.
		5	2.40	2.28	.	.
		6	2.44	2.32	.	.
		7	.	.	2.44	2.40
		8	.	.	2.36	2.60
		9	.	.	2.44	2.36
		10	.	.	2.44	2.52
		11	.	.	2.48	2.52
		12	.	.	2.48	2.48
		13	.	.	2.48	2.32
		14	.	.	2.40	2.28
3	AnimalID	1	.	.	.	.
		2	2.52	3.16	.	.
		3	.	2.84	.	.
		4	.	3.16	.	.
		5	3.12	3.16	.	.
		6	3.20	3.16	.	.
		7	.	.	3.36	3.20
		8	.	.	3.24	3.20
		9	.	.	3.20	3.16
		10	.	.	3.12	3.28
		11	.	.	.	3.16
		12	.	.	3.12	3.12
		13	.	.	3.20	3.24
		14	.	.	3.24	3.08
4	AnimalID	1	3.60	3.56	.	.
		2	.	.	.	.
		3	3.52	3.48	.	.
		4	3.52	.	.	.
		5	3.72	3.76	.	.
		6	.	.	.	.
		7	.	.	3.88	3.60
		8	.	.	3.80	3.76
		9	.	.	3.96	3.84
		10	.	.	3.56	4.00
		11	.	.	3.92	.
		12	.	.	3.52	3.84
		13	.	.	3.56	.
		14	.	.	3.92	3.72
5	AnimalID	1	4.32	4.12	.	.
		2	.	4.36	.	.
		3	4.00	4.20	.	.
		4	4.28	4.44	.	.
		5	4.16	4.32	.	.
		6	4.08	3.92	.	.
		7	.	.	4.20	4.24
		8	.	.	.	4.60
		9	.	.	4.60	4.36
		10	.	.	4.16	.
		11	.	.	.	.
		12	.	.	4.04	4.12
		13	.	.	4.32	4.40
		14	.	.	4.60	4.44

Table 4: ABR wave latencies (in ms) before and after NOE for NBN 8kHz stimuli, in Control (labelled as condition 1) and SD groups (labelled as condition 2).

Discussion

Tinnitus is a phantom percept and inherently hard to grasp through objective means. Its impact on tinnitus sufferers, however, is more than only tangible. It is devastating and can have serious consequences for their mental wellbeing: tinnitus is associated with depression, anxiety (Bhatt et al., 2017; Salazar et al., 2019) and even with suicidal ideation (Aazh & Moore, 2018; Szibor et al., 2019). Yet the potentially most immediate player mediating the wearing distress in tinnitus has so far been little acknowledged: disrupted sleep and changed sleep architecture have long been known to be associated with tinnitus (Attanasio et al., 2013; Burgos et al., 2005; Folmer & Griest, 2000; Hallam, 1996; Hébert et al., 2011c; Izuhara et al., 2013a; Koning, 2019a; Schecklmann et al., 2015) and may be central in mediating or facilitating some of its comorbidities. A causal link between tinnitus and changes in sleep is yet to be identified, even though it is known that the lack of sleep can facilitate stress (Nollet et al., 2020) and may promote depression (Rahmani et al., 2020), which are common comorbidities of tinnitus. Further, it has been shown that tinnitus is likely linked to increased spontaneous neural activity, as well as measures of hyperexcitability and increased local neural synchrony (Bauer et al., 2008; Berger & Coomber, 2015; Brozoski et al., 2002; Engineer et al., 2011; Kalappa et al., 2014; Kaltenbach et al., 2004; Manzoor et al., 2013; Noreña & Eggermont, 2003b; Seki & Eggermont, 2003b; Wu et al., 2016). It has long been acknowledged that such aberrant brain activity can interfere with natural brain-state dynamics, such as maintaining a global sleep state (Milinski et al., 2022; Vyazovskiy & Harris, 2013). Still, it remains unclear whether tinnitus leads to disturbed sleep or whether, as it has

previously been proposed sleep problems and tinnitus are just correlated by sharing a common neural substrate (Wallhäusser-Franke et al., 2013).

As outlined in the Introduction chapter (see also Chapters 3 and 4) we are proposing that altered sleep in tinnitus is – at least in part – due to the interference of tinnitus related aberrant brain activity with natural sleep dynamics. We propose, in turn, that the spatial and temporal overlap between functional changes in brain activity during sleep and tinnitus-related aberrant activity implies an important and yet underappreciated role of sleep in influencing such aberrant activity. We posit that due to such a bi-directional interaction, sleep may mitigate tinnitus-related neural activity when the physiological drive to enter and maintain a global sleep state is high. Conversely, when sleep pressure is low tinnitus-related activity may disrupt sleep.

The first experimental step was to assess when sleep changes emerge relative to the first symptoms of tinnitus after a triggering event. In human subjects the temporal relations between tinnitus trigger, the emergence of tinnitus and the onset of sleep disruptions are often unclear. Animal models of induced tinnitus allow to circumvent this problem by controlling the onset of tinnitus and the diagnostic regime. Still, animal models of tinnitus are commonly limited by the difficulty of objectively diagnosing a phantom percept (Brozoski & Bauer, 2016; McFerran et al., 2019; J. G. Turner et al., 2006) and of conducting longitudinal studies.

In the first part of this project, we aimed to characterise a model to investigate the long-term effects of induced tinnitus on behaviour and brain activity across sleep and wakefulness. Therefore, we needed an animal model allowing for the precise control of

the tinnitus trigger but also for reliable tinnitus identification and assessments over an extended period.

To allow for longitudinal assessment, we chose the ferret as an animal model and jointly used evoked auditory brainstem recordings and a battery of behavioural assessments to minimize the limitations of each individual paradigm (Chapters 1,2). Different to the mouse, which is a standard model for studying noise and drug induced tinnitus, ferrets can learn more sophisticated operant tasks, which widens the scope for behavioural tinnitus assessment. Furthermore, the lifespan of ferrets (6-8 years, Fox & Marini, 2014) as well as the age of senescence (Bakthavatchalu et al., 2016) considerably surpasses that of mice (1-2 years, McKillop et al., 2018; Yanai & Endo, 2021) and rats (2.5-3.5 years, Sengupta, 2013). Their longer lifespan allows for longitudinal assessment without age-dependent degeneration, which is essential for investigating the time course of persistent tinnitus and its comorbidities.

Using this model, we demonstrated that noise overexposure is associated with evidence of hearing impairment and, especially in the long term, with changes in behavioural performance indicative of tinnitus (Chapter 2). Further, we show that symptoms for tinnitus and hearing impairment after noise overexposure (NOE) were highly idiosyncratic (Chapter 3), in line with variable effects of noise trauma seen in rodent models (Shore et al., 2016) and the tinnitus heterogeneity in human patients (Beukes et al., 2021). This shows that the ferret could provide a powerful model to study persistent tinnitus on a case-by-case basis and help assess aspects of tinnitus that were so far separated by the constraints of human and animal experiments.

