

**Genetic Dissection of Hypothalamic Sleep
Circuitry**

by

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Declaration

I hereby declare that the contents presented in this thesis have not been submitted for any other degree, diploma or other qualification at other university or college, and are my own work. All information derived from the work of others has been acknowledged in the text and a list of references given.

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Abstract

Do sedative drugs engage the natural sleep circuitry to produce their effects? In particular, the sedated state induced by hypnotic drugs acting at adrenergic type 2A receptors (*adra2a*) is difficult to distinguish from natural deep non-REM sleep, but most researchers believe these are distinct vigilance states. Here I use pharmacogenetics to demonstrate that α_2 adrenergic receptor agonist (dexmedetomidine)-induced sedation, and the recovery sleep induced following sleep deprivation, do in fact share a common neuronal circuitry such that recovery sleep and sedation appear to be the same. To do this I used the TetTagging method. I expressed *c-fos*-promoter inducible hM₃D_q receptor genes selectively in the preoptic area (PO). I then gave mice sleep deprivation and allowed them recovery sleep or sedative doses of dexmedetomidine. Only neurons previously active during rebound sleep following sleep-deprivation or those disinhibited by dexmedetomidine expressed hM₃D_q receptors. Remarkably, NREM sleep with the accompanying drop in body temperature could be induced by selectively re-activating these “neural engrams” using systemic CNO a few days later. After sleep deprivation and recovery sleep, selective reactivation of neuronal ensembles in either MnPO or the LPO areas was sufficient to induce powerful NREM sleep; after dexmedetomidine-induced sedation, only selective reactivation of the LPO area could induce NREM sleep. Selective ablation of the vesicular GABA transporter gene in the LPO neurons hindered dexmedetomidine’s ability to induce sedation, suggesting that the neurons disinhibited by dexmedetomidine were GABAergic.

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List of publications during PhD

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2. Yu, X., Ye, Z.W., Houston C.M., Zecharia, A.Y., Ma Y., **Zhang Z.**, Uygun D., Parker S., Vyssotski A.L., Yustos R., Franks N. P., Brickley S.G. and Wisden W. Wakefulness is governed by GABA and histamine co-transmission. *Neuron* (In Press)

3. Yu X., Zecharia A., **Zhang Z.**, Yang Q. Z., Yustos R., Jager P., Vyssotski A. L., Maywood E. S., Chesham J. E., Ma Y., Brickley S. G., Hastings M. H., Franks N. P., and Wisden W. Circadian Factor BMAL1 in Histaminergic Neurons Regulates Sleep Architecture. *Current Biology* 24, 2838-2844, 2014.

4. Gelegen C., Gent T. C., Ferretti V., **Zhang Z.**, Yustos R., Lan F., Yang Q. Z., Overington D. W. U., Vyssotski A. L., Lith H. A., Wisden, W., and Franks, N. P. Staying awake - a genetic region that hinders alpha(2) adrenergic receptor agonist-induced sleep. *Eur J Neurosci* 40, 2311-2319, 2014.

Abbreviations

AAV	Adeno-associated virus
BNST	Bed nucleus of the stria terminalis
Bp	Base pair
cAMP	3,5-cyclic adenosine monophosphate
ChR2	<i>Chlamydomonas reinhardtii</i> rhodopsin
Clock	Circadian locomotor output cycles kaput (gene)
CMV	Cytomegalovirus
CNO	Clozapine- <i>N</i> -oxide
CNS	Central nervous system
Cre	Recombinase, Phage origin
Cry	Cryptochrome
DAPI	4',6-diamidino-2-phenylindole
DAT	Dopamine transporter
DBH	Dopamine beta-hydroxylase
DEX	Dexmedetomidine
Dox	Doxycycline
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
DR	Dorsal raphe
EEG	Electroencephalogram
EMG	Electromyography
eYFP	Enhanced yellow fluorescent protein
GABA	γ -aminobutyric acid
GAD	Glutamate decarboxylase
GPCR	G protein-coupled receptors
HDC	Histidine decarboxylase
hM3Dq	Human muscarinic subtype 3 G _q -coupled DREADD
hM4Di	Human muscarinic subtype 4 G _i -coupled DREADD
IEG	Immediate early gene
i.p.	Intraperitoneal
ITR	Inverted terminal repeat

LC	Locus coeruleus
LDT	Laterodorsal tegmentum
LH	Lateral hypothalamus
LORR	Loss of righting reflex
LoxP	Locus of crossing over
LPO	Lateral preoptic area
kb	Kilobase
µm	Micrometre
min	Minute
ML	Mediolateral
MnPO	Median preoptic area
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NREM	non-Rapid eye movement
pA	Polyadenylation signal
PAG	Pariequaductal grey
Per	Period gene
PB	Parabrachial nucleus
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PO	Preoptic
PPT	Pendunculopontine
PZ	Parafacial zone
RASSLs	Receptors activated solely by synthetic ligands
REM	Rapid eye movement
RN	Raphe nuclei
SC	Superior colliculus
SCN	Suprachiasmatic nucleus
SD	Sleep deprivation
SDS-PAGE	Sodium dodecyl polyacrylamide gel electrophoresis
SWS	Slow wave sleep
TMN	Tuberomammillary nucleus
tTA	Tetracycline-controlled transactivator
TRE	Tetracycline response element

vGAT	Vesicular GABA transporter
VLPO	Venterolateral preoptic area / nucleus
vPAG	Ventral periaqueductal gray
VTa	Ventral tegmental area
WPRE	Woodchuck posttranscriptional regulatory element
WT	Wild-type

Chapter 1 Introduction

1.1 Sleep: A historical perspective

Sleep is one of the most important behavioural experiences that we have. The average person spends about a third of their life asleep. Like hunger, thirst and sex, the urge to sleep is one of the fundamental and primal biological drives that builds and then dissipates. The word “sleep” refers to a reversible and arousable loss of consciousness. The first scientific description came up around 350BC by Aristotle. He wrote: “A person awakes from sleep when digestion is complete”. Although he was wrong, the answer of why and how we sleep is still a mystery.

It was not until the twentieth century that a scientific theory of sleep was really prompted. The discovery that there existed important regions in the brain for regulating sleep and wakefulness was made by Constantin von Economo (Von Economo, 1930). During the encephalitis lethargica world epidemic, he found that sleep disorders occurred in 85% of the patients with the encephalitis virus. Based on the observation of the dead patients’ brains, he suggested there must be a “sleep-regulatory centre”, which is the brainstem and the hypothalamus (See in Fig 1.1). He thought that this sleep-regulating centre must have a special affinity for the encephalitis virus, as it was so frequently affected (Von Economo, 1930). Constantin von Economo published the important paper to consider the “sleep-regulatory centre”, entitled *Sleep as a problem of localization*, and the text reads:

The search for a so-called sleep centre may at first sight appear a paradoxical idea. In the same manner as the waking state, so also does sleep appear as such a complex biological condition that the problem makes us at first sit up and take notice. Indeed, our entire life takes place in the alternating change of two biological conditions, the waking and sleeping state and in this way the problem might appear primarily of the same category as the problem of the centre of life itself (Von Economo, 1930).

The development of technology and clinical procedures in the field of sleep has enhanced the research, especially the electroencephalogram (EEG). In 1929, Hans Berger, a German physiologist and psychiatrist, reported the first human EEG to investigate brain electrical activity, and found differences between sleep and wakefulness (H Berger 1929). EEG has since become a popular method for sleep assessments.

