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Hypothalamic control of body temperature and sleep

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A thesis submitted for the degree of Doctor of Philosophy

Declaration of Originality

I declare that this thesis is the product of my own work. The data presented was collected during my PhD under the supervision of Prof. Nicholas Franks and Prof. Bill Wisden. Where literature is discussed it is referenced fully. Figures that rely on published material are attributed in the Figure legends. Any data produced in collaboration with others is directly specified in the text.

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ABSTRACT

The role of sleep is not understood but it is essential for life. The sleep cycle is highly correlated to the circadian control of core temperature which, in mammals, is lower during the sleep phase. General anaesthetics induce sedation with sleep-like features, but this often comes with hypothermia. The preoptic hypothalamus seems key to the successful regulation of sleep and thermoregulation. We hypothesise a convergence of neuronal circuits that regulate sleep and temperature regulation in the preoptic area. These may also form a neuronal target for general anaesthetics.

Using a pharmacogenetics technique called TetTagging it is possible to label neuronal circuits that respond to specific stimuli and then reactivate them later using a drug called clozapine N-oxide. I have used this technique to label neurons involved in the response to skin warming and used the reactivation to investigate the role of this circuit in sleep. I have also developed techniques to manipulate neuronal circuits through cooling to aid this investigation. By measuring EEG and core temperature, I found that recapitulation of activity in these warm-sensitive circuits induces strong delta oscillations, similar to those of natural sleep, followed by profound hypothermia more closely resembling the effects of general anaesthetics. A reduction in theta power was slower than the onset of delta and so may indicate an increase in sleep pressure. Following hypothermia, this state more closely resembled consolidated NREM, with REM sleep almost entirely absent. Immunohistochemistry has ruled out a GABAergic or cholinergic identity for these neurons.

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List of abbreviations

- 3V – 3rd Ventricle
- AAV – Adeno-associated virus
- Ac - Anterior commissure
- BAT- Brown adipose tissue
- BF - Basal forebrain
- BMAL1 - Aryl hydrocarbon receptor nuclear translocator-like protein 1
- Cg1 – Cingulate cortex
- CLOCK - Circadian Locomotor Output Cycles Kaput
- CMT – Central medial thalamus
- CRE - Causes recombination enzyme
- CVC - Cutaneous vasoconstriction
- CVD - Cutaneous vasodilation
- DEX – Dexmedetomidine
- DH - Dorsal Horn
- DMH - Dorsal medial hypothalamus
- DR - Dorsal raphe nucleus
- DREADD - Designer Receptors Exclusively Activated by Designer Drugs
- DRG - Dorsal root ganglion
- ECoG - Electrocorticography
- EEG - electroencephalogram
- EMG - Electromyography
- eVLPO – Extended Ventrolateral preoptic area
- GABA - Gamma-Aminobutyric acid
- GAD - Glutamic acid decarboxylase
- GFP – Green fluorescent protein
- hM3 – Human muscarinic acetylcholine receptor M3
- HR - Heart rate
- IML- Intermediolateral nucleus
- LBbd - Lateral parabrachial dorsal
- LC - Locus coeruleus
- LDT - Laterodorsal tegmental nucleus
- LFP – Local Field Potential

- LH - Lateral hypothalamus
- LOC – Loss of consciousness
- LORR – Loss of righting reflex
- LPbe - Lateral Parabrachial external
- LPO - Lateral preoptic area
- M2 – Motor cortex (2)
- MnPO – Medial Preoptic Area
- MPO – Median Preoptic Area
- NGS – Normal goat serum
- nNOS – Nitric oxide synthase 1 (neuronal)
- NREM – Non rapid eye movement
- Pb – Parabrachial nucleus
- PBS – Phosphate-buffered saline
- Pef - Perifornical area
- PGE2 - Prostaglandin E2
- PGE2R – Prostaglandin E2 receptor
- PnO - Pontine reticular formation
- PPT - Pedunculo pontine tegmental nucleus
- PV - Parvalbumin
- R-152 – 1,1-difluoroethane
- REM – rapid eye movement sleep
- RMS – Root mean square
- rRPA - Rostral raphe pallidus
- SCN - Suprachiasmatic nucleus
- SEM – Standard error of the mean
- SG - Sympathetic ganglion
- SMS - Skeletal muscle shivering
- SOM - Somatostatin
- TASK3 - TWIK-related acid-sensitive K⁺ channel 3
- TMN - Tuberomammillary nucleus
- TRE – Tetracycline response element
- TREK - TWIK-related K⁺ channel
- TRPA - Transient receptor potential - Ankyrin-type
- TRPC - Transient receptor potential - Canonical type
- TRPM - Transient receptor potential - Melastatin type
- TRPV - Transient receptor potential - Vanilloid type
- tTA – Tetracycline-controlled transcriptional activator
- VGAT - Vesicular GABA Transporter
- VGLUT - Vesicular glutamate transporter

- VLPO – Ventrolateral preoptic
- VLPO - Ventrolateral preoptic area
- vPAG - Ventral periaqueductal gray
- ZT – Zeitgeber time

Chapter 1: Sleep and Temperature Regulation

1.1. The Need to Sleep

Sleep is a physiological process that is necessary for life but little understood. It is observed in all mammals and has parallels across the evolutionary tree from fruit flies (*Drosophila melanogaster*) to fish (*Danio rerio*) (Hendricks et al. 2000, Shaw et al. 2000, Bushey et al. 2011, Zhdanova et al. 2001, Yokogawa et al. 2007). But why do we sleep? In evolutionary terms, to be inactive is to be vulnerable. It is also unproductive, when asleep we do not consume or produce offspring. Thus sleep is inherently paradoxical and yet there is no alternative - the consequence of not sleeping is death (Everson et al. 1989, Rechtschaffen and Bergmann 1995, Rechtschaffen and Bergmann 2002).

Sleep is a process of restoration, the removal of fatigue accumulated by day. How we become tired and what process removes our tiredness, has been the source of much research and many a hypothesis. Sleep may be for the brain itself, a process to remove waste products from tired networks of neurons. It may help these networks resynchronise or even allow neurons time to repair (Vyazovskiy and Harris 2013). Without sleep, perhaps we would forget to hold on to our memories (Diekelmann and Born 2010). But equally, it could be a time of rest and recovery for tired muscles, an undiscovered metabolic process or a state of involuntary inactivity - an evolutionary throwback that compels us to conserve energy (Driver and Taylor 2000, Siegel 2009, Schmidt 2014). For each hypothesis, serious questions remain. Sleep confers a modest energy saving but results in inherent vulnerability (Jung et al. 2011). High levels of muscular activity induce a physical exhaustion and an oxygen debt to be repaid, but do not clearly require more sleep (Driver and Taylor 2000). And yet, long periods awake result in a progressive cognitive fatigue that will demand more sleep on subsequent nights and chronic sleep deprivation is fatal (Rechtschaffen et al. 1989, Rechtschaffen and Bergmann 2002). But the cause of death is not obviously neurological. Instead, a combination of stress and infection results from not sleeping (Rechtschaffen and Bergmann 2002). So, is sleep for the body, the brain or both? Sleep is a confounding physiological state and its functions remain stubbornly elusive.

Sleep is conventionally split into two parts, the homeostatic and the circadian. Together these hypotheses try to explain the function and regulation of sleep. The first is the most fundamental. Over the course of a day an unknown homeostatic sleep pressure builds and culminates in sleep onset (Achermann and Borbély 2003). During sleep, this pressure is relieved and the process restarts. Hence, sleep is a restorative process. The circadian arm of sleep regulation allows the homeostatic system to be entrained with the light-dark cycle, under which it evolved. Thus, diurnal animals sleep more during the night and nocturnal animals more during the day (Saper et al. 2005). This circadian cycle also entrains a pattern of thermoregulation that facilitates cooling during the sleep phase of the cycle. A decrease in core temperature occurs in all sleeping mammals in broad synchronisation with sleep (Rechtschaffen and Bergmann 2002). The function of this temperature rhythm, beyond energy conservation, is unknown, although the cycle is independent of sleep, as are its clear impacts on metabolism. Are temperature and sleep synchronised to maximise energy conservation? Or, is a

decrease in core temperature a necessary part of restorative sleep, and if so, does waking fatigue originate from a metabolic or neuronal process?

In this thesis I will outline the architecture that is thought to govern sleep control and its parallels with the control of body temperature. Understanding why we sleep is a fundamental physiological question and there are two distinct ways to address it. The first is to understand how sleep developed - in what form it started and how it changed under unique evolutionary pressures in each branch of the phylogenetic tree. This is the evolutionary approach. The second approach is to study how sleep is controlled, how it is turned on or off and how it is regulated night after night. This is the mechanistic approach. In the following sections I will discuss both and their implications for why we sleep.

1.1.1. Vertebrates and the evolution of sleep

“If sleep does not serve an absolute vital function, then it is the biggest mistake the evolutionary process ever made.” Allan Rechtschaffen 1978

The evolution of sleep has been studied backwards. Mammals and then humans were the first to have electroencephalogram (EEG) and electrocorticography (ECoG) measurements made (*Caton 1875, Berger 1929*). The recordings had distinct characteristics of behavioural states from wake to sleep or anaesthesia (*Loomis et al. 1937, Aserinsky and Kleitman 1953, Jouvet 1962*). These observations were characterised by oscillations of defined power in certain frequency bands, leading to the discovery of delta oscillations and the classification of rapid eye movement (REM) and non-rapid eye movement (NREM) sleep (*Aserinsky and Kleitman 1953, Kales et al. 1968, Hori et al. 2001*). To understand the evolution of sleep we have to look across the phylogeny tree and compare organisms that have distant ancestors. Each group has had different evolutionary pressures on the development of sleep. For example, birds are a different branch of the evolutionary tree to mammals but they are also endothermic. Non-avian reptiles such as lizards, of the order *lepidosaurs*, are much closer to birds but are ectothermic. This approach is summarised in Figure 1.1

For a long time, it was thought that reptiles could not display NREM, also known as slow-wave sleep (SWS), and to date sleep in ectotherms is controversial (*Rattenborg 2006, Rial et al. 2007, Vorster and Born 2015*). Recent attempts to take ECoG recordings in iguanid lizards (*Iguana iguana*) were also inconclusive (*Ayala-Guerrero and Mexicano 2008*). New research has sought to change this view. To understand whether sleep is also present in cold blooded ectotherms, Shein-Idelson *et al* recorded local field potentials (LFP) and single units along with behaviour, in the central bearded dragon (*Pogona vitticeps*). Over 24 hrs the bearded dragon displays clear behavioural quiescence in the dark period as measured by heart rate, eye movement and head angular velocity (degrees per second). This physiology correlated with electrical measurements from the LFP recordings where higher power was seen in the <4Hz band during behavioural sleep, equivalent to the NREM signature seen in mammals and often referred to as delta power. Movement, particularly in mammalian rodents, is typified by 6-9 Hz theta power and sleep is scored on the ratio of theta to delta power in non-moving animals. In this lizard, there was little evidence of a theta band, but sleep scoring could be performed on the ratio of

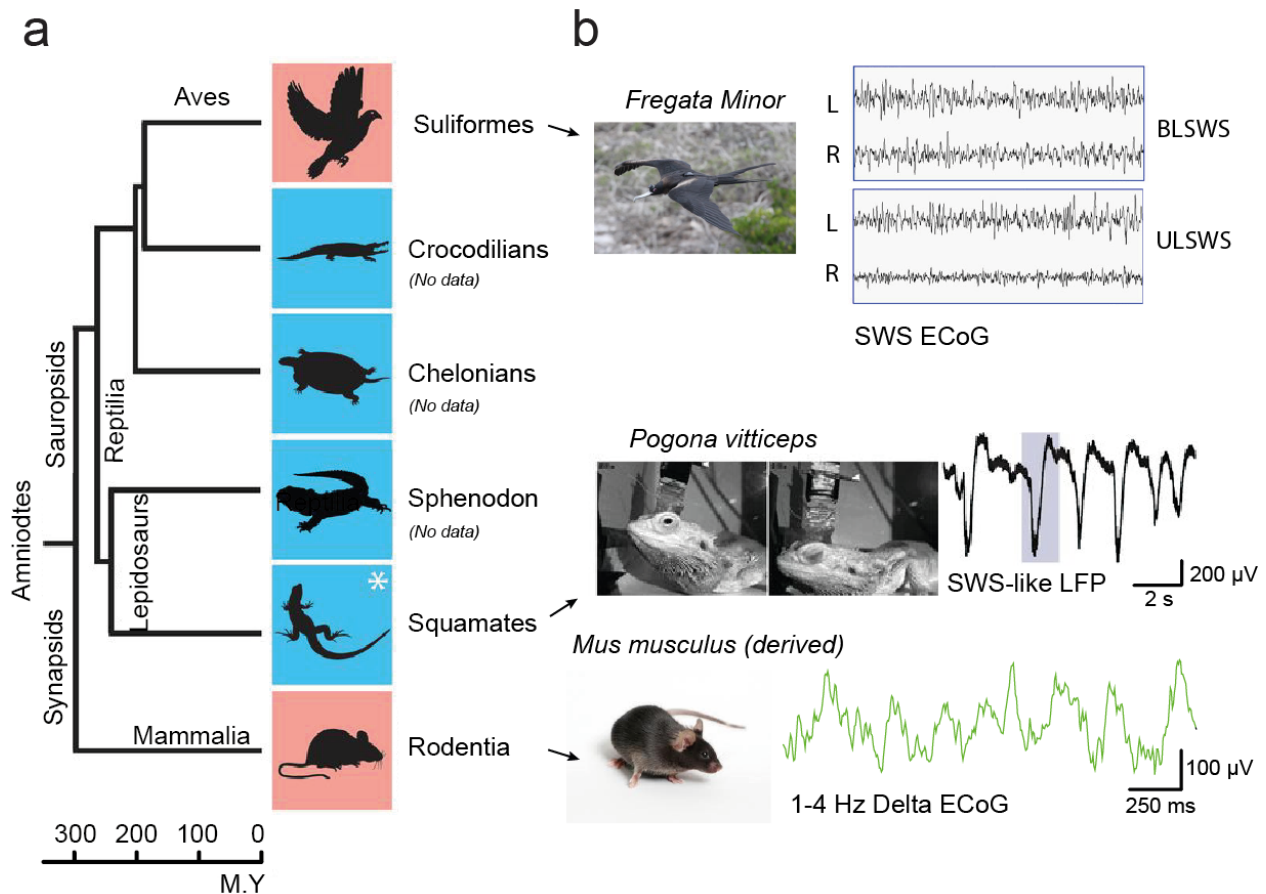


Figure 1.1 – Evolution of sleep. The evolution of different organisms from amniotes to present day. (a) The branches of the phylogenetic tree are classed as endothermic (red) and ectothermic (blue). (b) Shows examples of sleep from three different species on three different phylogenetic branches: From the Suliformes - the *Fregata minor*, from the Squamates - the *Pogona vitticeps* and from the Rodentia - *Mus musculus* (data from inbred C57BL/6). For each, examples of SWS are shown. In the *Fregata minor* this can be from the left (L) or right (R) hemisphere and bilateral (BLSWS) or unilateral (UNSW) SWS. Phylogenetic tree adapted from (Shein-Idelson et al. 2016). *Fregata minor* data from (Rattenborg et al. 2016)

power in 10-30 Hz band to the delta power. Periods of rapid eye-movements were shown to peak in the gaps between SWS scoring, where there was no movement, and were classified as REM. This produced an epoch of around 80 seconds and 350 SWS-REM cycles in total. Scored sleep lasted between 6-10 hours and ended approximately 30 mins before the end of behavioural sleep (Shein-Idelson et al. 2016). This is the clearest evidence for the early evolution of both SWS and REM prior to the evolution of thermogenesis and the warm blooded animal. The authors noted that the SWS-period was temperature dependent; warmer temperature reduced the period length. Animals in this experiment were kept at constant temperature but lizards in the wild would encounter environmental temperature changes natural to lizards that may produce different results. However, in previous studies this may also have masked the underlying EEG structure. Some electrical features of sleep in bearded dragons is unique and not seen in mammals. This might suggest that ancient sleep was a necessary component that has evolved to a more complex sleep seen in mammals and birds today. Its origin may reside in early evolved brain structures such as the brain stem, perhaps with a role for the hypothalamus and

thalamus (*Shein-Idelson et al. 2016*). Lastly, this has implications for the study of local sleep discussed later as lizards such as the bearded dragon do not have a neocortex.

Birds occupy a different branch on the evolutionary tree and provide a counter point to mammals and lizards. Birds, like non-avian reptiles do not have a neocortex but appear to have comprehensive ECoG features of sleep (*Rattenborg et al. 2009*). A study of the Zebra finch (*Taeniopygia guttata*) showed strong evidence of SWS and REM stages. The latter was defined by delta to gamma ratios although gamma power alone was not enough to separate REM from NREM. The use of k-means analysis, a form of automated clustering, to separate data for sleep stage classification in this study is a welcome addition. It is particularly important where little previous work exists. Curiously, in the Zebra finch, the propensity for each stage changes throughout the night, with progressively less SWS and more REM (*Low et al. 2008*). It is not known whether this is a generalised avian feature. More recently it was shown, using in-flight ECoG recording, that great frigate birds (*Fregata minor*) are able to sleep whilst airborne. The authors report that this SWS can be unihemispheric, but can also distribute to both hemispheres suggesting unequivocally that birds have evolved to sleep during flight. However, sleep time during flight is drastically reduced in these birds to less than 8% of their normal grounded sleep (*Rattenborg et al. 2016*). This is a crucial discovery as these birds are well known to fly for long periods of the time (*Weimerskirch et al. 2016*). Evolutionary pressures enabling longer flight appear to have either reduced the need for, or increased the tolerance to, sleep pressure.

Sleep architecture within mammals is often considered the most complex but is by far the most well studied. To date, in mammals where ECoG recordings have been made, evidence of SWS, NREM and REM episodes has been seen. Regardless of species, in the transition from wakefulness to sleep, NREM sleep always proceeds REM sleep. Despite this, sleep variation between mammals is often underestimated. In architecture, mammals are highly variable in total duration and in bout lengths for both REM and NREM. The most striking difference is between animals with monophasic sleep, like humans, and others with polyphasic sleep like rodents. Polyphasic sleep is characterised by normal interruptions of wakefulness throughout the sleep phase. It is possible that this sleep fragmentation is an evolutionary response to predation; however, the effect of body size / mass is hard to ignore. Whilst small mammals are polyphasic, larger mammals, even prey animals, have consolidated sleep bouts (*Capellini et al. 2008*). Why should the mouse or rat have longer polyphasic sleep whilst horses and cattle have much shorter consolidated sleep cycles? There is an inherent link between body size/mass (M) and metabolic expenditure (J kg^{-1}): $\text{Metabolic rate} = aM^b$, where b is approximately $\frac{3}{4}$. This is known as Kleiber's law. Thus, small animals have proportionally higher metabolic rates. Differences in the sleep architecture between species could be due to energy usage and this is illustrated by the common shrew (*Sorex araneus*). The Sorex displays remarkable sleep architecture that relates to food consumption. A study of three different Sorex species showed that whilst on average they spend 55 minutes foraging, under the same conditions they also sleep in bouts that are 64 minutes long. Furthermore, each period of activity contained shorter rest bouts of several minutes (*Jarmo Saarikko and Hanski 1990*). The authors found that the likelihood of sleeping was most correlated to the amount of food found in the gut of the shrew, although the duration of sleep was not. The small rests during the

active periods are comparable to daily torpor - an energy saving characteristic particular to small mammals and birds (*Ruf and Geiser 2015*).

A comprehensive meta-analysis of literature data by Capellini *et al* failed to find a relationship between the threat of predation and sleep bout length. If anything, animals at higher risk of predation during sleep appear to sleep slightly longer than those at lower risk. This effect remains even when accounting for body mass. Polyphasic sleeping is associated with small mammals, and perhaps inherently coupled to their energy requirements, but as a consequence they also have a longer total sleep time compared to their larger relatives. Perhaps polyphasic sleep is the inefficient ancestor of monophasic sleeping, although more data is required to support this conclusion (*Capellini et al. 2008*). So it appears that, at least within small mammals, energy usage is inherently coupled with sleep and the pressure of predation may be smaller than previously thought.

The lives of large predators are different. Energetically demanding, hunting comes with significantly higher costs for survival that might be reflected in longer sleep times (*Savage and West 2007*). Moving at speed or with endurance is the inherent advantage of the high-metabolic demands of endothermic animals and may come with an energy cost. Equally, prey animals spend necessarily large amounts of time consuming energy-sparse materials. These grazing times could not be accommodated by the waking hours of the predator. And so, even without the risk of direct predation, a predator-prey relationship may exist. However, sleep consolidation is known to increase with body size. This more efficient sleep may serve large prey animals, whilst smaller predators sleep longer, but have little pressure to consolidate further (*Capellini et al. 2008, Savage and West 2007*).

Sleep patterns in primates show variation across branches of the evolutionary tree. Coincidentally, this is seen in the evolution from sleeping in trees (arboreal sleeping) to sleeping on the ground. *Homo sapiens* sleep an average of seven hours, the shortest of all primates. In contrast, the genetically distant mouse lemur (*Microcebus murinus*) and the owl monkey (*Aotus trivirgatus*) sleep for between 13 to 17 hours. Interestingly, the most striking separation between us and other primates is found in the proportion of REM. Humans spend around 22% of sleep (~1.5 hr) in REM whilst all other primates spend proportionally less, from 18% in Rhesus macaque (*Macaca mulatta*) to 6% in the grivet or African green monkey (*Chlorocebus aethiops*). The same authors analysed the effect of seven distinct variables on sleep time using Bayesian methods. In particular, they focused on: primate position on the phylogenetic tree, body mass, diet composition, group size, birthing interval, endocranial volume and the activity period. When considering the predicted sleep duration for each primate, only *Homo sapiens* fall outside the 95% credible interval. The same discrepancy is true of proportions of REM sleep. Thus, in attempting to understand sleep we should consider that *Homo sapiens* may be outliers in our manner of sleep even compared to our closest relatives. It should be noted, of course, that no mention of excluded parameters is given in this work or evidence that this model may not be better predictive of human sleep using different variables (*Samson and Nunn 2015*). These additional variables could include features of modern living such as artificial light. Cognitive explanations for the observed differences propose that *Homo Sapiens* may have evolved to sleep more efficiently, and hence need less sleep. Perhaps more REM sleep is a consequence of a greater need for cognitive processing

during sleep (*Vyazovskiy and Delogu 2014*). For all animals, regardless of the way they sleep, the result of sleep-onset is the same - loss of consciousness is the defining shared characteristic of sleep.

1.1.2. Quantifying Consciousness

Sleep results in a loss of consciousness, but “consciousness” is often considered an esoteric concept, hard to define in scientific terms but easiest to comprehend through its absence. Still, reversible control over this process has revolutionised medicine by facilitating surgery under general anaesthetics. The process of sleep appears to necessitate loss of consciousness but being unconscious is insufficient for the sleep processes. So how can we define a consciousness transition such as sleep to wake?

Studying states of consciousness is intrinsically linked to the analysis of the electroencephalogram (EEG) in humans or electrocorticography (ECoG) in rodents. This is best described as an ‘epiphenomenon’ resulting from normal brain activity and providing a surrogate measure of behavioural states and the timing of their transitions. For example, an awake brain state is electrically different to a sleeping or anaesthetised brain. In this way EEG recording facilitates the measurement of activity inside the brain, information that cannot be achieved by observations alone.

Electrical measurement of neuronal networks can define a brain state as either awake or in one of many states of unconsciousness including NREM, REM, torpor, hibernation and, with the aid of other devices, even coma or a persistent-vegetative state. The electrical activity detected reflects synaptic synchronisation across the local network: currents generated, as ions move across membranes, are recorded as an electrical potential when compared to a ground or reference value. This observed potential, V_e can be recorded in different ways attributing different levels of sensitivity and spatial resolution. Whilst these measurements take the form of EEG in humans, in animals such as mice and rats the ECoG is essential. The key difference is the direct implantation of electrodes into the brain tissue. The trade-off is a loss of spatial resolution. Finer measurements with better spatial resolution can be made by the addition of bipolar electrodes to detect local field potentials (LFP) or special equipment for single unit spike recordings and more (*Buzsáki et al. 2012*). In recent years, progress has been made in understanding the molecular mechanisms behind ECoG signals, although some controversy remains. For the moment, it seems that while a sleeping animal is necessarily unconscious, unconsciousness is not sufficient for the process of sleep.

1.1.3. Why do we sleep?

The need to sleep is fundamental but there are good reasons not to. Periods of quiescence are vulnerable to predation and prevent the seeking of new food or mating behaviour. And yet sleep persists as a physiological trait and there is little convincing evidence for strategies to remove or significantly mitigate sleep entirely. Instead, the evolutionary pressure acts to restrict sleep at times of need, for instance, during the mating season of the Sand piper (*Calidris melanotos*) or during the long migrations of Frigate birds (*Fregata minor*) (*Lesku et al. 2012, Rattenborg et al. 2016*). Despite these adaptations

these animals must sleep and do so normally at other times. Thus within the varied sleep, observed across the evolutionary tree, there is a component that appears to be indispensable for complex life.

The two arms of sleep are known as Processes S and Process C (*Borbély 1982, Borbély et al. 2016*). The former is the homeostatic drive that ensures two things: first, the longer we are awake the more likely we are to sleep; and second, if we are awake too long we will sleep more to compensate. Process C is different, it represents the circadian rhythm and its entrainment to the light-dark cycle. This has been referred to as a gate-like mechanism, because it does not directly control sleep-wake transitions; instead, it realigns those transitions over time with cues from the master clock, the suprachiasmatic nucleus (SCN) (*Saper et al. 2005*). These processes are regulators but they can provide clues as to the functions of sleep. Principally, any restorative function of sleep must fulfil several characteristics. It must accumulate overtime and dissipate with time asleep. In doing so, it must couple to and facilitate process S. This is our key question.

Lack of sleep has propounded physiological effects. In a review of nine studies in rats deprived of total sleep, or selectively of REM or NREM, all animals lost weight, despite consuming more, and developed skin lesions. After a time, progressive hypothermia also develops. The end result of sleep deprivation is always death (*Rechtschaffen et al. 1989, Everson 1995, Rechtschaffen and Bergmann 2002*). We sleep because sleep is necessary for life but there are many competing theories as to why.

1.1.4. Sleep to move

Could the evolution of sleep be intrinsically tied to the evolution of the nervous system? Electrical signalling is an essential component of any system of cells that requires motility, and organisms that do not undertake movement do not require neuronal circuits. In evolutionary terms these first systems were simple, like the nerve net of the jelly fish, but developed into complex systems of nerve and muscle that could facilitate complex stimulus-orientated motion (*Arendt et al. 2008, Bucher and Anderson 2015*). This has obvious benefits, from seeking resources and mates to avoiding predators and danger, but this development also has a cost. Neural circuits and muscular activity are energy demanding processes which the host organism must satisfy. There are two reasons that this could necessitate a sleeping process. In the first instance, the system must be maintained and repaired and protected from deterioration (*Vyazovskiy and Harris 2013*). Secondly, higher energy consumption in short bursts of activity requires energy saving when movement is not required (*Siegel 2009*). In the case of nerves and musculature repairs, this may be best carried out when inactive and a sleeping state conserves energy. This is a rest and recovery hypothesis of sleep.

This energy hypothesis has several disadvantages. Consuming more food does not relieve tiredness - indeed physical tiredness from expending energy through muscular exertions does not directly induce sleepiness (*Driver and Taylor 2000*). Instead we feel muscular fatigue and lactic acid, which dissipates with our oxygen debt, whilst sleep effects are delayed. Whilst hormonal processes during sleep may be important for muscle repair, changes in sleep architecture following exercise are not proven. Where increases have been observed, they have been attributed to the increase in body temperature as

cooling to normal levels following exercise, can abolish the additional sleep drive (*Horne and Moore 1985, Montgomery et al. 1988, Driver and Taylor 2000*). And yet we feel progressively more tired through the course of the day, even without extreme muscular exertion. Our sleep debt appears to grow consistently and must be repaid. Lack of sleep, or sleep deprivation, leads to a directly proportional sleep rebound (*Rechtschaffen et al. 1989, Rechtschaffen and Bergmann 2002*). This process of tiredness and restoration is homeostatic.

A key component of the homeostatic regulation of sleep is the idea of accumulation and dissipation of a wakefulness marker. This may be a waste product to be removed, or a surrogate that tracked the progression of prolonged wakefulness. At the turn of the previous century, seminal work in dogs suggested the existence of a sleep-promoting hypnotoxin. This involved taking the cerebral spinal fluid of sleep deprived dogs and reinjecting it into control animals. To their surprise this made the controls sleep (*Ishimori 1909*). In 1997 it was suggested that adenosine might be such a marker. Adenosine is a primary metabolic component of the electron transport chain and energy storage unit for the nerves and muscles. *In vivo* micro dialysis experiments in the basal forebrain revealed that adenosine builds up with time awake and continues to do so under sleep deprivation. This increase is dissipated during recovery-sleep (*Porkka-Heiskanen et al. 1997*) *Ex vivo* experiments have shown that adenosine can inhibit cholinergic neurons in the basal forebrain (BF) that are involved in wake promotion. Follow up work has revealed that, on these neurons, adenosine activates the adenosine A1 receptor (A1R), which is G α_i coupled, inducing inhibition. Similarly, an injection of antisense oligonucleotides against A1R mRNA, into the BF, reduces the likelihood of NREM episodes and increases wakefulness (*Thakkar et al. 2003*).

This idea was extended in an interesting paper by Halassa *et al*, suggesting that astrocytes are involved in sleep homeostasis through the regulation of extracellular adenosine. Blocking gliotransmission with tetanus toxin light chain, using the Tet-off expression method, did not affect sleep architecture or total NREM or REM, but it reduced the appearance of sleep pressure measured in ECoG slow wave activity (SWA). This method also induced a tolerance to the sleep attenuating adenosine A1R antagonists, suggesting that direct coupling between adenosine and these receptors is a key factor in sleep pressure (*Halassa et al. 2009*). Whilst A1Rs are ubiquitous in the brain, others are more localised. The A2a receptor (A2aR) is expressed near the ventrolateral preoptic area (VLPO) (*Scammell et al. 2001*) This is interesting as A2aR knock-out mice are resistant to the adenosine receptor antagonist, caffeine (*Huang et al. 2005*).

The “rest and recovery” hypothesis of sleep may involve the build-up and dissipation of waste products. One of these homeostatic markers may be adenosine as a by-product of energy expenditure by nerves or the brain or muscles. These waste products may be removed most effectively by sleep.

1.1.5. Is sleep for cleaning the brain?

The idea of sleep as a process to remove waste products is an old argument that continues to advocate sleep as housekeeping process. For instance, it has been shown that β -amyloid levels in the interstitial

fluid (ISF) of C57BL/6 mice increases with time awake and experiments in mice that over express amyloid precursor protein (APP^{swe}/PS1^{dE9}) chronic sleep deprivation increases β -amyloid plaque formation (Kang *et al.* 2009). More recent research has investigated the role of cerebral spinal fluid clearance from the brain during sleep. Sleeping or anaesthetised mice injected with a tetramethylammonium tracer and imaged using a two-photon laser were found to have higher levels of diffusion in their neocortex during the sleep phase, compared to their awake control (Xie *et al.* 2013). Quantification- revealed a 60% increase in the interstitial space, which the authors suggest increases the rate of cerebrospinal fluid (CSF) exchange. As a consequence, they detected clearance of labelled β -amyloid (^{125}I -A β_{1-40}) at higher rates from the prefrontal cortex, during both sleep and anaesthesia. This is an exciting advancement in the understanding of possible roles for sleep. However, it is reasonable to ask whether an increase in metabolite clearance of just two-fold compared to waking is significant in biological terms: it must have sufficient value for an organism to accept long periods of inactivity (Xie *et al.* 2013). Equally, it is not clear why inactivity and unconsciousness would be necessary for this process. A study by the same authors has previously shown evidence for a novel paravascular network that envelopes brain arteries and allows subarachnoid CSF to enter the brain parenchyma. Tracer dyes were observed passing through the cortex and thalamus before being cleared via the rhino and deep cerebral veins (Iliff *et al.* 2012).