In the second part of this study, we found that evidence for tinnitus was associated with increased cortical excitability and reduced and disrupted sleep. On the other hand, strong hearing impairment was associated with prolonged and lighter sleep (Chapter 3). In line with the well-described association between tinnitus and altered or disrupted sleep in humans (Attanasio et al., 2013; Burgos et al., 2005; Folmer & Griest, 2000; Hallam, 1996; Hébert et al., 2011c; Izuhara et al., 2013a; Koning, 2019a; Schecklmann et al., 2015) these results suggest that the emergence of tinnitus after noise trauma is closely linked with sleep impairments. While not proving a causal relationship between tinnitus related aberrant brain activity and disturbed sleep, the results of this study are highly suggestive of such a relationship. Indeed, the elevated evoked cortical activity identified in animals with signs of tinnitus (Chapter 4) corroborates this possibility. Further support for this notion may be offered by assessing local brain activity in regions that are sensitive to shifts in vigilance state but specifically involved in tinnitus, such as auditory cortex (Engineer et al., 2011; Leaver et al., 2011; R. Salvi et al., 2000). As part of this study, terminal recordings with high density multichannel electrodes (Neuropixels) in auditory cortex were conducted in two of the chronically implanted animals but could not yet be analysed. Local spontaneous and evoked activity may provide further evidence for the relationship between tinnitus-related brain activity and sleep in these animals. In addition, future work entailing putative manipulations to alleviate tinnitus, e.g. multimodal (Conlon et al., 2022; Marks et al., 2018) or vagus nerve stimulation (Engineer et al., 2011) before, during or after sleep may uncover the causality in this relationship. This will help answer the question on whether altered sleep may indeed be a mediator for tinnitus comorbidities, such as depression and anxiety (Bhatt et al., 2017), which have previously been related primarily to the tinnitus itself.

It is possible that animals displaying signs of tinnitus in this study also developed other noise-induced conditions, such as elevated sensitivity to environmental sounds, hyperacusis (Liberman & Kujawa, 2017; S. E. Shore et al., 2016b). While animals showed behavioural deficits that cannot be fully explained by hyperacusis but are indicative of tinnitus, such as impaired gap detection ability, it cannot be ruled out that hyperacusis formed as an additional consequence of noise overexposure. It is, similar to tinnitus, thought to be related to a lack in afferent signals after peripheral damage and to a compensatory increase in neuronal gain to maintain firing homeostasis in the ascending auditory pathway (Auerbach et al., 2014; Gold & Bajo, 2014). It is possible that hyperacusis and tinnitus are complementary factors in mediating sleep impairments, with sleep disruption mediated by both persistent tinnitus related brain activity and increased perturbation of sleep by auditory evoked activity. Future work may address the relationship between specific symptoms after noise exposure in mediating sleep regulation and maintenance.

The results of this study further indicate a role of sleep in the transient mitigation of tinnitus. Specifically, increased evoked activity after noise overexposure that may be part of the functional basis of tinnitus (Engineer et al., 2011) was less pronounced during sleep (Chapter 4). Therefore, naturally occurring brain states that are known to interfere with sensory signal processing (Neckelmann & Ursin, 1993) may also mitigate aberrant changes in excitability after noise insult. As described in Chapter 4, this may be due to sleep of increased intensity, such as increased slow-wave activity as a consequence of persistent tinnitus-related activity during wakefulness (e.g. Bauer et al., 2008; Brozoski et al., 2002; Manzoor et al., 2013; Middleton et al., 2011). It remains to be shown whether sleep also interferes with aberrant spontaneous activity in tinnitus and can

possibly mitigate it. After progress in identifying functional alterations in awake or anaesthetized animal models of tinnitus (Bauer et al., 2008; Berger & Coomber, 2015; Brozoski et al., 2002; Engineer et al., 2011; Kalappa et al., 2014; Kaltenbach et al., 2004; Manzoor et al., 2013; Noreña & Eggermont, 2003b; Seki & Eggermont, 2003b; Wu et al., 2016) it is now possible to track such putative objective tinnitus markers, for example via high resolution recordings in regions of interest, e.g. auditory cortex (Engineer et al., 2011; Noreña & Eggermont, 2006c). Extension of such recordings across sleep and wake states will allow to identify the distinct role of natural brain state dynamics in the modulation of tinnitus markers, especially if sleep is manipulated in parallel, such as through artificial enhancement of slow waves (Ngo et al., 2013; Yamagata et al., 2021) or homeostatic increase of sleep pressure after periods of extended wakefulness (Huber et al., 2000b; Tobler & Borbély, 1986). It is possible that approaches such as these could pose as blueprints for future options of clinical tinnitus mitigation.

It is important to acknowledge that the association between sleep and tinnitus symptoms identified in this study is based on case studies in individual animals. Previous studies have reported various outcomes after noise trauma, some animals showed hearing loss, some developed tinnitus, others had a combination of both conditions, and some even had no impairment (Shore et al., 2016). Human tinnitus sufferers also show a high diversity of symptoms, with varying degrees of tinnitus severity and associated comorbidities (Beukes et al., 2021) which led to a recent proposal for strictly distinguishing between cases of tinnitus associated with and without additional suffering (De Ridder, Schlee, et al., 2021). Moreover, tinnitus is known to be a very heterogenous condition (Jarach et al., 2022) with a multicausality origin including genetic predisposition. Such diversity among tinnitus cases has been raised as a

fundamental challenge to experimental design and cause for variability in results in the tinnitus field (McFerran et al., 2019).

In this study, where an objective was to assess the tinnitus development alongside changes in sleep, it was paramount to identify each variant of impairment. Indeed, animals in this experiment each presented a unique combination of symptoms after noise overexposure. Only one animal developed signs of pronounced hearing loss with little or no accompanying tinnitus, and improved sleep maintenance after NOE, opposite to all other animals, who developed clearer signs of tinnitus. Among those animals, the time course of sleep disturbance differed throughout the experiment but was associated with the onset and development of tinnitus and hearing impairment in each case. To identify these specific cases of tinnitus and/or hearing loss and related changes in sleep, brain activity and tinnitus development after noise trauma, a detailed assessment of tinnitus and hearing impairment in each individual was paramount. The thorough completion of a complex set of paradigms for each animal throughout a long period of assessment was prioritised over a more temporally limited and less elaborate testing of a larger number of animals.

Finally, we addressed the possible role of sleep in tinnitus emergence and persistence. It is known that tinnitus can be transient but also last for months or years (Andersson et al., 2001) with hardly any spontaneous remission (Simões et al., 2021). Yet the mechanism of tinnitus development over time remains unclear. As outlined in Chapter 5, sleep-dependent synaptic plasticity may be involved in mediating or consolidating tinnitus. We, therefore, predicted that the amount of sleep immediately after noise trauma might affect tinnitus development. To address this, we sleep deprived mice after

noise overexposure and assessed signs of tinnitus and hearing loss. The well-established sleep deprivation paradigms added little additional stress (Huber et al., 2000; McKillop et al., 2018), and the entirety of the assessment was completed in a relatively short time, approximately 10 weeks per experimental group. Unlike the longitudinal assessment in ferrets, this allowed us to include two experimental groups and compare the effect of sleep deprivation to a control condition, where mice could sleep *ad libitum* after noise trauma. We found that sleep deprivation mitigated behavioural evidence for tinnitus and signs of hearing loss (Chapter 5).