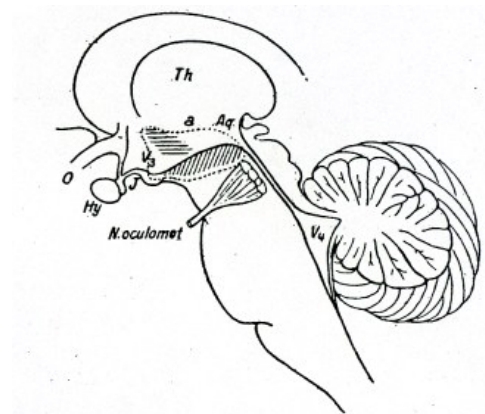


Figure 1-1 A drawing of human brain from von Economo's original work. Lesions to the brain region between the brainstem and midbrain caused excess sleepiness. Lesions in the anterior hypothalamus caused insomnia (Saper et al., 2005b).

In 1939, recognized as the father of modern sleep research, Nathaniel Kleitman published a standard reference book, *Sleep and Wakefulness*, which summarized years of research in the sleep field. He and his graduate student, Eugene Aserinsky, first introduced and described the "Rapid-eye movements sleep"

(Aserinsky and Kleitman, 1953). People describe this achievement as a monumental work in the modern sleep field. Later on, Allan Rechtschaffen and his colleagues defined the sleep stages in humans by EEG, which consist of rapid eye movement (REM) sleep and four non-REM (NREM) stages of sleep (Hori et al., 2001) (Fig. 1.2). Cortical desynchronization happens during wakefulness and REM sleep. In contrast, NREM sleep displays cortical synchronization, which manifests as slow waves. The deeper stages of NREM sleep are characterized by the amount of slow wave activity and delta power (1-4 Hz) (Hori et al., 2001). Rats and mice show polyphasic sleep with lots of short NREM-REM cycles throughout the whole day. There are no different stages of NREM in animals.

The research of brain circuits involving the sleep process has advanced rapidly. But what exactly the drive to sleep is in terms of biochemistry or circuitry remains unsolved, and understanding its mechanism would be a breakthrough.

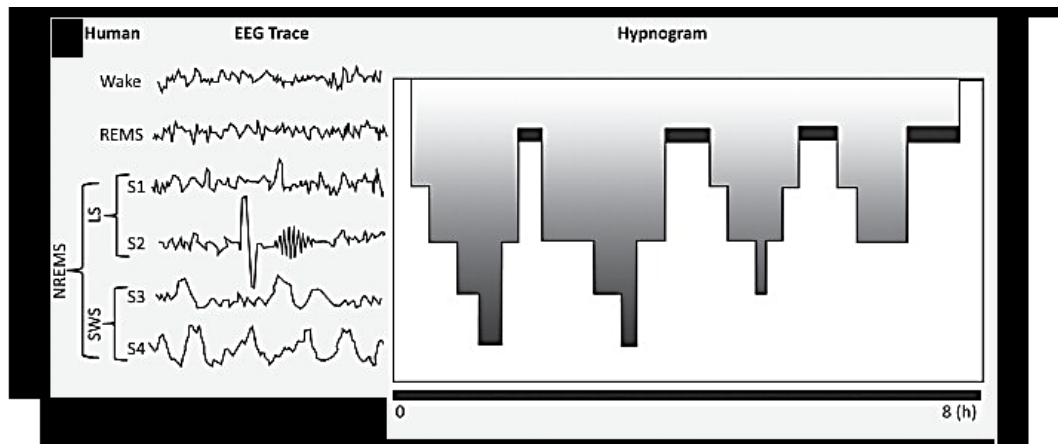


Figure 1-2 Each sleep phase has distinctive wave patterns. In humans there are two phases of sleep, NREM sleep and REM sleep. The four NREM stages are relevant to the depth of sleep, the arousal threshold increases from stage 1 to stage 4. Stage1 sleep is identified with low-voltage mixed frequency EEG activity and a shift from alpha waves (8 to 13 Hz) to theta waves (3 to 7 Hz). Stage 2 is characterized by sleep spindles (12 to 14 Hz) and K-complexes. Stage 3 and stage 4 are fused together and referred to as slow-wave sleep (SWS), delta sleep. The delta sleep is defined by slow wave (1-4 Hz) activity EEG. REM sleep's features include desynchronized EEG, and muscle atonia. The sleep hypnogram shows an eight-hour-night time sleep episode(Genzel et al., 2014).

1.2 Neural circuitry of wake and sleep

1.2.1 Neural circuitry of wakefulness

Noradrenaline, dopamine, histamine, orexin, serotonin, acetylcholine and glutamate are major neurotransmitters involved in arousal (Figure 1.3 & 1.4 A)(Lee and Dan, 2012). The locus coeruleus (LC) is the major source of noradrenaline. The LC, similar to other wake-promoting nuclei, also sends projections

throughout the brain to the hypothalamus, neocortex and cerebellum (Aston-Jones and Bloom, 1981).

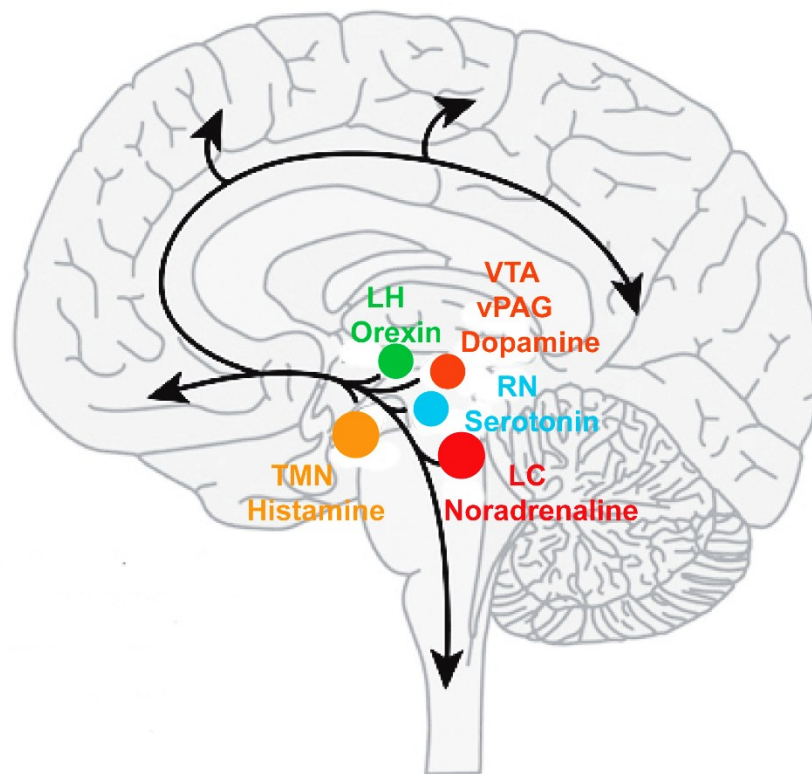


Figure 1-3 The key arousal pathways are shown for human brain. Abbreviations of neuromodulatory regions: LC, locus coeruleus; RN, raphe nuclei; VTA, ventral tegmental area; vPAG, ventral periaqueductal gray; TMN, tuberomammillary nucleus; LH, lateral hypothalamus. Modified from (Chiu and Prober, 2013)

Dopamine is mainly released from projections from the ventral tegmental area (VTA) and the substantia nigra pars compacta. However, wake-active dopamine neurons in a small recently identified nucleus, the dorsal raphe nucleus (DRN) and ventrolateral periaqueductal grey (vlPAG) regions, also contribute to promoting wakefulness (Dougalis et al., 2012; Lu et al., 2006). Dopamine β -hydroxylase knockout (*Dbh*^{-/-}) mice, which lack

noradrenaline and dopamine, need strong stimuli to wake up and have decreased latency after sleep deprivation (Hunsley and Palmiter, 2003, 2004). Dopamine enhances motor activity, which may promote waking. Mice with deletion of the dopamine transporter (DAT) gene show reduced NREM sleep and are hyperactive (Wisor et al., 2001).