At the heart of this hypothesis is the idea that unwanted products build up as a result of neuronal activity and must be removed for normal function. At the molecular level waste products have been hard to identify. Rates of turn-over of neurotransmitters do not appear to be affected by prolonged periods of wake and neurons do not clearly deteriorate in function. There is also emerging evidence that neurons may undergo periods of rest and these silent events could be a sign of local homeostasis (Vyazovskiy and Harris 2013). Furthermore, some recent work using electron microscopy has suggested that changes can be seen in the organelles of pyramidal neurons of sleep-deprived adolescent mice (de Vivo *et al.* 2016).

1.1.6. Is sleep for brain networks?

New techniques and methods of analysis have recently advanced our understanding of how sleep may be beneficial for neuronal networks. These hypotheses can be expressed as simple premise “Sleep is by the brain, for the brain” (Hobson 2005). These can be characterised in terms of information processing, synaptic plasticity and neuronal recovery.

The idea of local sleep was suggested by Vyazovskiy *et al.* Natural sleep, as previously discussed, is characterised by slow waves (SWS) and 1-4 Hz delta oscillations. However, multiunit activity in the cortex has shown that bouts of neuronal silencing, known as off-states, are high at the beginning of sleep and decrease over the course of sleep (Vyazovskiy *et al.* 2009). Analysis of the microstructure of ECoG signals in rats revealed that during wake, the pattern of neuronal activity was interspaced with similar neuronal silencing. Most importantly, prior to the onset of a NREM episode, neurons were observed undergoing periodic silencing with increasing likelihood - the more time spent awake, the

more of these events occur. It was proposed that this local sleep increases and culminates in global sleep (Vyazovskiy *et al.* 2011). It may explain the decline in cognitive function seen with sleep deprivation.

What process in the brain requires increased local sleep or neuronal silencing? The idea proposed by Vyazovskiy and Harris is a need for neurons to rest (Vyazovskiy and Harris 2013). Whilst other cells in the body can rest individually neurons form networks; its hard to rest if your neighbour is active, so groups of neurons fall silent together. This synchrony then spreads in a wave-like manner (Massimini *et al.* 2004). It may be that brain areas showing signs of local sleep are consequently less able to perform their functions. As this synchrony grows towards global sleep, the effects of sleep deprivation are apparent: loss of focus and cognitive function (Vyazovskiy *et al.* 2011). Then, local sleep is not sufficient but it may serve to protect neurons before they reach a critical level of metabolic stress. This stress has been likened to that of tired muscles that activate an unfolded protein response (UPR) pathway to prevent further damage and degrade unwanted proteins (Vyazovskiy and Harris 2013). Interestingly, if neuronal rest equates to down-states, and these downstates are causally responsible for the observation of 1-4 Hz slow waves, then this maintenance process is testable. Many anaesthetics can induce slow waves and it serves that administration of such a drug would be sufficient to replace sleep.

A final hypothesis attempts to explain why the brain is necessarily unconscious during the sleep process. This is called the synaptic homeostasis hypothesis and suggests that synaptic plasticity is inherently coupled to sleep. In this model, synaptic strength, acquired for some awake stimulus, is reset while the brain is disconnected from new stimuli. It is only when unconscious that these neurons can sample the brain in a quieter state and perhaps rationalise information that has been learnt and stored in memory (Vyazovskiy *et al.* 2008, Tononi and Cirelli 2014)

1.1.7. Circuitry of sleep and waking

Although the purpose of sleep has proved frustrating to uncover, researchers have made good progress in understanding the neuronal circuits that regulate NREM sleep timing, its onset and the transitions to REM and to waking. The hope is that understanding how sleep works might illuminate the why. But we seem to be quite a way from a full description of the circuitry that controls sleep induction, as new types of neurons involved are reported almost by the month.

Neurons whose activity induces NREM sleep or wakefulness

In healthy animals, the first stage of sleep following wakefulness is always NREM sleep. The homeostatic drive that results in NREM sleep is intrinsically slow, increasing with the hours spent awake and dissipating during sleep. This homeostatic pressure influences the timing of NREM by interacting with neural networks. Neural circuits regulate immediate wake to sleep transitions which occur over minutes not hours. This is important because slow progression in and out of sleep would be vulnerable and without the benefit conferred by sleeping. Fast transitions then lead to the hypothesis of a neural switch that could enforce these fast transitions between different states.

The preoptic hypothalamus is most widely implicated in the control of NREM sleep onset following the classical work of von Economo (*Economo 1930*). Early experiments using electrical stimulation in the basal forebrain (BF) were shown to illicit electrical and behavioural sleep in the cat (*Sterman and Clements 1962*). Subsequently, bilateral lesions in the preoptic area were shown to suppress sleep (*McGinty and Sterman 1968*). Recordings from the BF revealed separate populations of NREM and wake active neurons (*Szymusiak and McGinty 1986*).

Since then, studies by Saper *et al* have shown that c-Fos expression, a marker of recent activity, was increased in GABAergic neurons of the ventrolateral portion of the preoptic area (VLPO) during sleep, suggesting a role for the VLPO in sleep promotion (*Sherin et al. 1996*). Furthermore, VLPO's projections to the mammillary nucleus were indicative of an inhibition of arousal centres. Injections of cholera toxin B (CTB), which undergoes retrograde transport, into VLPO revealed reciprocal afferents from the tuberomammillary nucleus (TMN) as well as the raphe nuclei (*Sherin et al. 1998*). This laid the foundation for a simple model that became known as flip-flop - mutual inhibition that could account for stable state-transitions into and out of sleep. Single unit recordings from VLPO revealed tonic firing at <2 Hz during wake, increasing to 4-8 Hz during NREM and further with time spent asleep (*Szymusiak et al. 1998, Takahashi et al. 2009*). Lesioning of specific cell groups in this region decreases total NREM sleep (*Lu et al. 2000*). VLPO itself may have a high proportion of galanin positive neurons but their significance is unresolved (*Saper et al. 2010, Sherin et al. 1998, Lu et al. 2000*). Although wake-active neurons have been recorded in this region, it is not known how they contribute to the overall circuit (*Modirrousta et al. 2004, Takahashi et al. 2009*). It turns out that during sleep there are also many active GABAergic neurons in the broader lateral preoptic region, and these also promote sleep (*Zhang et al. 2015*). Thus, there may have been undue emphasis on VLPO in previous work. Figure 1.2 summaries the circuit connections for NREM and wake that have so far been dissected.

Histaminergic neurons in the TMN are wake-active neurons. Although not exclusive in this regard, histaminergic activity is positively correlated with vigilance. Histamine neurons increase their firing to approximately 10 Hz during wake, oscillating with behavioural correlates of arousal (*Takahashi et al. 2006*). The preoptic area has many nuclei that project posteriorly in the hypothalamus, some to the histaminergic neurons in the TMN and often to other wake-active nuclei. Histaminergic neurons in the TMN are able to stimulate arousal and are thought to mediate the sedative effects of 1st generation anti-histamines (*Reiner and Kamondi 1994, Thakkar 2011*). However, this story is perhaps more complicated than it seemed - histaminergic neurons also express GABA and directly innervate cortical areas. Removal of the vesicular GABA transporter (VGAT) from these neurons stimulates hyperactivity in these mice, and so inhibition may have a role in fine tuning arousal (*Yu et al. 2015*).

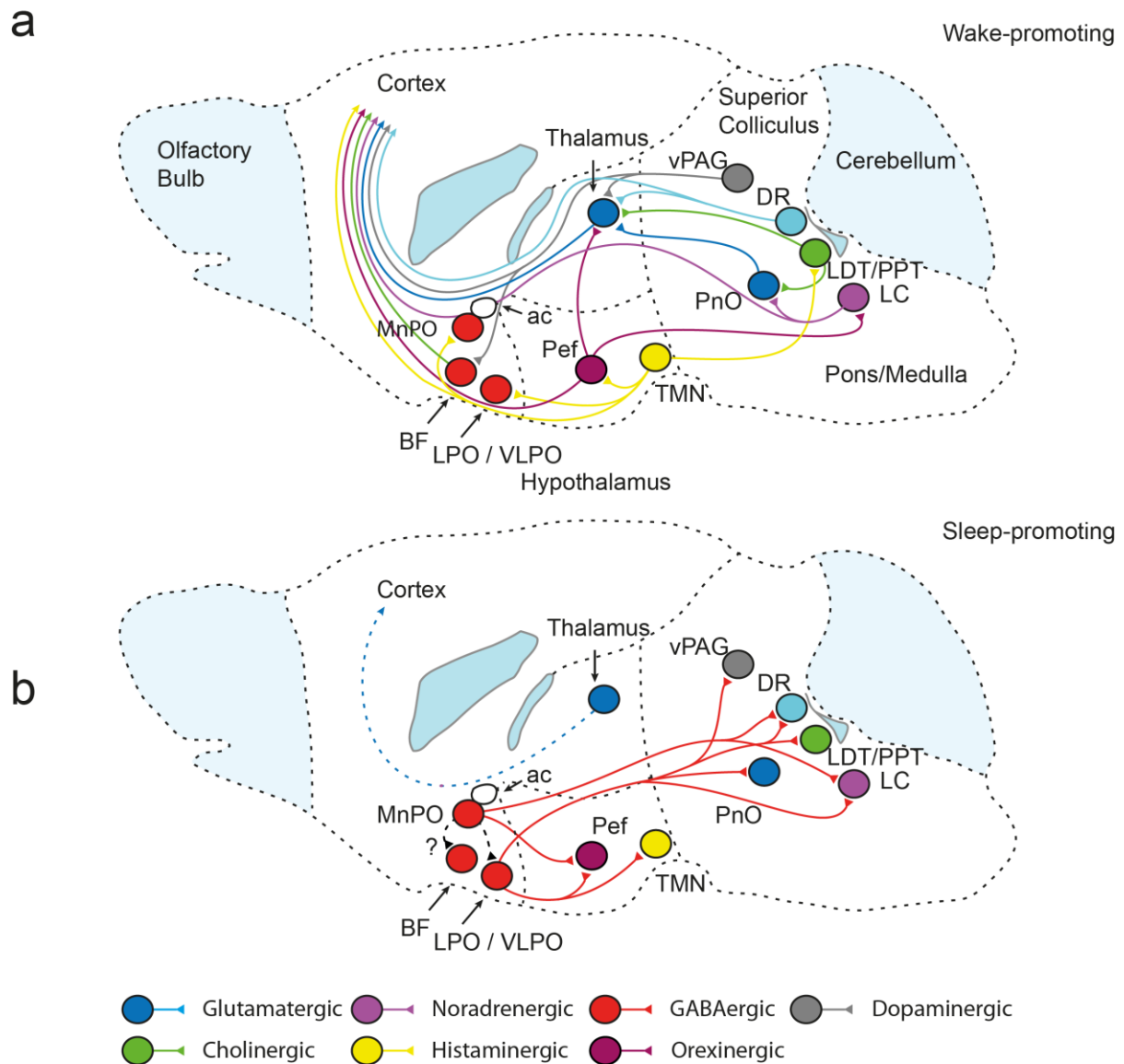


Figure 1.2 – Circuits of Sleep and Wake. Wake promoting nuclei and their projections contrasted against sleep promoting nuclei. (a) Circuits that promote wake. Projections from the reticular activating system (RAS) in the Pons, including LC, LDT, PPT and DR nuclei, innervate both thalamus and the neocortex. In the hypothalamus, orexinergic projections from the Pef and histaminergic neurons of the TMN project to the preoptic area and back to RAS nuclei. MnPO and LPO / VLPO firing is suppressed during wake. Pef also innervates the cortex as do cholinergic projections of the BF. The glutamatergic parabrachial nucleus (Pb) is not shown for simplicity but is active in wake and REM sleep and projects to the BF and LH as well as the cortex. (b) circuitry that promotes transitions from wake to NREM. Dominant GABAergic inhibition of wake active nuclei from the preoptic area. GABAergic projections from the LPO and VLPO region inhibit histaminergic neurons of the TMN and wake active Pef, LDT, PPT, LC, DR and vPAG nuclei. MnPO inhibits Pef, DR and LC. SOM⁺ GABAergic neurons of the BF also promote sleep. The connections between MnPO and LPO / VLPO are not well understood. Thalamocortical loop (dashed line) during sleep represents sensory isolation. Abbreviations: Laterodorsal tegmental nucleus (LDT); Pedunculo pontine tegmental nucleus (PPT); locus coeruleus (LC); dorsal raphe nucleus (DR); Pontine reticular formation (PnO); ventral periaqueductal gray (vPAG); tuberomammillary nucleus (TMN); Perifornical area (Pef); Lateral hypothalamus (LH); anterior commissure (ac); Median preoptic area (MnPO); Medial preoptic area (MPO); Basal forebrain (BF); Lateral preoptic area (LPO); Ventrolateral preoptic area (VLPO). Adapted from *Franks 2008, Saper et al. 2010*

A NREM sleep-active nucleus in the preoptic area that has substantial projections, but does not innervate the TMN directly, is the MnPO. MnPO contains sleep active neurons that have direct projections to the VLPO, as well as the LPO and BF (*Chou et al. 2002, Uschakov et al. 2007*). By one estimate, almost 75% of the total population is thought to increase its firing during the sleep phase (*Suntsova et al. 2002*). However, this firing is not linked to the transition to sleep but commences prior to sleep onset. There are two explanations. The first is that MnPO neurons have a role in sleep pressure, as evidenced through the induction of c-Fos during sleep deprivation. This effect is absent from VLPO (*Gvilia et al. 2006*). The second explanation is that MnPO neurons have a role in thermoregulation – they act to reduce core temperature. This would explain their sleep activity and circadian-like rhythm, but not their direct projections to VLPO. The role of MnPO in thermoregulation is discussed in more detail in section 1.2. Other MnPO projections have been found in the locus coeruleus (LC) and dorsal raphe (DR) nuclei, but the function of these networks is unresolved (*Uschakov et al. 2007*).

The lateral hypothalamus sends dense projections to the preoptic area both medially to MnPO, that might relate to its role in temperature regulation, and also to the area surrounding VLPO. Optogenetic stimulation of orexin neurons, also known as hypocretin neurons, in the lateral hypothalamus can wake mice from both NREM and REM sleep (*Adamantidis et al. 2007*). More recently, work by Xu *et al* discovered neurons in the BF that influence NREM sleep timing. They found neurons expressing Choline acetyltransferase (ChAT) and neurons expressing the vesicular glutamate transporter 2 (VGLUT2+), were active during wakefulness, consistent with their role in the ascending arousal system. But a subset of GABAergic neurons controls NREM onset. Of this subset, somatostatin (SOM⁺) neurons, but not parvalbumin expressing (PV⁺), neurons were active during the sleep period, and optogenetic stimulation of SOM neurons increased the probability of observing a NREM episode by almost 20%. Of the other three populations, all increased the probability of wakefulness when they were active (*Xu et al. 2015*). Another population of GABAergic neurons that promote wake has been discovered in the lateral hypothalamus. These cells densely innervate the VLPO region and expand the paradigm of sleep-wake regulation further (*Venner et al. 2016*). It has been found by other groups that similar, or identical GABAergic neurons in the lateral hypothalamus project directly to thalamic reticular neurons and activation of this inhibitory circuit is sufficient to wake mice from NREM but not REM sleep (*Herrera et al. 2016*). This effect could also be replicated under general anaesthesia. The subpopulation of these neurons is unknown, but may share features with wake promoting PV⁺ GABAergic neurons identified by Xie *et al*. The basal forebrain neurons initiate gamma band oscillations of around 40 Hz through the inhibition and synchronisation of cortical interneurons (*Kim et al. 2015*).

Neurons whose activity regulates REM sleep

The TMN histamine neurons, locus coeruleus (LC), dorsal raphe (DR) are silent during REM (*Takahashi et al. 2010, Saper et al. 2010*). Some evidence suggests that the pedunculo pontine tegmentum (PPT) and laterodorsal tegmentum (LDT) increase their activity during REM and, when preceded by NREM, activation of cholinergic subpopulation of either the PPT and LDT using optogenetics, is sufficient to initiate REM (*Van Dort et al. 2015*). Curiously, previous work in the preoptic area showed that stimulation of MCH neurons can lengthen REM episodes primarily through inhibition of histaminergic

arousal neurons in the TMN. Inhibition of the same MCH neurons modulated hippocampal theta rhythms but with no further REM effect (*Jego et al. 2013*). Orexin neurons, as discussed previously, are also able to inhibit MCH neurons and may do so as part of an arousal pathway (*Apergis-Schoute et al. 2015, González et al. 2016*). They also project to the LC (*González et al. 2012*). Stimulating the PPT or LDT was not able to extend REM episode duration suggesting separate circuitry is involved in REM initiation and maintenance. Recent work by the Yang Dan lab has suggested different circuits for REM control. Optogenetic stimulation of GABAergic populations in the ventral medulla (VM) of GAD2-cre mice can promote REM onset when preceded by a NREM episode. Stimulation trials during wake did not produce REM onset, only the probability of further wake-episodes (*Weber et al. 2015*). GABAergic subpopulations of these neurons are as yet unidentified. REM circuits are less well understood than those for NREM but, in general, an interplay between cholinergic, monoaminergic and GABAergic populations around the pons is thought to regulate REM timing (*Saper et al. 2010*).

Additional questions, still unsolved, about the circuitry governing sleep-wake transitions include: how do homeostatic mechanisms interact with the sleep circuitry to facilitate recovery-sleep? What is the key difference that separates natural sleep from a state of simple unconsciousness?

1.2. Staying Warm

1.2.1. Why did endotherms evolve?

Generating warmth is an unusual physiological trait. More animals rely on other means to establish sufficient warmth to move and function appropriately. While the endotherms can generate their own heat and are not reliant on their environment, ectotherms are unable to regulate their core temperature. But being endothermic has inherent advantages and principal of these is the ability to move fast and with endurance. Additionally, thermogenesis allows animals to move at night when the warming power of the sun is unavailable. This is an unrivalled advantage in gathering energy, particularly via predation. Understanding the evolution of this trait is particularly challenging because few markers exist in the fossil record, although, some progress has been made. Several hypotheses have been suggested and at their heart is the advantage granted by an enhanced metabolism. For example, an environmental demand for ever increasing aerobic capacity may have resulted in an evolutionary pressure to raise resting metabolic rates to the high level seen in mammals and birds today. Alternatively, cold pressure may have brought about a similar upward shift in basal metabolism or it may have been a consequence of parental warmth that improved the prospects of their offspring (*Bennett and Ruben 1979, Farmer 2000, Hillenius and Ruben 2004, Nespolo et al. 2011, Little and Seebacher 2014*).

Whichever driver resulted in endothermic branches in the phylogenetic tree, it is a fundamental part of mammalian physiology today. The preoptic hypothalamus is responsible for many autonomic functions and the regulation of temperature is no exception. Internal and external sensory information is integrated here to discern the right effector outcome. The way this integration is performed and the basis for thermogenic control is the focus of this section.

1.2.2. What is thermogenesis?

Thermogenesis is the production of heat and its molecular machinery are based in brown adipose tissue (BAT). BAT is sometimes described as a 'multidepot' organ because it is found in several distinct locations throughout the body. In the mouse there are two subcutaneous depots, referred to as anterior and posterior, as well as four visceral referred to as: mediastinal, mesenteric, retroperitoneal and abdominopelvic depots. Together, these stores of fat are the central heaters that keep core temperature elevated (*Cannon and Nedergaard 2004*).

The BAT is uniquely positioned to increase heat generation as it contains large numbers of mitochondria. Mitochondrial oxidation is the primary source of non-shivering thermogenesis. Crucially, these mitochondria express UCP-1 (*Argyropoulos and Harper 2002*). This protein is inducible and able to subvert the efficient function of the electron transport chain (ETC) for adenosine triphosphate (ATP) synthesis. Normal respiration generates a proton gradient across the inner membrane of the mitochondria, which is dissipated by the ATP-synthase and the energy stored in the production of ATP

(*Rolfe and Brown 1997*). UCP-1 forms a membrane pore that opens at high ratios of fatty acid to di and trinucleotides, and allows protons to flow back down their gradient bypassing the ATP-synthase. In the first instance, heat is produced through respiration as the protons flow back through UCP-1 - their electrochemical energy is not stored as ATP (*Nicholls 1983, Ricquier 2011, Busiello et al. 2015*). Furthermore, fatty acid oxidation is also increased, which produces more NADH. This stimulates the proton gradient by donating electrons to the electron transport chain to repeat the process. This has been likened to a 'short-circuit' as it diverts metabolic energy production away from molecular synthesis and instead it is lost as heat. This added warmth is recirculated through the body by the blood (*Ricquier 2011, Busiello et al. 2015*).

The nuclear receptor Rev-erb α strongly regulates the circadian control of temperature. This is through transcriptional repression of UCP-1 that is highly expressed in BAT (*Argyropoulos and Harper 2002, Gerhart-Hines et al. 2013*). Changes in Rev-erb α expression alter the sensitivity of mice to cold challenge. During the day-night cycle, when the mice are more tolerant to cold at ZT22, Rev-erb α is low. This contrasts with ZT10 when Rev-erb α is at its peak. These events also coincide with highest and lowest points in UCP-1 expression. Rev-erb α knock-out (KO) mice also lose their daily circadian temperature rhythm as well as the circadian oscillation of UCP-1 itself, where levels remain high. Cold tolerance throughout the day is then markedly increased (*Gerhart-Hines et al. 2013*). Rev-erb α is one of a family of nuclear receptors previously discussed - its transcription is induced by the molecular clock, heterodimer CLOCK- BMAL1, and it forms a core part of the peripheral circadian rhythm (*Guillaumond et al. 2005, Ko and Takahashi 2006*).

1.2.3. Temperature sensing: From the skin to the brain

Transient receptor potential (TRP) channels on the skin are sensitive to changes in temperature. These ion channels induce depolarisations in sensory neurons under the skin, that synapse with the spinal cord, and transmit thermal information to the brain. TRPV1-4 channels in particular are warm-sensitive with Q₁₀ values of 5 or greater. Variants within this channel family that also have warm-sensitivity, include TRPM2-5, whilst TRPM8 is cold-sensitive (Q₁₀ <0.2) along with TRPA1 and C5, although each respond to a different thermal range (*Bautista et al. 2007, Wang and Siemens 2015*). These receptors also play significant roles in noxious stimulus sensation from inflammatory pain to injury and sickness. The signal is transduced from cutaneous thermoreceptors on the skin to primary somatosensory fibres that synapse with lamina-1 neurons of the spinal dorsal horn.

The ascending afferents to the brain split into spinothalamic and hypothalamic pathways. The former projects directly to the thalamus and onto the primary somatosensory cortex. Before this split sits the lateral parabrachial nucleus (LPb), accepting ascending cutaneous temperature signals and transmitting them to both thalamus and hypothalamus. The LPb does not require the thalamic relay to transmit cutaneous temperature signals to the hypothalamus (*Nakamura and Morrison 2008*). It is now thought that the thalamic relay is solely to transmit temperature perception. The LPb nucleus is activated by cold-challenge as determined by c-Fos expression. Retrograde labelling from the preoptic area

double labels the same LPb neurons. These LPb neurons use glutamate as a transmitter: the projections of the LPb nucleus to the MnPO can be blocked by glutamatergic antagonists that, in turn, block observed responses to cold-challenge (*Nakamura and Morrison 2008*). Other pathways transmit thermal information from around the body, like the abdominal viscera for example, to the LPb nucleus; however, many sensory signals representing blood pressure, satiety and thirst converge on the same nucleus (*Davern 2014, Alhadeff et al. 2014*). Thus, the LPb may act to integrate these signals to produce more coherent responses or to relieve physiological conflicts (*Morrison and Nakamura 2011*)

MnPO in the preoptic area receives LPb projections. Neurons in MnPO determine the LPb input as either too hot or too cold. This determination is probably mediated by separate populations of local neurons. The circuitry is summarised in Figure 1.2. Warm stimuli are processed from glutamatergic afferents where two efferents from MnPO to MPO determine the response. Warm stimuli continue as glutamatergic projections, whereas cold stimuli are thought to be mediated by GABAergic projections (*Nakamura and Morrison 2008, Nakamura and Morrison 2010*). In the MPO, these synaptic connections determine different effector outcomes by modulating secondary GABAergic projections between the MPO and dorsal medial hypothalamus (DM). These can either inhibit or disinhibit thermogenesis in BAT, as well modulate skeletal muscle activity (shivering) and produce either vasodilation or constriction. They may also influence species-specific behaviour such as panting and paw-licking (*Morrison and Nakamura 2011*). Transections just external to the preoptic area induce non-shivering BAT thermogenesis and it is suggested that this descending GABAergic circuit has tonic activity that under normal environmental conditions suppress excess heat production as well as other mechanisms, such as skeletal muscle thermogenesis (*Chen et al. 1998*). Projections from MPO to the rostral raphe pallidus nucleus (rRPa) also contribute to cold defence (*Morrison and Nakamura 2011*).

The response to temperature change is comprehensive. Changes in heart and respiratory rate and blood pressure are normal and inherently controlled through preoptic signal integration. How these separate processes are demarcated at the circuit level is hard to unravel but progress has been made in deterring functional subpopulations that are sufficient to initiate responses to temperature challenge in the MnPO-MPO axis.

1.2.4. The Warm-Sensitive and Cold-Sensitive Neurons

The first study to identify a region of the brain responsible for temperature defence was performed in 1938 by HW Magoun *et al.* These experiments identified regions sensitive to direct warming within the cat hypothalamus (*Magoun et al. 1938*). Sometime later, directly warm-sensitive (WSN) and cold-sensitive neurons (CSN) were found within the preoptic area (*Nakayama et al. 1961, Nakayama et al. 1963*). This appears paradoxical, as it is not strictly necessary for a sensory pathway to detect stimuli down-stream of the initial source. However, this may indicate the sensitivity, in general, of neurons to changes in temperature. Indeed, it turns out that many neurons throughout the brain increase their firing in response to heating, and not just those in the PO area (*Van Someren 2000*). Nevertheless, in the classical model, MPO neurons directly receive and integrate afferents from MnPO, such that a

GABAergic WSN will disinhibit a GABAergic projection neuron (Nakamura 2011). The WSN plays the role of a preoptic interneuron. And so direct sensing of temperature by these neurons may act as an integration signal to determine the magnitude of the response required. For instance, when the brain temperature is physiological but the environment is cold, only small adjustments are needed.

New ground has been broken recently with the discovery of putative markers for WSNs and their associated CRE mice. In the first of these papers, Song *et al* used Fura-2 based calcium imaging to identify WSNs (Song *et al.* 2016). Approximately 16% of cultured preoptic neurons were thermally sensitive under these conditions. A series of pharmacological experiments were then used to deduce

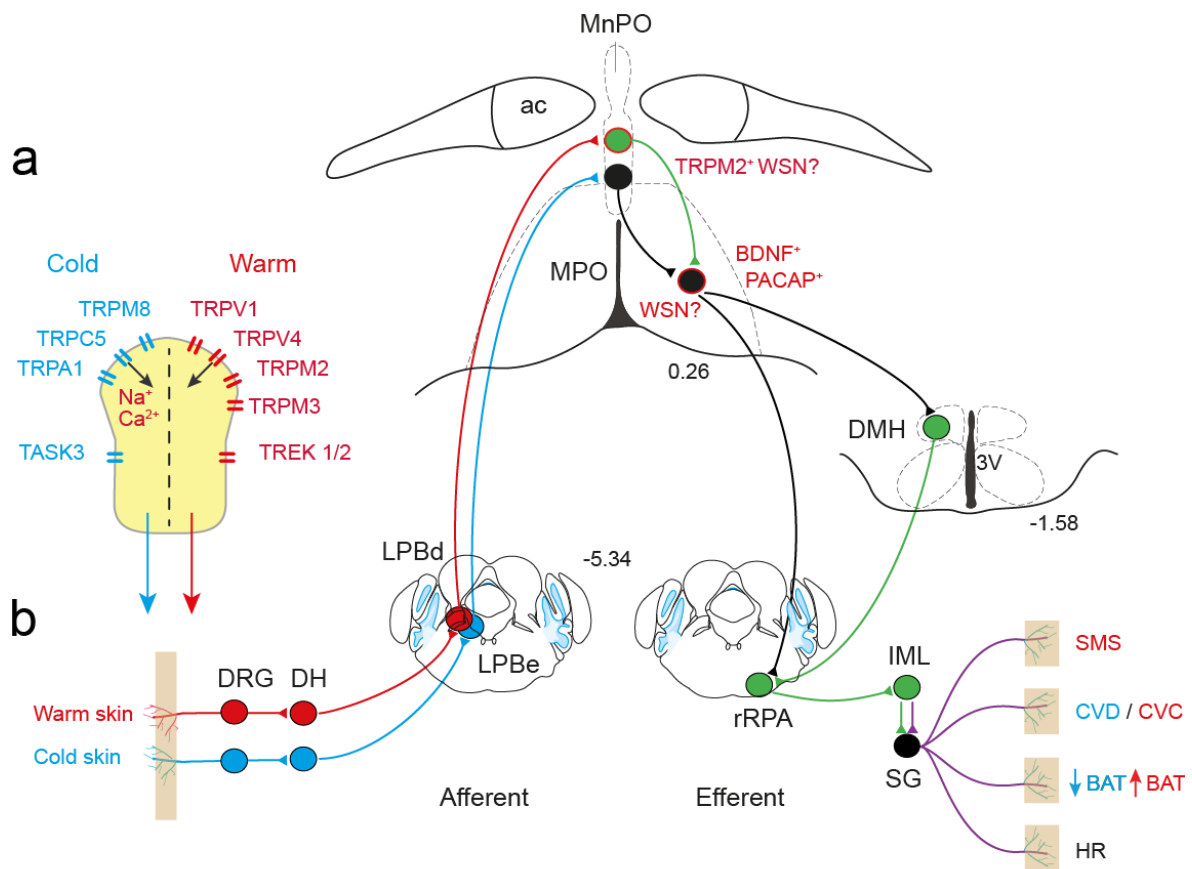


Figure 1.3 – Circuits of temperature regulation. (a) Detection of warm or cold temperature on the skin by sensory neurons expressing temperature sensitive channels. (b) sensory signals travel to DRG and DH to and on to the LPb. Cutaneous warm sensation is processed through the LPbd whilst cold sensation is processed through LPbe. Both glutamatergic afferents continue to the MnPO where temperature signals are discriminated. These neurons may be directly warm sensitive (red outline) and express TRPM2*. Warm signals are transmitted by glutamatergic afferent (green) to MPO whilst cold signals are relayed by GABAergic neurons (Black). In MPO, neurons that may also be warm sensitive (red outline) and expressing either BDNF or PACAP send GABAergic projections to the DMH and the rRPA. Glutamatergic (green) as well as serotonergic and adrenergic neurons (both purple) relay information to effector organs through the IML and the SG. Warming leads to CVD and a down regulation of BAT thermogenesis. Cooling leads to CVC, SMS and an increase in BAT thermogenesis. HR increase with both warming and cooling (Morrison and Nakamura 2011, Song *et al.* 2016, Tan *et al.* 2016). Abbreviations: Transient receptor potential (TRP) – Melastatin type (M), Vanilloid type (V), Ankyrin type (A) and Canonical type (C); TWIK-related acid-sensitive K⁺ channel 3 (TASK3); TWIK-related K⁺ channel (TREK); Dorsal root ganglion (DRG); Dorsal Horn (DH); Lateral Parabrachial external (LPbe); Lateral parabrachial dorsal (LPbd); Median preoptic nucleus (MnPO); Medial preoptic nucleus (MPO); Dorsal medial hypothalamus (DMH); rostral raphe pallidus (rRPA); Intermediolateral nucleus (IML); Sympathetic ganglion (SG); Skeletal muscle shivering (SMS); Cutaneous vasodilation (CVD); Cutaneous vasoconstriction (CVC); Brown adipose tissue (BAT); Heart rate (HR).

potential mediators of this response, resulting in TRMP2 as the putative candidate for preoptic warm sensation. Cultured preoptic neurons from *Trpm2*^{-/-} mice could not demonstrate a calcium response when warmed. A follow up assessment of *Trpm2-CRE* mice showed that *Trmp2*⁺ neurons in the preoptic area were also positive for both VGLUT2 (30%) and GAD67 (60%). However, only DREADD activation with CNO in *VGLUT2-CRE* mice, but not *GAD67-CRE*, was able to induce hypothermia (Song et al. 2016). This goes directly against previous work that WSNs are GABAergic (Nakamura and Morrison 2010).