While it will need to be addressed which aspects of sleep may be involved in mediating the consequences of noise trauma, future work may also start to explore the long-term effects of sleep after a tinnitus trigger. It is possible, for example, that delayed degeneration of the cochlear nerve after noise trauma (Kujawa & Liberman, 2009) results in hidden hearing loss or tinnitus long after the initial trigger. There is also increasing evidence that neuroinflammation plays a role in the development of tinnitus (Adcock & Vanneste, 2022). It remains open, how acute sleep deprivation influences such long-term effects or whether sleep would also need to be manipulated on a longer timescale.

An obvious way to reduce sleep-dependent plasticity further would be to use repeated instances of sleep deprivation. Yet, a careful balance must be struck between putative positive effects of such approaches on tinnitus emergence and indirect negative effects mediated by cognitive impairments (Philip et al., 2012) and raised stress levels (Nollet et al., 2020) associated with repeated sleep deprivation. These adverse effects could predispose to develop tinnitus (Ciminelli et al., 2018b) and be detrimental to the

subjects wellbeing. That said, schedules of restricted sleep are an established treatment for insomnia patients and have been shown to benefit patients by improving insomnia severity and sleep continuity (Maurer et al., 2021). Similar paradigms could be explored to be tested in the context of tinnitus development.

As an alternative to repeated sleep deprivation or restriction and its detrimental side effects is to target the modulation of sleep-dependent plasticity more directly. For example, previous studies indicate that enhancement of NREM slow-wave activity can affect experience-dependent brain plasticity (Bendor & Wilson, 2012; Ngo et al., 2013). Similar methods may be explored to manipulate tinnitus-specific plasticity during sleep and potentially shape long-term health outcomes.

When developing such protocols, be it behavioural sleep manipulation or artificial manipulation of activity or plasticity during sleep, it should be taken into account that the effect of sleep on mediating consequences of noise trauma may be time-dependent relative to the tinnitus trigger. It is believed that consolidation of structural and functional changes in the brain after a triggering event eventually produces persistent tinnitus (McFerran et al., 2019). The exact time course of that plasticity is unclear. However, tinnitus is associated with long-term brain-wide structural and functional changes (Elgoyhen et al., 2015a), especially in the limbic system (Leaver et al., 2011; Rauschecker et al., 2010a). While experience-dependent brain plasticity can be observed already after one sleep period (Clawson et al., 2018; Frank et al., 2001), it seems unlikely that the global changes seen in long tinnitus sufferers take place exclusively during the early days after the tinnitus trigger. In fact, tinnitus can develop with a delay relative to noise trauma (J. Turner et al., 2012) and, after its onset,

comorbidities tend to improve over time with some sufferers (less than 1%) experiencing complete remission of their tinnitus (Simões et al., 2021), although this may depend on a variety of factors, including genetic predisposition (Lopez-Escamez & Amanat, 2020). Nevertheless, this suggests an ongoing change in the underlying structural or functional networks after initial tinnitus emergence. Thus, the potential to affect tinnitus progression may be present over a long period after a noise trauma. Ultimately, sleep manipulation on multiple time scales may offer a way to harness this potential, possibly by hindering the emergence of tinnitus or limiting its progression at later stages.

Conclusion

The experiments in this thesis provide evidence for a bi-directional interaction between tinnitus and natural brain state dynamics. This highlights a new angle in tinnitus research, which could prove fruitful in explaining tinnitus comorbidities and offer opportunities for new experimental approaches. Future work may even foster tinnitus as a model to study sensory disconnection during sleep and bring new insights into local and global sleep regulation. Yet, most importantly, this project shows that sleep has the potential to help explain and, ultimately, treat the evasive condition that is tinnitus.

Methods

All procedures were in accordance with the Animal (Scientific Procedures) Act 1986 (amended in 2012) and performed under a UK Home Office Project Licence in accordance with institutional guidelines. Ferret and mouse electrophysiological and behavioural experiments were approved by the Committee on Animal Care and Ethical Review of the University of Oxford.

Animal husbandry

Ferrets: 8 adult female pigmented ferrets (*Mustela putorius furo*) were housed in small groups of at least 3 animals in standard laboratory enclosures or large pens housing up to 10 animals. Food and water were available *ad libitum* except during periods of operant behavioural testing, where water access was temporarily regulated. Periods of water regulation lasted for a maximum of 5 consecutive days before at least two days of *ad libitum* access to water. While under water regulation, animals received performance-dependent amounts of water during the behavioural task, topped up to an amount of 60 ml/kg BW per day with water provided subsequently so animals maintained a bodyweight within 15% of their free-feeding weight. Animals were housed under a 15/9h light-dark cycle from mid-March until mid-November (summer light cycle) and otherwise under an 8/16 light-dark cycle (winter light cycle) to mimic natural seasonal changes in light exposure. To suppress the oestrus ferrets were routinely

injected with Delvosteron (MSD Animal health, Proligestone 100mg/ml) 1 month before the change towards the summer light cycle.

Behavioural experiments were conducted predominantly during the light phase, and chronic EEG recordings took place only during the summer light cycle. During times of continuous EEG recordings (lasting 2-3 days), animals were single housed in a custom-made enclosures with *ad libitum* access to water and food. Equivalent bedding and nesting material to the home cage was provided, and lighting and temperature conditions to their habitual housing were maintained. Before the first chronic recording, ferrets were progressively habituated to the recording chamber and to the tethering between recoding setup and the cranial implant. Recording enclosures were cleaned after each recording session.

Mice: 14 male adult C57/BL6ntac-cdh23h-9 mice were single housed with water and food available *ad libitum* for the duration of the experiment. Bedding and nesting material were provided. For the duration of chronic EEG recordings and habituation to the recording cages (a minimum of 6 days in total) mice were single housed in custom-made clear Plexiglas cages (20.3 × 32 × 35 cm). Cages were housed in ventilated, sound-attenuated Faraday chambers (Campden Instruments, Loughborough, UK, two cages per chamber) under a standard 12/12h light-dark cycle. Mice were habituated to both the recording cage and to the tethering between recoding setup and the cranial implant for a minimum of two days prior to the start of chronic recordings. Whenever mice were transferred into new home/recording cages throughout the experiment, the nest or nesting material and portions of the bedding were transferred as well to maintain environmental context as much as possible and reduce procedure-related distress.

Surgery

Ferrets: The preparation of the surgical procedure largely followed the methodology described by Homma and colleagues (Homma et al., 2017) using aseptic techniques. Anaesthesia was always induced with a single intramuscular injection of medetomidine hydrochloride (0.022 mg/kg body weight; Domitor, Orion Pharma) and ketamine hydrochloride (5 mg/kg; Narketan10, Vetoquinol). Maintenance of anesthesia followed two protocols depending if was a recovery experiment (e.g. implantation of electrodes) or a terminal experiment (e.g. terminal electrophysiological recordings). For terminal procedures a stable level of anaesthesia and hydration was maintained through an intravenous infusion (3–4 ml/h) of medetomidine hydrochloride (0.022 mg/kg/h) and ketamine hydrochloride (5 mg/kg/h) in 0.9% saline solution supplemented with 5% glucose. For recovery experiments, anaesthesia was maintained using Isoflurane (1–3%) (IsoFlo, Abbot Laboratories) using 100% Oxygen as a carrier.