The glutamatergic drive from the parabrachial nucleus (PB) and the adjacent pre-coeruleus region to cortex is critical for promoting wakefulness (Fuller et al., 2011). The external lateral and lateral crescent parabrachial nuclei are important for hypercapnia-evoked arousal (Kaur et al., 2013). Deletion of the *Vglut2* in that area increases the latency to EEG arousal from hypercapnia (Kaur et al., 2013).

The PPT and the LDT serve an important role in waking and REM regulation, and also project to areas including the basal forebrain, hypothalamus, LC and raphe nuclei (RN) (Hallanger et al., 1987; Satoh and Fibiger, 1986). The PPT/LDT contain different populations of cells, including cholinergic, GABAergic and glutamatergic neurons (Boucetta and Jones, 2009; Wang and Morales, 2009). All cholinergic neurons are maximally firing during wake and REM (Boucetta et al., 2014). The PPT/LDT may activate the cortex both wakefulness and REM sleep.

The basal forebrain, anterior to the preoptic hypothalamus, receives input from the brainstem and projects to the neocortex. Optogenetic stimulation of cholinergic basal forebrain neurons

during NREM sleep can induce an immediate transition to wake or REM sleep and prolong wakefulness (Han et al., 2014).

The posterior and lateral hypothalamus, containing orexin and histamine neurons, receive inputs from the basal forebrain and the preoptic area and project widely to all brain regions (Saper et al., 2010). Orexin mediates arousal. When rats are injected with orexin, wakefulness was increased and NREM was reduced (Sakurai, 2007). The histaminergic neurons in the tuberomammillary nucleus (TMN) are thought as “wake-on, sleep-off” neurons which receive an inhibitory input from VLPO. TMN neurons become active just after waking, fire at an average rate of about 5 Hz, and their activity is suppressed during sleep (Sakai et al., 2010; Takahashi et al., 2006). Lack of histamine in mice impairs wakefulness and results in shorter sleep latency in novel environments (Parmentier et al., 2002). Adenosine, one of the candidate sleep homeostatic molecules, promotes sleep through the activation of adenosine A₁ and A_{2A} receptors (Berridge, 2006). Activation of A₁ receptors in the TMN promotes NREM sleep, but activation of A₁ receptors in the lateral preoptic area promotes wakefulness (Oishi et al., 2008; Zhang et al., 2013).

The serotonergic neurons in the raphe nuclei (RN) modulate sleep. Lesions of the RN reduce the sleep time (Jouvet, 1972, 1999). Blocking serotonin formation also reduces NREM sleep (Borbely et al., 1981). Depressive patients suffer insomnia or fragmented sleep. The changes can be reversed by treatment with antidepressive selective serotonin re-uptake inhibitors (SSRIs) (Wilson and Argyropoulos, 2005).

1.2.2 Neural circuitry of NREM sleep

The preoptic area (PO) in the anterior hypothalamus serves as one of the most important sleep promoting areas (Fig1.4A). The PO area, a mixture of sleep-active, wake-active, temperature-sensitive and state-indifferent neurons (Alam et al., 2014; Szymusiak et al., 2007; Takahashi et al., 2009), houses circuitry which initiates and/or maintains sleep, and regulates body temperature (Lu et al., 2000; Saper et al., 2010; Sherin et al., 1996; Szymusiak et al., 2007). Classical experiments in cats showed that direct electrical stimulation of the PO hypothalamus induced powerful NREM and behavioural sleep (Clemente and Serman, 1963). Recently, another key sleep promoting nucleus in the parabrachial region in the brainstem has also been characterized (Fig1.4B) (Anaclet et al., 2014).

The ventrolateral preoptic nucleus (VLPO) projects to the arousal system and is thought to promote NREM sleep (Saper et al., 2010). The more sparsely located VLPO neurons in the PO, termed the extended VLPO, are suggested to innervate the noradrenergic locus coeruleus (LC) and dorsal raphe promoting REM sleep. VLPO neurons also target orexinergic neurons, the glutamatergic parabrachial nucleus, dopaminergic PAG, as well as the PPT/ LDT nuclei (Saper et al., 2010). In rats with about 80% lesions in the VLPO cluster, the amounts of NREM and delta power were reduced by half. The loss of neurons in the VLPO cluster correlated closely with the loss of NREM (Lu et al., 2000).

Additional circuitry for promoting NREM may exist outside the VLPO (Anaclet et al., 2014). Pharmacogenetic activation of the GABAergic neurons in the parafacial zone (PZ) in the brainstem increased NREM sleep and delta power, and decreased beta-gamma power. By contrast, inhibition these neurons increases wakefulness and decreases NREM and REM sleep (Anaclet et al., 2014). These neurons directly inhibit wake promoting parabrachial nucleus-basal forebrain-cortex circuit. They release GABA onto the basal forebrain-projecting parabrachial nucleus.

nucleus; PFC: prefrontal cortex; PZ: parafacial zone (Anaclet et al., 2014).

1.2.3 Control of REM sleep

The studies for investigating the mechanism associated with REM sleep help us to better understand sleep-wake behaviour. Early studies found REM sleep generated in the pons (Jouvet, 1962). Cholinergic neurons in the LDT/PPT have been identified as promoting REM sleep. Optogenetic activation of cholinergic neurons in the PPT/LDT nuclei increase the number of REM episodes, but not the duration of REM sleep (Van Dort et al., 2015). Stimulation of cholinergic neurons in the basal forebrain during REM sleep increased the duration of REM sleep episodes (Han et al., 2014). More recently, neurons in the lateral hypothalamus were also found to regulate the REM sleep state (Clement et al., 2012). The lateral hypothalamus contains orexin. The orexin neurons target the monoaminergic neurons and REM-off neurons to stabilize the waking stage and transition to REM sleep, which makes the transition to REM sleep difficult. Consequently, people who have lost orexin neurons fall sleep easily during the day. The lateral hypothalamus also contains GABA and melanin-concentrating hormone (MCH), which inhibits REM-off regions (Jego et al., 2013; Tsunematsu et al., 2014).

1.3 Sleep-wake dynamics

A flip-flop switch in electronics exists when a circuit has two stable states with the existence of one state inhibiting the second (Saper et al., 2010; Saper et al., 2005b). Using this model, Saper and his

colleagues proposed an influential hypothesis about sleep-wake regulation. In the sleep system, one subsystem inhibits the other subsystem and acts to stabilise its own firing; hence the most popular model in the sleep field taking the name “flip-flop switch” (Saper et al., 2005a; Saper et al., 2005b). The wake promoting system is suggested to have two main pathways. One acts to inhibit the reticular nuclei to open the thalamocortical connection (Saper, 2013). Another based on the monoaminergic neurons, including the LC, DR, PB, and TMN, inhibits the basal forebrain and PO area (Saper et al., 2010; Saper et al., 2005b). On the other hand, the neurons in the wake promoting system are inhibited by input from the VLPO neurons and the median preoptic (MnPO) nucleus. At sleep onset, GABA/galanin neurons in VLPO and GABA neurons in MnPO, increase their firing rate and inhibit wake-promoting aminergic-type neurons, like TMN and LC (Saper et al., 2005a; Saper et al., 2010; Saper et al., 2005b). Orexins stabilize the behavioural state of wakefulness because they send strong excitatory signals to wake-promoting neurons. The orexin knockout mice have more transitions between wake, NREM and REM, which may support this model (Mochizuki et al., 2004)

Although many studies support the model, this model has a few weakness. First, some proposed interactions between different neurons are not accurate. Inhibition of histaminergic neurons via GABA is dispensable in the regulation of the sleep–wake cycle (Zecharia et al., 2012). Second, the model does not well represent the firing changes in the brain regions. The PO neurons fire fast around the state-transition, but the firing rate of wake-active neurons in the basal forebrain changes slowly. Histamine neurons

start to fire after the onset of NREM-Wake transition. Besides, the mechanism responsible for turning the switch is unclear. Indeed, the circadian drive and homeostatic need for sleep are not considered in the model.