A different approach was taken by Tan *et al* using a novel method known as phosphoTRAP. Activated neurons, such as those that respond to a warm stimulus, phosphorylate ribosomal protein S6. The ribosomes in these activated neurons can be extracted, immunopurified and their associated transcripts sequenced (Tan et al. 2016). This identified Brain-derived neurotrophic factor (BDNF) or Pituitary adenylate cyclase-activating polypeptide (PACAP) gene expression as markers for WSNs. BDNF-CRE and PACAP-CRE mice allowed both neuronal types to be optogenetically activated to induce hypothermia; the mechanism of heat loss was a combination of tail-vein vasodilation and a reduction in BAT thermogenesis (Tan et al. 2016). It is not clear to what extent these two populations of neurons overlap and what degree of similarity they have with the TRPM2⁺ neurons. The BDNF and PACAP genes are expressed in the VMPO and also in the MPO region. The work by Song *et al* only identifies the preoptic region as a whole and a large portion of the MnPO and MPO, as containing WSNs (Song et al. 2016). It is clear that these markers are all sufficient for induction of hypothermia in response to a warm environment but it is still plausible that they represent, at least in part, non-overlapping populations. Considering only VMPO-BDNF⁺ neurons with projections to the dorsal medial hypothalamus (DMH), more than 90% double-label with the GABAergic marker, Glutamate decarboxylase 2 (GAD2, also known as GAD65). Furthermore, optogenetic stimulation of their target, DMH-VGLUT⁺ neurons, inhibits the down regulation of BAT thermogenesis confirming the need for inhibitory projections (Tan et al. 2016). Thus the dorsal-preoptic area of MnPO and upper-MPO may contain a directly warm-sensitive population of VGLUT2⁺-TRMP2⁺ neurons that relay sensory information, but also monitor brain temperature to protect against exaggerated responses such as fever. In the ventral-preoptic area of MPO and VMPO, BDNF⁺ and PACAP⁺ populations of GABAergic neurons relay sensory information to glutamatergic neurons of the DMH to inhibit BAT thermogenesis. The following Chapter considers the relationship between sleep and temperature, and in particular the role that WSNs may have in the regulation of sleep. This is the core topic I attempt to address experimentally in this thesis.

1.3. To sleep in a warm bed

Thermoregulation is at the core of mammalian physiology. The circadian clock synchronizes the temperature oscillation with the day night cycle such that we are colder when we sleep. This rhythm is intrinsic to the peripheral clock and yet preoptic neurons show sleep-wake specific firing patterns. Anesthetic mechanisms recapitulated within the preoptic area show both sedation and hypothermia, suggesting a direct neuronal connection. (*Zhang et al. 2015*). Research on people, measuring sweat responses under warm blankets, has shown that we actively regulate our temperature during NREM but less efficiently in REM sleep (*Bach et al. 2002*). Some mammals have no REM thermoregulation at all (*Parmeggiani 2003*). Warming prior to sleep increases NREM consolidation and reduces REM (*Bach et al. 2002*). Lastly, chronic sleep deprivation induces progressive hypothermia (*Bergmann et al. 1989, Everson 1995*). Are there deeper connections between these two physiologies, and if so, what function is served by linking these autonomic behaviors?

1.3.1. Thermoregulation during sleep

In endothermic animals the sleep cycle is strongly correlated with the circadian temperature profile. However, the relevance of a neuronal circuit that links the temperature cycle to a healthy sleep physiology remains contentious. Studies of mammals and birds have suggested a torpor hypothesis of daily energy conservation over the circadian day, through lowering core temperature and metabolism (*Ruf and Geiser 2015*). This explains the inherent circadian temperature cycle without an additional coupling to sleep-wake regulation, and in fact, the temperature cycle can be uncoupled from the sleep-wake cycle by using forced desynchronisation protocols (*Wright et al. 2002*). Nevertheless, neuronal connections between sleep and temperature regulators, under this model, provide, at best, a timing signal to ensure these events correlate efficiently. The SCN is important here, guiding the rhythms of sleep onset, temperature regulation and even induction into hibernation (*Ruby et al. 2002*). Further, circadian temperature regulation is intrinsically linked to nuclear receptors and the peripheral clock and does not appear to require a reciprocal connection to sleep circuitry (*Gerhart-Hines et al. 2013*). A signal from the thermoregulatory centre in the preoptic area to the drivers of sleep, however, is an interesting prospect.

In contrast to the circadian studies (see above), many experiments suggest a more inherent connection between sleep and temperature regulation. In several studies on humans, warming, but not cooling, at different times of the day produced consistent increase in sleep that have not yet been explained. Warming in the afternoon, at least 1.5 hours prior to sleep and not more than 8 hours before, increases SWS time and encourages greater sleep consolidation with a decrease in REM (*Horne and Reid 1985, Bunnell et al. 1988, Shapiro et al. 1989, Jordan et al. 1990, Dorsey et al. 1999, Bach et al. 2002*). These results are well replicated in studies on rats (*Morairty et al. 1993*). Cooling during the day in various studies did not alter sleep architecture consistently, and warming during sleep was more likely to cause

waking and sleep fragmentation. Further human studies have successfully replicated this result using gentle, cutaneous warming and demonstrated its utility in improving the sleep of elderly and narcoleptic patients (Raymann *et al.* 2005, Fronczek *et al.* 2008, Raymann *et al.* 2008).

Despite this, the relationship between temperature and sleep is not understood. It has been suggested that an increased temperature during the wake phase may increase the speed at which waking-fatigue is accumulated. However, the opposite effect has not been observed from cooling (Horne 1981). An alternative hypothesis suggests that sleep improvement is induced by forcing a delay in the cold phase of the circadian temperature cycle (Dorsey *et al.* 1999). As yet, how these physiologies interact has not been satisfactorily answered. The attempts of McGinty and Szymusiak to answer these questions are addressed in the introduction to Chapter 2.

1.3.2. Torpor as a state of unconsciousness

Torpor is defined as a state of inactivity accompanied by a decrease in metabolic activity or core temperature. This is observed in animals under extreme conditions such as hypothermia, but also as an explanation for the inactivity of hibernating animals like the arctic ground squirrel (*Spermophilus parryi*) (Buck and Barnes 2000). In contrast to the arctic ground squirrel, the fat-tailed dwarf lemur (*Cheirogaleus medius*) has been reported to lack NREM sleep during natural hibernation. Instead, bouts of REM sleep coincide with slight elevations in temperature. This underlines the difficulties of assessing sleep states through ECoG recordings during unusual physiological conditions such as torpor. For the dwarf lemur, the authors report large periods of very low voltage ECoG data, principally below 75 μ V (Krystal *et al.* 2013). However, absolute cut-off values may not have much value if the ECoG power is artificially suppressed. Equally, the frequency components of low voltage data may be missed. Whilst classically accepted, it seems unlikely that an animal has no sleep stage of any kind – a sleepless-wakeless state. Even a slight rise in temperature to above 30°C can initiate bouts of REM (Blanco *et al.* 2016). I suggest that whilst the frequency content of the ECoG may be harder to interpret at lower temperatures, a significant portion may be retrievable by accounting for the effects of temperature on the recording. This may shift our perspective on the relationship between hibernation and sleep, particularly if 2-4 Hz power dominates within the low voltage region, and the low amplitude (power) is simply a function of temperature. In the first study by Krystal *et al.*, the authors accepted that they did not have the means to assess the temperature effect on the recording amplitude and so felt unable to account for it. This should be key to future studies.

The effects of torpor on ECoG recording were almost certainly seen in data on the arctic ground squirrel (Daan *et al.* 1991). The important distinction when assessing this data is whether these low metabolic states of hypothermia are closer to sleep or wake. This is particularly important as we know that unconsciousness *per se* is not sleep. Is the need to sleep abolished during torpor? - The arctic ground squirrel has shown a need to enter NREM sleep during arousals from hibernation, suggesting otherwise (Daan *et al.* 1991). The key feature of hibernation is a low temperature and metabolic output but this does not remove the need to sleep. Although it is feasible that the process of accumulating fatigue is

dampened during torpor, it would be interesting to know if less sleep was required. Ultimately, a question remains: is hibernation a low metabolic form of natural sleep or something else altogether?

1.3.3. Brain states of hibernation and hypothermia

Naturally occurring torpor during hibernation has distinct neuronal characteristics that might suggest a unique state, neither awake nor asleep. This state is characterised by structural plasticity of synapses. At low brain temperatures of less than 4°C, ground squirrels (*Spermophilus undulates*) have less thin-type dendritic spines, whilst shaft and stubby spines increase in number and mushroom-type spines are unchanged (Popov *et al.* 2007). These changes are reversed when the animals are rewarmed. This demonstrates the acute effect of low temperatures on brain structure. Similar changes have been seen in the dendritic tree of CA3 pyramidal neurons of European hamsters during hibernation. In general, less apical branching and less spines are seen during hypothermia but are recovered to normal levels within two hours of waking (Magariños *et al.* 2006). It has been suggested that brief arousals from natural hibernation may be a mechanism to prevent permanent damage to dendritic structure as post-synaptic densities increase just prior to an arousal event and peak at normal levels before torpor resumes (Ruediger *et al.* 2007). Quantification of localisation of pre and post synaptic markers by von der Ohe *et al.* found a 50 to 65% reduction in synapses during torpor. Translocation, not degradation, appears to be the cause of this reduction, and the reason that fast reconstitution can occur as temperature rises (von der Ohe *et al.* 2007). Interestingly, a substantial microarray study of 5,109 genes compared summer time expression with winter levels during activity and torpor in ground squirrels and found the greatest differences were seasonal, often resulting in downregulation. Torpor did not alter the liver profile to detectable levels but a small increase in cardiac tissue expression was observed in 59 of 115 cDNAs. It is difficult to know if these changes, that were not more than two-fold over baseline, hold a biological significance (Williams *et al.* 2005). One protein was notable for its winter expression - RNA binding protein-3 (RMB3). This has recently been shown to be protective of synaptic regeneration. 5XFAD mice, also known as Alzheimer's mice, do not reconstitute synaptic structure following deliberate cooling in the laboratory setting. Where levels of RMB3 should rise during a cold challenge they do not in these mice. Lentiviral delivery of RMB3 to the hippocampus of these mice is able to inhibit synaptic loss during cooling as well as rescue synaptic reconstitution on rewarming (Peretti *et al.* 2015).

1.3.4. Sleep deprivation induces hypothermia

In a study of REM-only sleep deprivation in rats over 20 days, an increase in food consumption of almost 200% was reported, but interestingly, it was accompanied by an 11% reduction in body weight (Koban and Swinson 2005). A possible explanation is seen in the O₂ consumption data, that rose by 166% over baseline. Collectively this suggests that sleep deprivation drives an increase in metabolic rate (Koban and Swinson 2005). However, in most experiments on sleep deprivation, slow and progressive hypothermia is observed in core temperature measurements (Bergmann *et al.* 1989, Everson 1995, Rechtschaffen and Bergmann 1995). This contradiction is not easy to explain. RT-PCR

and western blot analysis in these animals showed that uncoupling protein-1 (UCP1) was elevated during sleep deprivation, in BAT (Koban and Swinson 2005). As discussed, this protein is important in BAT thermogenesis for switching ATP production to heat production and suggests this is the origin of the increase in metabolism. Certainly, more energy is being used and temperature would be expected to rise. Unfortunately, core temperature was not measured directly during these particular REM experiments. It is plausible that mechanisms to counteract the hypermetabolic state are activated; decreasing energy usage and its negative effects by lowering core temperature. It is unclear how mechanisms to alter temperature from neuronal circuitry, and from the molecular machinery within the BAT, might compete to produce such an outcome. The author of this paper suggested the response is similar to that seen in cold defence. It is clear from this work that REM sleep is not dispensable in sleep architecture and BAT thermogenesis is a fundamental part of the energy balance between normal sleep and the hypermetabolism induced by REM-deprivation (Koban and Swinson 2005).

Sleep deprivation protocols in humans are notably less severe and often harder to interpret. In particular, one recent study of sleep deprivation in 20 healthy adults (17 men, 3 women, age 26 ± 3 years) over 60 hours demonstrated a progressive drop in heart rate (75 ± 2 to 67 ± 2 bpm $p < 0.001$) and core temperature (36.4 ± 0.4 to $35.9 \pm 0.4^\circ\text{C}$) (Vaara et al. 2009). It should be pointed out, however, that the standard errors reported in this paper for each group's core temperature are greatly overlapping and cast doubt on the claim that the observed differences are attributable to $p < 0.001$. No changes were observed in blood pressure (Vaara et al. 2009).

1.3.5. Hypothermia under general anaesthesia

General anaesthesia is medically essential but not without risks. From the neuroscience perspective, the most interesting of these risks is hypothermia; a progressive drop in temperature that is dose dependent and observed with every drug considered a clinical sedative or general anaesthetic (Taylor et al. 1985, Tang et al. 1993, Díaz and Becker 2010). Clinically, hypothermia is often attributed to a “core-to-peripheral” redistribution of heat, meaning vasodilation. Whilst small decreases in temperature are seen in the surgical setting, the mechanisms that bring this about have not been resolved. In part, this is because patients are frequently warmed, masking the effect size and the mechanisms responsible. But we have some evidence that central thermoregulatory mechanisms may be involved. One study in mice by Zhang et al, recapitulating the sedative effects of dexmedetomidine (DEX), could induce sedation and hypothermia from the same brain region, the MnPO (Zhang et al. 2015). Without warming the subject, hypothermia observed with sedative agents is severe and, when central mechanisms are involved, substantial inhibition of BAT thermogenesis is the likely cause (Tan et al. 2016).

The relationship between anaesthetic mechanisms and hypothermia provides a clear link between sedative pathways and temperature control. One explanation is that natural sleep-pathways and thermoregulatory circuits necessarily function together. This has some precedence. Propofol (2,6-diisopropylphenol), perhaps the most widely used intravenous general anaesthetic, has been shown

to replace sleep during a 12 hr infusion in rats without signs of sleep deprivation and rebound (*Tung et al. 2001*). Similarly, rats that have been sleep deprived for 24 hrs recovered no better with natural sleep than they did under propofol sedation (*Tung et al. 2004*). Whilst unconsciousness may not be sufficient to replace natural sleep, the actions of some sedative drugs may do just that. In contrast, the inhalational anesthetic isoflurane does not appear able to replace natural sleep (*Mashour et al. 2010*). This also suggests that it must act via a different neuronal mechanism.

Understanding this link may help us understand the functions of the sleep. The role of circuits originating in MnPO or MPO region of the hypothalamus are not well understood. Thus far, sleep deprivation is thought to induce c-Fos in neurons that project to the VLPO region, whilst glutamatergic and GABAergic neurons of the MnPO and MPO respectively, are sufficient to induce hypothermia. An overlap between neurons that respond to sleep and those that suppress thermogenesis would provide an explanation for the sedative properties of anaesthetics.

Chapter 2: Activity- tagging warm- sensitive neurons

Introduction

2.1. Temperature neurons of sleep

Sleep is associated with lower core temperature as part of the natural circadian cycle. General anaesthetics induce sedation and loss of consciousness, but also hypothermia and warming can increase the probability and continuity of sleep. This suggests that there is a neuronal link between the physiologies of sleep and temperature.

Understanding these links is challenging from an experimental point of view. For instance, we know that sedatives, like the $\alpha 2$ -adrenergic receptor agonist DEX, can lower core temperature (*Zhang et al. 2015*). We also know that the time course is different and that sedation occurs faster than the drop in core temperature. In fact, the animal will stop moving before losing more than 2°C (unpublished observations). Whilst this tells us that hypothermia is not the cause of loss of consciousness, this wasn't the real question. That is, was a temperature control circuit activated to cause loss of consciousness (LOC)? Hypothermia may be a consequence of this sedative-temperature circuit but, because of its physical relationship to the environment, is simply not observed until later.

Work primarily by McGinty and Szymusiak was the first to investigate the neuronal link directly, and expand on the idea that warming could interact directly with the sleep circuitry. They showed that warming the preoptic area of cats, using an implanted 'thermode' or warming-probe, could induce changes in the recorded EEG, particularly in the 0.5-4 Hz (*McGinty et al. 1994*). This resulted in a hypothesis, that thermoregulatory circuits were an integral part of 'depth' attributed to delta power in NREM sleep. Follow-up experiments, using multiple recording electrodes on an implanted microdrive, identified distinct populations within the preoptic hypothalamus that control this effect. Of 308 neurons, 21% demonstrated a response to temperature change and were subsequently classified as either warm-sensitive ($n = 31$, WSN – $Q_{10} > 2$) or cold-sensitive ($n = 34$, CSN – $Q_{10} < 0.5$). Subpopulations of these neurons displayed sleep-wake dependent firing patterns, 64% of WSN increased their firing rate during NREM compared to wake by a factor of 1.2, whereas 74% of CSN were reduced to 0.8 (*Alam et al. 1995*). One observation was particularly striking, if harder to interpret; the degree of thermosensitivity of these neurons changed over the sleep-wake cycle such that WSN themselves were markedly more sensitive to temperature during NREM sleep than wake, and CSN less so. Curiously, of the remaining non-thermosensitive neurons only 25% did not display sleep-wake discharge patterns. In fact, 60% were correlated to wake activity (NREM / Wake ratio approx. 0.4) and 15% to NREM sleep (NREM / Wake ratio approx. 2.4) (*Alam et al. 1995*). Subsequent work revealed that the same preoptic warming process could induce inhibition in posterior hypothalamic and dorsal raphe neurons – these are both active in waking and components of the ascending arousal system (Guzmán-Marín et al. 2000, Steininger et al. 2001). This further progressed the idea of directly coupled thermoregulatory and sleep-wake systems. More recent experiments have confirmed the sleep/wake activity of neurons in the MnPO (*Suntsova et al. 2002*). Additional experiments using anterograde and retrograde tracers, have confirmed that diverse projections leave MnPO for nuclei involved in sleep-wake control, including VLPO and extended VLPO, the lateral hypothalamus (LH) and the DR (Uschakov et al. 2007). Finally,

while VLPO neurons express c-Fos during ongoing sleep, a subset of MnPO neurons were found to be activated by sleep deprivation. These neurons are thought to be the same that project to VLPO, although this picture is far from clear (*Chou et al. 2002*).

So what role does MnPO serve in the preoptic area? Some light was shed on this by Zhang *et al* using a relatively new technique of TetTagging to label neurons involved in a recent stimulus. TetTagging is discussed in more detail later but, in brief, it involves expressing a fluorescent transgene under the control of c-Fos. This mechanism can be suppressed by doxycycline (DOX) until the stimulus is ready to be administered. In this experiment, DEX was able to label neurons in the MnPO and MPO region. When these neurons were reactivated with clozapine N-oxide (CNO) they recapitulated sedation, but also hypothermia. This is a well-known side effect of DEX (Zhang et al. 2015). This result is direct evidence that the actions of the sedative directly activated thermoregulatory neurons in the MnPO and MPO region as well as sedative neurons. A questions remains, are these neurons the same or distinct populations?

2.2. Aims and Objectives

Primary Aims:

Sleep and temperature share physiological connections but do they share a neuronal connection? The aim of the following Chapter is to answer this question. We will use TetTagging pharmacogenetics to study the connections between temperature circuits and sleep-wake control.

Primary Objectives:

- First I will use c-Fos dependent TetTagging to label neurons that respond to a warm-stimulus
- I will then reactivate warm-labelled circuits and record core temperature, ECoG and behavioral responses from these animals.
- I will collect histological brain samples and perform IHC to understand the distribution of WSNs within the preoptic hypothalamus.

Secondary Objectives

- I will use different IHC markers to attempt to characterise the molecular identity of WSNs.
- I will characterise and account for the effect of temperature in ECoG recordings.

Results and Discussion

2.3. Activity-tagging in the preoptic hypothalamus

Neurons that control our response to a temperature challenge may also have a role in regulating our sleep. To investigate this response, I established a protocol to label neurons that respond to a warming challenge. This technique is called TetTagging and utilises two viral components that interact to couple c-Fos expression, a marker of cellular activity, with the expression of a fluorescent receptor or effector (*Zhang et al. 2015*). This transgene is a receptor known as a Designer Receptor Exclusively Activated by Designer Drug (DREADD) that allows activation of labelled neurons and the observation of their behavioral output (*Armbruster et al. 2007, Urban and Roth 2015*). This modified muscarinic receptor can no longer respond to acetylcholine but on binding clozapine N-oxide (CNO), can activate G α_q signaling pathways that release intracellular calcium and make action potentials more likely (*Armbruster et al. 2007*). This ligand is otherwise physiologically inert.

2.3.1. Warming increases delta power but not sleep

A change in environmental temperature will elicit many physiological responses as well as behavioral aversion to the stimulus – but does persistent warmth influence our need to sleep? To investigate this, I warmed groups of C57BL/6 mice at $32 \pm 1^\circ\text{C}$, 50-60% relative humidity (RH) for two hours whilst recording untethered ECoG with the Neurologger 2A and core temperature from an implanted thermologger (Figure 2.1a). Surprisingly, these mice show a rapid rise in core temperature, averaging at $1.57 \pm 0.16^\circ\text{C}$ ($n = 16$), followed by a slow compensation that returns them to normal, just prior to their exit from the warming box (Figure 2.1b and c). Evidently, large physiological changes are effected to reduce heat production and facilitate compensation. This is supported by the sudden reduction in temperature as the mice leave the warm environment and overcompensate for ambient room temperature of 20°C . Whilst passive heat loss through vasodilation, particularly from the tail, is a factor in this process it seems likely that a suppression of thermogenesis in brown adipose tissue would be required for adaptation of this magnitude.

A hypotheses put forward by McGinty and Szymusiak *et al* over several years suggest that a form of coupling exists between these WSNs and other neurons inherent to sleep circuitry. Of most importance is the observation that warming from the environment, or directly with an implanted thermode, can influence ECoG recordings and even induce sleep. In our mice, warming ($n = 5$) induces a gradual but substantial increase in delta power over two hours of about 50%, compared to controls ($n = 3$), but this is not accompanied by a reduction in theta, and the change in the theta / delta ratio is also not different to controls (Figure 2.1d, e and f). Thus, warming under these group conditions does not appear to induce natural sleep, but may increase sleep pressure. Changes in delta power during waking have been linked to homeostatic mechanisms that influence sleep timing (*Vyazovskiy et al. 2011, Vyazovskiy*

and Harris 2013). Whilst no changes observed in the 9-14 Hz alpha band, which is more closely aligned to theta, strong evidence of alert behaviour and vigilance was evident from the warm-dependent effects seen in the 15-25 and 25-40 Hz range. However, this work is preliminary: greater n numbers are needed to fully describe the effects of warming on the ECoG.

At best, we can conclude that there is some evidence that links circuits that respond to temperature to those that influence the ECoG, and particularly the power of the delta band. This may influence sleep, but does not induce it acutely under these conditions. So the question that remains is as follows: does the preoptic WSN provide the link between the detection of warmth and the change in ECoG?

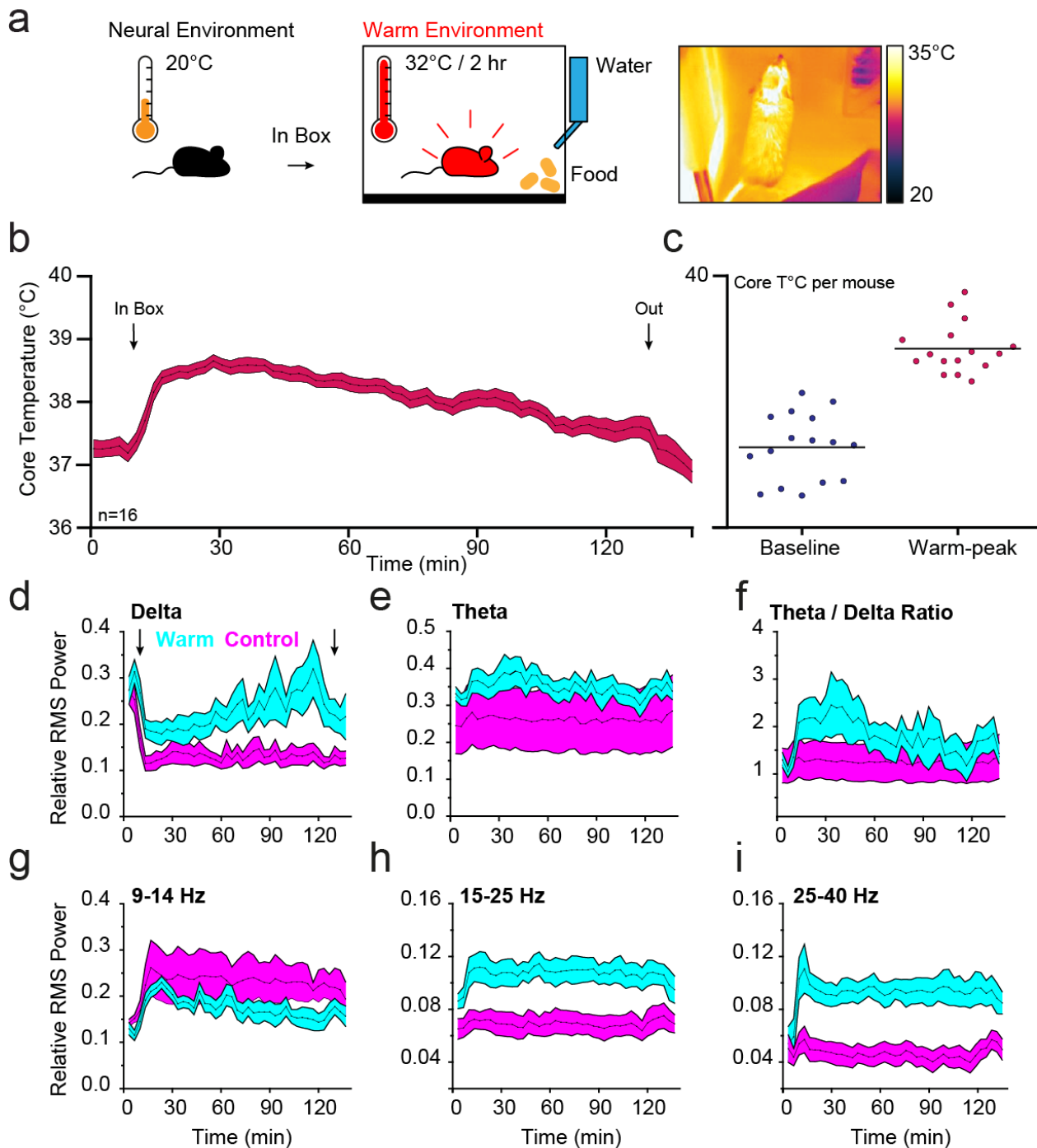


Figure 2.1 – The effect of a warm stimulus. Recordings of core temperature and ECoG. (a) male C57BL/6 mice were placed into a warm box at $32 \pm 1^\circ\text{C}$ for 2 hrs at 50-60% relative humidity with ad libitum food and water. An example image is shown from thermal imaging camera. (b) core temperature recordings from warmed-mice including a 10 min baseline and 10 min post-warming period. Warming increases core temperature over the first 15 mins before compensation is evident and temperature slowly decreases towards normal values. Mice removed from the box overcompensate and cool briefly below normal levels ($n = 16$). (c) absolute temperature of individual mice from baseline average to peak temperature. Individuals have large variations but mice with higher baseline averages warm to higher values (not shown). (d-i) ECoG recordings during warming expressed as an RMS of the banded power normalised to total power (1 - 99 Hz). Each has a 10 min baseline and 10 min post-warming period. Warm (cyan, $n = 5$), control (magenta, $n = 3$). Arrows indicate the beginning and end of the warming. (d) RMS power in the delta band (1 - 4 Hz). (e) RMS power in the theta band (5 - 9 Hz). (f) Theta / Delta ratio of RMS power from each band calculated from individual mice. (g) RMS power in 9 - 14 Hz band (h) RMS power in 15 - 25 Hz (i) RMS power in 25 - 40 Hz.

2.3.2. Levels of preoptic c-Fos rise during a warm stimulus

To understand the neuronal activity associated with a warm environmental stimulus, I perfuse-fixed C57BL/6 mice that had been warmed in groups, as previously described and prepared the brains for c-Fos IHC. The immediate early gene c-Fos is upregulated by neuronal activity and, with a half-life of 30-40 minutes, provides a surrogate record of recent stimulus-evoked activity (*Morgan et al. 1987, Hunt et al. 1987, Wisden et al. 1990*). Counting c-Fos positive neurons provides a quantitative measure of neurons and nuclei that were active at the time of the stimulus.

Consistent with previous literature on temperature responses, the preoptic area was highly reactive to c-Fos IHC as seen in Figure 2.2. A large portion of this IHC was performed by Nathan Andrews, an undergraduate student under my supervision. The MnPO showed the greatest change in c-Fos activity compared to control mice, more than fourfold higher (Figure 2.2a-c). This agrees with the most recent literature on temperature circuits that highlights MnPO as the key center for integration of glutamatergic afferents, carrying both warm and cold stimulus signals. Nearby MPO was also found to have elevated c-Fos, with almost twice as many in the warm group over controls and yet, in absolute terms, far more active neurons are found in MPO than in MnPO (Figure 2.2d-f). In part this is due to the relative size of each region, but it also might suggest a functional relationship. Under the classical model, in a warm environment, these receiving neurons in MnPO are glutamatergic and project to MPO to synapse on GABAergic interneurons. From this data we cannot be sure whether positive neurons are activated by cutaneous afferents directly from warming, as in the case of a WSN, or from downstream excitation from the aforementioned populations. From the large number of c-Fos⁺ neurons in the control group, approximately 1500, MPO appears to have high tonic activity. Equally, MPO may receive dense afferent projections from excited cells in MnPO that work to amplify the number responding to warmth. This population may be further enhanced by directly WSNs, as postulated by Nakamura *et al* (*Morrison and Nakamura 2011*). As an experimental control, c-Fos levels in the cortex (M2 and Cg1) were quantified between neutral and warm environments. As expected, large numbers of c-Fos positive neurons were observed in this highly active region but little difference could be attributed to a change in ambient temperature (Figure 2.2g-i). The level of c-Fos activity seen in MnPO and MPO gives an indication of how the brain responds to temperature changes but it does not reveal the output of the stimulated neurons and the many functions they may influence. Neurons can be excited by the expression of DREADD receptors that can be activated by CNO but the key is to only activate the specific population of interest - in this case, the warm sensitive circuit. The large difference of c-Fos activity raises the possibility of stimulus labeling, also known as TetTagging (*Reijmers et al. 2007, Reijmers and Mayford 2009, Garner et al. 2012, Zhang et al. 2015*). The Tet-tagging system requires a strong and consistent signal to induce the Tet-response element (TRE) sufficiently through the Tet-transactivator (tTA). Only some stimuli are suitable for tagging and Figure 2.2 provides us with confidence that a warming stimulus under this protocol is sufficiently strong to attempt to label WSNs with a tTA-driven transgene expressing a fluorescent DREADD (hM3Dq-mCherry)

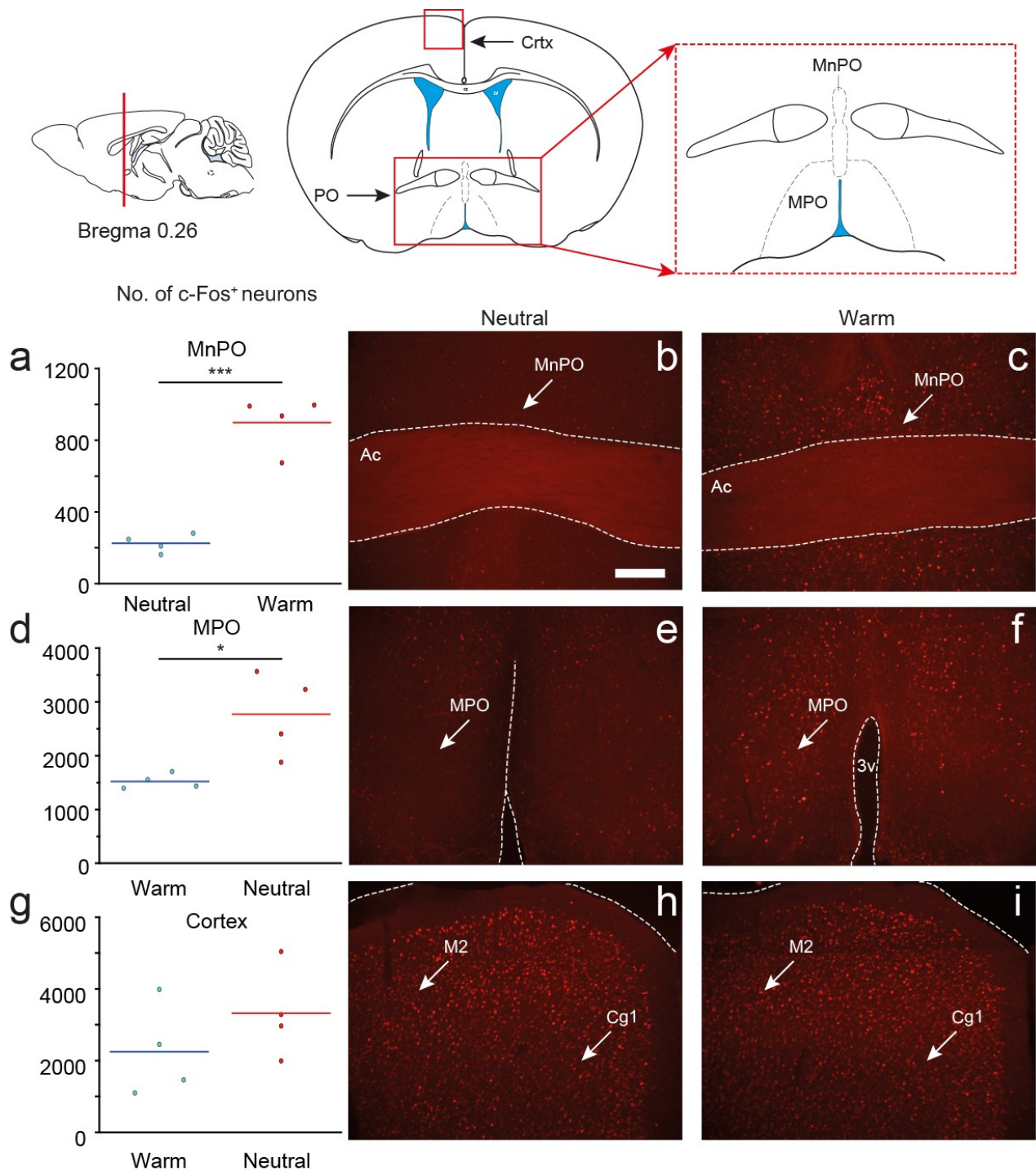


Figure 2.2 – c-Fos induction in the preoptic hypothalamus. c-Fos staining different brain regions, following a warm stimulus compared to neural controls (a) Quantification of c-Fos staining from of the MnPO region. A warm stimulus increases detectable c-Fos by almost fourfold compared to control mice (***P<0.001, n = 4). (b) and (c) show a comparison of representative images from MnPO either side of the anterior commissure (Ac) in neutral and warm conditions. (d) shows the same quantification of c-Fos in MPO where a small difference was detected between stimuli (*P<0.05, n = 4), (e) and (f) compare images of neutral and warm conditions in MPO respectively. The third ventricle is visible on the midline. (g) compares c-Fos staining in the cortex around M2 and Cg1. No difference between stimuli was detected although baseline c-Fos is always high (P>0.05). (h) and (i) compare images of neutral and warm conditions in the M2 / Cg1 region respectively. All images have the same scale. The scale bar is 200 μm.