Atropine sulfate (0.06 mg/kg, s.c.; Atrocare, Animalcare) was administered to minimize pulmonary secretions and dexamethasone (0.5 mg/kg, s.c.; Dexadreson, Intervet UK) to prevent cerebral edema. Doxapram hydrochloride (4 mg/kg, s.c.; Dopram-V Injection, Pfizer) was administered to maintain respiratory rate. Perioperative analgesia was provided with buprenorphine hydrochloride (0.01 mg/kg, s.c.; Vetergesic, Sogeval UK) and meloxicam (0.2 mg/kg, s.c.; Metacam, Boehringer Ingelheim). Antibiotics to prevent infections were used during the surgery (Augmentin 0.02 mg/Kg i.v. every 2 hours;

Bechaam) and daily 5 days after the surgery (Xynulox 0.1 mg/Kc i.m.). Depth of anaesthesia, respiratory rate, EKG, and end-tidal CO₂ were monitored and maintained throughout the experiment. The animal's temperature was monitored using a rectal probe and maintained at 38°C using a homeothermic electrical blanket (Harvard Apparatus), and a forced-air warming system (Bair Hugger, 3M Health Care). The ferret was placed in a stereotaxic frame, the eyes were protected with a carbomer liquid eye gel (Viscotears, Alcon Laboratories), the skull was exposed, and a stainless-steel bar was attached above the mid-sagittal ridge using dental cement. EEG cables were soldered (prior to implantation) to custom-made headmounts (Pinnacle Technology Inc. Lawrence, USA) and attached to stainless steel EEG screw electrodes were placed unilaterally over the right frontal (AP 4 mm, ML 4 mm) and occipital (AP 7mm, ML 5mm) cortical areas. A reference screw electrode was implanted over the cerebellum. Two tip-blunted stainless-steel wires were placed into the nuchal muscle to record electromyography. Wires and screws were fixed to the skull surface using dental acrylic. A craniotomy was placed contralateral to the occipital EEG screw electrode (AP approx. 7mm, ML approx. -5mm). The dura mater was removed. A single-shank silicon probe (NeuroNexus) with 32 recording sites, vertically arranged and spaced at 100 µm intervals, was inserted 1.8 –2.5 mm anterior to the caudal end of the ectosylvian gyrus and 3.9 – 4.7 mm lateral from the midline. The probe was slowly advanced perpendicularly to the pial surface. The craniotomy was sealed using a low toxicity silicone adhesive (Kwik-Sil, WPI) and closed with dental acrylic. Externalised probe connectors and EEG head mounts were protected with accessible plastic enclosures secured to the dental acrylic. To expedite recovery from anaesthesia after the end of the procedure, animals received Antisedan via intramuscular injection (atipamezole

hydrochloride, 0.06mg/kg, s.c.). A minimum 2-week recovery period was ensured prior to further procedures.

Mice: The surgical procedure largely followed the protocol described in Milinski et al. 2021. Surgical procedures were carried out using aseptic techniques under isoflurane anaesthesia (3–5% induction, 1–2% maintenance). During surgery, animals were head-fixed using a stereotaxic frame (David Kopf Instruments, CA, USA). Liquid gel (Viscotears, Alcon Laboratories Ltd., UK) was applied to protect the eyes. Metacam (5mg/kg, s.c.) and Vetergesic (0.1 mg/kg s.c.) were administered preoperatively. Stainless screw electrodes were placed unilaterally in the left frontal (AP + 2 mm, ML - 2 mm) and occipital (AP - 3.5–4 mm, ML - 2.5 mm) cortical regions. A reference screw electrode was placed above the cerebellum. EEG screws were soldered (prior to implantation) to custom-made headmounts (Pinnacle Technology Inc. Lawrence, USA), and all screws and wires were secured to the skull using dental acrylic. Two single-stranded, stainless-steel wires were inserted on either side of the nuchal muscle to record the electromyogram (EMG). At the end of the surgery, animals were administered saline (0.1 ml/20 g body weight, s.c.). The animal's temperature was monitored and maintained throughout the procedure using a rectal probe and a homeothermic monitoring system (Harvard Apparatus), and a heated chamber for recovery afterwards until the animal readily walked around. Metacam (1–2 mg/kg) was orally administered for at least 3 days after surgery. A minimum 2-week recovery period was ensured prior to further procedures.

Noise overexposure

Ferrets: Noise (1/2 octave narrowband pink noise centred at 8 kHz, 98dB SPL at ear level) was presented for 120 minutes unilaterally via an earphone to the right ear while the

left ear was fitted with an ear plug and silicone impression material (Otoform, Dreve). To reduce the chance of inducing lasting cochlear damage, a relatively low intensity (98 dB SPL) was used for NOE. Noise of 100 dB and 97 dB SPL presented for 2 hours in mice and guineapigs, respectively, have been shown to induce only temporary hearing threshold shifts (Dehmel et al., 2012; Kujawa & Liberman, 2009). The procedure was carried out under general anaesthesia. Anaesthesia was induced through intramuscular injection of medetomidine hydrochloride (0.022 mg/kg body weight; Domitor, Orion Pharma) and ketamine hydrochloride (5 mg/kg; Narketan10, Vetoquinol). Depth of anaesthesia and the respiratory rate were monitored throughout the experiment. Anaesthesia was maintained through injection of half of the initial dose after 60 minutes or when the animal showed signs of arousal. Temperature was maintained at 38°C using an homeothermic monitoring system (Harvard apparatus). To revert the effect of Domitor and expedite recovery from anaesthesia, animals received Antisedan via intramuscular injection (atipamezole hydrochloride, 0.06mg/kg, s.c.). Noise over exposure (NOE) took place during the light phase, and animals were given at least 48hrs of rest before any other procedure.

Mice: Noise (1/2 octave narrowband pink noise centred at 8 kHz, 98dB SPL at ear level) was presented for 120 minutes via a free-field speaker. The left ear was fitted with an ear plug and silicone impression material (Otoform, Dreve). The procedure was carried out under isoflurane anaesthesia (3–5% induction, 1–2% maintenance). Liquid gel (Viscotears, Alcon Laboratories Ltd., UK) and patches of aluminium foil were applied to protect the eyes and shield from light exposure. The animal's temperature was monitored and maintained throughout the procedure using a rectal probe and a homeothermic monitoring system (Harvard Apparatus), and a heated chamber for

recovery afterwards until the animal readily walked around. NOE took place during the last two hours of the dark phase (ZT22-24) for all animals.