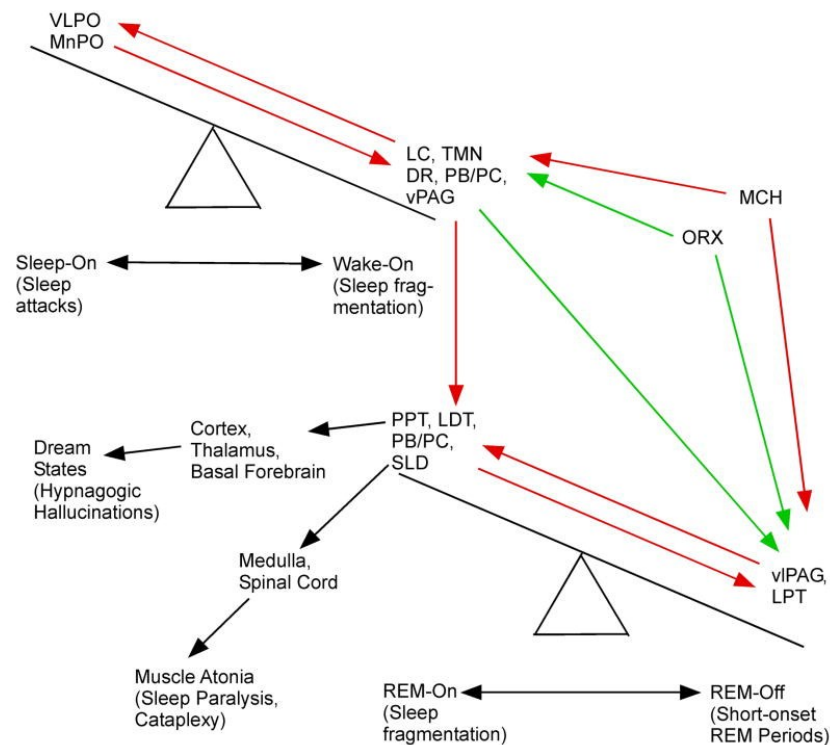


Figure 1-5 The flip-flop switch hypothesis for controlling the sleep-wake transition and REM-NREM transition. Red arrows show inhibitory projections, green arrows show excitatory ones. During wakefulness, the arousal system inhibits the VLPO and REM-on neurons, which can prevent from the wake to REM transition. During sleep, VLPO and MnPo inhibit the arousal system. The switches are reinforced by the orexin neurons and melanin-concentrating neurons. DR: dorsal raphe, LC: locus coeruleus, LDT: laterodorsal tegmental nuclei, LPT: lateral pontine tegmentum, MnPO: median preoptic nuclei, PB/PC:parabrachial/precoeruleus nucleus, PPT: pedunculo pontine tegmental nuclei, SLD: sublateralodorsal nucleus, TMN: tuberomammillary neurons, vPAG: ventrolateral periaqueductal gray matter, VLPO: ventrolateral

preoptic nuclei, vPAG: ventral periaqueductal gray matter(Saper, 2013).

In the early 1980s, Alexander Borbély first put forward the two-process model of sleep-wake regulation. He provided a novel theory that the sleep-wake cycle is regulated by circadian rhythm Process C and sleep homeostasis Process S. These two processes tightly interact to determine the timing of sleep onset, duration of sleep and even the depth of sleep (Borbely and Achermann, 1999).

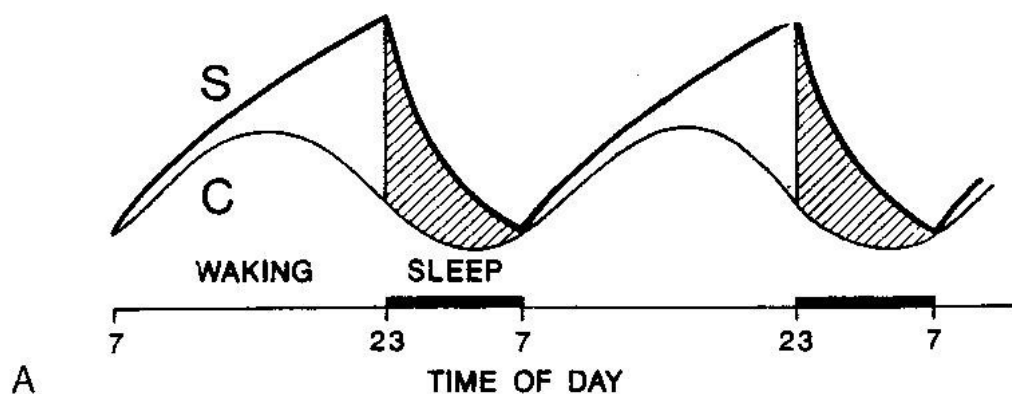


Figure 1-6 Borbély's Two-process model of sleep regulation. Process S indicates the homeostatic driver and Process C represents the circadian driver. A 24-hour circadian rhythm of sleep and arousal results from process C. Process S determines the duration of sleep and wakefulness. Sleep pressure builds as wakefulness is maintained. During sleep it reduces to a level where wakefulness is resumed once more(Borbely and Achermann, 1999).

Circadian rhythms contribute mostly to the timing of sleep (Franken and Dijk, 2009). In the mammalian brain, the suprachiasmatic nucleus (SCN) in the hypothalamus, functions as

an internal clock, controlling the 24 hour rhythm of metabolic, physiological and endocrine processes, including temperature regulation and production of hormones such as melatonin and cortisol(Huang et al., 2011; Pandi-Perumal et al., 2007).

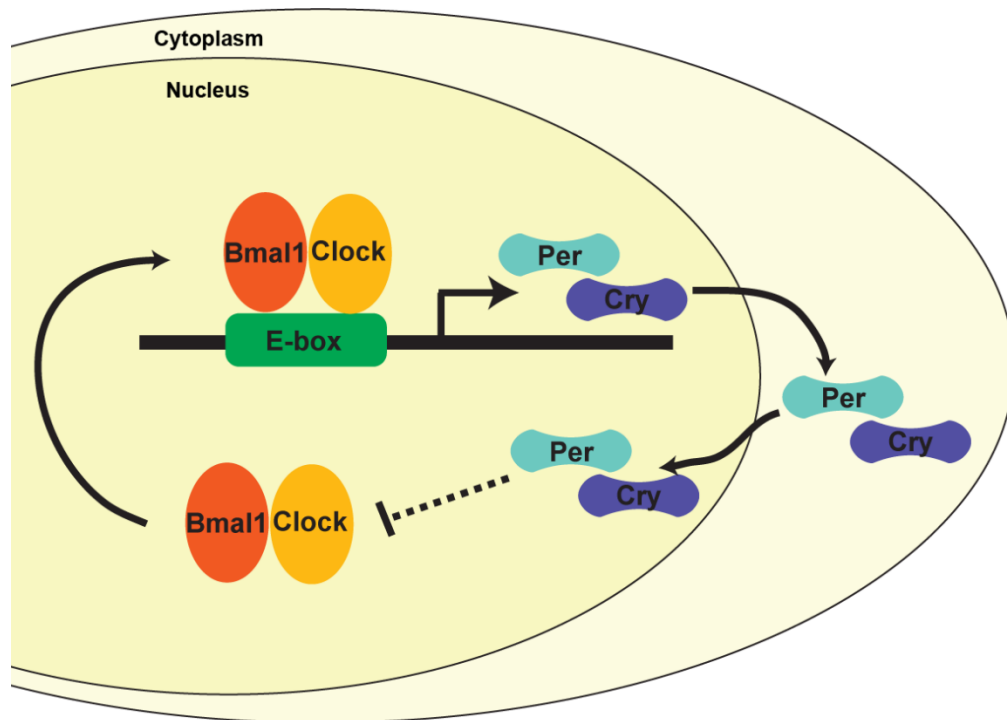


Figure 1-7 A simplified cartoon of the circadian clock in mammals. Bmal1 and Clock regulate the transcription of factors by binding on the circadian E-box element. Bmal1 and Clock form a dimer and then active the expression of Per and Cry genes. Per and Cry become negative elements of the transcription feedback loop to inhibit activity of Bmal1 and Clock. Modified from (Kondratova and Kondratov, 2012).