2.3.3. Activity-tagging with a warm stimulus

Activity tagging allows neuronal populations to be labelled with DREADD receptors. The labelled populations are those that responded to a stimulus by firing action potentials and, as a consequence, expressed c-Fos. These populations should function normally until the mouse is administered CNO to activate the circuit in question. After TetTagging, warm labelled cells are part of warm labelled circuits. These circuits may activate other circuits downstream; these are known as partner circuits. The activation of a circuit that results in a behavior is known as a recapitulation. To TetTag warm-sensitive circuits of male C57BL/6 mice I injected MnPO and MPO, the regions of high c-Fos activity, with two AAVs. These each contained an element of the TetTagging system - the activity dependent vector containing the tetracycline transactivator (tTA) (AAV-c-Fos-tTA), and the DREADD vector containing the modified hM_{3Dq}-mCherry fusion construct (AAV-TRE-hM_{3Dq}-mCherry) (Figure 2.3a). The behavior protocol for TetTagging-warmed mice and their controls is illustrated in (Figure 2.3b). The mice were kept on doxycycline (DOX 200 mg / kg) following surgery, to inhibit tTA activity, whilst the constructs were allowed four weeks' for viral expression. During this time, I expected only the activity dependent vector to express significantly in the area of virus infection. Whilst the tTA is inhibited from binding the tetracycline response element (TRE) only weak basal (leak) expression of the DREADD vector should occur. This has been noted for this TetO promoter. To understand the contribution of this non-specific leak expression, I undertook pre-stimulus control injections of CNO at the end of the expression period. Prior to the warm stimulus, the mice were removed from DOX for two days, an optimum period previously determined in our lab (Zhang *et al.* 2015). After the stimulus, I placed the mice on high DOX (1g / kg) for one day to prevent further DREADD expression. As our previous work had shown high levels of background c-Fos in the neocortex, which could induce hM_{3Dq} expression if the both viruses were present, it was important to see if DREADD expression here would produce the same results as preoptic expression. To control for this, I injected a separate group in the motor (M2) and cingulate cortex (Cg1) and these underwent warming with the stimulus group. Three days after warming, CNO or saline vehicle was administered in a randomised regime, with the mice remaining on DOX for home cage recordings of ECoG and core temperature, as well as locomotion experiments in the open field arena for up to ten days. The warm-stimulus for these animals was delivered in small groups to prevent TetTagging of sleep neurons in mice that slept naturally when kept alone and without stimulation in a habituated environment (*unpublished observation*).

To assess whether the TetTagging was successful, I compared mCherry expression from the warm-stimulated group with the OFF-DOX no stimulus control group. The group that did not receive a warm stimulus showed some non-specific background expression, largely in the form of DREADD expression in the fiber background, with little cell body expression evident. The warm stimulus group, however, showed high numbers of mCherry-positive neurons in midline of the preoptic area in MnPO and MPO. A comparison of the off DOX control with the warm stimulus group is shown in shown in Figure 2.3c &d.

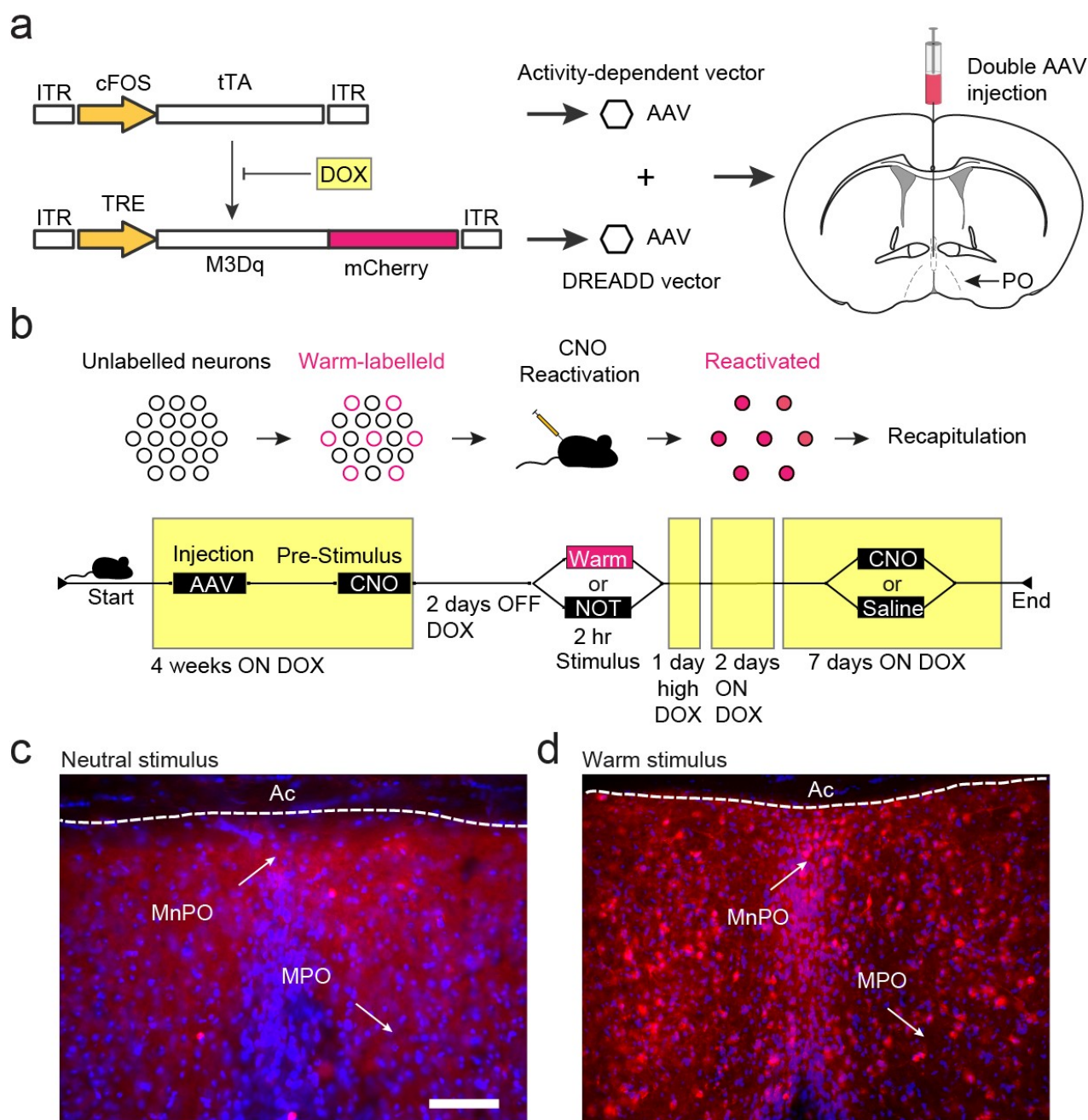


Figure 2.3 – Activity tagging warm-sensitive neurons. (a) Schematic of the TetTagging expression vectors. The Activity dependent vector (AAV-*c-Fos-tTA*) is mixed with the DREADD vector (AAV-*TRE-hM3Dq-mCherry*) and injector into the preoptic area. (b) the experimental protocol and doxycycline (DOX) schedule that allows neurons to be warm-labelled. Mice are given an AAV injection and allowed 4 weeks for expression but are maintained on 200 mg kg⁻¹ DOX. Before any stimulus mice are injected with CNO (Pre-STIM CNO group) to assess basal activity. The warm stimulus is given after 2 days off DOX. Control mice were taken off DOX but did not receive a stimulus (OFF-DOX group) to assess endogenous c-Fos activity. After the stimulus mice are placed on 1 g kg⁻¹ DOX for 24 hrs then 200 mg kg⁻¹ DOX. After another 2 days, 5 mg kg⁻¹ CNO or saline injections began under a randomized protocol. (C) Expression of the hM3Dq-mCherry (Red) in MnPO and MPO in mice removed from DOX but not stimulated (neutral). The image is merged with a DAPI stain (blue). There are no cell bodies present, although a high level of background fiber staining is seen (d) shows that same conditions but with a warm stimulus. Warm-labelled cell bodies can be seen over the background staining. The scale bar is 200 μ m.

To confirm that the fluorescent signal observed in the histology was the result of the functional expression of the hM₃D_q-mCherry fusion protein some mice were killed for electrophysiology, instead of receiving injections of CNO. This work was performed entirely by Drs Zhiwen Ye and Catriona Houston and is shown in Figure 2.4. Only neurons that were mCherry, as a result of warm-stimulus TetTagging, were tested. These responded to 5 μ M CNO through an 8 ± 2 mV shift in membrane potential that was sufficient to induce depolarization ($p < 0.01$, $n = 5$).

These data confirm that our warm stimulus raised c-Fos expression, promoting transcription of the tTA, and without inhibition by DOX, the tTA protein was free to activate the TetO promoter and allow transcription and translation of the hM₃D_q-mCherry fusion protein. Any mouse that repeated the experiment was allowed a 1-month recovery on DOX before the stimulation protocol was repeated. Previous work from this lab has shown this is sufficient to abolish a previous TetTag (Zhang et al. 2015).

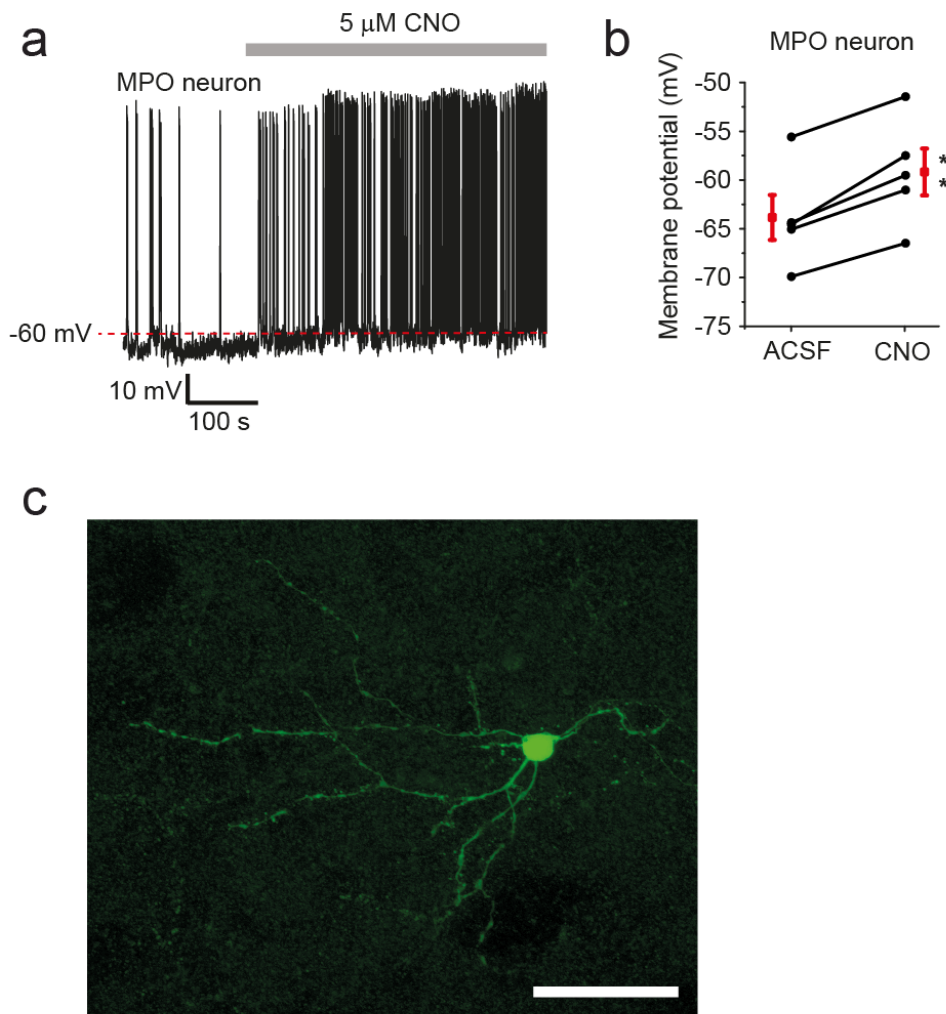


Figure 2.4 - Electrophysiology: Warm-labelled neurons respond to CNO. Recordings made entirely by Drs Zhiwen Ye and Catriona Houston. Electrophysiology of warm-tagged neurons in whole-cell current clamp responding to CNO. (a) Example of MPO neurons. (b) shows the population response of four neurons compared to ACSF controls. CNO induces depolarisation (** $P < 0.01$) (c) Confocal image of a Biocytin Alexa Fluor 488 fill of one neurons that responded to CNO. The scale bar is 20 μ m.

2.3.4. Recapitulation induces hypothermia

Mice that were TetTagged with a warm stimulus were fitted with mock-Neurologgers one day prior to recording to allow habituation. On the day of the experiment, the mock-Neurologger was replaced with the real Neurologger 2A device to record ECoG between 15 and 120 mins prior to the CNO injection. Each experiment was carried out in the dark phase when the animals are most active between zeitgeber time (ZT) 18 and 20. Administration of CNO (5 mg/kg) produced significant and prolonged hypothermia compared to controls and induced a state of torpor characterised by a hunched or curled-up posture and behavioral immobility. An example recording of core temperature over five days from an implanted thermollogger in a warm-stimulated mouse given CNO, is shown in Figure 2.5a. The mouse is injected twice, two days apart. On each occasion CNO induced hypothermia, followed by a slow recovery to normal temperature. In fact, full recovery is not seen until the start of the next lights-off period. The circadian temperature cycle is clearly visible in-between these events and appears undisturbed.

The average response of each group to CNO or vehicle over the first 400 minutes is summarised in Figure 2.5b. As expected a vehicle injection of saline produced little change in temperature. After injection of CNO, TetTagged mice displayed hypothermia with an average drop of $8.66 \pm 0.62^{\circ}\text{C}$ ($n = 12$), much greater than the three control groups. Small differences were observed in the two CNO control groups that serve to validate the TetTagging protocol. The mean minimum core temperature of the WARM-CNO group was $28.1 \pm 0.62^{\circ}\text{C}$ ($n = 12$) whilst the main control groups have smaller responses, OFF-DOX CNO 33.9 ± 0.22 ($n = 6$) and Pre-Stimulus 34.6 ± 0.13 ($n = 3$). As expected the OFF Target CNO response was the same as the vehicle control at $35.0 \pm 0.39^{\circ}\text{C}$ ($n = 4$). Pre-Stimulus CNO mice have a small response over saline injected mice representing basal hM₃D_q expression, most probably from a leak expression through the TetO promoter as previously discussed. The OFF-DOX CNO group has a slightly greater response still, representing the added expression hM₃D_q receptors to neurons with high basal c-Fos during the OFF-DOX period. This response is short-lived and returns to similar levels seen in the Pre-Stimulus CNO group.

To compare the spread of responses for the mice in each group, the lowest temperature of each individual mouse is shown in Figure 2.5c. For comparison, the lowest temperatures recorded during normal sleep and the wake period prior to the injection are also plotted, as is a new group, the OFF-target CNO group where virus injections were made to the neocortex instead of the preoptic area. Surprisingly, saline injections produce a reduction in core temperature to levels observed during sleep. The mean core temperature minimum from a vehicle injection was $35.3 \pm 0.16^{\circ}\text{C}$ ($n = 13$) compared to natural sleep, 35.4 ± 0.10 and wake, 35.8 ± 0.10 (both $n = 21$). Note that mean waking temperature is higher at $36.6 \pm 0.08^{\circ}\text{C}$ ($n = 21$). Saline injections may reduce core temperature for one of two reasons, the simplest explanation is that the saline solution is at room temperature and briefly lowers the visceral temperature around the logger. The second possibility is that the injection of vehicle results in an increase in sleep as a consequence of stress – this sleep is accompanied by a reduction in core temperature (*unpublished observations*).

Recapitulating the activity of a warm-tagged circuit in the preoptic area validates our initial hypothesis that MnPO and MPO are sufficient to fully activate a warm defense circuit. Neurons in these regions responded to the increase in environmental temperature by signaling to downstream effectors of thermoregulation, and in doing so, increased their levels of c-Fos promoter activity resulting in expression of the M₃Dq-mCherry tag. The administration of CNO reactivated the same circuit and recapitulated the same cooling mechanisms used to compensate for the warm environment. The depth

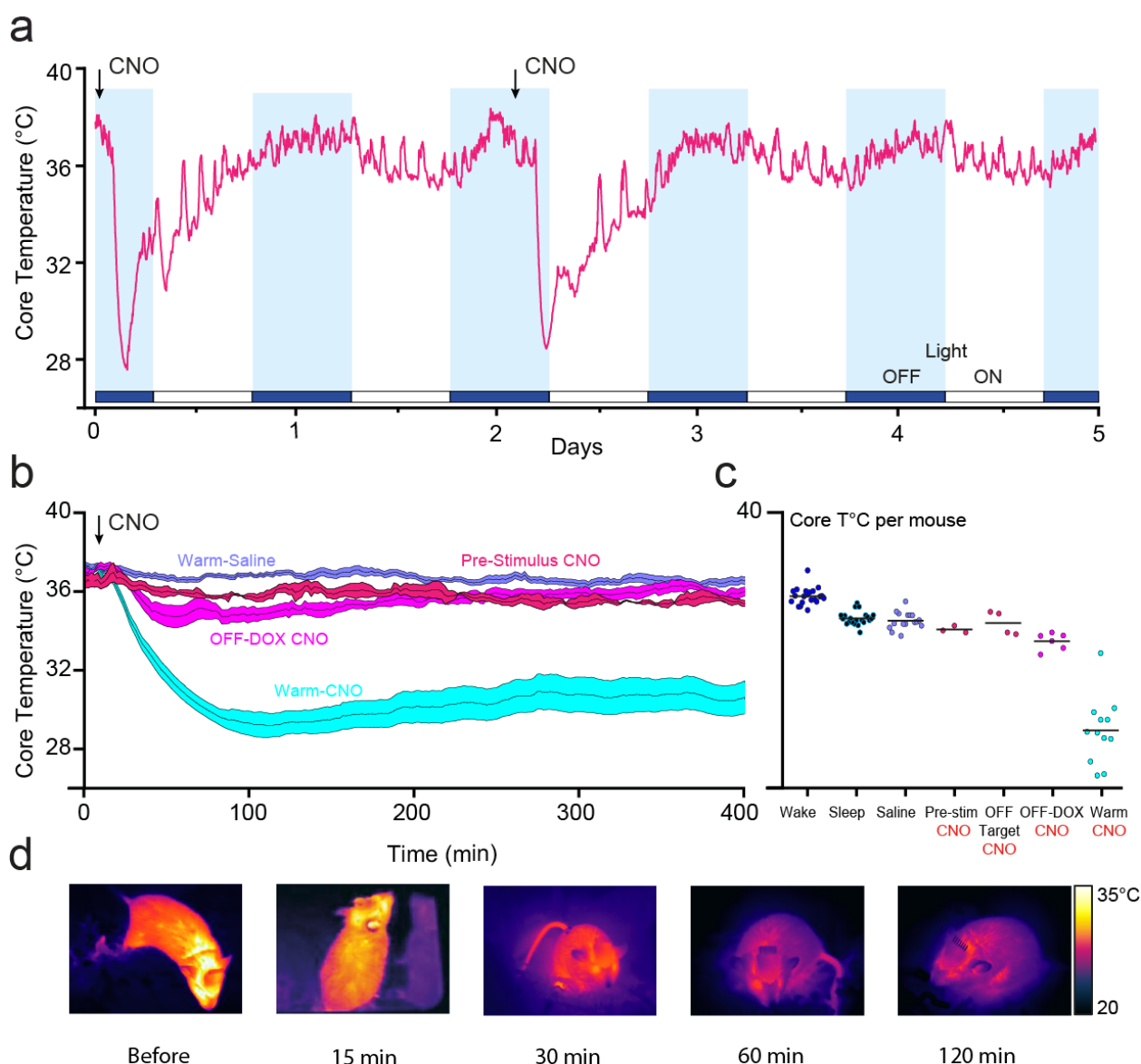


Figure 2.5 - CNO recapitulates the warm defence. Core temperature recordings of TetTagged mice following CNO injections. (a) Example of a mouse tagged with a warm stimulus given CNO (Warm-CNO) twice over a five-day period. On both occasions the mouse recapitulates hypothermia lasting more than 24 hrs. The circadian temperature is visible in the background. (b) Initial response to CNO over 400 mins of Warm-CNO mice (cyan, n = 12) and the three control groups, OFF-DOX CNO (magenta, n = 6), Pre-Stimulus CNO (burgundy, n = 3) and Saline (blue, n = 12). Warm-CNO mice recapitulate substantial hypothermia compared to control groups. A small response is evident from OFF-DOX CNO mice. (c) show the lowest temperature recorded in each animal following recapitulation. The lowest temperatures observed during sleep and wake area added for comparison. A fourth control, OFF-Target CNO is also shown (d) thermal imaging of a the CNO response from a Warm-CNO mouse over 120 mins.

of hypothermia observed here may be more than could be achieved by passive means, such as vasodilation, and suggests that BAT thermogenesis may have also been inhibited.

Under the classical model, the warm-tagged neurons that induced this effect may be either the glutamatergic cell bodies of MnPO or their projection targets, the GABAergic neurons of MPO. Recent evidence has suggested that it is the glutamatergic population, expressing TRPM2, that can directly sense changes in brain temperature (*Song et al. 2016*). However, this circuit also receives extensive input from peripheral temperature sensors and both methods of detecting temperature change could have resulted in TetTagging and contributed to the recapitulated hypothermia. It is known that GABAergic projections from MPO neurons project to glutamatergic effectors in the DMH to influence BAT thermogenesis.

My primary aim was to assess the neuronal output of the temperature circuit to detect changes that may suggest the activation of sleep-like mechanisms. To do this we must look at the ECoG during recapitulations.

2.3.5. Recapitulation induces changes in the ECoG

I recorded freely moving ECoG in each mouse following injections of CNO with the aid of the Neurologger 2A. For each animal ECoG recordings started during the light phase, when the animals are most active, (ZT17-19), and injections were given 1-2 hours later and continued for 24 to 48 hours. Later experiments were recorded for longer, when the length of the response was better understood. Our ECoG recordings showed distinct changes in the power in each frequency band, relative to the ECoG power baseline. The time course was consistent with previous work using the same drug with a response within 20 mins. The effect of a reduction in core temperature is, however, noticeable within the raw ECoG data (Figure 2.6).

Figure 2.6a and b compares a saline and CNO injection with its associated sleep scoring (CED Spike2). An injection of CNO induces an increase in delta power compared to a saline control but, whilst the saline recordings can be scored normally, the CNO cannot. Whilst differentiating between wake and non-wake is feasible, the remaining states do not score reliably and result in a NREM-like-torpor state, with suppressed REM (Figure 2.6c, d and e). This is a product of the reduction in ECoG power, as a consequence of hypothermia, that disrupts the threshold for NREM scoring. This is most clearly visible in the ECoG root mean square (RMS), shown in the examples, where a reduction in power coincides with the drop in core temperature (Figure 2.6b). As a consequence, this state has a highly variable epoch length.

The effect of hypothermia on ECoG power is consistent with previous studies. For example, and as previously discussed, recordings from hibernating dwarf lemurs during hypothermia are described as “low voltage” EEG (*Krystal et al. 2013, Blanco et al. 2016*). It is possible that suppression of ECoG power by a lowered core temperature may have masked the underlying frequency architecture. However, the context is also far from clear, as hibernators have far lower core temperature than the animals in our experiments. Thus, our animals are far more likely to have preserved valuable ECoG signal while maintained just above 20°C. We believe that this architecture is important and retrievable and I have accounted for the change in temperature by normalizing the power in each frequency band to the total power between 1-99 Hz, using a running power spectrum (Figure 2.6f-k). The frequency bands are delta (1-4 Hz), theta (5-9 Hz), alpha (9-14 Hz), low gamma (15-25 Hz) and high gamma (25-40 Hz). CNO injections in mice TetTagged with a warm stimulus caused the largest increase in relative delta power and the largest fall in theta (Warm-CNO, $n = 7 - 8$) As a consequence, the theta-delta ratio falls substantially (Figure 2.6e, f and g). When this ratio coincides with low signal in the electromyogram (EMG), it fulfils the classical criteria for NREM sleep. The Pre-Stimulus CNO group ($n = 5$) is indistinguishable from injections of vehicle ($n = 11$) and suggest that leak expression of the hM₃D_q receptor through the TetO promoter is not sufficient to influence these ECoG recordings. However, the OFF-DOX CNO controls ($n = 7-11$) have elevated delta power over the Pre-Stimulus group suggesting that basal activity, without DOX inhibition of the tTA, can induce expression. Interestingly, a parallel change in theta does not occur. In the CNO group, the change in theta power is slower than the change in delta and may be influenced by the onset of hypothermia (Figure 2.6f and g). This is discussed in

more detail further on. In the 9-14 Hz alpha band the warm stimulated group displays a fast drop in power that is more closely aligned to that of the delta (Figure 2.6i). For the other frequency bands of 15-25 and 25-40 Hz, no change was elicited by recapitulation (Figure 2.6j and k). This is despite differences evident during the warm stimulus.

Recapitulation of activity in the warm-tagged circuit elicited changes in the ECoG alongside changes in hypothermia. The predominant change is an increase in the power of the delta oscillation. This parallels the increase in delta power seen during the warm stimulus although recapitulation occurs much faster. The change in theta is slow and suggests that the rise in delta power may not be sufficient to induce sleep but may represent an inherent link between raised temperature and sleep pressure. This would be an interesting finding that would suggest that sleep acts to limit energy expenditure. Much of the interpretation of this result rests on the timing of the change in the ECoG. Once torpor has set in by 50 min, and theta power has dropped, these animals enter a state of NREM-like torpor. Whilst hypothermia defines torpor, these animals have both a low theta / delta and low EMG power – this passes the definition for NREM classification. Whether this is sleep, in the sense of the process of recovery is unclear. Nevertheless, a neuronal connection links the response to warm environment to an increase in delta power.

Literature on human subjects and rats has suggested that a warm stimulus induces sleep approximately 1.5 hours after the event, encouraging the normal sleep process by increase SWS and consolidating sleep bouts (*Horne and Reid 1985, Horne and Moore 1985, Bach et al. 2002*). This is consistent with my data that shows an increase in delta power. The temperature stimulus may act to shorten sleep latency and consolidate natural sleep. In this way, the induced sleep would be acting more like recovery sleep. My results are also consistent with the work of McGinty and Szymusiak, that showed that warming of the preoptic area with an implanted thermode could elicit sleep-like signatures in the ECoG (*Morairty et al. 1993, McGinty et al. 1994*).