Auditory brainstem response measurements

Ferrets: Auditory brainstem responses (ABRs) were obtained in lightly anaesthetised (immobile and lack of response to light noxious stimulus, absence pedal reflex) ferrets using sterile subcutaneous monopolar needle electrodes (0.35 x 12 mm, MN3512P150, Spes Medica). Sedation was induced through intramuscular injection of medetomidine hydrochloride (0.022 mg/kg body weight; Domitor, Orion Pharma) and ketamine hydrochloride (5 mg/kg; Narketan10, Vetoquinol). Depth of anaesthesia and respiratory rate were monitored throughout the experiment. Sedation was maintained through injection of half of the initial dose every ~60 minutes, or when the animal showed signs of arousal. Temperature was maintained at 38°C using a forced-air warming system (Bair Hugger, 3M Health Care). To expedite recovery after completion of the recordings the effect of Dormitor was reversed by Antisedan injection (atipamezole hydrochloride, 0.06mg/kg, s.c.). Stimuli were presented with earphones (Sennheiser CX300 II) inserted into the ear canal (left and right, in subsequent recordings) and fixated in place with silicone impression material (Otoform, Dreve). Auditory stimuli were generated using an RP2.1 Enhanced Real-time processor (Tucker Davies Technologies, TDT) with a sampling frequency of 100 kHz connected to a TDT PA5 programmable attenuator. The earphones were calibrated using SigCalRP TDT calibration software to generate compensation filters ensuring stable intensity levels for a frequency range from 250 to 30000Hz. Click stimuli (rarefaction click trains, rectangular voltage pulse, 100 μ V, low-pass filtered) were presented at a rate of 17/sec for 700 repetitions per intensity level

(40, 50, 60, 70, 80, 90dB SPL). Narrow-band pink noise stimuli (centred around 1, 4, 8 and 16 kHz) of 5 ms duration were presented at a rate of 21/sec for 700 repetitions per intensity level and frequency combination. Signals were recorded from two active subcutaneous electrodes, placed close to the left and right auditory bullae, respectively, and referenced to an electrode placed at the vertex of the skull. A ground electrode was placed on the back of the animal. The signal was routed to a preamplifier (Medusa TDT RA16PA) via a low impedance headstage (TDT RA4LI) and recorded by an RZ2 Bioamp Processor controlled by BioSigRP software (TDT).

ABR signal detection: Data analysis was performed offline based on average signals (averages over 700 individual ABR traces) for each stimulus and intensity level using a custom-written MATLAB script: local maxima and minima in the signal were detected using the *findpeaks* function (signal processing toolbox, MATLAB) and ABR waves I to V were defined depending on predetermined response windows for each sound level (40-90dB SPL): waves were always detected first for the 90dB SPL signal for any given stimulus (clicks or NBN stimuli) and following previous descriptions (Buran et al., 2020; Moore, 1993; Moore & Hine, 1992). The *findpeaks* function was applied on the entire signal. During the baseline condition (BL) the peak of the consistently most prominent wave, wave II, was defined as the maximum peak between (and including) 1.56ms and 2.4ms. Peaks of waves I, III, IV and V were selected relative to the latency of wave II with the following criteria: $-1.08\text{ms} \leq \text{peak I} \leq -0.4\text{ms}$; $0.04 \leq \text{peak III} \leq 0.92\text{ms}$; $0.96\text{ms} \leq \text{peak IV} \leq 1.52\text{ms}$; $1.56\text{ms} \leq \text{peak V} \leq 2.2\text{ms}$. As response latencies increase with decreasing sound level, the response windows for sound levels below 90dB SPL were shifted (delayed) by the following time spans: 0.12ms (80dB), 0.23ms (70dB), 0.32ms (60dB),

0.48ms (50 dB), 0.6ms (40dB). Artefactual bias was minimised by excluding detected peaks: (1) if there was no detected peak for the next higher sound level (e.g. a wave V at 40db was deemed artefactual if no detectable wave V was observed at 50dB) or (2) if a detected peak had a shorter latency than the corresponding peak at the next higher intensity with a tolerance of ± 0.12 ms (3 data points, dps).

After detection of waves I to V, wave magnitudes were defined as the difference between the wave peak and the immediately following trough (local minimum). Wave latencies were defined as the time of each wave's peak (maximum) relative to stimulus onset. In addition, as a readout for the magnitude of the entire ABR signal across all waves, the root mean square (RMS) of the signal was calculated by applying the MATLAB function *rms* on the signal in the predefined response window, 1.6ms to 4ms, to include only the ABR signal. To account for longer response latencies at low sound levels, the response window was shifted by 0.16ms (4 dps) for each 10 dB decrement. Statistical comparisons were conducted for wave magnitude, latency, RMS, across sound levels and time of recording (conditions).

Because of the potential disruption to the structure of the ABR after NOE (Post1 and Post2 conditions) and to ensure that equivalent waves were compared across conditions in each animal, the timing of wave II in the basal conditions (BL) was used as time reference. For the analysis of Post1 and Post2 assessments, after applying the *findpeaks* function on the entire ABR signal, the closest peak to the reference time (wave II in BL) was defined as wave II peak. If two peaks were equally close to the latency of the BL wave II peak, the peak with the longer latency was selected. If wave II had been undefined in the BL condition, it was in the post NOE condition detected as described

for the BL condition above (based on the maximum peak in a predefined response window).

The measure for the sound level modulation of both latency and response magnitudes was obtained by fitting a linear function of the respective measure (latency, wave II magnitude or RMS) for each animal and condition at the different sound levels. Wave II was chosen as representative measure for changes in hearing ability, as it was consistently the most prominent ABR component across intensities and also since wave II represents activity in an early structure in the auditory brainstem (the cochlear nucleus, Henry, 1979) and therefore reflects a degradation of cochlear or auditory nerve activity more directly than higher-level nuclei, represented by ABR waves III to V. In addition, elevated activity in the cochlear nucleus can be an indicator for tinnitus (Brozoski et al., 2002; Kaltenbach et al., 2004; Middleton et al., 2011).

Mice: Auditory brainstem responses (ABRs) were obtained in lightly anaesthetised (immobile and absence pedal reflex) mice using subcutaneous electrodes. Anesthesia was induced through intraperitoneal injection of medetomidine hydrochloride (0.022 mg/kg body weight; Domitor, Orion Pharma) and ketamine hydrochloride (5 mg/kg; Narketan10, Vetoquinol). Depth of anaesthesia and respiratory rate were monitored throughout the experiment. Animal temperature was monitored and maintained using a rectal probe and a homeothermic monitoring system (Harvard Apparatus). To expedite recovery from anaesthesia after the end of the procedure, animals received Antisedan via intraperitoneal injection (atipamezole hydrochloride, 0.06mg/kg, i.p.). Sound stimuli were presented to one ear, while the other ear was fitted with an ear plug, respectively. Stimuli were presented from a free field speaker

positioned 8 cm in front of the animal. Auditory stimuli were generated using an RP2.1 Enhanced Real-time processor (Tucker Davies Technologies, TDT) with a sampling frequency of 100 kHz connected to a TDT PA5 programmable attenuator. The speaker was calibrated using SigCalRP TDT calibration software to generate compensation filters ensuring stable intensity levels for a frequency range from 250 to 30000Hz. Click stimuli were presented at a rate of 17/sec for 700 repetitions per intensity level (40, 50, 60, 70, 80, 90dB SPL). Pure tone stimuli (1, 4, 8 and 16 kHz) of 5 ms duration were presented at a rate of 21/sec for 700 repetitions per intensity level and frequency combination. Signals were recorded from two active subcutaneous electrodes, placed close to the left and right auditory bullae, against a reference electrode placed at the vertex of the skull. A ground electrode was placed on the back of the animal. The signal was routed to a preamplifier (Medusa TDT RA16PA) via a low impedance headstage (TDT RA4LI) and recorded by an RZ2 Bioamp Processor controlled by BioSigRP software (TDT).