Lesions of the SCN disrupt the 24 hour rhythm of the sleep-wake cycle. In rats with lesions, there is no significant change in the total length of sleep or wakefulness during a 24 hour period compared to those without (Ibuka and Kawamura, 1975; Liu et al., 2012b).

However, both the number of episodes of NREM to wake and the duration of wakefulness change significantly (Liu et al., 2012b). At the molecular level, from *Drosophila* to humans, the 24 hour rhythm is controlled by positive and negative regulators. Brain and muscle ARNT-like 1(Bmal1) and circadian locomotor output cycles kaput (Clock), as the positive regulators, affect the transcription of period genes (Per) and Cryptochrome (Cry) (Albrecht, 2012; Reppert and Weaver, 2001). On the other hand, *PER* and *CRY* inhibit the transcription of Bmal1 and Clock, as the negative regulators. Deletions or mutations of these genes in mice generally alter the timing or pattern of sleep (Albrecht, 2012; Reppert and Weaver, 2001).

Sleep homeostatic regulation is less well understood than circadian regulation. We are far from a general consensus about what makes us sleepy. When we do not get enough sleep, we become sleepy and only recovery sleep can reverse this. In fact the drive to "recovery sleep" after prolonged sleep deprivation seems so strong that it is similar to taking a sedative drug. Some studies focus on the rebound sleep in response to sleep deprivation. After sleep deprivation, sleep "pressure" accumulates greatly, and the amount of NREM sleep needed increases (Borbely and Achermann, 1999). During sleep, sleep pressure is discharged until it drops to a level low enough to allow the organism to wake.

The mechanism(s) and neural circuitry that makes sleep inescapable if we are deprived of sleep is not well understood. Investigating neurochemistry and sleep circuitry would help us to understand the sleep homeostatic mechanism. The neuronal

activity during wakefulness may be the driving force of sleep. Local neurons in the cortex can “go offline” as sleep after a prolonged wakefulness (Vyazovskiy et al., 2011a; Vyazovskiy et al., 2011b). Changed intrinsic activity in hypothalamus and basal forebrain may trigger the drive to sleep by excessive wakefulness (Porkka-Heiskanen, 2013; Saper et al., 2010; Saper and Lowell, 2014).

Extracellular messengers that build up. For example, adenosine, prostaglandins or interleukins have been proposed to constitute the sleep drive (Porkka-Heiskanen, 2013). Extracellular adenosine concentration increases in some brain regions (although not in the PO) during a prolonged period of wakefulness and blocking the A1 receptors in the basal forebrain can reduce the amount of recovery sleep and delta power (Gass et al., 2009; Porkka-Heiskanen et al., 1997). Prostaglandin D2 is also increased during sleep deprivation in the region below hypothalamus and the basal forebrain (Urade and Hayaishi, 2011).

However, the two-process model assumes these two processes are independent. Recent research found process C and process S cycles interact with each other to form a 24-h rhythm at a molecular and physiological level. Sleep deprivation can affect the activity of SCN neurons. Mice lacking *bmal1*, *cry1* or *cry2* genes have less NREM sleep after sleep deprivation (Laposky et al., 2005; Wisor et al., 2002). NREM sleep consolidation was increased in *cry1, 2*^{-/-} mice but decreased in clock-mutant mice (Wisor et al., 2008). Delta power in the EEG, which is an indicator of the homeostatic need for NREM, was reduced in both *bmal1*^{-/-} and clock-mutant mice and increased in *cry1, 2*^{-/-} mice (Dudley et al., 2003; Laposky et al.,

2005; Wisor et al., 2008). *Per2* also responds to homeostatic sleep need and regulates sleep pattern (Shiromani et al., 2004). A local circadian clock in the brain, controlled by the clock protein BMAL1, regulates histamine synthesis by controlling level of HDC mRNA. Removing BMAL1 from histaminergic neurons in the TMN locally also increase wakefulness in the night, have more fragmental sleep, and reduce NREM sleep after sleep deprivation (Yu et al., 2014). Most neurons may have local clocks, which sleep-active neurons can use local clocks that measure something that inhibits or activates them.

1.4 Sleep and sedation

Several anaesthetic and sedative drugs at low concentrations can induce a state that resembles natural sleep. Learning how sedation works in the brain may bring some insights on the mechanism of sleep. A number of sedative drugs have been developed that act on the noradrenergic system. Activation of inhibitory $\alpha 2A$ receptors with agonists such as clonidine, xylazine or dexmedetomidine, induces, with increasing dose, sedation and loss of righting reflex (Drew et al., 1979; Nelson et al., 2003). Dexmedetomidine is a sedative drug used in the clinic. It produces an arousable state, when patients administered dexmedetomidine still can respond to modest stimulation such as shaking (Kamibayashi and Maze, 2000). Dexmedetomidine lowers body temperature and enhances slow wave activity in the neocortex (Baker et al., 2014; Gelegen et al., 2014).

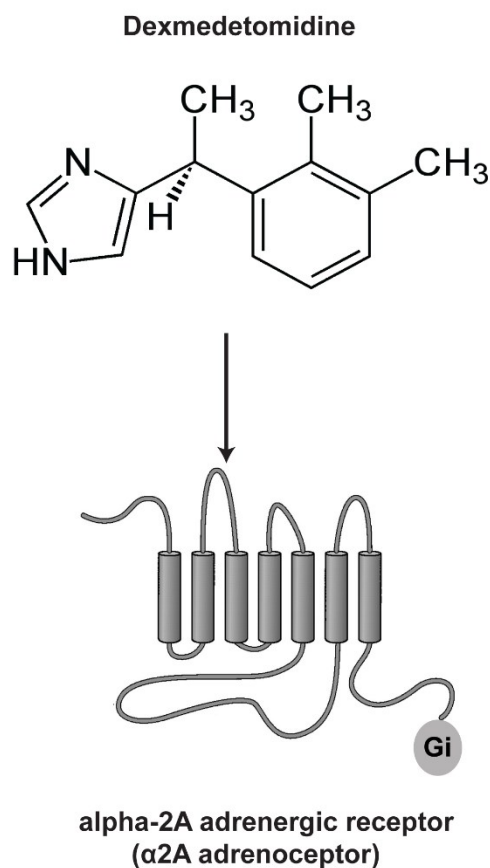


Figure 1-8 The chemical structure of dexmedetomidine. Dexmedetomidine, an agonist of α 2-adrenergic receptors, is a sedative medication used in the clinic for long-term sedation.

At the circuit level, it is unclear how α 2 agonists induce sedation and loss of consciousness. The most popular hypothesis is that dexmedetomidine primarily acts on α -2a adrenoceptors in the LC, where there is a reduction in noradrenaline release into the neocortex and thalamus, thus producing sedation and sleep-like states (Aghajanian and VanderMaelen, 1982; Baker et al., 2014; Correa-Sales et al., 1992; Lakhlani et al., 1997; Nelson et al., 2003). Activation of α -2a receptors causes intracellular signalling via G proteins, which decreases levels of 3, 5-cyclic adenosine monophosphate (cAMP) that prevents the phosphorylation of ion

channels by cAMP-dependent kinases. The membrane becomes hyperpolarised. G-protein activation also prevents Ca^{2+} entry through voltage-gated calcium channels (Gertler et al., 2001).