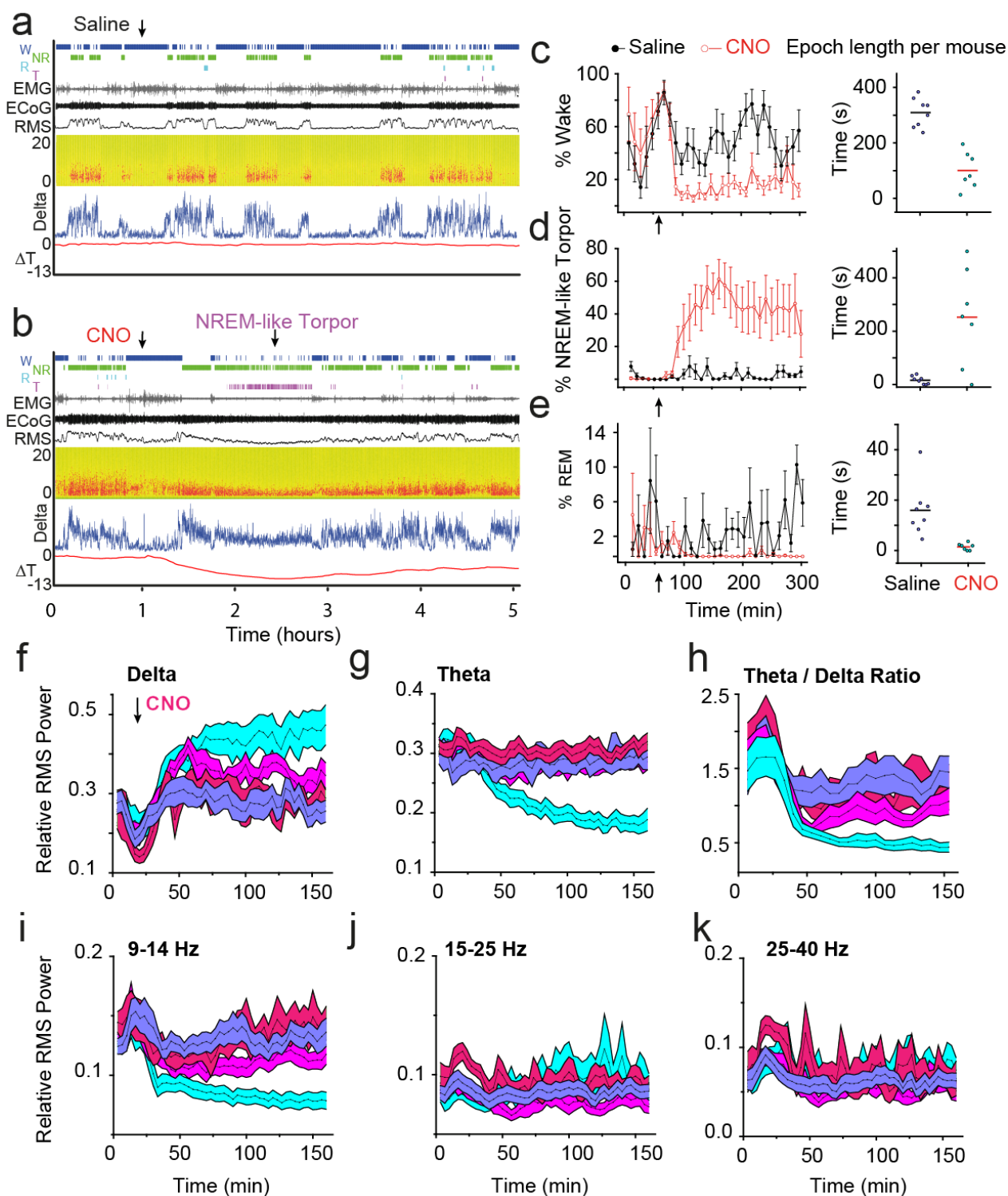


Figure 2.6 – CNO recapitulates an increase in delta power. ECoG response of Warm-CNO mice. (a - e) show five hour recordings after CNO or saline and the results of sleep scoring without power normalisation. (f - k) show ECoG power after normalisation. (a) examples of the response of Warm-CNO mice given saline. Sleep states labelled: wake-blue (W), NREM-green (NR), REM-cyan (R), Torpor-purple (T). Power in the spectrogram is arbitrary and the scale is the same for both examples. (b) shows the same data for a Warm-CNO mouse given CNO. At 2 hrs sleep scoring fails to classify NREM or REM. This coincides with low core temperature and reduced ECoG power (see RMS and delta). We call this torpor. Rapid switching between torpor and NREM is called NREM-like torpor. (c) average sleep scoring for wake states for animals given saline or CNO. The average epoch length for individual mice is also plotted. (d) shows the same for NREM-like torpor. (e) REM sleep. (f) ECoG response of each group to CNO or saline expressed as an RMS of the banded power, normalised to total power (1 - 99 Hz). Each has a 15 min baseline before injection (arrow). Warm-CNO (cyan n = 7-8), OFF-DOX CNO (magenta n = 7-11), Pre-Stim CNO (burgundy n = 5), Saline (blue n = 11). (d) RMS power in the delta band (1 - 4 Hz). (e) RMS power in the theta band (5 - 9 Hz). (f) Theta / Delta ratio of RMS power from each band calculated from individual mice. (g) RMS power in the 9 - 14 Hz band. (h) RMS power in the 15 - 25 Hz (i) RMS power in the 25 - 40 Hz band.

2.3.6. The distribution of warm-labelled circuits

In principle, the position of each fluorescently-tagged neuron provides an indication of its function. Clusters of neurons activated together may serve a collective purpose with defined roles but, importantly, where expression differs between mice, it may help to explain variations in the behavioural responses.

For a neuron to be successfully tagged it must fulfil several conditions. Firstly, it must be transduced by, and express, both the activity-dependent vector and the DREADD vector. This mCherry staining relates to neurons transduced with, and expressing, both viral components and subsequently warm-stimulated whilst the mouse was in an OFF-DOX behavioural phase. Each mouse brain was serially sectioned and stained with an anti-mCherry primary antibody (to amplify the tagged signal - after perfusion, primary staining was often weak or not visible) The pattern of cell body expression and the most dense areas of fiber background staining, were mapped to the Paxinos and Watson mouse brain atlas (*Paxinos and Franklin 2004*). Quantification of the histological results are shown in Figure 2.6a, b and c, and a schematic of the virus distribution for each of 11 out of 12 animals in the Warm-CNO group is presented in d.

To quantitatively assess the histology data, I developed a matrix-method to map the relative behavioural responses of each mouse to the histological distribution which the mice had in common. This highlights the relative significance of each area to features of the data recorded from recapitulation. To do this, each mouse brain's hypothalamic area was divided into an individual histology-response matrix, with each preoptic nuclei on one axis and an anterior to posterior coordinates relative to bregma on the other. Expression in a given region is represented as a cell in the matrix. This can hold physiological data. Areas without expression are left blank (white). Physiological parameters from the temperature response, including relative change, time to temperature trough and recovery duration, are then added to each available cell in the corresponding matrix. A group matrix was then compiled from the median response at each point in the matrix (Figure 2.6a, b and c). These matrices show that early MnPO and more posterior MPO have the dominant effect on the behavioral results. A signal is also seen in the subnuclei of the bed nucleus of the stria terminalis (BST). Surprisingly, whilst Figure 2.6a and b are similar, the time to recovery in Figure 2.6c is also influenced by lateral expression towards LPO and VLPO. This suggests that the initial temperature circuit may not control recovery and rewarming of the animals from torpor. This analysis provides an estimate of the important parameters that affect the recapitulation. However, some questions remain such as the relative importance of the BST subnuclei. This may be answered by a process of qualitative exclusion.

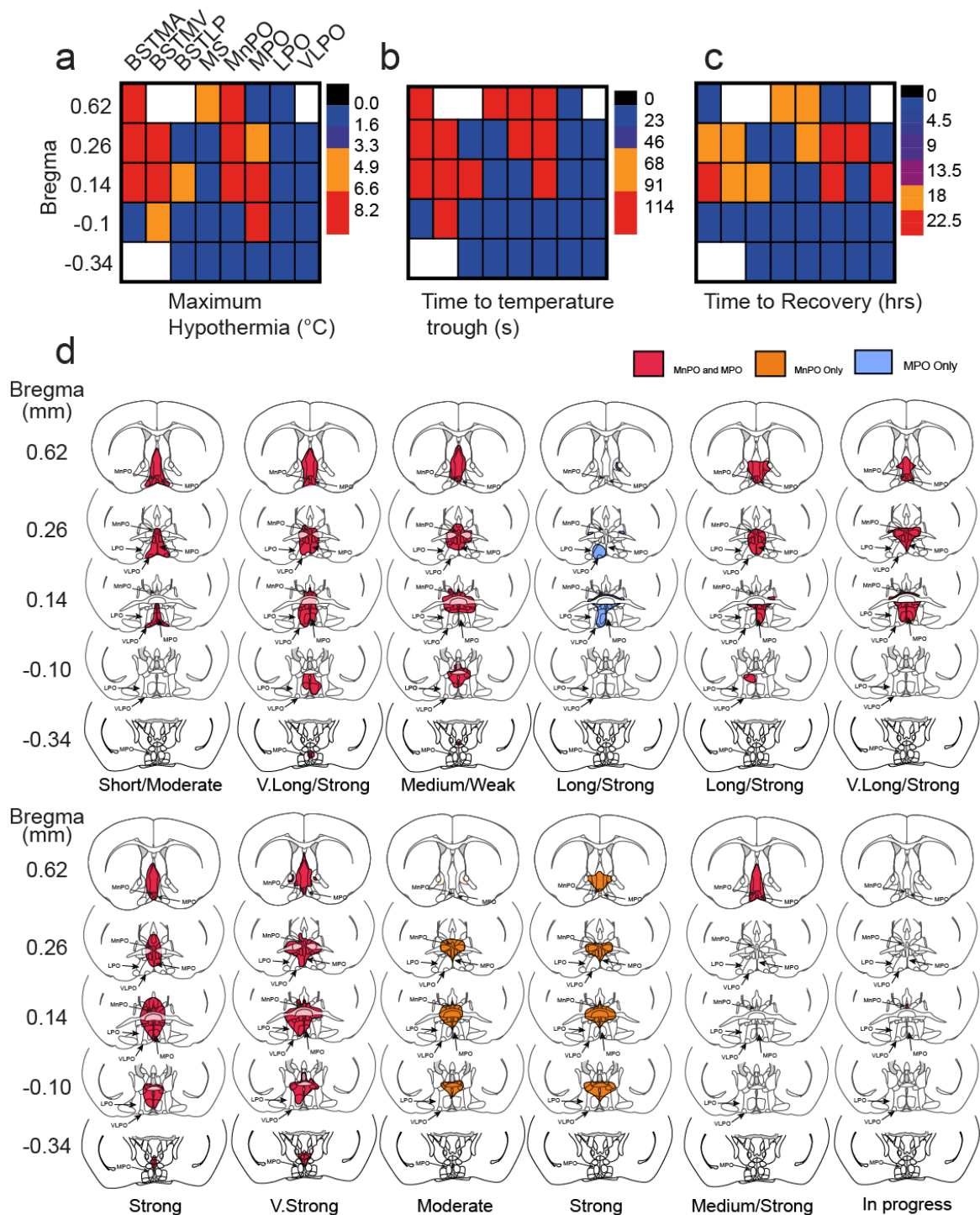


Figure 2.7 – Distribution of warm-labelled circuits. Distribution of warm-labelled neurons from immunohistochemistry on Warm-CNO mice after recapitulation experiments. (a-c) shows a quantitative assessment on the contribution of warm-labelled neurons to the recapitulation of the warm defence. In each case, a binary assessment is made on the presence of warm-labelled neurons in each nuclei in each mouse. If positive, a behavioural value i.e maximum hypothermia, is added to that cell of the matrix. (a) Median matrix for maximum hypothermia (°C) (b) shows the time to temperature trough (s). (c) the time to recovery from hypothermia (hrs). schematic of the distribution of warm labelled neurons in each mouse across the preoptic area (+0.62 to -0.34 from Bregma). Expression in MnPO and MPO (red), MnPO only (orange), MPO only (blue). Each mouse has two qualitative descriptors - length of the effect (hrs): Short (< 10), Medium (10 - 20), Long (20 - 30), V.Long (> 30) and strength of the effect ($\Delta T^{\circ}\text{C}$): Weak (< 4), Moderate (4 - 8), Strong (8 - 10), V.Strong (> 10). Where no value is given there was insufficient data. One animal is still in progress.

Surprisingly, virus expression from the stereotaxic injection spreads preferentially in the dorsal ventral axis, but not mediolateral axis across the coronal sections resulting in little lateral diffusion either side of the intended MnPO to MPO target (Figure 2.6d). However, a larger viral spread was observed anterior to posterior, covering as much as 400 μm . Nonetheless, expression was still confined to the preoptic hypothalamus. Most expression is seen within the MnPO and MPO boundaries as intended by the injection coordinates, but some expression is also seen in the subnuclei of the BST. Occasionally, expression reaches the LPO and VLPO region, but this is rare. Previous work in our lab has shown that c-Fos expression is high in the BST irrespective of the stimulus (*unpublished observation*). Neurons, in the diagonal band, a part of the BST for example, have a high firing rate during sleep, so our result is not inconsistent (*Van Someren 2000*).

Twelve TetTagged mice recapitulated their warm stimulus, resulting in a degree of hypothermia. All data quoted here are the mean of the minimum temperatures $\pm$ SEM of the group in question. In each mouse with successful recapitulation the virus distribution covered the target area, although each virus has its own unique distribution. For instance, two mice had no expression in MPO and yet I recorded distinct responses to CNO with hypothermia of 29.3 and 28.8°C. One single mouse did not have tagged neurons present in MnPO but retained MPO expression. Surprisingly, this mouse also recapitulated a behavioral response of 28.0°C (Figure 2.6d). Thus, despite the low numbers, neither region appears necessary for recapitulated hypothermia, instead both are sufficient. This is consistent with the idea that MnPO and MPO form two links in warm-sensitive circuit that passes through the preoptic area, and stimulation of the either link can activate the warm defense.

Expression in both MnPO and MPO together was not obviously predictive of a strong response ($27.9 \pm 0.82^\circ\text{C}$, $n = 9$), although this accounts for most of the data. Mice with additional expression in LPO were no different ($29.0 \pm 1.64^\circ\text{C}$, $n = 4$) and compare well with mice grouped by additional expression in VLPO ($28.4 \pm 0.59^\circ\text{C}$, $n = 3$). Approximately half of all mice displayed some medial septum expression, but irrespective of LPO or VLPO labelled neurons, this was not a contributing factor to hypothermia ($28.86 \pm 1.04^\circ\text{C}$, $n = 6$). Finally, almost all mice ($n = 10$) expressed the hM₃D_q-mCherry receptor in subnuclei of BST, namely the medial anterior (BSTMA) and medial ventral (BSTMV). For the two mice that did not, core temperature after CNO reached a trough of 28.02 and 29.55°C respectively. This confirms that, although some expression was detected in BST nuclei, it was not a necessary factor in the recapitulation of hypothermia (but could still be involved in the sleep response). To confirm that activating MnPO and MPO are the necessary condition for recapitulation and not the activation of hM₃D_q signalling pathways in other neurons in the brain, four off-target controls were recorded. These neocortex-CNO mice received an injection to the neocortex (M2 and Cg1), were removed from DOX and warmed in the same manner as Warm-CNO mice. As seen in the c-Fos IHC experiments, high basal levels of endogenous c-Fos are present in these regions. This is true even under neutral conditions and thus c-Fos promoter activity would be expected to induce hM₃D_q-mCherry expression. Overall, these areas could not recapitulate a response following CNO administration ($35.03 \pm 0.39^\circ\text{C}$, $n = 4$) and were comparable to the saline response discussed previously ($35.28 \pm 0.16^\circ\text{C}$, $n = 12$). This is strong evidence that off-target regions are not able to induce hypothermia even with DREADD

expression. By using a process of qualitative exclusion, we can suggest on histological grounds that MnPO and MPO are the necessary components of the temperature response. Each of the other infected nuclei does not appear to contribute. Further work is needed to assess these regions in NREM-like torpor.

2.3.7. Which neurons make up the warm-sensitive circuit?

Historically, attempts to reveal the molecular identity of neurons in the warm-sensitive circuit have been unsuccessful. This is particularly true of directly warm-sensitive neurons, the WSN itself. However, until recently all the literature on this circuit suggested it to be inhibitory and Tet-Tagging this circuit provides unique opportunity to attempt to double label with common GABAergic markers. To understand the composition of the warm-sensitive circuit we undertook extensive immunohistochemistry of likely markers that might co-label with our hM₃D_q-mCherry. These were primarily GABAergic subpopulations. Immunohistochemistry was successfully carried out on Choline acetyltransferase (ChAT) as a marker for cholinergic neurons and Glutamate decarboxylase 67 (GAD67) for GABAergic neurons. In addition, several markers for GABAergic subpopulations were also stained including: Parvalbumin (PV), Calretinin (CR), Somatostatin (SOM), and Nitric oxide synthase 1 - neuronal (nNOS). These results are seen in Figure 2.7. For these experiments, the immunohistochemistry was performed by undergraduate David Miao and Clinical Research Fellow Leda Lignos, under my supervision.

Of these markers, PV and SOM were shown by Xu *et al* to mark important populations of neurons that regulate sleep in the nearby basal forebrain (Xu *et al.* 2015). In their work, SOM neurons were recorded as NREM active whilst PV and ChAT neurons were wake and REM active. But the role of these neurons in the midline preoptic nuclei is unknown, particularly with regard to the control of temperature. With the exception of SOM, all the markers successfully tested were detected in neurons of the MnPO and MPO, although distributions showed distinct changes through these nuclei across the anterior to posterior axis. Cholinergic neurons have not been implicated in this part of the temperature circuit or in sleep induction in the preoptic area, although they are found in this region. In this way, staining for ChAT serves as a negative control for these data. In general, very few double labelled ChAT⁺ neurons were identified (not greater than 1%) and these were not easy to distinguish from background (n = 2). Whilst small populations of neurons can have large effects because such neurons can have extensive innervations, I believe this sporadic double labeling is due to the background expression of the TetTagging system (Figure 2.7a).

Previous work on temperature circuits found that afferents relaying the sensory detection of warmth would synapse at glutamatergic neurons in the MnPO nucleus and relay information to GABAergic recipients in the MPO. In our staining of GABAergic markers, we could find evidence of a strong GABAergic presence in the MPO and LPO regions but, in each case, these were separate from our warm-labelled population. Instead, our staining frequently revealed warm-labeled neurons surrounded by dense GAD67⁺ fibers and distinctly separate GABAergic cell bodies (n = 2) (Figure 2.7b). It should be noted that the merge of GABAergic staining in green, with a dense fibrous background, with the mCherry of warm-labeling produces a distinct yellow – this is not true double labelling.

Staining for SOM revealed a lateral distribution that was frequently either side of the warm-labelled area. On occasion, where population intermingling occurred, by chance it was in the region closest to the BST and yet still no double labelling was observed (n = 3) (Figure 2.7c). Recent work has suggested that nNOS expression in certain GABA neurons in the neocortex is proportional to sleep pressure and

nNOS is also a well-known GABAergic marker expressed throughout the hypothalamus (*Morairty et al. 2013, Dittrich et al. 2015*). The MnPO region is rich in nNOS-positive cells but these do not appear to be warm-sensitive despite the appearance of sparse double labelling (Figure 2.7d). Staining for nNOS

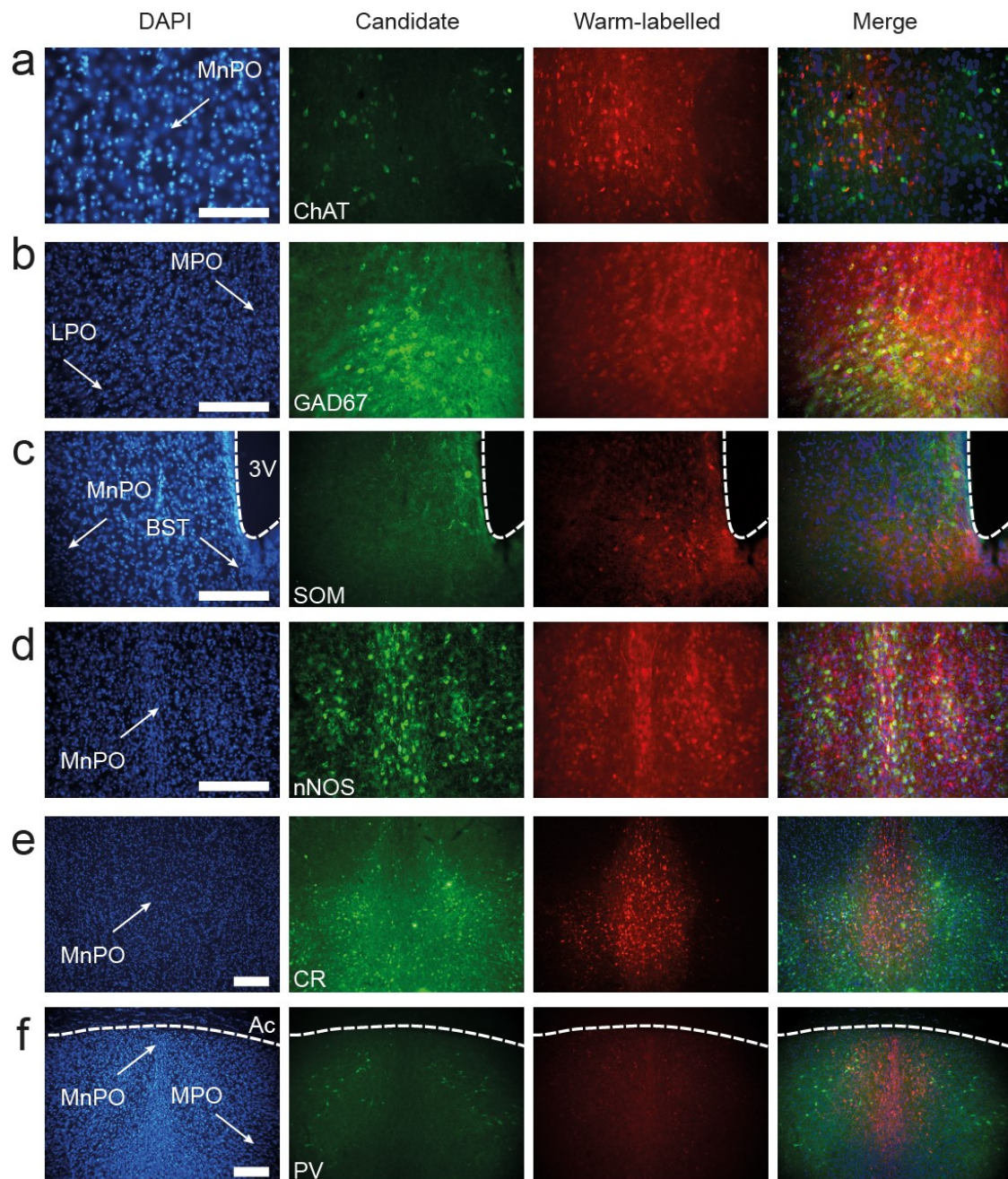


Figure 2.8 – Warm-labelled neurons are not GABAergic. Immunohistochemistry of warm-labelled neurons in the preoptic hypothalamus with six candidate markers. (a-f) DAPI nuclear staining (blue), candidate marker (green), warm-labelled (red) and a three channel merge are shown for each candidate. (a) shows immunohistochemistry against Choline acetyltransferase (ChAT). Only very sparse labelling observed ($n = 2$). (b) Glutamate decarboxylase 67 (GAD67) staining using a tyramide amplification protocol. At high magnification, yellow merged cells are not clearly double labelled ($n = 2$). (c) Somatostatin (SOM) showed mostly lateral staining towards the BST ($n = 3$). (d) Nitric oxide synthase 1 - neuronal (nNOS). Less than 10 nNOS+ observed cross all sections ($n = 2$). (e) Calretinin (CR) ($n = 3$). (f) Parvalbumin (PV) ($n = 6$). n numbers represent independent mice. All scale bars are 200 μm .

in MnPO revealed mostly non-overlapping populations, but across all stained sections no more than 10 nNOS⁺mCherry⁺ neurons were detected (3 x sections, n = 2). At this stage it is not known if these are staining above hM₃D_q-mCherry background expression. Further work will be carried out to quantify this staining and assess the importance of this potential nNOS subpopulation.

The calcium binding proteins PV and CR are also well known markers for GABAergic interneurons and both have expression in the preoptic area including both MnPO and MPO (*Zaborszky and Duque 2000, Xu et al. 2010*). PV is unique in that large numbers of positive neurons change their distribution between anterior and posterior sections of the preoptic area (Bregma +0.6 to 0.02 mm). Further forwards, PV⁺ neurons are found in the midline nuclei but progressively move to the sides of MPO and out towards LPO, at posterior coordinates. Mindful of this change in distribution, no positive double labelling was detected for either CR (n = 3) or PV (n = 6) (Figure 2.7e and f).

Despite evidence in the literature that suggests a GABAergic identity for the WSN our staining does not support this conclusion. The preoptic area is strongly positive for GABAergic markers but these did not convincingly double label with our warm-labelled neurons. Sparse labelling of ChAT and nNOS may be biologically significant but more work is needed to quantify these results. We also intend to perform confocal microscopy to reduce the bleed-through effects that dominate GAD67 staining. Whilst multiple attempts were made to co-label with other common markers including: anti-galanin, Neuropeptide Y (NPY), Vasoactive intestinal peptide (VIP), Brain-derived neurotrophic factor (BDNF), Early growth response protein 1 (EGR1), sometimes with several different antibodies, none were successful.

2.4. Summary of findings

In both humans and rodents, studies have shown that a warm stimulus in the hours before bed consolidates and increases the likelihood of sleep (*Horne and Reid 1985, Bunnell et al. 1988, Shapiro et al. 1989, Jordan et al. 1990, Morairty et al. 1993, Dorsey et al. 1999, Bach et al. 2002*). We postulated that there may be a connection between circuits that control temperature and circuits that regulate sleep, and data in this chapter provides evidence of such a connection. By activity tagging neurons in the preoptic hypothalamus with a warm stimulus and then reactivating these neurons, we were able to observe an increase in delta power as a direct consequence of this circuit. This was followed by hypothermia and a state of NREM-like torpor.

The connection between sleep and thermoregulation can be illustrated by comparison with the effects of general anaesthetics, which induce unconsciousness and hypothermia (*Taylor et al. 1985, Tang et al. 1993, Díaz and Becker 2010*). In our results, the appearance of an early rise in delta power followed by progressive hypothermia, is similar to the features of the sedative DEX (unpublished observation). However, we see loss of movement only with the onset of hypothermia. So, while a warm stimulus can activate neurons that increase delta power, they do not appear to initiate sleep directly. This is also consistent with human and rodent studies that found that warming does not induce sleepiness *per se*, if anything warming during sleep is disruptive (*Bach et al. 2002*). Instead, the latency to sleep is reduced. It has been shown that a change in delta power relates to sleep pressure that builds during wake and dissipates during sleep (*Tobler and Borbély 1986, Dijk et al. 1987, Werth et al. 1996, Vyazovskiy et al. 2009*). The increase in delta power seen here may be equivalent to an increase in sleep pressure as a consequence of being warm.

2.4.1. Biological implications

Why should being warm increase the pressure to sleep? There are well known connections between the regulation of temperature and the regulation of sleep that might shed light on the biological implications for this circuit. One circumstance is associated with an increase in delta power more than any other, this is sleep deprivation. Most interestingly, chronic sleep deprivation induces hypothermia, suggesting a requirement for a thermoregulatory response to an increase in sleep pressure (*Bergmann et al. 1989, Everson 1995*). Furthermore, this suggests that a connection between temperature and sleep circuits is activated by prolonged wakefulness. Is there a role for this circuit in natural sleep? Mammals have a circadian temperature cycle and are colder during their sleep phase (*Rechtschaffen and Bergmann 2002, Mohawk et al. 2012*). A circuit that relates warmer waking temperature to sleep pressure may help to regulate the timing of sleep, to ensure that temperature and sleep cycles are in phase. Circadian temperature cycles have been abolished in the *Rev-erb α* knock-out mouse and, although this may be because BAT thermogenesis is disrupted and not because the drive to change core temperature has been removed, these mice have disrupted sleep homeostasis and reduced sleep

consolidation following sleep deprivation (*Gerhart-Hines et al. 2013, Mang et al. 2016*). Finally, thermoregulation is less efficient during REM than NREM in humans and absent in REM sleep of small mammals (*Bach et al. 2002, Parmeggiani 2003*). This suggests that a drive to increase delta power, that might occur through our warm-sensitive circuit during NREM, is suppressed during REM. Consistent with this idea, NREM consolidation in humans and rodents following a warm stimulus is accompanied by a reduction in REM sleep (*Bach et al. 2002*). It is possible this warm-sensitive circuit has a role in the microarchitecture of sleep.

2.4.2. Future directions

To understand the role of this warm-sensitive circuit there are several questions to answer. The first and most important is what type of neuron has been activity tagged. We have strong evidence from immunohistochemistry that these neurons are not GABAergic and attempts to TetTag in VGAT-CRE mice have not been successful (not shown). These results are consistent with the population of neurons described as warm-sensitive by *Song et al.* in MnPO and dorsal portions of MPO (*Song et al. 2016*). But it is not consistent with the BDNF or PACAP GABAergic populations of VMPO and ventral MPO described by *Tan et al.* (*Tan et al. 2016*). Whilst both these preoptic populations can induce hypothermia, only the former is directly warm-sensitive by virtue of TRPM2 expression. It will be interesting to see if our tagged-WSNs express this channel. We may TetTag in specific glutamatergic neurons, with the aid of VGLUT2-CRE mice, to assess whether the population identified by *Song et al.* is the same that can increase delta power. However, this would not help us understand the heterogeneity of our warm-sensitive circuit. Instead, it may be more informative to TetTag in VGLUT2-CRE mice using the same AAVs as for wild type animals but include a third virus. This AAV would contain hSyn-Flexed-GFP and express only in VGLUT⁺ neurons and so tell us what proportion of our TetTagged circuit was glutamatergic.

We interpret our data as suggesting that our tagged neurons are normally involved in thermoregulation, but are also directly responsible for the increase in delta power that we observe. However, it is possible, that separate sleep-neurons, that are themselves warm-sensitive, drive this response. We can utilise the properties of sedative agents to help us better understand this circuit. For example, we know that DEX-induced sedation (100 $\mu\text{g kg}^{-1}$) occurs quickly (within 3 minutes of injection) whilst hypothermia takes much longer (unpublished observation). Our recapitulation experiments did not show fast-acting sedation, although the experiments of *Zhang et al.* in the same part of the brain could recapitulate this component of DEX (*Zhang et al. 2015*). This suggest two things, that WSNs do not act as sedative pathways and that, while sedatives like DEX may act through them, they also induce sedation through a different mechanism. We hope to be able to separate the components of sedation and hypothermia. I hope to record photometry signals of GCaMP6 in VGLUT2 neurons in the MnPO during sleep and anaesthesia. Comparing the timing of these neurons during sedation, and the onset of hypothermia, may help us understand if they are separate populations. Similarly, if these neurons are solely responsible for thermoregulation and sleep pressure, but not sedation, blocking their output using

tetanus toxin light-chain may separate the hypothermic and sedative components of sedative drugs like DEX.

One problem associated with the study of hypothermic agents, like general anaesthetics, is particularly important for our study. ECoG power is suppressed by lower temperatures and there may also be frequency dependent changes. We have accounted for this through a normalisation to total power, but it is hard to account for changes in frequency. To understand this relationship better, I intend to give DEX to animals in both a neutral and warm environment and compare the ECoG changes we observe. If this is successful, I will attempt to recapitulate warm-tagged mice in a warm environment. I expect this will prevent torpor and raise the power of the ECoG but its effects on behaviour will be most interesting. Finally, it is crucial to better characterise the effect of a warm-stimulus induced delta power on sleep. I will extend the experiments on the effects of a warm-stimulus, described in this chapter, by warming mice and then recording their sleep over the following 24 hrs.

Methods

2.5. Animals and Surgery

2.5.1. Use of Animals

Animals used in these experiments were done so following ethical approval and under Home Office regulations regarding the use of animals in scientific research. Animals were housed under 12-hour light dark cycles with *ad libitum* food and water. These were multiply-housed prior to surgery and then singly housed to protect surgical implants. Unless absolutely avoidable, animals did not undergo reuse under home office guidance. Strict adherence was paid to the 3R's guidelines on the effective and ethical use of animals under experimentation.

2.5.2. Surgery

Surgery for experimental procedures was carried out under 1-2% v/v isoflurane anaesthesia. During surgery and recovery analgesia was provided by subcutaneous (SC) injection of buprenorphine at 50 µg / kg and carprofen (Rimadyl), 0.004% v/v in the drinking water for seven days. All animals were given a course of broad-spectrum antibiotic, 0.01% v/v enrofloxacin (Baytril) in drinking water. Each animal was given no less than 1 week to recover after surgery.

2.5.3. TetTagging-DREADD AAV injections

For TetTagging, two viruses AAV-c-Fos-tTA and AAV-TRE-hM₃D_q-mCherry were stereotactically injected into adult C57BL/6 mice in a 1:1:1 ratio with 20% w/v mannitol, using either a silica micro-capillary pulled in house or a steel injector (10 µl-Hamilton). Specific injection volumes and infusion rates were set digitally on an electronic pump and ranged for 0.1-0.4 µl at 0.25 µl min⁻¹. Injection coordinates were AP +0.4 mm, ML 0 mm, DV -4.5 mm (MnPO) DV -4.8 & 5.2 (MPO) relative to Bregma. Viruses were allowed four weeks for normal expression. Viral preparation is detailed in 2.5.8

2.5.4. TetTagging Protocol

Animals were maintained on 200 mg kg⁻¹ doxycycline (Harlan TD.09265) until the beginning of the experimental protocol. The mice were removed from doxycycline 48 hrs before a warm stimulus of 2 hrs 32 ± 1°C, 50-60% relative humidity and immediately placed on 1 g kg⁻¹ doxycycline (Harlan TD.09295) for 24 hrs, before returning to 200 mg kg⁻¹ doxycycline. 72 hrs after stimulation mice were given intraperitoneal injections, in the dark phase at (zeitgeber time 18-20), of 5 mg kg⁻¹ clozapine N-oxide (Sigma C0832 or Tocris 4936) whilst recording ECoG and core temperature.

2.5.5. Recording ECoG

ECoG recordings were performed a Neurologger2A device allowing behavioural experiments in freely moving animals. ECoG screw were implanted at the same time as the virus injection under the surgery protocol at coordinates: Reference - AP +1.5 mm, ML -1.5 mm relative to Bregma, 1st - AP -1.5 mm, ML +1.5 relative to Bregma, 2nd Lambda -1 mm, ML 0 mm. Data was collected at 200 Hz with four times oversampling.