ABR signal detection was identical as in ferrets (see above) except from the definition of response windows: wave II was defined as the maximum peak between (and including) 1.6ms and 2.6ms. As response latencies increase with decreasing sound level, the response windows for sound levels below 90dB SPL were shifted (delayed) by 0.12ms/10 dB decrement. Discrepancies between ABR latencies in ferrets and mice were likely due differences in animal size and anatomy.

Gap induced inhibition of the acoustic startle (GPIAS) testing

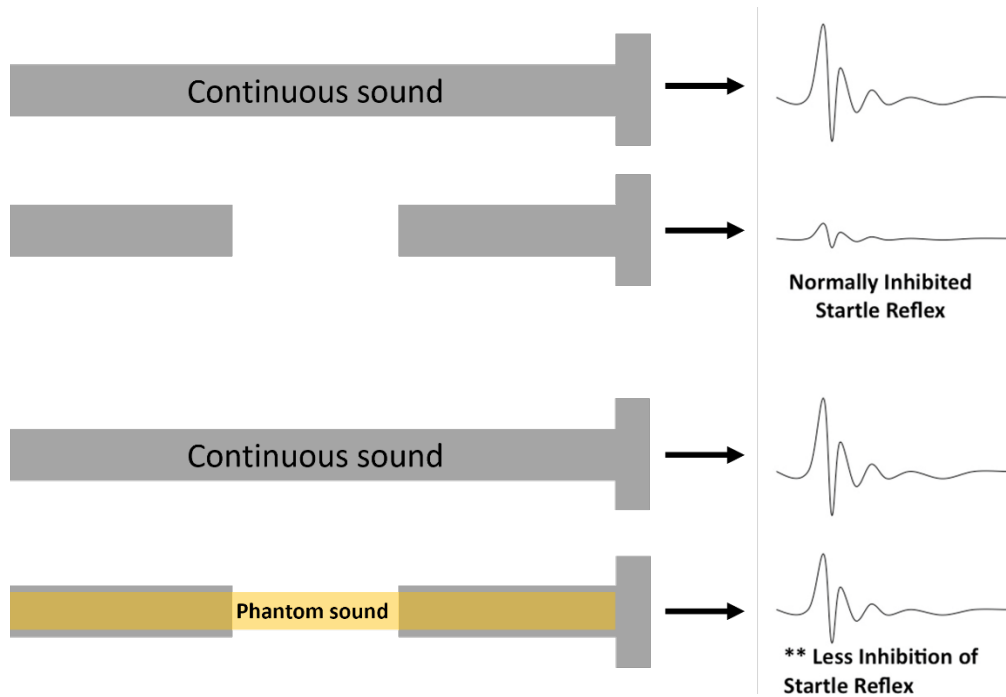


Fig. 1: Gap induced inhibition of the acoustic startle: schematic depiction of startle responses in a normally hearing animal (top) and in a tinnitus animal (bottom). The graphic is adapted from Fig.1 of Turner and colleagues 2012 (J. Turner et al., 2012).

Ferrets: Animals were placed in a custom-made chamber within a sound isolated compartment and presented with continuous low intensity white noise (broad band noise, 0.5-30 kHz, intensity level 40 dB SPL). The relatively low intensity of background noise compared to GPIAS assessment in rodents (J. Turner et al., 2012) was chosen to maximise the contrast between background and startle stimuli and therefore counteract

strong habituation or a low startle response that is often observed in carnivores (e.g. (Gruner, 1989)). Once acclimatised to the chamber, 40 brief high-intensity noise bursts (10ms, 120 dB SPL) causing a startle response were presented with a randomized inter-trial interval of 3 to 13 seconds. The startle response was recorded using a load cell sensor positioned below the floor of the behavioural chamber. Half of the startle stimuli (20 trials) were preceded by 50ms by a silent gap (gap length 50ms) in the background noise, serving as a predicting signal for the high-intensity noise bursts. Therefore, theoretically, in animals who are able to detect the gap, the startle response to 'gap trials' is reduced relative to 'non-gap trials' (Fig.1) (J. Turner et al., 2012) although gap induced facilitation of the startle response has also been reported where the startle response is enhanced in gap trials (Longenecker et al., 2018). Gap and non-gap trials were presented in a randomised order. The startle amplitude was calculated for each trial offline, defined as the difference between minimum and maximum of the load cell signal within a predefined response window (10-50ms after stimulus onset). To avoid a bias in recorded responses caused by artefacts unrelated to the startle response, trials with extreme values were excluded from further analysis. Extreme values were defined where the startle magnitude exceeded the mean $\pm$ the standard deviation across gap or non-gap trials, respectively. Gap detection ability was assessed by evaluating the inhibition of the startle in gap as compared to non-gap trials. Startle inhibition was evaluated in 4 sequential blocks containing 5 gap and 5 non-gap trials each. This block-based analysis has been suggested previously, in order to capture effects of within-session habituation (Longenecker et al., 2018). For each block, the mean startle response magnitude for gap and non-gap trials was used to calculate the startle inhibition, *mean startle (Gap trials) / mean startle (Non-gap trials)*. Note that the

parameters for the GPIAS paradigm were selected based on previous studies, since pilot experiments to establish parameters would have required a high number of animals, in particular due to the quick habituation of ferrets in this paradigm, and were therefore beyond the scope of this study. A gap duration of 50 ms was chosen since it reliably induces GPIAS in multiple animal models such mice (Hickox & Liberman, 2014), guinea pigs (Coomber et al., 2014) and rats (J. G. Turner et al., 2006). The interval between gap and startle stimulus was chosen as it has been shown in human subjects that with short (<60ms) intervals between the prepulse and the startle stimulus PPI relies predominantly on preattentive mechanisms (Kohl et al., 2013). Since the objective for the GPIAS paradigm in this study was to provide a tinnitus assessment that is not affected by motivation, a short lead interval of 50 ms was used.