Selectively stimulating LC neurons induces waking, but the converse is not true: acutely inhibiting LC neurons does not produce strong sleep, even after 1 hour of stimulation (Carter et al., 2010), an unexpected result if α_2 agonists are supposed to acutely inhibit the LC to induce hypnosis. Moreover, dopamine beta-hydroxylase (DBH) is the essential enzyme to convert dopamine to norepinephrine. In the dopamine β -hydroxylase knockout (Dbh $^{-/-}$) mice, significant reduced latencies and prolonged duration of dexmedetomidine action was found. However, the loss of righting reflex (LORR) still can be induced in the knockout mice (Hu et al., 2012). The sedative effect may not mediate via $\alpha_2\text{A}$ -adrenoceptors to inhibit noradrenaline (NA) release, but via $\alpha_2\text{A}$ -adrenoceptors in non-adrenergic cells. $\alpha_2\text{A}$ -adrenoceptors are not only in the LC, but also in the layer VI of the neocortex, the lateral septum, the ventral medial hypothalamic area and the spinal cord (Gelegen et al., 2014). Dexmedetomidine may induce loss of movement through these areas (Gilsbach et al., 2009; Hu et al., 2012), making interpretation of this observation difficult (Sanders and Maze, 2012).

We gained unexpected insight into this issue by selective genetic knockdown of $\alpha_2\text{A}$ -adrenoceptors from adrenergic neurons in the LC. Initially, it was proposed that activating $\alpha_2\text{A}$ -adrenoceptors on LC terminals in VLPO would disinhibit sleep-active neurons (Nelson et al., 2003). Thus low doses of dexmedetomidine would

give light inhibition of the LC and sedation; high doses give strong LC inhibition and LORR (Fig 1.4) (Zhang et al., 2015). In our lab, we found that knocking down $\alpha 2A$ - adrenoceptors in the LC completely, and surprisingly, spared the drug's capacity to induce sedation, but did, on the other hand, abolish dexmedetomidine's ability to induce the apparently deeper LORR state. This suggests that these two states, sedation and LORR are distinct, and that sedation induced by dexmedetomidine does not depend on it reducing NA release from LC terminals.

Recent research has demonstrated dexmedetomidine may exert its anaesthetic effects through the sleep pathway. During dexmedetomidine induced sedation, EEG activity shows sleep spindle and slow-wave sleep in humans (Huupponen et al., 2008). Administering GABA_A receptor antagonist, gabazine, alongside dexmedetomidine attenuated anaesthetic effects in rats, which has a similar effect to the GABA release from VLPO during physiological sleep (Nelson et al., 2003). For both dexmedetomidine administration and NREM sleep, in the initial period, the local field potentials from central medial thalamus, ventrobasal thalamus, cingulate cortex and barrel cortex showed higher power in the δ band of frequencies and later a sudden drop in frequency from 40Hz to 20Hz (Baker et al., 2014).

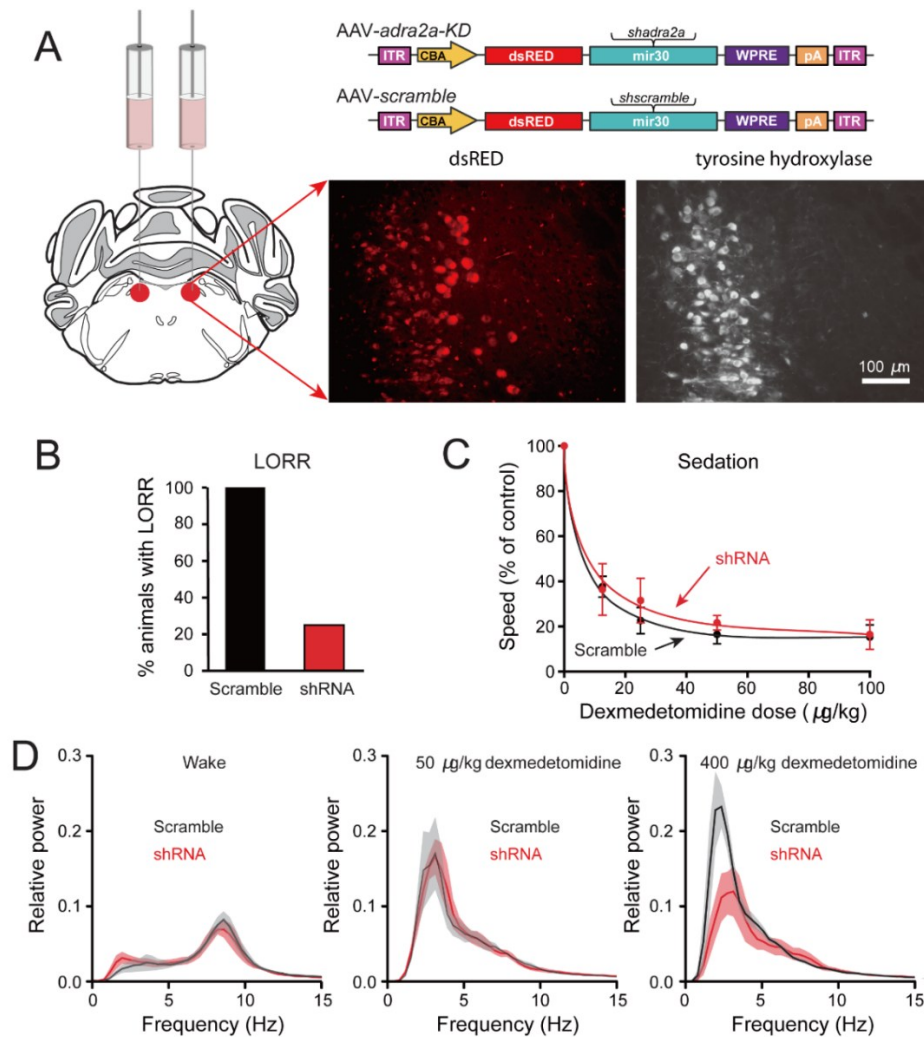


Figure 1-9 Knock down of adrenergic α 2A receptors in the locus coeruleus blocks dexmedetomidine-induced LORR, but not sedation. (A) AAVs expressing either dsRED-mir30-*shadra2a* or dsRED-mir30-*shscramble* transgenes were bilaterally injected into the LC of adult mice. Photographs illustrate AAV transgene expression (dsRED) in the LC as confirmed by co-staining with tyrosine hydroxylase antisera (white). ITR, inverted terminal repeats; CBA, chicken- β -actin enhancer/promoter. (B) Percentage of animals with LORR induced by 400 μ g/kg dexmedetomidine. LC-scramble (black; n=9) and LC-*adra2a*-KD (red; n=8) mice showed a significant difference (Fisher's exact test; $P=0.0023$). (C) Fourier transform power spectra for LC-scramble (black), and LC-*adra2a*-KD (red) mice in the waking state (left; n=5) and in response to 50 μ g/kg dexmedetomidine (middle; n=4) and 400 μ g/kg

dexmedetomidine (right; n=4). Lighter shaded envelopes indicate the s.e.m. (D) Movement of LC-scramble (black; n=4-6), and LC-adra2a-KD (red; n=4-7) mice in response to sedative doses of dexmedetomidine were not significantly different (two-way ANOVA, $P=0.91$). Data provided by Ferretti and Steinberg (Zhang et al., 2015)

Nelson et al. also found that c-fos expression in the VLPO, LC, and TMN during physiological sleep and dexmedetomidine administration was similar, indicating the activation of the same neural circuits under both conditions (Nelson et al., 2003). When the VLPO was lesioned, dexmedetomidine was unable to induce the same proportion of time rats spent in NREM sleep and c-fos expression did not decrease in the TMN suggesting that the VLPO was unable to inhibit it (Nelson et al., 2003). We hypothesized that dexmedetomidine cause loss of consciousness through a common mechanism, by hijacking the PO hypothalamic circuitry to mimic deep recovery sleep.