2.5.6. Recording Core Body Temperature

Core body temperature was measured using an implanted temperature logger (DSTnano, Star-Oddi) recording on a pre-defined programme sampled every two minutes for CNO or drug injections and every 30 mins for sleep wake experiments. A thermal imaging camera was borrowed from the laboratory of Dr. David Clements, Department of Physics, Imperial College London.

2.5.7. Electrophysiology

Electrophysiology measurements were obtained according to the protocols in Zhang *et al* by Drs Zhiwen Ye and Catriona Houston (Zhang *et al.* 2015). In brief, whole-cell current clamp recordings were obtained from mCherry+ neurons in the preoptic area of acute 250 µm-sections from warm-TetTagged mice. CNO was added at 5 µM and responses compared to ACSF (in mM: NaCl 125, KCl 2.5, CaCl₂ 2, MgCl₂ 1, NaH₂PO₄ 1.25, NaHCO₃ 26, glucose 11).

2.5.8. Data

2.5.9. Virus Production

Molecular biology and virus production for the TetTagging protocol was conducted under the protocol in Zhang *et al* and carried out by Raquel Yustos (Zhang *et al.* 2015). In brief, virus particles were produced by co-transfection of the AAV transgene plasmid with adeno-associated virus helper plasmids pH21, pRV1 and the adenovirus helper plasmid pFΔ6 in HEK293 cells. After 60 hrs these were purified by centrifugation and purified over 1-ml heparin columns (Sigma #5-4836). Samples were then concentrated using AmiconUltra-4 with Ultracel-100 membrane (100 kDa) at 2000 g for 10 min. Each virus was then aliquoted and stored at -80°C.

2.5.10. Immunohistochemistry

Animals were terminally anaesthetized and killed by transcardial perfusion of 4% PFA (w/v). Perfused-fixed brains from experimental animal were sliced at 35 µm thickness on a Leica microtome (VTS1000S)

and stained free-floating with primary antibodies under a standard protocol. Specific conditions for each antibody are given in the following table. Sections were washed three times in PBS, permeabilised in 0.4% (v/v) Triton X-100 PBS for 30 min and then blocked in 0.2% (v/v) triton, 4% (v/v) normal goat serum (NGS) for 30 min before incubation with primary antibody overnight at 4°C. The sample are then washed three times in PBS, each 10 min, and then incubated at room temperature with secondary antibody in PBS 2% (v/v) NGS for two or three hours. The secondary antibodies were Alexa Fluor 594 goat anti-mouse IgG (H+L) (Molecular Probes, A11005), Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes, A11034), Alexa Fluor 594 goat anti-Rat IgG (H+L) (Molecular Probes, A11007, Alexa Fluor 488 donkey anti-goat IgG (H+L) (Molecular Probes, A11055). All secondary antibodies were used at 1:1000 dilution except SOM (1:500) and AChT (1:200). GAD67 staining required a tyramine amplification step (Molecular Probes T20912) and the protocol followed the manufacturers specification using a kit of H₂O₂ (0.0015% v/v) HRP goat anti-mouse conjugate (1:200) (Alternative: 31470 Thermofisher, 1:200) and Alexa Fluor 488 tyramide (1:100, 45-90 mins). Triton X-100 prevents this protocol from working and NGS is replaced by 5% w/v bovine serum albumin (BSA).

2.5.11. Specific antibody conditions

Primary Antibody Target	Order Code	Company	Species	Dilution	Initial Condition
GFP	A6455	Thermofisher	M	1:1000	4°C, ON
mCherry	632543	Clontech	Rb	1:1000	4°C, ON
mCherry	M11217	Thermofisher	Rt	1:1000	4°C, ON
Parvalbumin	N.K	N.K	Rb	1:1000	4°C, ON
Calretinin	ab16694ab702	abcam	Rb	1:1000	4°C, ON
Somatostatin	MAB354	Millipore	Rt	1:500	4°C, ON
GAD	MAB5406	Millipore	M	1:1000	4°C, ON
nNOS	N2280	Sigma	M	1:1000	4°C, ON
ChAT	BP144P	Millipore	Gt	1:200	4°C, ON
c-Fos	sc-52	Santa Cruz	Rb	1:1000	RT, ON

2.5.12. Data Analysis

ECoG data was analysed using spike2 software (CED) using custom scripts (Appendix 3.11). Specific detail is given in the figure legends and the text. 'Power in band' was calculated as running power spectrums of 1024 bins for each band, 1-4 Hz (delta), 5-9 Hz (theta), 9-14 Hz, 15-25 Hz, 25-40 Hz and dividing by the power between 1-99 Hz. Images were analysed using the Fiji v.1.47a. Graphs and statistics were generated in OriginPro v8.6.

Chapter 3: Slowing neuronal circuits by cooling

Introduction

3.1. How do anaesthetics work?

Anaesthetic mechanisms are diverse with each molecule interacting with different molecular targets. But these drugs converge on the same outcome - a state of unconsciousness. How can different molecules, as diverse as propofol, isoflurane and even Xenon produce the same outcome? One primary hypothesis is that this convergence is at the level of neuronal circuits, in particular on sleep pathways (*Nelson et al. 2002, Franks 2008, Zhang et al. 2015*). This hijack of the endogenous control of consciousness would explain the sleep-like states observed under anaesthesia. But despite different mechanisms, the states of unconsciousness appear the same in behavior and often, ECoG. Could these convergent pathways act in different ways but on the same 'switch'?

3.1.1. Neural mechanisms for general anaesthesia

There are many channels and receptors that are anaesthetics sensitive, but within this diverse group, a small number of candidates are thought to be the principal mediators of the anaesthetic effect. Principally, these are the GABA_A receptor, N-methyl-D-aspartate receptor (NMDA-R) and the two-pore-domain potassium channel (2PK) (*Franks and Lieb 1994, Franks 2008, Yip et al. 2013*).

The GABA_A receptor is widely expressed in the central nervous system. It is comprised of five subunits two α , two β and a γ subunit most often in an $(\alpha_1)_2(\beta_2)_2\gamma_2$ conformation but also as $(\alpha_2)_2(\beta_3)_2\gamma_2$ and $(\alpha_3)_2(\beta_{1-3})_2\gamma_2$ channels (*Franks 2008*). The γ_2 subunit is speculated to be involved in synaptic localisation, but extrasynaptic receptors are also found. Levels of γ_1 expression throughout the brain are lower than γ_2 , but distinct patterns of expression are still found throughout the brain. For example, the MnPO and MPO regions of the anterior hypothalamus appear to have distinct γ_1 expression. (*Wisden et al. 1992*). These expression patterns govern the way general anaesthetics like propofol act on specific neuronal circuits, but the nature of this relationship is unresolved.

Understanding how loss of consciousness is initiated has been the subject of much research. Comparisons of local field potentials across the cortex and thalamus have provided evidence of a centralised 'hub' that may facilitate loss of consciousness during sleep and similarly may be sensitive to the actions of general anaesthetics (*Baker et al. 2014*). Propofol is the drug of choice for anaesthetic induction. The high frequency activity (20-40 Hz) seen under propofol or natural sleep changes during loss of righting reflex (LORR) or NREM onset. The timing of this change in the CMT is 5-10 seconds before changes in the barrel and cingulate cortex (*Baker et al. 2014*). Furthermore, isolation of the thalamocortical loop from external stimuli during propofol anaesthesia has also been observed in human subjects with magnetic resonance imaging (MRI) (*Ní Mhuircheartaigh et al. 2013*). Although, thalamocortical timing is not different in DEX sedation, it may share a similar thalamic mechanism through changes in phase relationship (*Baker et al. 2014*).

General anaesthetics act on different molecular targets, but these different targets may inhibit the same common circuitry. Equally, anaesthetics may act on quite different circuits that ultimately result in the same end point. Dissecting these questions remains challenging but classical methods of analyzing the contribution of a local circuit has yielded interesting information. Injecting low volumes of drugs or other pharmacological agents to target nuclei helps to dissect their role in the large system. For example, microinjections of pentobarbitone into the PnO region of rats suggests that a distinct anaesthesia-sensitive region exists for barbiturates. This was named the mesopontine tegmental anaesthesia area (MPTA) (*Devor and Zalkind 2001, Sukhotinsky et al. 2007*). Interestingly, this area overlaps with a newly discovered glycinergic ascending pathway to the thalamus (*Giber et al. 2015*). Injections of GABA_A receptor agonists such as muscimol or CNQX a competitive antagonist at the AMPA receptor, are also common. These alter circuit dynamics, usually by changing the balance of excitation or inhibition. However, even with small injection volumes it is well known that diffusion is fast and wide and the concentrations used are often high. Quantification of this effect by Edeline *et al* using radiolabeled muscimol suggests that injecting a volume of just 50 nl, distributes to a volume of 50-143 μ l (equivalent to mm³) within 15 minutes of the injection (*Edeline et al. 2002*). To control for this, behavioral results must be observed very soon after of the injection to have confidence in the specificity of the data. Furthermore, repeated application requires a washout period for the animal.

3.1.2. Cold neurons are slow neurons

My research here aims to assess the validity of localised cooling as an alternative method to achieve the same goal of understanding the contributions of specific nuclei. Neurons are highly sensitive to changes in temperature. Cooling reduces their intrinsic firing rate and low temperatures can induce full inactivation before permanent cell damage occurs. Temperature distribution is finely regulated within the brain to a narrow physiological range to mitigate these effects, but deliberate local cooling has been used for many years to disrupt these circuits because it can be spatially focused and brief in time.

The idea of the refrigerant cooled probe was used by Bénita and Condé in 1972 and successfully further adapted by Zhang et al in 1986. In both cases Freon was used as the refrigerant and actively pumped into the inner barrel of a “cryoprobe”. After vaporisation the gas then leaves via the outer compartment which is usually under vacuum to aid the process. Figure 3.1a shows the design of the 1986 Freon cooling circuit and the cryoprobe design for implantation in the cat. The probe comes with a heater wire, to reduce off-target cooling, and an internal thermocouple to provide temperature feedback. Tested in the anaesthetised cat, this method can reduce brain temperature, at a distance of 1 mm from the probe surface, to 10°C in less than 1 min (*Bénita and Condé 1972, Bénita et al. 1979, Zhang et al. 1986*). This is sufficient for complete inactivation of the neuronal circuit. Effects would likely result from a larger region still but the probe could be adjusted to warmer temperatures if required. As the probe reference was set to zero, any lower may cause tissue damage and so this represents the maximal cooling that can be achieved. In classical studies, also by Bénita and Condé, inhibition of the saphenous nerve action potentials by cooling probe could only be achieved at extremely low temperatures of -10°C at 1 mm distance. Whilst the amplitude of their recordings was quite stable, latency to firing was dramatically

different from temperatures lower than 15°C (*Bénita and Condé 1972*). Unfortunately, intermediate temperature up to 37 were not tested.

Alcohol-loop cooling, or cooling with similar non-freezing mixtures has been used for many years and with significant success (*Skinner and Lindsay 1968*). The most recent paper to adapt this method was published by Cooke *et al* and focuses on efficient use of this system with low cost components - data acquisition through the commonly used CED1401 hardware and a temperature feedback loop through Spike2 software (CED) (*Cooke et al. 2012*). The goal of this cooling system was to completely inactivate the targeted neuronal circuits. The “cooling-chip” they designed was adapted for use in small animals such as the ferret or larger but still occupies a volume of 21 mm³ and so would require further miniaturisation for use in rats. The basic design and implantation of this device can be seen in Figure 3.1b.

The temporal dynamics of neuronal circuits can be manipulated by temperature. Long *et al* showed that particular motor neurons in the song bird brain were directly responsible for the timing of vocalizations. Cooling these neurons slowed their firing and the bird song itself. The acoustic structure remained unaltered. This important finding was achieved through precise control of an implanted Peltier device (*Long and Fee 2008*). Subsequently, the same lab published detailed analysis of their method including the optimization of cooling probes, heat removal from the warm side of a Peltier device and detailed modelling of heat distribution around the cooling probe, in the bird brain. The latter can be generalised to mammalian brains with minor alterations to physiological parameters (*Aronov and Fee 2011*) The design of the Peltier is shown in Figure 3.1c. The limitation of this device is the maximal extent of cooling, which may modulate neuronal circuits but be insufficient for inactivation, particularly of deep structures.

As discussed, the thalamus is thought to be a key hub in the regulation of the conscious behavior because it integrates sensory awareness. Changes in ECoG during sedation, sleep or torpor may represent changes in the thalamocortical loop (*Baker et al 2014*). But is this structure necessary and to what extent to are these changes causative of the observed behavior? A cooling probe that could slow the electrical dynamics of the thalamocortical loop, through a thalamic hub such as the central medial thalamus, would help to answer this question. A reduction from 40 to 20 Hz in gamma oscillations is associated with anaesthetic transitions. A cooling probe may help to bring about this change artificially in an attempt to recapitulate the behavior of a sedative.

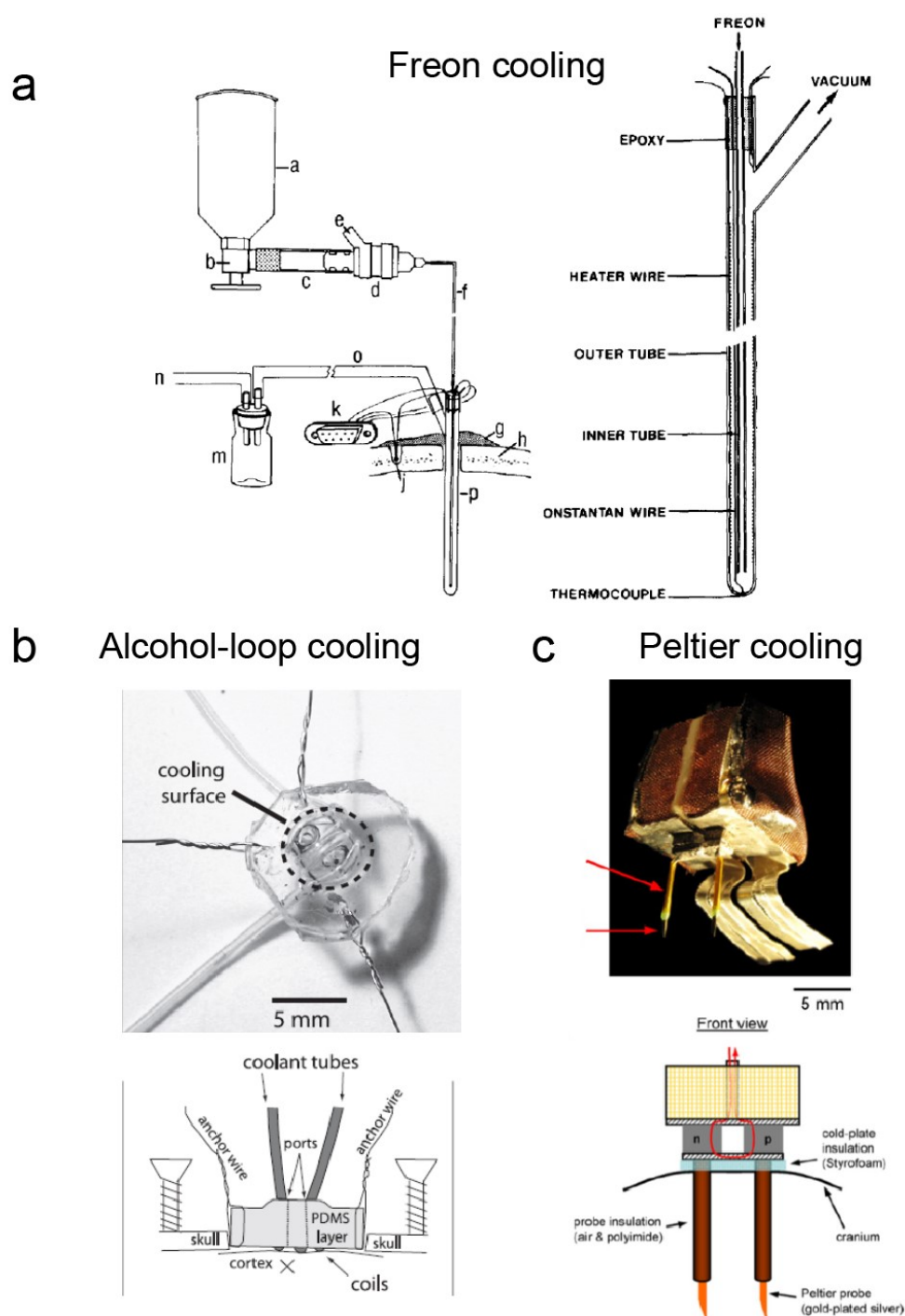


Figure 3.1 – Historical Methods. Examples of technologies used for cooling the brain. (a) Refrigerant cooling system based on pumped Freon, controlled by a fuel injector. The cryoprobe itself is under vacuum and contains a thermocouple and a heating coil (Zhang *et al.* 1986). (b) shows a recent example of alcohol-loop cooling with implantation of tubing on the cortical surface (Cooke *et al.* 2012). (c) Design of a Peltier device for shallow cooling in the song bird brain. Bilateral probes are visible as are cooling ‘fins’ attached to the hot plate (Long and Fee 2008, Aronov and Fee 2011).

3.2. Aims and Objectives

Primary Aims:

What does it mean to be unconscious? Sleep and anesthesia are clearly different, the things that wake us from sleep must not wake us from anaesthesia. And yet, both are unconscious states with similar ECoG. In both sleep and anesthesia, the thalamocortical loop is disrupted - synchronised so that information cannot be relayed. If the thalamic nuclei integrate sensory information, then without it we may fall into unconsciousness. I will investigate methods of local brain cooling that might allow me to answer this question.

Each method discussed has unique advantages and challenges but retains some core features. Firstly, a method of cooling must be controllable and flexible so that it can be delivered to a tethered-awake animal. Secondly, the cooling effect must be focused to a target region, whilst also protecting off-target areas. And thirdly, an experimental measure such as ECoG or LFP recording, must be feasible under the two previous conditions. Three distinct methods have been used in the literature and I will investigate them in turn. The first is indirect refrigeration, using both a cryoprobe and a solid probe. The second is the alcohol-loop cooling method and finally there is cooling with an implanted Peltier device. I will discuss the development of each method, how they are attached during surgery and what data they ultimately generated. I will then weigh each method and attempt to generalize their experimental value.

Primary Objectives:

- I will investigate three different methods of cooling deep brain nuclei in the rat
- I will choose a cooling probe methodology to cool midline thalamic nuclei
- During cooling I will collect ECoG data
- Following the experiment I will use histology to confirm probe placement.

Results and Discussion

3.3. Technologies of cooling

3.3.1. Inactivation by refrigerant cooling probe

Cooling by refrigeration uses the principles of vaporisation to remove heat from a target region. In previous studies, hollow thermodes with liquids running through them, have been inserted into animals and liquids passed through them (*Bénita and Condé 1972, Bénita et al. 1979, Zhang et al. 1986*). We hypothesised that a liquid refrigerant might be used with our own hollow cooling probe or 'cryoprobe' and, under the right conditions, vaporise at the tip allowing warmed refrigerant gas to escape. In doing so, the cryoprobe could be used to cool a deep brain region of interest.

Refrigerants are liquids that, when undergoing a phase transition, remove heat from the environment. Thus, these substances have high enthalpies of vaporisation and low boiling points. Whilst there are many commercial refrigerants, most need to be certified and retrieved after use. However, there are other chemicals with similar thermodynamic properties that are not classed as refrigerants. 1,1-difluoroethane ($C_2H_4F_2$) (R152) is one such compound and is most commonly known as dust-off®, an aerosol canister used for cleaning. At moderate pressures, of around 5.1 bar (510 kPa) difluoroethane is a liquid but at normal temperature and pressure it is a gas. This phase transition requires large amounts of energy, approximately 275 kJ kg^{-1} . This enthalpy of vaporisation provides the cooling 'power' of our refrigerant.

Refrigerant circuits take advantage of the thermodynamic properties of refrigerants to control and direct their cooling. This is achieved by circulation of the liquid through a cycle of evaporation, to produce cooling, and recompression to reuse the refrigerant. For our system, a closed circuit would not have added benefit and R152a is cheap and not harmful if released to the environment. Figure 3.2a shows an open refrigeration circuit connected to a cryoprobe. The cryoprobes were purchased as custom made steel tubes with a single bullet-end and combined with a polyimide inner tube to separate liquid from gaseous refrigerant. These tubes form an outer and inner compartment. The cryoprobe would sit mounted in a bracket specifically designed for surgery (Figure 3.2b). I conceived of all the designs shown, but Andrew Rochester (CAD technician, Mechanical Instrumentation Workshop, Department of Physics) directly used the CAD software to produce the schematics. I have used these schematics to produce images and figures that show my designs.

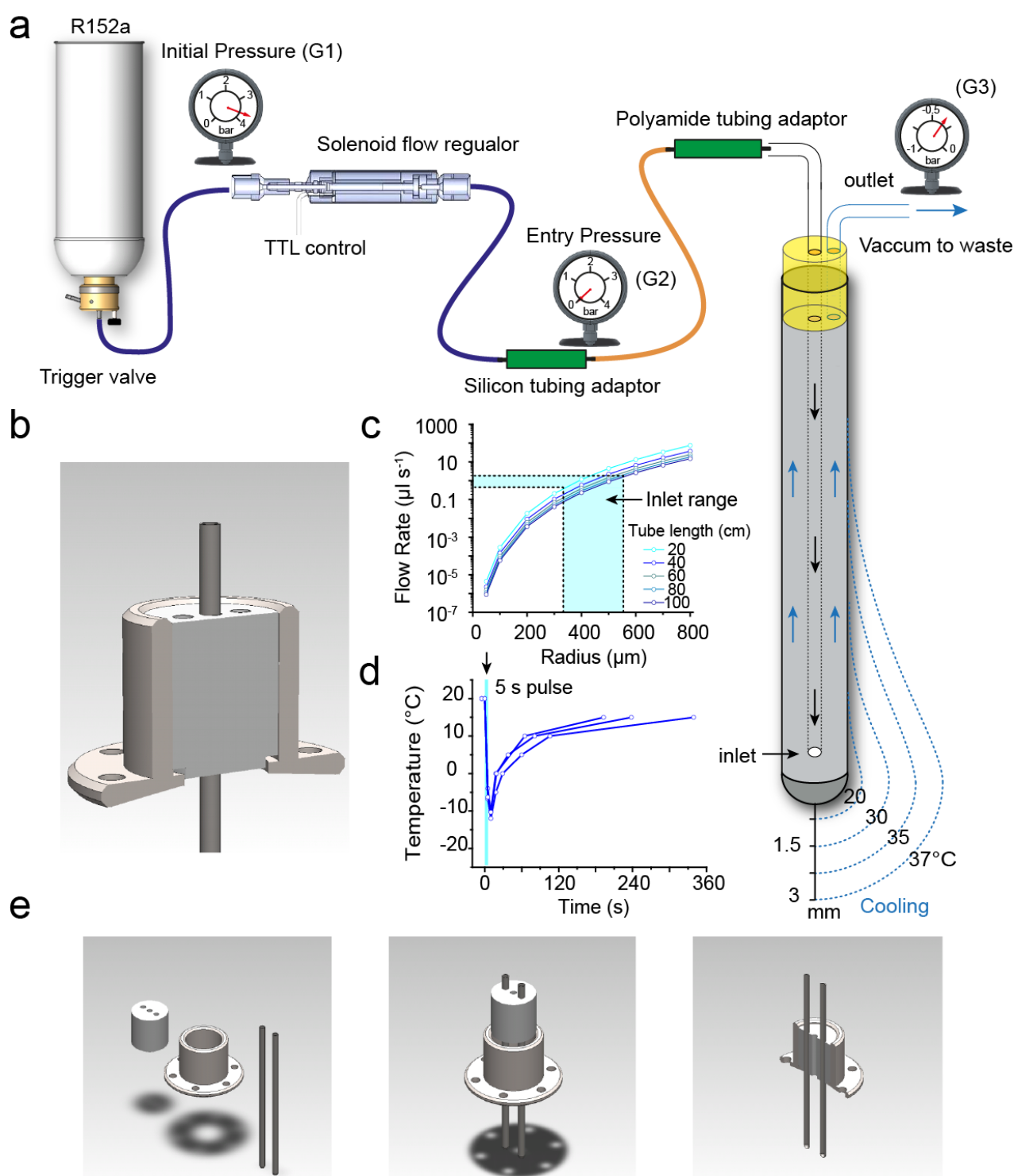


Figure 3.2 – Hollow Cryoprobe. (a) Configuration of the hollow cryoprobe circuit from the R152 canister. G1, 2 and 3 present pressure gauges in the system. The isotherm lines represent an exponential distribution of temperature values assuming an average cooling of 33°C in a sphere of $117\ \mu\text{l}$ ($r = 3\ \text{mm}$), as described in the text. (b) Cross-section for the head-mounting bracket design with polystyrene insert. (c) shows the minimum and maximum range for the inlet radius given the Poiseuille relationship between flowrate and radius (d) shows a cooling test in triplicate initiated by 5 second pulses of refrigerant through the trigger valve. The solenoid was not connected for this experiment. (e) shows the assembly of the head-mounting bracket in a bilateral probe configuration. The designs were envisaged by the author, but the CAD software was handled by Andrew Rochester (CAD technician, Department of Physics).

The key to our system is to induce a pressure-drop at the point that cooling is required, in this case inside the hollow cryoprobe. In this method, the liquid-phase refrigerant, from an upturned R152a canister, is held at a constant pressure by a trigger valve. The experimenter sets the initial pressure of the system by limiting the flow of liquid at the trigger valve, but keeping the gauge pressure just above the R152a vapor pressure. This restricts the flow rate to a manageable level and reduces vaporisation and freezing in the tubing. To begin the experiment, the solenoid flow regulator-valve is activated by a transistor-transistor logic (TTL) signal. The timing of the TTL signal can then be adjusted, to switch the valve at a predefined cycle rate and regulate the flow further. The refrigerant passes from the Teflon tubing to smaller silicon tubing, through an adaptor, and finally to polyimide tubing directly into the cryoprobe. The resistance provided by the tube increases linearly with length and helps to reduce the flow rate, as well as maintain the pressure and the refrigerant in the liquid phase. A pressure drop, when comparing the initial (G1) and entry pressures (G2), would signify a leak in the system.

Using the thermodynamic properties 1,1-difluoroethane (PubChem CID 6368), it is possible to estimate a viable flow rate for cooling. If we assume an enthalpy of vaporisation of 275 kJ kg^{-1} , and a liquid density at 4 bar of 990 kg m^{-3} , then vaporisation consumes $0.27 \text{ J } \mu\text{l}^{-1}$ of refrigerant. If the flow rate was $1 \text{ } \mu\text{l s}^{-1}$ then the cooling power of the cryoprobe would be 0.27 W . How much brain tissue would this cool down? If we consider that the main constituent of brain tissue is water, then we can use the specific heat capacity of water ($4.178 \text{ J g}^{-1} \text{ K}^{-1}$) to estimate the effect of cooling. If we further assume that our target temperature is no less than 4°C to induce neuronal inactivation, and that we will cool for one minute, then:

Eq.1

$$\frac{0.27}{4.178 \times 33} \times 60 = 0.117 \text{ g}$$

we will cool approximately $117 \text{ } \mu\text{l}$ of tissue around the tip of the cryoprobe to 4°C . This is the equivalent of a sphere around the cooling probe of 3 mm radius. In practice, this would not be evenly distributed. In Figure 3.2a, isotherm lines describe the estimated relationship between temperature and radial distance from the source, plotted to a more realistic exponential decay. Solutions to the three dimensional heat equation, in the vicinity of a cooling rod in the brain, were solved recently and the solution is a zero order modified Bessel function of the second kind, more often known as a Bessel-k function (Aronov and Fee 2011). However, this can be approximated to a simple exponential of the form: Ae^{-x} .

Our calculations suggest that $1 \text{ } \mu\text{l s}^{-1}$ is a reasonable rate to connect an experiment of at least 60 seconds for sufficient cooling of brain tissue. To set the flow rate, the circuit was adapted to the parameters of Poiseuille's equation. This governs the relationship between the physical properties of the liquid and its performance under pressure. The two most important parameters that can be varied are the length of tubing, between the source and the inlet, and the radius of the tube at its exit. The exit here is the polyimide inlet to the cryoprobe. The relationship between these parameters is shown in Figure 3.2c and sets a minimum and maximum range for the radius of the inlet. In the open, the pressure

drop is determined by the refrigerant and atmospheric pressure. I optimised these parameters in an attempt to reduce the flow rate inside the cryoprobe to value between 0.5 and 2 $\mu\text{l s}^{-1}$. Further adjustment of the solenoid valve cycle rate is then used to optimize vaporisation - this is discussed in more detail in the next section. An example of cryoprobe cooling *in vitro* is shown in Figure 3.2d imitated with five second burst of refrigerant. The solenoid was not used for these experiments

Finally, the refrigerant moves from the smaller volume of the polyimide inner tube to the larger outer tube, which is also under a small vacuum (0.3 bar), and vaporises, thus taking heat from the environment. The vacuum is important because of the small volumes in the cryoprobe and the rate of expansion for this refrigerant. For each 1 μl of liquid refrigerant vaporised, approximately 246 μl of gaseous refrigerant must be extracted through the exit port. If it cannot leave, the pressure rises and vaporisation will stop. When the circuit functioned as it should, the refrigerant was removed from the behavioral arena and vented to air. However, a buildup of pressure and liquid phase refrigerant in the cryoprobe was a frequent problem. Any leaks that resulted would also freeze on the equipment further compounding technical challenges of this system. Whilst this method would allow high levels of cooling, it is not easy to set up or operate. In particular, it requires precision control of a pressurised refrigerant as well a wide diameter of metal probe that must be surgically implanted.

3.3.2. Challenges of implanting a cryoprobe

The first design for holding a cryoprobe was envisaged as a titanium attachment screwed to the skull and holding a cooling probe in place using a rubber or polystyrene insert. The probe itself would be discarded after use but the attachment retrieved and reused. Figure 3.2e shows a computer-aided design (CAD) of this first attachment, housing twin cryoprobes for the bilateral cooling of deep brain structures. It has three components, the first is a titanium housing that can be attached to the skull of a rat using the same screws used for ECoG recordings. The second is a polystyrene foam insert that holds guide paths for each cryoprobe and helps insulate the cortex from the cooling effect. A single cryoprobe for unilateral cooling can be mounted as easily as two and implanted stereotaxically.

Despite the challenges of operation, the principal difficulty with this design was the surgical implementation. Previous experiments have focused on larger animals such as ferrets and cats, but the rat brain is far smaller. A width of 1 mm on the metal shaft of the probe was too extreme for surgery and especially in deep targets. Furthermore, this measurement did not include an insulating layer that would make the probe thicker still. The inclusion of a heater wire would be essential, but inside a much smaller barreled probe – this would be a significant challenge to overcome.

I considered alternative configurations that might allow a small probe diameter, particularly focusing on combinations of inner tubes that would both create air gaps for insulation and extract waste gas. Unfortunately, given the small scale I could not control the refrigerant through an inner tube of less than 500 μm diameter. Ultimately, I could not prepare a device of this configuration that was both functional and small enough for implantation in the rat brain. To overcome this, I started investigating the idea of

a solid probe or spike as a conductor of heat thus removing the need for flow-rate control deep in the brain.

3.3.3. Progression to solid probes

The use of solid probes carries many advantages over the hollow design. Firstly, the diameter of the probes can be greatly reduced and space savings used to incorporate an insulator, whilst maintaining surgical viability. The material could also be switched for one of greater thermal conductivity in this case steel ($16 \text{ W m}^{-1} \text{ K}^{-1}$) is replaced by gold-plated silver ($420 \text{ W m}^{-1} \text{ K}^{-1}$) and, without a need for manufacturing, these are much easier to source. This method has already been used in the literature by Fee *et al*, albeit for shallow cooling of less than 2 mm in the brains of song birds (*Long and Fee 2008*). However, the properties of this probe design can be adapted for rat brain targets of greater than 6 mm by making two adjustments. Firstly, by increasing the temperature gradient between the exposed end of the probe and the target i.e. having a colder source; and secondly, by exposing a larger surface area of the probe to cooling, thereby increasing the rate of heat flux away from the target. To make a solid probe, I cut gold plated silver wire of 200 μm diameter into lengths of 15 mm and placed them inside a polyimide sheath of 300 μm inner diameter, leaving a 50 μm airgap. Both ends of the probe were then sealed with cyanoacrylate (Superglue). At each end an exposed section allows heat transfer, 1 mm at the bottom and 5-8 mm at the top depending on the depth of the brain target. Surgical procedures for implantation are discussed in a latter section.