Mice: Animals were placed in a small experimental chamber on top of a motion-sensitive platform (San Diego Instruments, SR-LAB Startle Response System) and presented with a white noise acoustic background at 67 dB SPL, similar to the paradigm used by Turner et al. 2012. The beginning of each session consisted of a 3-min acclimation period (only background noise) followed by two startle-eliciting noise bursts (20ms, 105 dB), which serves to habituate the animal's startle response to a more stable baseline (J. Turner et al., 2012). Data from the habituation phase were not used in subsequent analysis. After habituation, startle-eliciting noise bursts were presented at random intervals (3-13s) over the background noise. Half of the startle stimuli were preceded by a silent gap (50ms gap duration) in the background noise 100ms before. This commonly leads to a reduced startle of the animal relative to startle responses evoked by startle stimuli not preceded by a silent gap. This relative decrease of the startle response is referred to as

GPIAS (gap-induced inhibition of the acoustic startle) and is thought to reflect gap detection ability (Turner et al., 2012; Turner et al., 2006). Since animals are prone to adaptation in this paradigm (i.e. a reduced startle after repeated exposure to the startle stimulus), each assessment consisted of only 40 trials (20 trials with a silent gap preceding the startle stimulus and 20 trials without; trials were randomly interspersed). In addition, consecutive GPIAS assessments were interleaved by at least 14 days. The startle amplitude was calculated for each trial offline. To capture different aspects of the startle response in the analysis, startle magnitude was defined based on two different parameters of the signal and using a short and a longer response window, respectively: startle magnitude was defined as maximum - minimum value of the signal within the response window, and as the average of the signal within the response window. Response windows were defined within 0-50ms and 0-1000ms after stimulus onset. Gap detection ability was assessed by evaluating the modulation of the startle in gap as compared to non-gap trials. Startle modulation was assessed in sequential blocks of 5 gap and 5 non-gap trials. This block-based analysis has been suggested previously, to capture effects of within-session habituation (Longenecker et al., 2018). For each block, consisting of 5 gap and 5 non-gap trials, the average startle response magnitude was calculated for gap and non-gap trials. Startle modulation was then defined as *mean startle (Gap trials) / mean startle (Non-gap trials)*. Extreme values were excluded from further analysis. Extreme values lay outside the mean $\pm$ 5 standard deviations (based on a 5000-iteration bootstrap) and were defined for each combination of experimental group, time, block, response window and startle parameter.

Operant gap detection

Ferrets were trained by positive reinforcement using water as a reward to carry out a gap-detection task in an operant arena as described in previous work (Bajo et al. 2010; Gold et al., 2014). The setup consisted of a circular arena (radius, 75 cm) housed in a double-walled sound-attenuated room. Animals were trained to initiate a trial by activating infrared sensors on a centre platform of the arena by licking a spout, ensuring that the animal positioned its head at the centre of the arena. This triggered the presentation of one of two types of sound stimuli presented through a single speaker (Audax TW025MO) facing the platform at 0 degrees. The two stimulus types were a continuous sound and the same sound but including silent gaps. After stimulus presentation, the animal had to leave the central platform and approach a response location at the periphery of the arena at 30° to the left in 'gap trials' and 30° to the right in 'non-gap trials' to get a water reward. There was no time limit for the animals to respond. Incorrect responses were not rewarded. After an incorrect response, trial initiation triggered the identical sound stimulus up to two more times (correction trials) before trial initiation would trigger a novel trial. Correction trials were not included in the data analysis. Sound stimuli were generated by TDT System III hardware (Tucker-Davis Technologies). The test sessions were controlled by a custom-built program (MATLAB) that registered the position of the ferret at the centre and response locations, presented the stimuli and delivered the rewards accordingly. Sound stimuli were broadband gaussian noise bursts (BBN, 30 kHz bandwidth) and one octave narrowband noise bursts (NBN) centred at 1, 4, 8, and 16 kHz. In gap trials 4 equally spaced, identical silent gaps were introduced in the stimulus. Across trials the length of these gaps differed and were either 3, 5, 10, 20, 50, 100 or 270 ms duration. Stimuli were generated *de novo* for each trial, cosine ramped with a 10 ms rise/fall time and had a total duration

of 2080 ms. All stimuli were filtered using the inverse transfer function of the speaker to obtain stable intensity levels across included frequencies at 76 ± 5 dB SPL. Animals were assessed twice daily in blocks of 5 consecutive days separated by at least two days of *ad libitum* access to water. Within each assessment sound stimuli were constant in terms of frequency composition (only gap lengths were randomised across trials within an assessment) but stimuli were varied between assessments to obtain approximately equal numbers of trials for all stimuli (1, 4, 8 and 16 kHz NBN and BBN). Animals performed *ad libitum* in each session until ceasing to initiate trials. Initially, animals were trained using only the longest gap length (270ms) until they reached at least 80% correct responses in two consecutive sessions before being tested in the paradigm but now using all gap lengths. Animals had to complete 600-1000 trials for each stimulus type. Analysis was based on average performance for each session. All trials per session were included to calculate average session performance for each gap length (0, 3, 5, 10, 20, 50, 100, 270 ms), except when animals had response times longer than 5 seconds. Those trials were excluded from further analysis. Entire sessions were excluded from the analysis if more than 5 gap lengths had been presented less than 5 times. For analysis of gap detection thresholds, a sigmoid function (R P (2022). *sigm_fit* (mathworks.com/matlabcentral/fileexchange/42641-sigm_fit, MATLAB Central File Exchange) was fitted on hit rate (between 0 and 1) vs gap length, calculated for each animal, stimulus (1, 4, 8 and 16 kHz NBN and BBN) and condition (BL, Post1, Post2). Thresholds were defined as the gap length in closest proximity to a hit rate of 0.5 (chance level) on the fitted function. Fits with slopes at threshold of more than 10 times the median slope across all samples were excluded from the analysis (this applied to 2 out of 78 samples).

Operant silence detection

Operant silence detection (modified from Stolzberg et al. 2013) took place in the same operant arena as the operant gap detection paradigm, with the same initiation of individual trials via activation of infrared sensors on the centre platform. Upon initiation, a light emitting diode opposite the initiation platform lit (signalling the start of the trial) while either of the three stimulus types was presented: a narrowband noise (NBN, one octave bandwidth with centre frequencies at 1, 4, 8, and 16 kHz and randomised across trials), an amplitude modulated BBN (AM stimulus, 100% modulation depth, 5 Hz) or silence (no sound). Stimuli in 50 % of trials were NBN, in 30 % AM and in 20% Silence to ensure equal probability of reward in accordance with the following criteria. In AM and silence trials responses to a sensor located +30° relative to the initiation platform were rewarded with water whereas in NBN trials responses to the -30° sensor were rewarded. There was no time limit for the animals to respond. A trial was completed whenever the animal responded to either of the two sensors. During the training phase for this paradigm, reward probability of correct trials was gradually reduced from 1 to 0.7. After animals reached the performance criterium (>80% correct in two consecutive sessions), the paradigm was modulated in that Silent trials were never rewarded ('testing phase' of the paradigm) to measure the animals' performance without a further training effect. To keep the overall reward probability consistent with the training phase non-silent trials' rewards probability was 0.9. Animals were assessed two times per day for maximally 5 continuous days before a minimum of two days without assessment and *ad*

libitum water access. Animals performed *ad libitum* in each session until ceasing to initiate trials and completed a total of 1000 trials in this paradigm during the baseline testing block, as well as during assessment blocks after noise overexposure (Post1 and Post2). To refamiliarize animals with the paradigm before each testing block, animals performed in the training paradigm until reaching the performance criterium. While this retraining might have allowed animals to adjust to a new 'perceptual baseline' after noise overexposure and therefore mask subtle effects of tinnitus or hearing impairment on performance, it was meant to prevent effects of time on the animal's performance (e.g. the animal forgetting aspects of the paradigm). Animals proceeded to completed 1000 trials in the testing phase of the paradigm over multiple sessions to minimize the effect of day-to-day variability, such as due to motivation and behavioural state of the animal in each session. To assess performance, hit rates were compared across stimuli and conditions (Baseline, Post1 and Post2). Sessions were an animal completed less than 5 trials for more than 5 gap lengths were excluded from further analysis. Trials with long response times (>20s) were also excluded. For statistical analysis, a generalised linear mixed measures model was built using IBM SPSS Statistics Version 28.0.0.0 (190). The program only excluded cases (animals) that have data for only one time point. Cases with data for two or more time points were included in the model. In this instance, data from 7 animals contributed to timepoints BL and Post1, but only 2 animals contributed to timepoint Post2.