1.5 Pharmacological manipulations of gene expression

The heart of the method, TetTagging, which I use in this thesis to investigate sleep nuclei is based on inducible gene expression. Inducible gene expression systems in mammals mostly use a tetracycline-responsive transcriptional regulator developed by Bujard and colleagues (Gossen and Bujard, 1992). The two basic systems are the Tet-Off (tTA) and Tet-On (rTA) systems. tTA is composed of the Tet repressor of *Escherichia coli* (cloned from the tetracycline resistance operon) and the virion protein 16 activation

domain; tTA binds to an operator sequence, tetO, in promoters to activate transcription (Gossen and Bujard, 1992; Gossen et al., 1995). In the presence of tetracycline or doxycycline (Dox), tTA undergoes a conformational change and cannot bind to the tetO sequence, resulting in termination of transcription. In contrast, in the absence of tetracycline or doxycycline, transcription is activated. Therefore, the tTA system is also called the Tet-Off system (Gossen and Bujard, 1992; Gossen et al., 1995). In most cases, a double transgenic approach has been used in mice (Garner et al., 2012; Liu et al., 2012a; Reijmers et al., 2007). In this system, a specific promoter drives expression of tTA and the tetO promoter drives expression of the transgene of interest. By controlling the presence of tetracycline or doxycycline, transgenes expression is made reversible. *In vivo*, to control the timing of transgene expression, mice consume either food or water that contains doxycycline (Garner et al., 2012; Liu et al., 2012a; Reijmers et al., 2007).

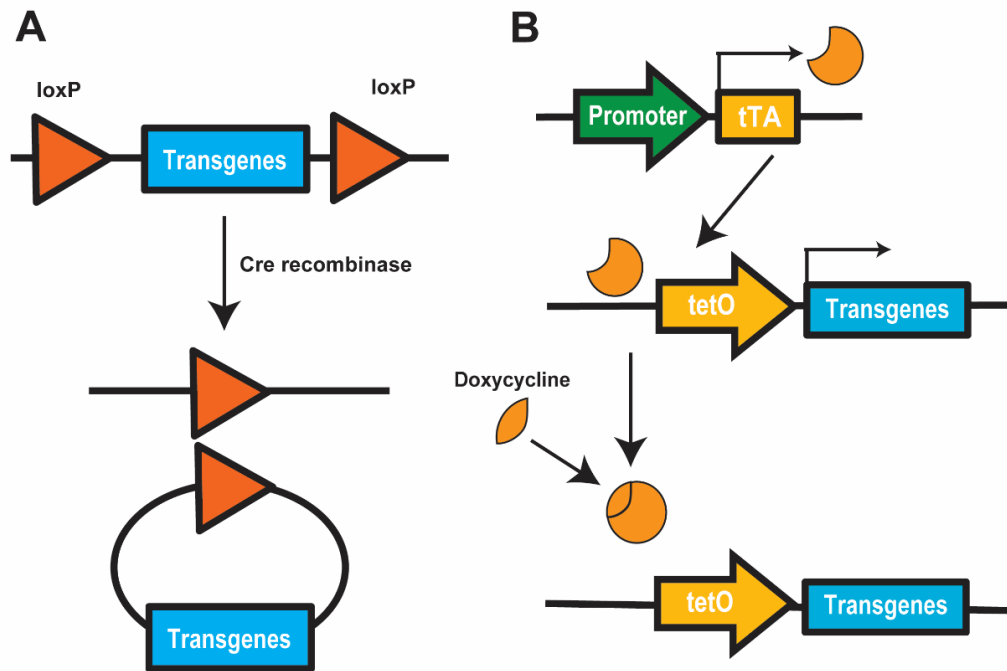


Figure 1-10 A: Schematic showing site-specific recombinase system. For the Cre-LoxP systems, LoxP sites are usually placed in introns without compromising gene expression. Before recombine, the conditional allele should have wild-type activity. During recombination, the Cre protein must locate and bind to the LoxP sites to break and rejoin the DNA to recombinant genes. B: Tet-Off inducible gene expression system. Promoter induces the expression of tTA. In the absence of doxycycline, tTA binds the responsive elements tetracycline-resistance operon tetO, which can drive the downstream target genes. In the presence of Dox, tTA dissociates from tetO stopping transgene expression.

Pharmacogenetics is the modulation of transgenic receptors via pharmacological agents, which can directly activate or inhibit a specific receptor and as a consequence induce a behavioural outcome. A recently developed strategy is pharmacogenetic modulation *in vivo* by the receptors activated solely by synthetic ligand (RASSLs) and designer receptors exclusively activated by designer drugs approach (DREADD) (Alexander et al., 2009; Alvarez-Curto et al., 2011; Coward et al., 1998). In most cases,

engineered G-protein coupled receptors (GPCR) were used to interact with exogenous ligand rather than endogenous ligand (Alexander et al., 2009; Alvarez-Curto et al., 2011; Coward et al., 1998). DREADDs and RASSLs respond through G proteins to exogenous ligands, such as clozapine-n-oxide (CNO), and induce responses that last hours (Alexander et al., 2009; Rogan and Roth, 2011). CNO, a pharmacologically inert agonist, is modified from clozapine which has a high affinity to this receptor family. Furthermore, CNO can cross the blood brain barrier and is bioavailable in rodents and humans. Bryan L. Roth and his colleagues used directed molecular evolution to create designer receptors based on random mutagenesis via error-prone polymerase chain reaction. Introducing just two mutations enabled Gq-coupled human M₃ (hM₃) muscarinic receptor to respond to CNO but not the native ligand acetylcholine. In addition, the receptors show no baseline activity (Armbruster et al., 2007; Pei et al., 2008).

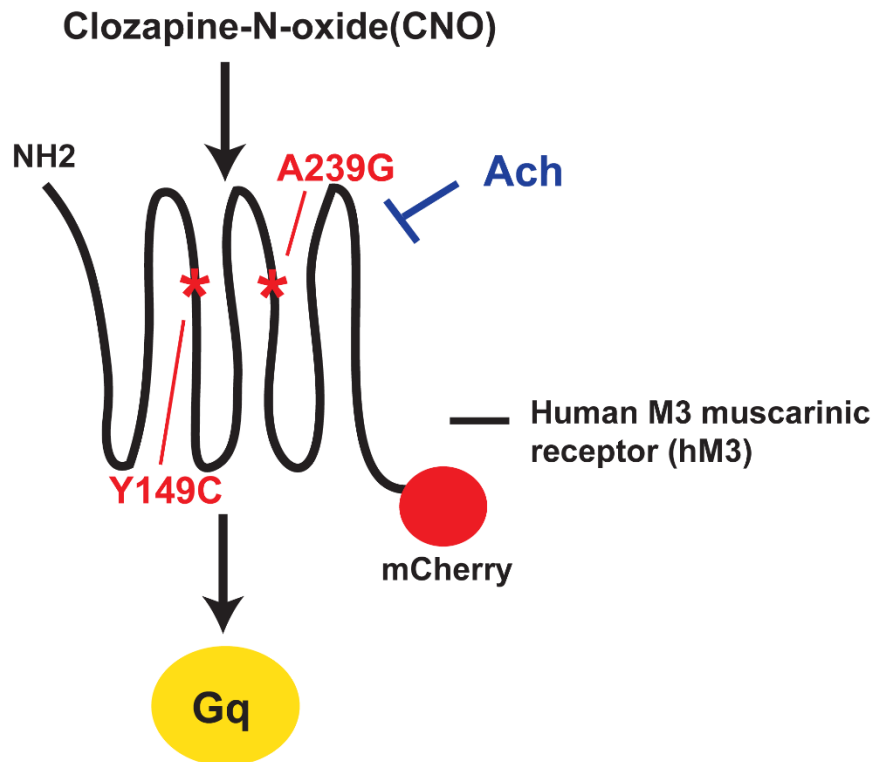


Figure 1-11 Schematic diagram of the hM3Dq receptor. The receptor is formed by point mutations in the third and fifth transmembrane regions of muscarinic receptors, Y149C and A239G (indicated by the red asterisk). The mutant receptor cannot bind acetylcholine (Ach), the endogenous agonist, but can bind CNO to activate the Gq pathway with high efficacy (Armbruster et al., 2007). The receptor is fused with mCherry at its C-terminus.