3.3.4. Designing a vaporisation chamber

Extracting heat from the probe can be achieved with a small chamber for vaporisation. Designs for the chambers to hold a solid probe are shown in Figure 3.3. I decided to have these chambers 3D printed to keep them small, light and easy to modify. They were also quick and inexpensive to prototype in the early stages. The first reservoir for the solid probes is depicted in Figure 3.3a. After the probes have been implanted into the skull, using a stereotaxic frame, the base section is placed on top and attached to the skull using screws that normally record ECoG. The ECoG wires are then soldered to the same screws. This reuse of the screws reduces the surgical impact. The cap section of the chamber has two sockets that function to secure the upper portion of the probes and maintain probe alignment in the brain. When the top section is secured with tissue glue, a reservoir is formed between the top and bottom. Two ports allow refrigerant to enter and exit, with the smallest hole for pressurised liquid R152a and the larger for the gaseous R152a. Vaporisation in the chamber induces cooling, removing heat from the probes. The addition of a domed recess under the reservoir provides an air gap that insulates the cortex from the chamber, however, when this chamber was tested on the bench the reservoir was clearly too small and the area of exposed probe insufficient. At even moderate flow rates, liquid refrigerant would fill the chamber and prevent further cooling. Nonetheless, some cooling would occur and the chamber was surgically implantable. The concept seemed promising although substantial design changes would be required to better control the way the refrigerant passed through.

Figure 3.3b shows the second iteration of the chamber design. I increased its volume by extending the cylindrical portion upwards. Whilst the refrigerant entry port through the top remains narrow it is also now threaded to prevent leaks. The gas exit port is still larger than the entry to try and facilitate vaporisation in the correct place in the circuit. The increased total height also allows a larger portion of the probe to extend into the reservoir and thus more surface area for extracting heat. The domed recess is retained to provide an essential air gap but increased in height to allow insulating layers of polystyrene to be placed directly on the skull. The connection between the baseplate and the top section now has a universal orientation so that the gas exit port can be rotated during surgery and not interfere with the ECoG attachments. This was an essential addition that resulted directly from the challenges of surgery.

Figure 3.3c shows a chamber I modified to accommodate two probes in a bilateral configuration. Whilst not always required, many interesting structures have bilateral symmetry in the brain. A good example is the MPTA. Unfortunately, in this setup the upper torso cannot be rotated as the design for the base section was taken from 3.3a and is stronger when attached to the skull. Two probes require more stability than one and they are more likely to be positioned further back on the skull requiring more screw positions. On this version, a slight camber was added to the base section to better fit the curvature of the skull at posterior coordinates. In each case the chambers were intended to be surgically mounted over an insulating layer. After several rounds of testing, the chosen configuration was a layer of polystyrene foam, which has very low thermal conductivity ($0.2 \text{ W m}^{-1} \text{ K}^{-1}$) and an aerated insulator that has fine air pockets. These were in addition to the air gap built into the evaporation chamber.

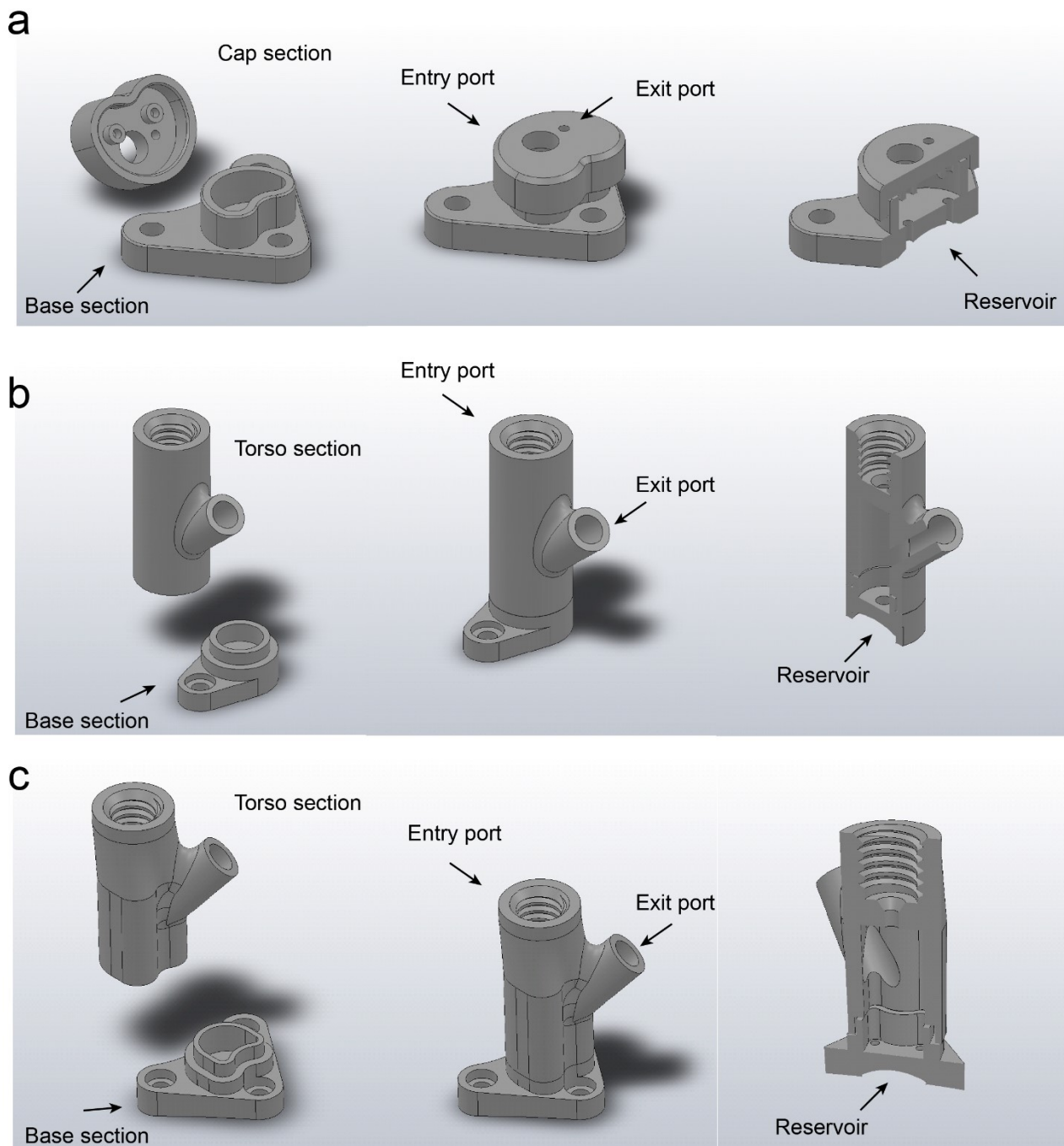


Figure 3.3 - Designing a vaporisation chamber. Illustrates the design and testing of vaporisation chambers. (a) one of the very first designs, consisting of a base section and a cap section. The base has screw attachments and is glued to the cap section to form the reservoir. The cap section includes sockets to maintain the stability of the probes. (b) A more recent design incorporating several changes including an extended torso section and threaded entry port. The entry port is substantially narrower than the exit port. This was the first design to include a universal orientation and a smaller base section to improve surgical outcome. A domed recess is included for cortical insulation. (c) an alternative design for bilateral cooling. The more stable base section from (a) was adapted to the longer torso. The domed recess is larger and the base section cambers to the curvature of the skull. The designs were envisaged by the author, but the CAD software was handled by Andrew Rochester (CAD technician, Department of Physics).

3.4. Cooling by refrigeration

Refrigerant circuits require control of flow rate and crucially a pressure drop as the refrigerant passes to a region of high volume. In an open circuit, the inlet side of the reservoir is at high pressure whilst the outlet side is low pressure. The key to control is restricting the midpoint of these two arms, the point at which cooling occurs, to the vaporisation chamber. The principle design and testing of the circuit itself, along with the solenoid flow regulator, is shown in Figure 3.4. This includes all components although many amendments and configurations were necessary to improve consistency and reliability. It is intended to be seen as an evolution of the circuit in Figure 3.2a, but the way it works is different in principle. Reliability and the difficulty of troubleshooting during an experiment are the defining challenges of this circuit.

As in the previous circuit, the canister of R152a is opened by depressing the custom-made trigger valve and held open by an adjustable screw. The solenoid valve remains closed and the pressure gauge value can be read. The adjustable screw is used to set the initial pressure to 4 bar (G1). It should be noted that all of the pressure gauges used showed small signs of leakage at high pressures, but not enough to disrupt their operation. To start the refrigeration, the first solenoid valve is opened by a switch, away from the behavioral arena, and a pressure drop is recorded across the first two gauges, reading 2 bar each (G1 and G2). Instead of just liquid, a liquid-vapor mix travels first through a flow restrictor before entering the solenoid flow regulator, which now acts as an expansion valve. A second pressure drop produces more vapor, now cold from its phase transition, but much closer to the vaporisation chamber. A non-return valve (V1) prevents its backflow. This also protects the solenoid expansion valve from the liquid refrigerant if the pressure rises unexpectedly. Vaporisation should occur immediately after the non-return valve (V1). By moving the solenoid closer to the animal, the vaporisation is much closer to the target and more controlled. Cooling occurs in the chamber and refrigerant gas leaves via the exit port and through a second non-return valve (V2). Although this valve raises the pressure in the tubing (T3), a small vacuum pump on the exit side increases the pressure drop by 0.3 bar (G3) to prevent condensation and improve gas extraction.

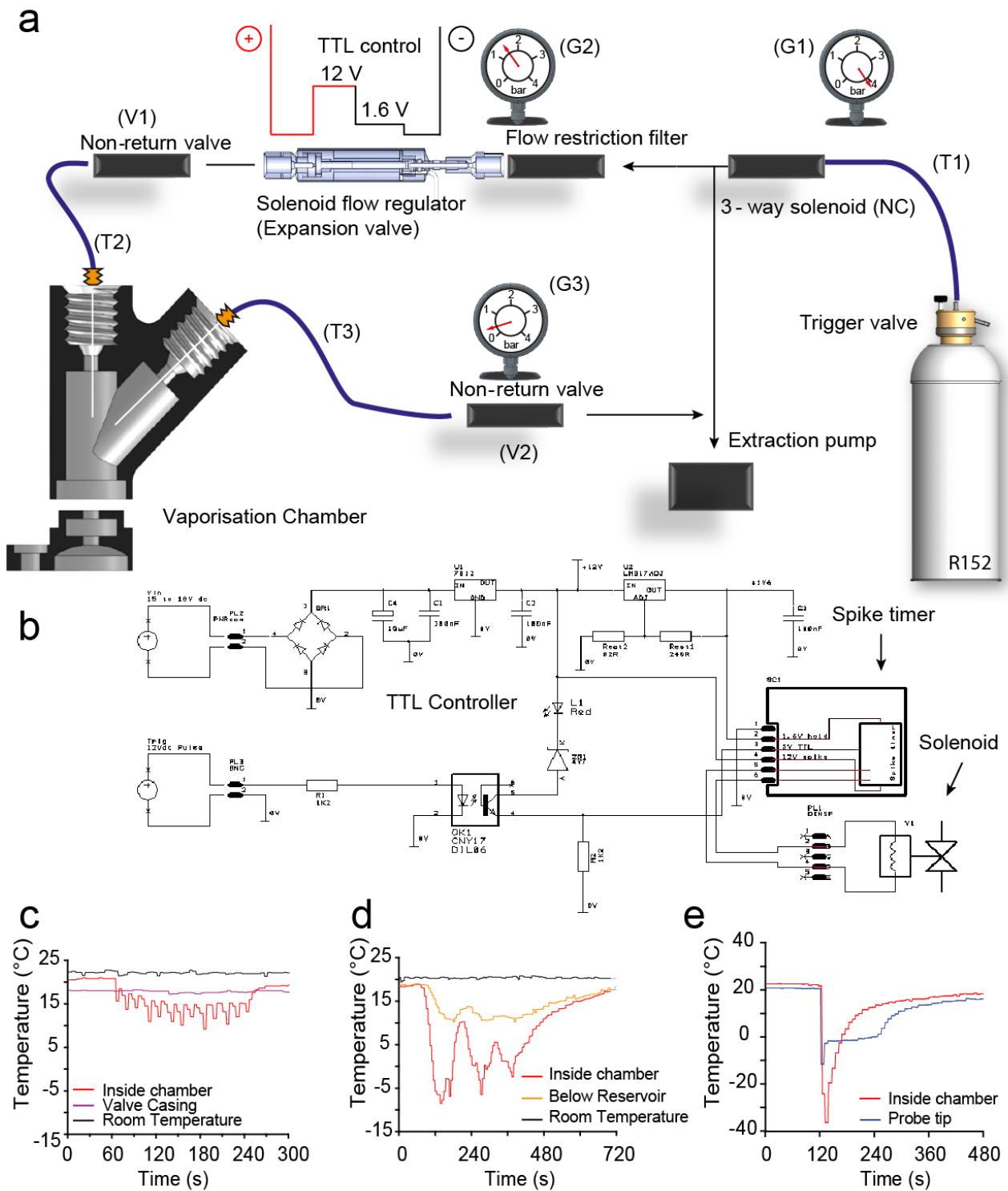


Figure 3.4 - The refrigeration circuit. Shows a refrigeration circuit complete with control and calibration. (a) shows the circuit itself with the solenoid flow regulator acting as an expansion valve. It is shown with a normally closed (NC) 3-way solenoid valve that can redirect gas flow to waste during an experimental off-period, this prevents having to recalibrate during behaviour recordings. The non-return valves are labelled V1 and V2 in order of operation. The vaporisation chamber is shown with both ports threaded. (b) Shows the circuit diagram for the TTL controller that was built in house. This was constructed by the author under the guidance of Susan Parker (Electrical technician, Department of Physics). (c) operation of the solenoid valve during benchtop testing. (D) shows an example of another test without the solenoid. Substantial off target cooling is also detected. (e) relationship between chamber cooling the cooling to the tip of an 8 mm silver probe.

Figure 3.4b shows the electronic control circuit of the expansion solenoid. This takes the input of a spike generator and produces a 5 V TTL signal, which is fed to the commercial spike timer that came with the valve. The circuit also takes a separate input voltage and outputs at two constant voltages, 12V and 1.6V, that are also sent to the spike timer. On receiving the TTL signal, the spike timer allows a 12V square wave of fixed duration to open the solenoid valve; the duration of which is set by a potentiometer. This is immediately followed by the 1.6 V holding voltage applied until the TTL signal is turned off. In operation, a sequence of spikes from the generator produces a sequence of defined and precise openings in the solenoid valve and a precise flow of refrigerant vapor. For this experiment I tested solenoid valves with fluorinated elastomers (FKM type) and ethylene propylene diene monomer (EPDM) seals. These O-ring link components are key to valve function but by very sensitive to swelling, crystallization of salts or water-ice and corrosion. I made temperature recordings of the solenoid valve and the reservoir to calibrate the equipment and these can be seen in Figure 3.4c. The opening of the expansion valve produced periodic drops in reservoir temperature but was also accompanied by cold vapor that contributes greatly to the cooling effect, depressing the temperature baseline. It is possible that some vapor escapes the valve at these pressures even when closed, and that once opened, the valve fails to close fully and a small but constant flow of vapor passes through. Measuring the temperature of the solenoid valve itself showed a constant temperature and helps to ensure that vaporisation is only occurring after the non-return valve (V1). Unfortunately, consistent replication of this protocol was profoundly difficult.

An example of refrigerant cooling without the solenoid valve is shown in Figure 3.3d. Whilst not as controlled, far greater cooling is facilitated without the valve and the experiment is easier to conduct. This also revealed a significant problem with off-target cooling, recorded by a thermocouple below the chamber, that would need to be addressed. A further complication during these experiments was that absolute temperature values in the reservoir may not be low enough for cooling in deep targets. To gauge the tip temperature of the silver probe I recorded from bench top assemblies of cryoprobes in reservoirs held together with clamps. An example is shown in Figure 3.4e. The goal of this experiment was to calculate the maximum load in the reservoir that would lower the temperature at the tip of an 8 mm probe to 0°C. Even though cooling a silver probe room at room temperature requires far less energy than active brain tissue, the result was still very unexpected. A reservoir of -35°C would be required. At this point it was obvious that several difficulties needed to be overcome. The loss of cooling power down the length of a probe was exponential and so targets that were high in the brain would also be disproportionately easier to inhibit. The very cold temperatures in the reservoir also create significant challenges for controlling off target effects that may seriously compromise the interpretation of behavioral data.

3.4.1. Surgical implantation and testing

Surgical implantation of the probe and chamber involves construction of the reservoir around the implanted probe, whilst leaving enough space for ECoG electrodes and connectors. A schematic of this arrangement is shown in Figure 3.5a. Probes were made beforehand, as previously described. Images of the completed probe tip and the insulation are shown in Figure 3.5b.

The images for each surgical step are shown in Figure 3.5c and are labelled 1-4. First, the silver probe is placed at the target region using a stereotaxic frame and secured with tissue glue. A polystyrene insulator, shown in orange, is placed around the probe in preparation for the base section of the vaporisation chamber (1). After this bracket has been screwed into the skull, the torso section is attached using tissue glue with further insulation already in place around the side (2). This lateral insulation reduces residual cooling of the dental cement and the head of the animal (3). Finally, the apparatus is covered with a protective layer of dental cement and only the connections for EEG, LFP and temperature are exposed along with the refrigerant entry and exit ports (4). During development, thermocouples were placed in different locations to record both on-target and off-target temperatures. This was particularly useful in learning how to control the refrigerant and improve the insulation. The results of these temperature measurements are shown in Figure 3.5e, with recordings made both inside and underneath the chamber, as well as on the skull at distances of 1 and 3 mm from the base section. Off-target cooling, below the chamber, is substantial and drops from 34 to 19°C, which is too extreme even for even transient cooling of the cortex and particularly whilst close to the ECoG screws. The lateral thermocouples recorded a different time depended effect. Thus, off target cooling can be separated in to two categories: direct and indirect. The former results from insufficient insulation and occurs at the same time and is proportional to the cooling of the reservoir. The latter, which appears time shifted, may results from gradual cooling of the head stage cement during long experiments This is much harder to protect against and suggests shorter cooling periods are preferable during behavior experiments.

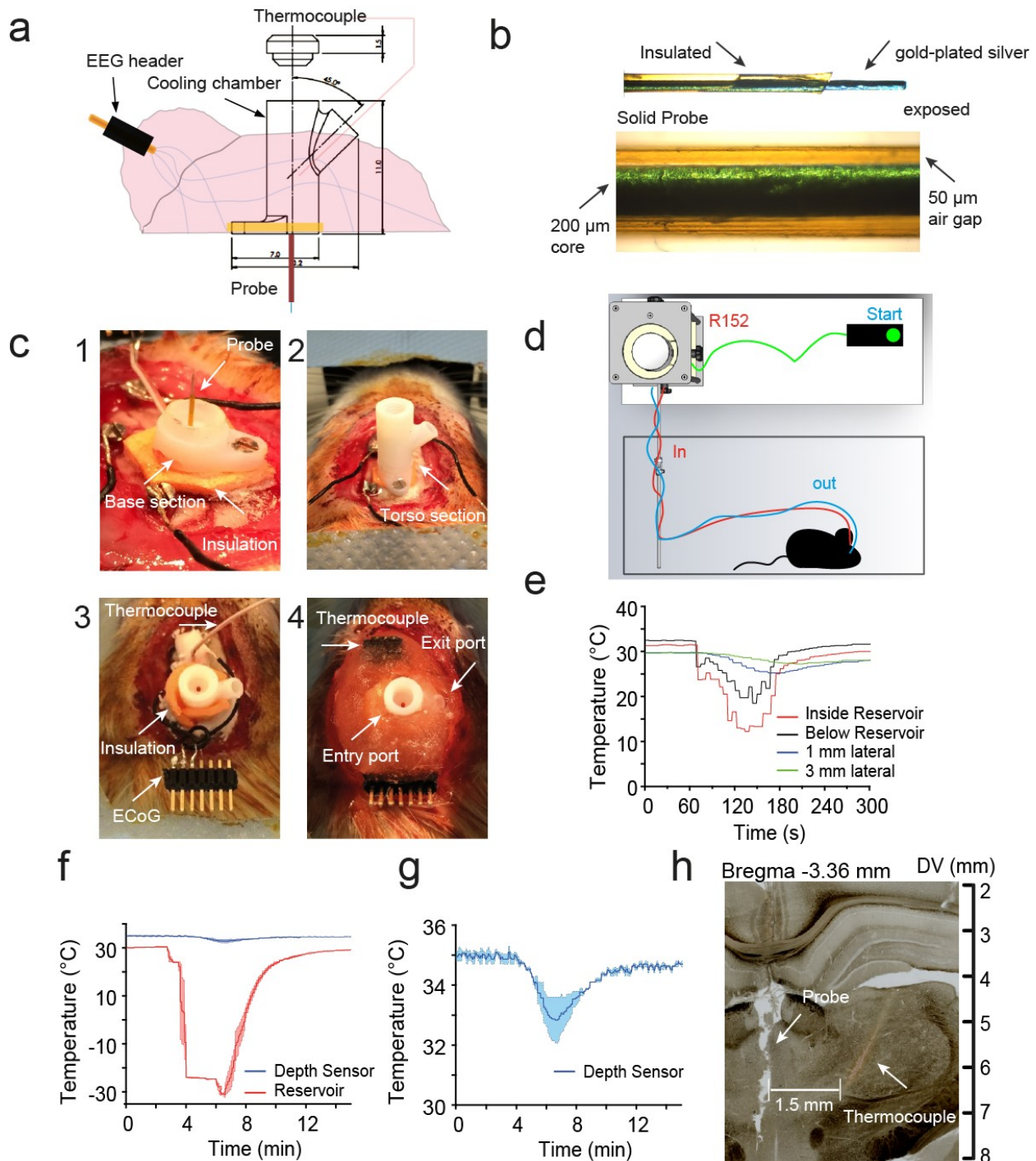


Figure 3.5 – Surgical implantation. This details the arrangement of the vaporisation chamber on the skull of a male Sprague Dawley rat with a CMT probe (a) Schematic of the vaporisation chamber with ECoG and cooling probe in dental cement. (b) Solid probe of gold plated silver of 200 µm diameter with a polyimide insulator of 350 outer diameter. Insulator wall thickness was 25 µm leaving a 50 µm air gap around the probe. Each end was sealed with Superglue. 1 mm was left exposed at the target end and 5-8 mm at the top. (c) shows the progression of surgery from ECoG, probe, thermocouple and base section attachment (1), torso attachment (2), insulation and ECoG completion, (3) dental cement covering. (d) shows a schematic of the behavioural experiment with R152 canister mounted outside the arena. (e) shows the temperature inside the reservoir during a three-minute cooling. Also shown are off-target temperature below the reservoir and adjacent on the skull at distances of 1 and 3 mm. (f) Temperature recording from inside the reservoir and from an implanted thermocouple 1.5 mm mediolateral to a CMT probe. (g) shows the same depth measurement rescaled. (h) histology showing the placement of the probe in the CMT (Bregma -3.36, DV -6.5) and the adjacent deep thermocouple.

Figure 3.5f shows an example of reservoir cooling *in vivo* at its maximum. This experiment was performed in triplicate. The lowest temperature of the reservoir is just under the boiling point of R152 - 25°C. As discussed previously, very low reservoir temperatures are required for deep brain cooling. Interestingly, the boiling point acts as a buffer and stabilizes the rate of vaporization around this temperature. This results in cleaner, more consistent temperature profiles, if only at this one temperature. During this experiment, a thermocouple imbedded in a needle was stereotaxically directed at an angle of 25° to a site adjacent to the probe. The track mark of the probe sits just off center to the CMT. The thermocouple entered the brain at ML -4.6 mm. This allowed me to take measurements of radial cooling directly. These are shown at full scale in Figure 3.5f and enlarged in Figure 3.5g. The triplicates are plotted as mean $\pm$ SEM and show a distinct if small drop in deep brain temperature to approximately 33°C. The brain of this animal was sliced and track marks for both the solid probe and the thermocouple are clearly visible. The distance from the probe surface at which the change in temperature was measured was approximately 1.5 mm. This is roughly consistent with the isotherm modeling shown in Figure 3.2a, despite the large differences in the methodologies. Nonetheless, the area of effect where sufficient temperature was reached may be very small. The effector temperature on nerve conduction and the experiments of Bénita and Condé was discussed previously. In my own experiments, a critical temperature of 15°C, that would affect the latency to action potentials, would be reached at a distance of about 0.5 mm from the probe, assuming an exponential decay. Although, these distributions are more readily modeled by the steeper Bessel-k function and so the distance is likely to be smaller (Aronov and Fee 2011).

In summary, the refrigeration method produces a high degree of cooling but even this may not be sufficient to inactivate deep brain structures. Whilst choosing shallower targets may be beneficial there is an alternative. I think a smarter approach may be to intentionally slow neuronal dynamics using more moderate cooling. Modulating neuronal circuits activity in this way may be a more subtle method of understanding their contributions to behavior. The use of refrigerants is difficult and the challenges of using these *in vivo* should not be underestimated. The use of a solid cooling rod and vaporisation chamber has proved effective although care must be taken to minimize off target effects.

3.5. Cooling by conductive fluid

If cooling the brain to alter circuit dynamics can be gentle, then perhaps another method of cooling should be considered. The most frequently used method is loop-cooling and involves circulating a fluid, usually an alcohol that resists freezing, that can be actively cooled. Typically, the coolant used is either ethanol or methanol, or combinations with slightly different properties. The main advantage of these systems is that they are cheap and easy to use and maintain. It requires only a pump and is generally unsealed. The alcohol passes through a cooling apparatus, usually wet or dry ice, and then through the target before recirculating or being collected. The disadvantage is a limit to the cooling available, particularly as the coolant warms quickly during transit in narrow tubing. Equally, increasing the flow rate to remove more heat from the target decreases the time available for cooling the solution. Nonetheless, the system is generally precise. The system I developed was built on the previous design work for the evaporation chamber but attempts to simplify the control of cooling by replacing the refrigerant with a constant flow of chilled ethanol. These experiments were modeled on cooling the CMT as a reduction in frequency may result in a change in behavior, and possibly LORR.

An ethanol pumping circuit is shown in Figure 3.6a. A larger reservoir of ethanol is placed inside a container of ice and allowed to cool. The experiment can be run in either of two ways displayed as (A) or (B). In (A), the ethanol is pushed out of the reservoir by gas-displacement and leaves via silicon tubing. Ethanol travels across the circuit and into PTFE tubing, via an adaptor with specialist connections to ensure good seals (062 MINSTAC - Lee company), and through the adapted vaporisation chamber, now labelled the cooling-chamber. As the ethanol passes through the cooling chamber, it removes heat from the exposed silver probe. The ethanol is then collected (Route A) as it cannot enter the pressurised reservoir. In Option (B), a rotary pump pushes chilled ethanol around the circuit and through the reservoir and the implanted chamber. In this method, the ethanol returns to the reservoir to be re cooled (Route B). For the purposes of testing this method *in vivo*, option A was used however, if Route B is implemented, electronic feedback through a rotary pump would be advised but is beyond the scope of this assessment. For both options I used a cooling chamber that was threaded at both ports and well insulated. In addition to the polystyrene foam on previous designs I added an additional layer of thin bubble to create airgaps between the insulators and the dental cement and further reduce total head cooling (Figure 3.6b) This was also present on the skull.

I used method (A) whilst developing this system as this was the most straightforward way to reconfigure my previous work. During development, I found that increasing the pressure in the reservoir from 0.5 to 2 bar resulted in a linear increase in the flow rate through the chamber, from 40 to 90 ml min⁻¹ (Figure 3.6c). If the ethanol was chilled to $5 \pm 1^\circ\text{C}$ using wet ice, then adjusting the flow rate would determine the temperature of the reservoir to a minimum of 8°C (Figure 3.6d). Experimentally, I decided I would record a two-minute baseline and then set and maintain the pressure of the system for a period of three minutes. This would be long enough to look at changes in neuronal excitability. This protocol with different pressures is illustrated in Figure 3.6e. To assess the reliability of cooling at a given pressure,

I repeated the experiment at 2 bar four times and the result is shown in Figure 3.6f. The variation is within an acceptable margin of $\pm 2.5^\circ\text{C}$ and, while this is greater than that shown with the refrigeration method at -20°C , this system is considerably more reliable, reducing experimental failures. Importantly, these simpler methodologies are easier to troubleshoot when problems arise and may not result in

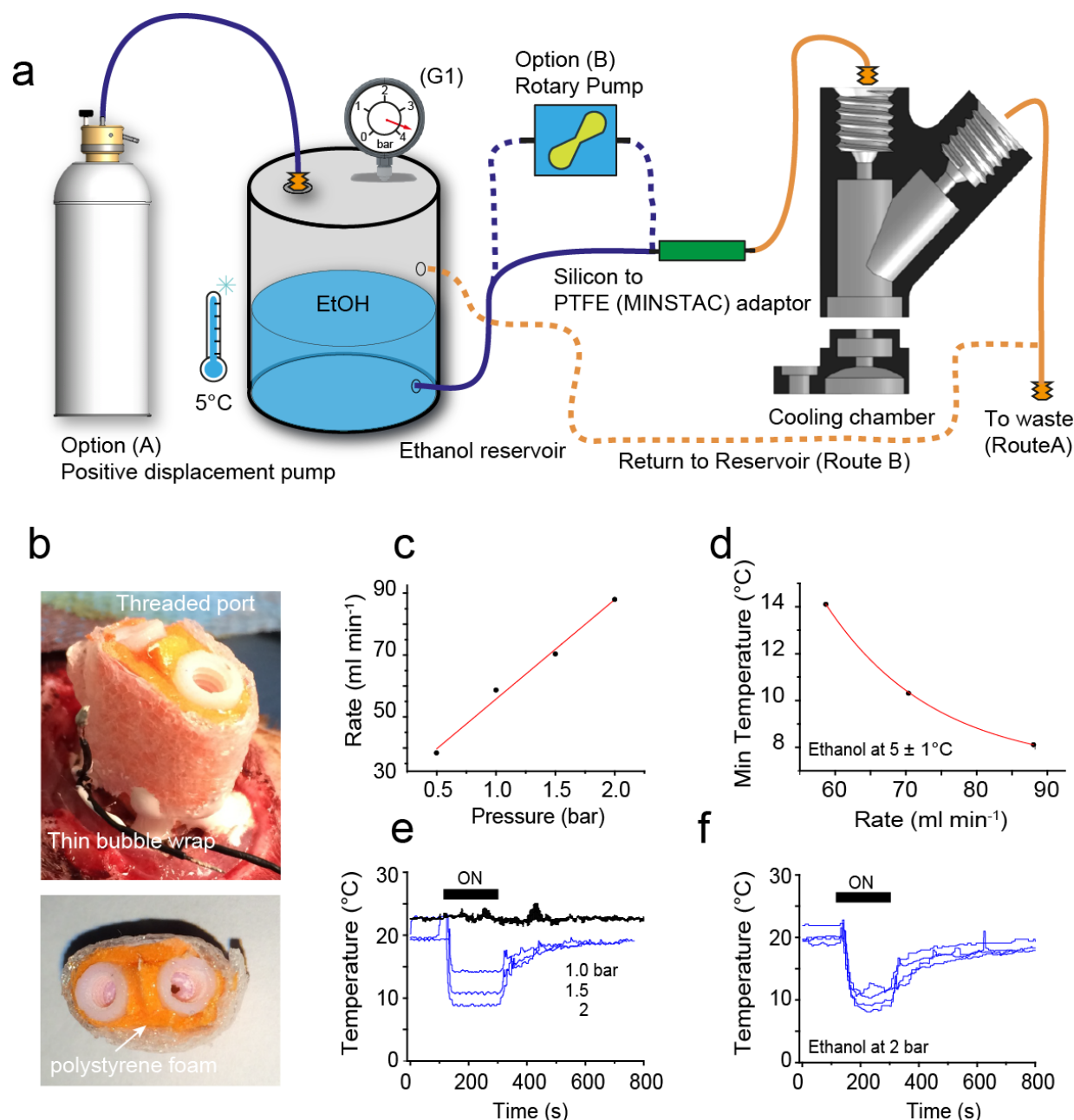


Figure 3.6 – Alcohol-loop method. Alcohol-loop system for brain cooling with a gold-plated silver solid probe (previously described). (a) schematic of alcohol loop system with two different mechanisms. Option (A) uses positive displacement to pump chilled ethanol at $5 \pm 1^\circ\text{C}$, out of a reservoir and through the cooling chamber. Ethanol is then collect separately. Option (B) uses a rotary pump to control the flow of ethanol in a circuit through the cold reservoir and back to the reservoir. (b) shows the cooling chamber mounted on the head of a male Sprague Dawley rat. It is covered by two layers of insulation, polystyrene foam and thin bubble wrap. (c) shows the relationship between pressure in reservoir and the resulting flow rate. (d) the relationship between the flow rate and the temperature of the cooling chamber. (e) Change in temperature over time with different pressures. (f) shows four replicates at the same pressure to assess experimental variability.

ending the experiment. This system could cool to a greater extent by running the ethanol through dry ice, however, the rate of flow could not exceed that tested here because of the likelihood of leaks even with precision made seals. Deep tissue measurements of cooling were not performed with the ethanol loop system, as the dynamics of that response are unlikely to change. Examples of ECoG recordings during refrigeration, but not alcohol-loop cooling, are discussed later in Figure 3.8.