EEG signal processing and vigilance state scoring

Data acquisition was performed using a Multichannel Neurophysiology Recording System (TDT, Alachua FL, USA). Cortical EEG was recorded from frontal and occipital derivations. EEG/EMG data were filtered between 0.1 and 100 Hz, amplified (PZ2 preamplifier, TDT, Alachua, FL, USA) and stored on a local computer at a sampling rate of 1017 Hz, and subsequently resampled offline at 256 Hz. Signal conversion was performed using custom-written Matlab (The MathWorks Inc., Natick, MA, USA) scripts. Signals were then transformed into European Data Format (EDF). Vigilance state scoring was performed manually offline prior to spectral analysis and assessment of sleep architecture.

Manual vigilance state scoring was based on visual inspection of consecutive 4s epochs of filtered EEG and EMG signals (SleepSign, Kissei Comtec Co, Nagano, Japan). Frontal and occipital EEG and neck muscle EMG channels were displayed simultaneously to aid manual scoring and video recordings of the animal were consulted for further validation of the behavioural state at any given time. 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, characterised by high amplitude and low frequency EEG), REM sleep (low-voltage, mid-frequency EEG with a low level of EMG activity) or REM2 (low-voltage, high-frequency EEG with a low level of EMG activity, see Chapter 3, Fig.2D for illustration). Epochs containing an EEG signal contaminated by artefacts (such as due to gross movements of the animal, eating or drinking) were excluded from subsequent analysis.

For each 24h-recording, EEG power spectra were computed by a fast Fourier transform (FFT) routine for 4-s epochs (Hanning window), with a 0.25-Hz resolution (SleepSign

Kissei Comtec Co, Nagano, Japan). Artefacts in specific frequency bins that had remained unnoticed during manual data scoring were excluded offline: for each 0.25 Hz frequency bin of a given vigilance state during a 24h recording, the mean and standard deviation across all epochs was calculated based on a 500-iteration bootstrap. Values outside the average across all epochs ± 600 standard deviations were excluded from the analysis (to exclude only extreme outliers). Exclusion in this case applied not to all frequency bins of the respective epoch but just to the specific frequency bin identified as an outlier. The objective of this was to prevent extreme outliers from distorting the spectral power estimate in particular frequency bins.

Sound presentation during sleep and wakefulness

During this paradigm ferrets were kept in identical conditions as during chronic electrophysiological recordings: animals were housed individually in a custom-made recording chamber in a 15/9h light-dark cycle. Frontal and occipital EEG and neck-muscle EMG recordings were obtained in the freely-moving animal over approximately 48 hours per recording session. In the first 24 hours per recording session the animal was left undisturbed. Over the course of the second 24 hours of the session auditory stimuli were presented via a free-field speaker installed over the recording cage. Presented sounds were one octave narrow-band stimuli with centre frequencies of 1, 4, 8 and 16 kHz. Stimuli had a duration of 820ms and had a silent gap of 38ms at the centre of the stimulus. Stimuli were presented at 40, 50, 60 and 65 dB SPL (as measured at floor level in the centre of the recording cage). Inter-stimuli intervals (ITIs) were of a random duration between 10 and 42s and each stimulus-intensity combination was presented

200 times. Stimuli were generated via MATLAB and produced using an RP2.1 Enhanced Real-Time Processor (TDT) and an Alesis RA150 Amplifier. Stimulus presentation was controlled via a custom written MATLAB script.

Analysis of evoked responses

EEG: Raw data were transformed into a MATLAB compatible format (.mat) using the *tdtbin2mat* MATLAB function (provided by TDT). EEG Evoked responses were analysed within a time window set between -0.5s and +5s relative to sound stimulus onset. Each stimulus presentation is referred to hereafter as a trial. Trials that fell into an epoch that contained an artefact as defined during the manual vigilance scoring procedure were excluded from further analysis. To aid computing efficiency the signal was down sampled by a factor of 2 (from an original sampling rate of 3051Hz). Trials were then sorted into groups based on stimulus frequency, intensity, and vigilance state. Due to high variability in the EEG signal of individual trials, for each group of trials (all trials for the same condition, vigilance state, stimulus and stimulus intensity), 20 bootstrapped means were drawn to serve as a representative signal sample for subsequent analysis. Peaks and troughs of evoked potentials were detected in each signal sample after smoothing the signal using a moving average of ~8 points (implemented by the *smooth* MATLAB function). As the shape of the evoked potential was not uniform across animals, criteria for identification of peaks and troughs were defined based on the individual animal and derivation. Peaks and troughs were automatically detected in a time window after stimulus onset that was predefined using a custom written MATLAB script and the *findpeaks* MATLAB function. Time windows for early (R1), mid (R2) and late (R3) response components were defined based on the average latency of peaks and troughs

in the respective animal. Note that for Case1 R1, R2 and R3 could be identified, for Case2 R1 and R2, and for Case3 only one response component, R1.

LFP: Auditory cortex local field potentials were obtained in parallel to EEG recordings via a single-shank silicon probe (NeuroNexus) with 32 recording sites (see surgery). Raw data were acquired with a sampling rate of (24414Hz), down sampled by a factor of 2 before evaluating evoked potentials. Evoked potentials were analysed in the same manner as EEG signals (see above). However, these results were not included in the analysis due to insufficient signal quality.

Sleep deprivation

Sleep deprivation in mice was conducted via provision of novel objects, which is an established method for sleep deprivation in mice (e.g. Huber et al., 2000; McKillop et al., 2018) that makes use of the animals' natural tendency to explore novel objects and keep animals therefore engaged. This paradigm is thought to be less stressful for the animals compared to other forms of sleep deprivation, because the exploratory behaviour mimics naturalistic waking conditions (McKillop et al., 2018). During sleep deprivation, animals were continuously monitored by the experimenter and novel objects were provided whenever the animal showed signs of drowsiness (behavioural immobility or increasing delta activity in the EEG, which was continuously recorded and monitored). Sleep deprivation was conducted during the initial 6 hours of the light period (the animals' inactive phase) to ensure that the difference in the amount of sleep between sleep deprived animals and undisturbed control animals was maximal.

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