3.6. Cooling by the Peltier effect

The final method of cooling that I assessed was the use of a Peltier device. This methodology was used to great effect by Long *et al*, as discussed previously. Peltier cooling is potentially the easiest to control as can be considered entirely electronic, but it is also likely to be the weakest in absolute cooling. The power input to the Peltier device is not the limiting factor experimentally. The amount of cooling that can be produced is equivalent to:

$$Q = PIt$$

where t is time (s), I is current (A) and P (V) is the Peltier coefficient of the device - the resulting Q is in joules (J). Note that the Peltier coefficient is the Seebeck coefficient (V / K), multiplied by the temperature (K). Instead, the heat sink is the limiting factor. As long as heat is effectively removed from the system, cooling is effective. In the original work by Long *et al*, a tethered water cooling system was used but they have subsequently shown that air cooled 'fins' are sufficient. I attempted to recreate a version of their device, using commercially available parts, and make it suitable for chronic implantation in the rat (Figure 3.7).

3.6.1. Operation and calibration of Peltier device

A Peltier device consists of alternating semiconductors (N) and (P), with different electrical properties placed between ceramic plates. The Peltier effect details how the application of voltage across the device induces a thermal gradient from top to bottom, forming one hot plate and one cold. In this experiment the cold plate faces down, directed by screws to cortical cooling, while the hot plate faces up. A heat sink is attached by a thermal adhesive, to stabilize the temperature gradient. ECoG screws are then implanted with dental cement holding the apparatus together (Figure 3.7b).

Temperature recordings of the hot and cold plates with and without the heat sink are seen in Figure 3.7c. The heat sink suppresses the heat buildup from almost 40 to less than 25°C. Heat rebound can also be seen clearly without a heat sink as the hot surface starts to warm through the device. Adjusting the current to the device effectively regulates the degree of cooling and allows fine control over the experimental paradigm. Whilst the changes in temperature are small it is consistent and easy to control. The effect is also reproducible with little expertise. The control circuit shown in Figure 3.7e is a simple arrangement of resistors and potentiometers made in our laboratory, that allows the experimenter to decide both the initial range and the power to the device. This was used in place of a commercial current source that is often expensive, but has the advantage of being adjustable over a wider range and programmable if required.

3.6.2. Surgical implantation of the Peltier device

The surgical arrangement of the Peltier device is shown in Figure 3.7f where it is positioned over the cingulate cortex of a male Sprague Dawley rat. The skull was prepared for surgery as detailed in the methods. The hot plate of the Peltier device is aligned with the screws and thermal adhesive applied between them. Tissue glue is required around the edges. Whilst the plate cannot be physically attached to the bone, a combination of tissue glue and thermal adhesive are sufficient. It should be noted that the latter takes several hours to harden fully. This is expected during the recovery from surgery. The

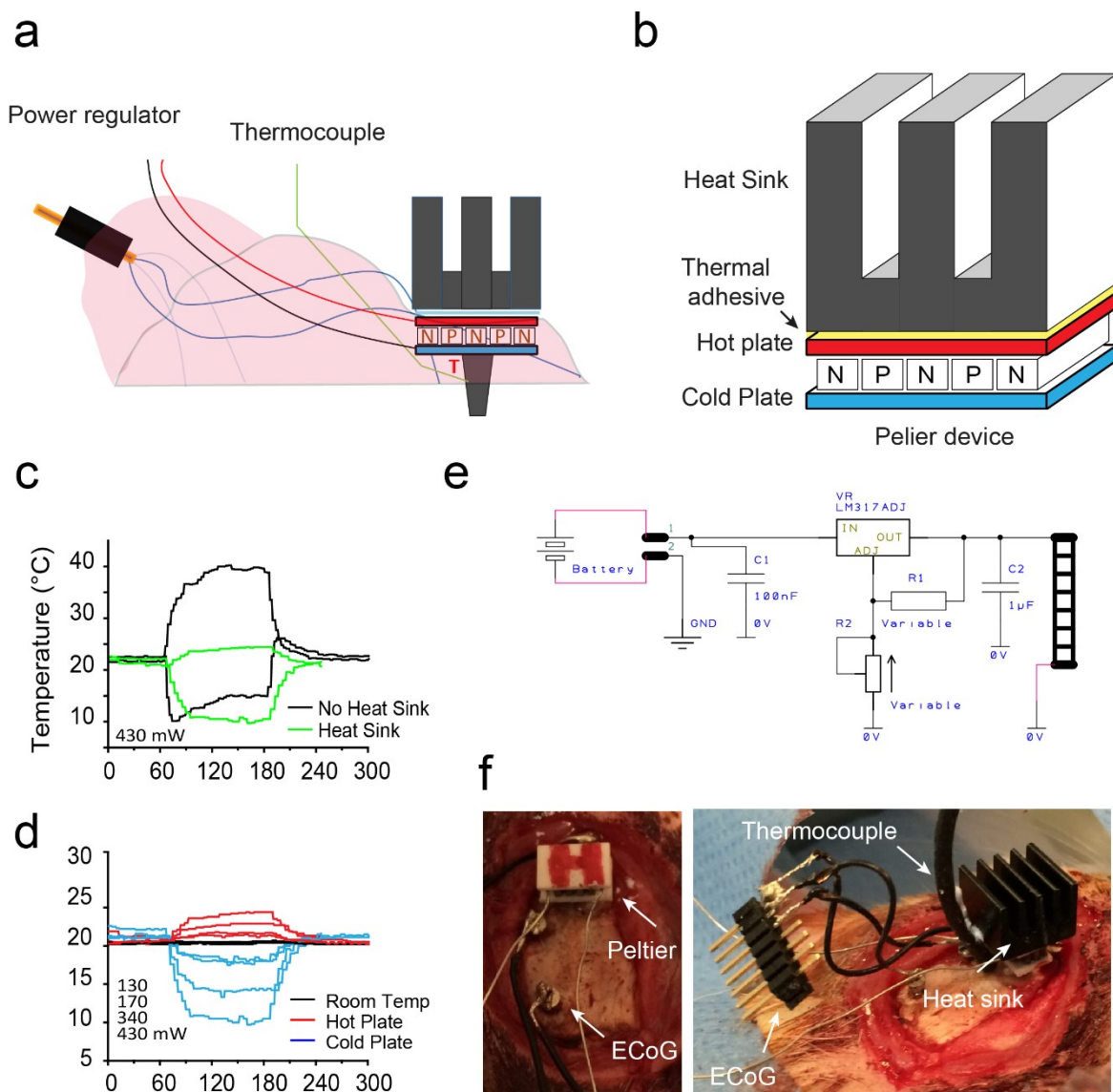


Figure 3.7 - The Peltier Device. (a) Schematic arrangement of the Peltier implanted on the head of a rat. (b) Peltier device complete with heat sink attached by thermal adhesive. (c) contrasts the operation of the Peltier device with and without a heat sink. (d) Shows the relationship between applied voltage and thermoelectric cooling. Note values are given in mW. This acts as a calibration curve to future experiments. (e) shows a control circuit. Manually adjustable potentiometers allow the experimenter to change the input resistance and as a result the temperature of the cold plate. (f) surgical procedure both with and without the heat sink in place.

heat sink should have an appropriate size, covering as much of the hot plate as possible. It was attached to the Peltier device prior to surgery with thermal adhesive and allowed to hardened.

3.6.3. Considerations

As previously discussed, the limitation of this device is the relatively low level of cooling. The maximum effect here was a drop to 10°C, which would be further reduced against the heat load of a warm animal. Nonetheless, this methodology has much potential. Unfortunately, controlling the Peltier whilst implanted in dental cement proved unexpectedly difficult. An implanted thermocouple could not record a change in temperature when voltage was applied and I cannot be sure at this stage whether the heat load of the skull was too high to allow an effect, or if there was another issue. I suspect that dental cement may have caused some problems with the wiring that was included during surgery, but this will need to be investigated further.

3.7. Comparison of methods

In this chapter, three distinct methods of brain cooling have been developed for use in Sprague Dawley rats. Each has significant advantages but comes with its own challenges. Whilst refrigerant cooling yields the coldest temperatures, it is the most technically challenging. Refrigerants are inherently difficult to handle and calibrations of flow rates through solenoid valves, under significant pressure, require either expertise or substantial practice. Ethanol-loop cooling requires neither and is more reliable in the expected response than the refrigerant system. The ease of use of this system and its consistency, are a direct trade-off against the maximum temperature drop. However, on the basis of deep brain measurements from the refrigeration experiments, even maximum cooling with this ethanol-loop system would not sufficiently cool our silver probe for deep targets beyond a few millimeters. The limit of the refrigeration system by our measurements is 7 mm, but in practice may be less. It is clear, however, that for shallower targets, of perhaps less than 3 mm, the alcohol-loop system is far superior to using refrigeration.

The use of 3D printed parts in these projects eliminated the need for polydimethylsiloxane (PDMS) molding prior to experimentation. This has made prototyping fast and efficient, whilst also facilitating the use of complex tools, such as the vaporisation chamber, which have multiple components and threaded ports. This should be a frequently used technique when designing tools for neuroscience research. It is worth considering that these experiments in rats would not encounter similar problems of cooling in the mouse, which has a far smaller brain. If surgical limitations could be overcome, then it is feasible. The Peltier device may be most suited for adaptation to the mouse.

For cooling shallow targets, the Peltier device must be considered alongside the alcohol-loop. Peltier cooling is weaker than both other methods, but highly controllable. It is the most accurate, returning to the same absolute value on different occasions with high levels of precision. Once configured circulating ethanol is precise, but it is hard to reconfigure the same way each time as the arrangement of tubing has a large impact. The Peltier device is suitable for slowing neuronal activity in shallow targets and thus studying temporal dynamics, but is unlikely to silence neuronal activity altogether (*Bénita and Condé 1972*). The success in analyzing circuit dynamics, seen in the literature, suggests that complete inactivation may not be necessary for the observance of behaviors. Perhaps the subtler Peltier cooling of shallow structures should be revisited with this in mind. The prefrontal cortex has recently surfaced as a target of sleep homeostasis and hypothermia is a paradoxical correlate of sleep and anesthesia, the effects of which are unknown. The Peltier device may be the right approach to shed light on these problems.

3.8. Thalamic cooling and ECoG

Lowering the temperature of neuronal circuits may slow or inhibit temporal dynamics. Sleep and anaesthesia are associated with a shift to lower frequencies in the 20-40 Hz band of the ECoG. I have attempted to recreate this shift by cooling the CMT, a key nucleus in the thalamocortical circuit. This would help us understand whether a loss of thalamocortical integrity was sufficient for LORR in the rat.

Recording ECoG data during the cooling of the CMT in awake behaving animals was the most challenging aspect of this project. Over many experiments and several different methods, some preliminary data was collected, although the n numbers are low. I first refined the cooling protocol in sedated male Sprague Dawley rats, under either 2% v/v ISO or DEX ($20 \mu \text{ kg}^{-1} \text{ I.V.}$) and then moved to testing each methodology in awake rats. To cool the CMT in awake animals, I briefly anaesthetised the subject rat with 2% v/v isoflurane and attached the refrigerant tubing and the ECoG recorder, a Neurologger 2A. The latter, unlike with mice, needs to be secured with tape in the rat. Anaesthesia was never longer than 10 minutes, and the rat was allowed 20 mins to recover from the effects in the behavior arena. I recorded baseline ECoG for 5 mins before each cooling event, which lasted 1 to 3 minutes, depending on the target level of cooling. I attempted to increase the cooling on each successive attempt although technical issues sometimes prevented this. The results of this cooling are shown in Figure 3.8. Using the refrigerant cooling method, I made 19 attempts to record ECoG, LFP and temperature data across 8 rats implanted with cooling probes, and 3 control rats without probes. I achieved $n = 3$ with replicates in 2 out of 3 for the probe group and $n = 1$ from the controls.

An experimental schematic shows the implantation site of the probe in the CMT at Bregma -3.36 mm (Figure 3.8a). The first example is shown in Figure 3.8b of an ECoG recording a no-probe control animal. This displays ECoG power banded, plotted at 200 Hz, in segments between 1 and 40 Hz. Each band was normalised by dividing by the power between 1 and 99 Hz over the same running window. The corresponding ECoG, EEG and ECoG RMS are plotted above. The RMS, calculated over a 30 second sliding window, is an indicator of the effects of absolute changes in power, on the recording. The sonogram illustrates the changes in frequency content between 1-20 Hz, but without further normalisation to total power. Cooling in this animal was successful on a single occasion (blue shaded region) and no change in ECoG can be seen (Figure 3.8b). A typical example recording from an animal with a probe implanted to the CMT can be seen in Figure 3.8c. Four consecutive rounds of cooling were carried out but, like the control animal, little evidence for cooling dependent change were observed. The histology confirmed that the probe was in the correct place and close assessment of the video failed to reveal any changes in behavior. As no changes occurred from cooling it was important to have another type of control, in this case a positive control for action in the midline thalamic nuclei. Injections of small volumes using guide cannulas and precision syringes is a classical technique that has revealed much about the brain. Here, I injected 500 nl of 1 mM muscimol, a GABA_A agonist, into the CMT (Figure 3.8d). This induced loss of righting reflex in less than 60 seconds and was paralleled by a theta to delta transition characteristic of natural sleep and non-inhalational anesthesia. These are clearly visible in

both normalized banded power and the sonogram. Drug injections are known to diffuse widely making their effects hard to interpret. We cannot be sure which thalamic nuclei was ultimately responsible. This is where a well-functioning cooling probe would be useful. Fast, reversible inhibition with a more localised effect would be easier to interpret. Unfortunately, I was not more successful with the other methods. Using the alcohol-loop, from three attempts I recoded just $n = 1$ (probe) with temperature and ECoG and $n = 1$ control (data not shown). The degree of cooling may also not have been sufficient for the experimental aims. As discussed previously, the Peltier device failed post-surgery on two attempts. Despite the value that a cooling probe may bring to this project reaching this technical threshold has proved difficult and has not yet helped understand thalamic contributions to the ECoG or behaviour.

3.8.1. Experimental challenges

Whilst recording temperature was robust and consistent, performing the cooling experiment or recording ECoG with the Neurologger 2A was not. The principal experimental difficulty was in achieving consistently low temperatures while reducing movement noise in the recording. On most attempts, cooling in the reservoir was moderate ($\Delta T < 15^{\circ}\text{C}$), but at its peak it fulfilled the design of the protocol ($\Delta T 50^{\circ}\text{C}$). To overcome this, I amended my protocol to short cooling of 1-3 mins repeated several times in the same experiment so increase the change of recording 'cooling events'. Unfortunately, small refrigerant leaks, changes in pressure or a sudden movement of the animal around its tether created large ECoG artifacts that disrupted the signal. This was the largest source of experimental error. A further four attempts, across three animals, had to be excluded from analysis entirely, because of refrigerant leaks in the behavioral arena. R152 can induce anesthesia-line ECoG at high concentrations, possibly as a result of hypoxia (unpublished observation). Finally, and despite the issues discussed, these were not the biggest reason for loss of data over these experiments. A technical issue with the Neurologger 2A in our laboratory, that at the time was not understood, resulted in approximately 50% of experiments not recording data. We now know that a hardware fault resulted in sporadic failures to turn on correctly and record. For our experiments, this would only become apparent when attempting to download the data.

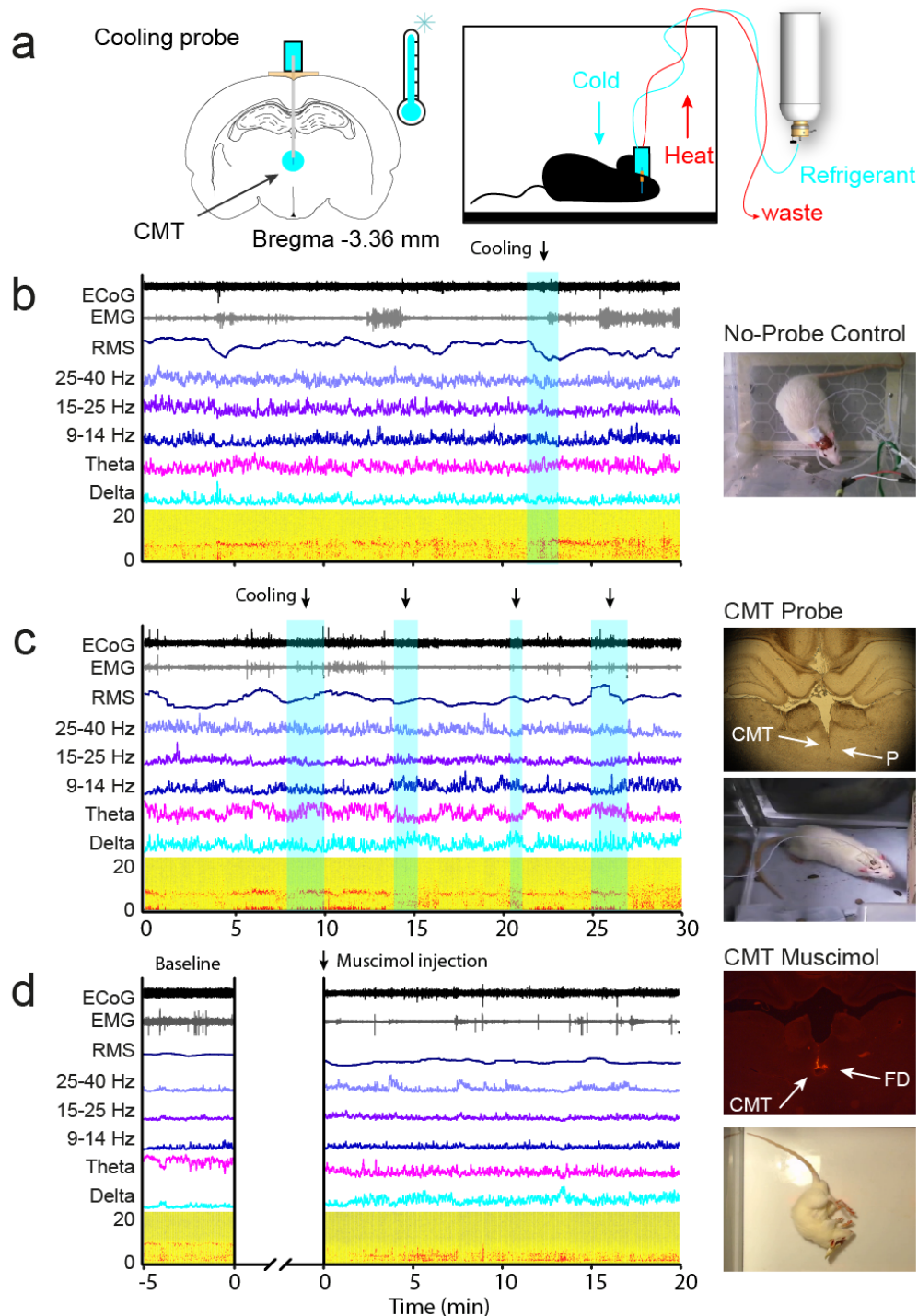


Figure 3.8 – CMT Cooling and ECoG. Examples of ECoG data recorded from animals undergoing thalamic cooling. (a) a gold plated silver probe was surgical implanted into the CMT and vaporisation chamber secured over it. Refrigerant gas was used to cool the probe in freely moving animals whilst recording ECoG. (b) the no-probe control animal underwent surgery and had a vaporisation chamber secured over the CMT target, but no probe was present. This controls for off target cooling and the behavioural effects of the protocol e.g. equipment noise or refrigerant leak. The blue bar represents a period of cooling. No change in ECoG or behaviour was observed. (c) Shows repeated cooling of an animal with a CMT probe but no change in ECoG or behaviour was observed. The histology confirmed the correct placement of the probe tip in the CMT nucleus. (d) shows a positive control for the CMT target area. An intracerebral injection, via a guide cannula, of 500 nl of 1 mM of the GABA_A agonist muscimol, in saline. A dye containing beads fluorescent at 594 nm (FD) was included to confirm the injection site in the CMT (histology). The gap in the recording is 11 mins during which the injection cannula was inserted into the brain and the ECoG shows only movement artefacts. Post injection at t(0), this animal demonstrated an almost immediate theta to delta transition in the ECoG which coincided with a loss of righting reflex. No changes were observed at higher frequencies.

3.9. Summary of findings

I had two primary aims for my experiments in cooling the brain nuclei. The first was to understand the limits of the technologies available and how they might be applied to the science of sleep and consciousness. The second was to apply the technology to a brain target, in this case the CMT. I have taken three methods and adapted them for use in the rat, a rodent model far more common than larger mammals. These methods can be used simultaneously with recording of temperature, ECoG and LFP. I have then tested their functionality in alternating neuronal circuits in the central medial thalamus.

Refrigeration is the most effective means of removing heat but this degree of cooling may not be required. I believe the focus on neuronal inactivation in these methods may be overstated and, given the level of difficulty with this technology, it must be necessary to justify its use. It has been shown that subtler cooling, changing temporal dynamics without inactivation, is capable of yielding significant circuit level understanding and behavioral results (*Long and Fee 2008*). Whilst hollow thermodes have been used in larger animals like cats, smaller solid probes are effective and worth the trade-off for a simpler protocol and easier surgery, with less tissue damage. This method is most useful for inactivation of shallow to moderately deep targets of 4 - 7 mm.

Ethanol-loop cooling is simple to set up and cheap in components. It is also the most reliable given its simplistic mode of operation and, crucially, troubleshooting problems that invariably develop requires little technical skill to solve. Feedback mechanisms are useful but are not required for small reductions in temperature, lasting only two to three minutes. Whilst a gas pump was used here, a rotary pump would be equally effective and necessary for longer experiments where feedback becomes essential. This method is most suited for inhibition of neuronal circuits in shallow targets of less than 4 mm, or over larger areas of cortex where other methods cannot spread their effect.

Cooling by the Peltier effect is the most controllable and most consistent. For cooling brain targets of less than 2 mm deep it should be the first choice method. Furthermore, Peltier cooling may be suitable for work in mice. Cooling can be directed to the surface of the cortex or directed, via implanted screws or shallow probes, into targets like the cingulate cortex. Cooling beyond this depth remains a challenge and for targets at greater than 2 mm the Peltier may require its own water cooling system.

Cooling neuronal circuits is a useful method that continues to produce interesting data. It does not require genetic manipulations and is highly local and reversible. However, the strength of the data obtained is undermined by lack of genetic specificity to particular neuronal types. The most significant weakness is that inhibition by cooling affects both excitatory and inhibitory neurons in the same manner. Secondly, observed changes in circuits and behavioral outcomes cannot be attributed to specific neurons or mechanisms without further work. Changing temporal dynamics is more useful in this regard, but requires recordings of the target or surrogates such as ECoG or LFP at the least. It is clear that these technologies cannot be used in isolation but must complement other genetic techniques to answer

specific questions. The best questions for these technologies are perhaps not about circuit functions but circuit dynamics.

Methods

3.10. Cooling the brain: Protocols

As this chapter details methodological developments, only technical aspects not described in the results and discussion are listed in this method section.

3.10.1. Surgery

Surgery was performed on rats given 15 $\mu\text{g kg}^{-1}$ buprenorphine and 150 μl of 2.5 % v/v enrofloxacin in 10% v/v ethanol. The skull was cleaned with 30% v/v H_2O_2 to reveal suture lines. Cooling probes were implanted at the following coordinates for the CMT AP -3.2, ML 0.0, DV -6.8. For intracranial injections rats were instrumented with 20 or 22-gauge guide cannula at the same coordinates as the cooling probe. 31 gauge injectors attached to 1- μl Hamilton syringes were used to administer the drug. For surgical attachment of the Peltier device, the skull bone was prepared as previously described, before 6x4 mm Peltier device (RS components 490-1216) was glued just ahead of the coronal suture and above the cingulate cortex. The device is too large for stereotaxic placement.

3.10.2. Intracranial drug injection

A Hamilton syringe was used to inject 500 nl of 1 mM muscimol in saline into the CMT using the implanted guide cannula. Injections were given at a constant rate and completed with 60 seconds.

3.10.3. Awake rodent – Refrigerant or alcohol cooling

Following surgery and a two-week recovery period, 3-month old male Sprague Dawley rats were attached to cooling apparatus, for either ethanol or refrigerant, in a tethered arrangement. This was usually completed in less than 10 mins, under 2% v/v ISO. Anaesthesia was essential to minimise stress. At least 20 mins of recovery time was allowed before starting baseline ECoG recordings. Each baseline was 5 mins, followed by 1 to 3 mins of cooling between 10 and -25°C measured from the vaporisation chamber. This was repeated after a short recovery period. Temperature measurements were recorded using 36 to 40-gauge T-type thermocouples (Omega Engineering Ltd)

3.10.4. Measuring deep brain temperature

To measure deep brain temperature a special thermocouple-needle (Omega Engineering Ltd) was implanted, using a stereotaxic frame, at the Bregma 3.2, ML +4.6 and rotation 25° from horizontal. The exact position was confirmed by histology. This was performed just after cryoprobe implantation before recovery. The procedures were performed under terminal anaesthesia. Gentle warming was provided to account for hypothermia. Thermocouples recorded the temperature in the reservoir, at off-target locations and at a site adjacent to the side of the probe.

3.10.5. ECoG Recording

ECoG recordings were made at 200 Hz in rats implanted with screws for at AGND: AP +7.0, ML -1.0, Reference (to lambda): AP +4.0, ML +2.0, Signal 1 (to bregma): AP +2.0, ML +2.0, and Signal 2 (to lambda): AP -2.0, ML 0.0. EMG wires were also included. All recordings were made at 200 Hz, 10-bit resolution and ± 500 μ V range using the Neurologger 2A. No oversampling was used.

3.10.6. Histology

Brains were perfuse fixed in 4% v/v PFA for 24 hours and then washed in 30% w/v sucrose in PBS for two days before serial sectioning through the thalamus at 40-45 μ m thickness on a Leica microtome. The position of the probe and thermocouples was confirmed by bright-field microscopy.

3.10.7. Data analysis

ECoG data was analyzed using spike2 software (CED) using custom scripts (Appendix 3.11). 'Power in band' was calculated as running power spectrums of 1024 bins for each band, 1-4 Hz (delta), 5-9 Hz (theta), 9-14 Hz, 15-25 Hz, 25-40 Hz and dividing by the power between 1-99 Hz. Images were analysed using the Fiji v.1.47a. Graphs were plotted in OriginPro v8.6.

Concluding Remarks

Neuroscience can be fascinating in its complexity but it is stubborn in giving up its secrets. The science of sleep is no exception. We may spend a third of our entire lives unconscious and yet we have no convincing explanation as to why. We can only be sure that we have little choice in this matter.

In completing this thesis, I hope to have unravelled a small part of the sleep mystery. Focusing on circuitry in the hypothalamus, I have shown that neurons that are normally involved in temperature regulation can influence sleep circuits. The relationship between temperature and sleep may relate to sleep pressure.

“There is a time for many words, and there is also a time for sleep”

- Homer, The Odyssey

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Appendix

3.11. Appendix: Spike2 Scripts

3.11.1. ECoG and EMG Filter

'Script applies FIR 5-45 Hz Band pass to EMG Ch(4). Creates EEG 0.5 low pass (new Ch(10)) and subtracts from original signal Ch(1). Changes colour'

```
var v12% := ViewFind("FILENAME");
var ch1%; 'Created virtual channel
FrontView(v12%);
ChanSelect(4, 1);
FiltCreate(-1, 4, 1.8, 5, 45); 'FIR Band pass
ChanShow(FiltApply(-1, 7, 4, 0.000000, 105282.55, 1)); 'Apply created filter
ChanTitle$(7, "EMGFIR");
ChanHide(4);
ChanSelect(4, 0);
ChanSelect(1, 1);
FiltCreate(-1, 2, 1.6, 0.5); 'FIR Low pass
ChanShow(FiltApply(-1, 10, 1, 0.000000, 105282.55, 1)); 'Apply created filter
ChanTitle$(10, "EEG Noise");
ChanTitle$(1, "EEG RAW");
ch1% := VirtualChan(0, ""); 'Create new virtual channel
ChanShow(ch1%); 'Make it visible
VirtualChan(ch1%, "Ch(1)-Ch(10)", 1); 'Edit virtual channel
Optimise(ch1%);
ChanTitle$(ch1%, "EEG FIR");
ChanHide(10);
ChanHide(1);
ChanSelect(1, 0);
ChanColourSet(701, 1,0,0,0.501961); 'Set channel colour
ChanColourSet(7, 1,0,0,0); 'Set channel colour
```

3.11.2. ECoG power in band

'Script generates power in band from Ch(v1) in ranges 1-4, 5-9, 9-15, 15-25, 25-40 Hz and divides by 1-99 Hz.

'1024 bit resoltuon less that 0.3 Hz'

```
var v12% := ViewFind("FILENAME");
var ch5%; 'Created virtual channel
var ch4%; 'Created virtual channel
```

```

var ch3%; 'Created virtual channel
var ch2%; 'Created virtual channel
var ch1%; 'Created virtual channel
FrontView(v12%);
ch1% := VirtualChan(0, ""); 'Create new virtual channel
ChanShow(ch1%); 'Make it visible
VirtualChan(ch1%, "Pw(v1,0.3,1,4,1,99)", 1); 'Edit virtual channel
ChanTitle$(ch1%, "RDelta");
ch2% := VirtualChan(0, ""); 'Create new virtual channel
ChanShow(ch2%); 'Make it visible
VirtualChan(ch2%, "Pw(v1,0.3,5,9,1,99)", 1); 'Edit virtual channel
ChanTitle$(ch2%, "RTheta");
ch3% := VirtualChan(0, ""); 'Create new virtual channel
ChanShow(ch3%); 'Make it visible
VirtualChan(ch3%, "Pw(v1,0.3,9,14,1,99)", 1); 'Edit virtual channel
ChanTitle$(ch3%, "9-14");
ch4% := VirtualChan(0, ""); 'Create new virtual channel
ChanShow(ch4%); 'Make it visible
VirtualChan(ch4%, "Pw(v1,0.3,15,25,1,99)", 1); 'Edit virtual channel
ChanTitle$(ch4%, "15-25");
ch5% := VirtualChan(0, ""); 'Create new virtual channel
ChanShow(ch5%); 'Make it visible
VirtualChan(ch5%, "Pw(v1,0.3,25,40,1,99)", 1); 'Edit virtual channel
ChanTitle$(ch5%, "25-40");
Optimise(ch3%);
Optimise(ch2%);
Optimise(ch1%);
Optimise(ch4%);
Optimise(ch5%);
ChanColourSet(705, 1,0.752941,0.25098,1); 'Set channel colour
ChanColourSet(704, 1,0.501961,0,1); 'Set channel colour
ChanColourSet(703, 1,1,0,1); 'Set channel colour
ChanColourSet(702, 1,1,0,0); 'Set channel colour
ChanColourSet(701, 1,0,0,0.501961); 'Set channel colour

```