DREADD strategies are widely used to map neuronal circuits controlling many fundamental functions of the brain, such as memory formation, regulation of metabolism, sleep and so on (Garner et al., 2012; Saito et al., 2013).

1.6 Aims and hypothesis

Previous studies have identified the PO hypothalamic area as being a key brain region controlling sleep, but functional details of these neurons – especially their roles in sleep homeostasis and

regulation require further clarification. For example, do certain sedatives act at the circuitries of preoptic area or more widely? (Franks, 2008; Rihel and Schier, 2013). I hypothesized that the heavy but arousable state

dexmedetomidine produces is similar to a particular type of deep natural sleep, the NREM sleep experienced after sleep deprivation, so-called “recovery sleep”. In this thesis, I address these questions by genetically manipulating and tracing the hypothalamic neural circuits involved. Our key tool involves TetTagging (Garner et al., 2012; Reijmers et al., 2007; Zhang, 2015). Firstly, I would like to test whether neurons in the PO area would turn on c-FOS expression following systemic administration of the α_2 agonist dexmedetomidine and which neuronal subtypes are activated during recovery sleep and sedation. Secondly, by using TetTagging method, we would like to hypothesize that this PO circuitry is sufficient to sense and respond to the stimuli that trigger sleep and hypothermia, and that drug-induced sedation and recovery sleep appear to be the same state. Thirdly, we need to find out whether GABAergic neurons in the PO area are needed for the induction of sedation. Last but not the least, I would like to identify the projections of the neurons which are activated during dexmedetomidine-induced sedation.

Chapter 2 Materials and Methods

2.1 AAV constructs

First, the TetTag transgene components, AAV- P_{c-fos} -tTA and AAV- $P_{TRE-tight}$ -hM3Dq-mCHERRY, were placed into two separate AAV transgenes. As a building block, I started with the plasmid pAAV-ITR- P_{cmv}/β -actin-iCre-2A-Venus-WPRE-pA-ITR (Tang et al., 2009). A PacI site was first introduced just upstream of the CMV/ β -actin promoter in this plasmid using Quick Change Mutagenesis (Agilent) (Primers 5'-CTGGAAGCTCCTTAATTAACGCTCTCCTGTTCCGACC-3' and 5'-GGTCGGAACAGGAGAGCGTTAATTAAGGAGCTTCCAG-3'), and the " P_{cmv}/β -actin-Cre-2A-Venus" fragment then removed with PacI and NotI digestion. To generate the AAV- P_{c-fos} -tTA construct, the c-fos-tTA cassette, from a plasmid containing a c-fos promoter/first intron -764/+918 fragment (P_{c-fos}) linked to the tTA reading frame (Reijmers et al., 2007) (Addgene plasmid 34856; gift from Mark Mayford), was PCR-amplified (the forward primer with a NotI site: 5'-GCATTCCACCACTGCGGCCGCTCATCAGTTCCATAGG-3' and reverse primer with a PacI site: 5'-GGTGCGGGCCTCTTCTTCTTAATTAAGCCAGACGGCCGCAGC-3') and subcloned into the PacI/NotI digested Cre-Venus vector, to give pAAV-ITR- P_{c-fos} -tTA-WPRE-pA-ITR.

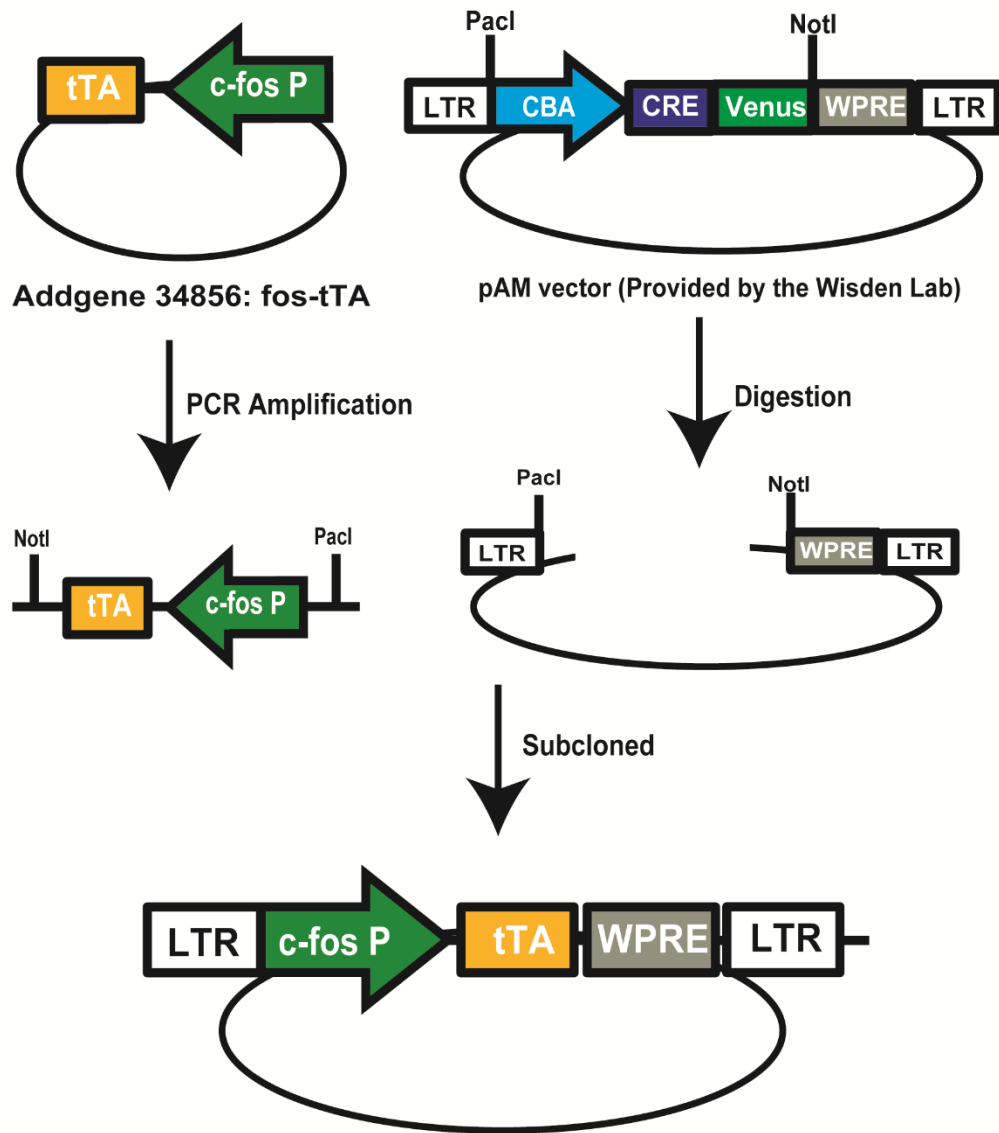


Figure 2-1 Restriction map of pAM vector (Abraham et al., 2010) and the original plasmid cFos-tTA which the backbone and insert were ligated. CBA, chicken beta-actin promoter; c-fos P, c-fos promoter; CRE, Cre recombinase; Venus, a variant of yellow fluorescent protein; ITR, inverted terminal repeat; WPRE, woodchuck hepatitis post-transcriptional regulatory element.

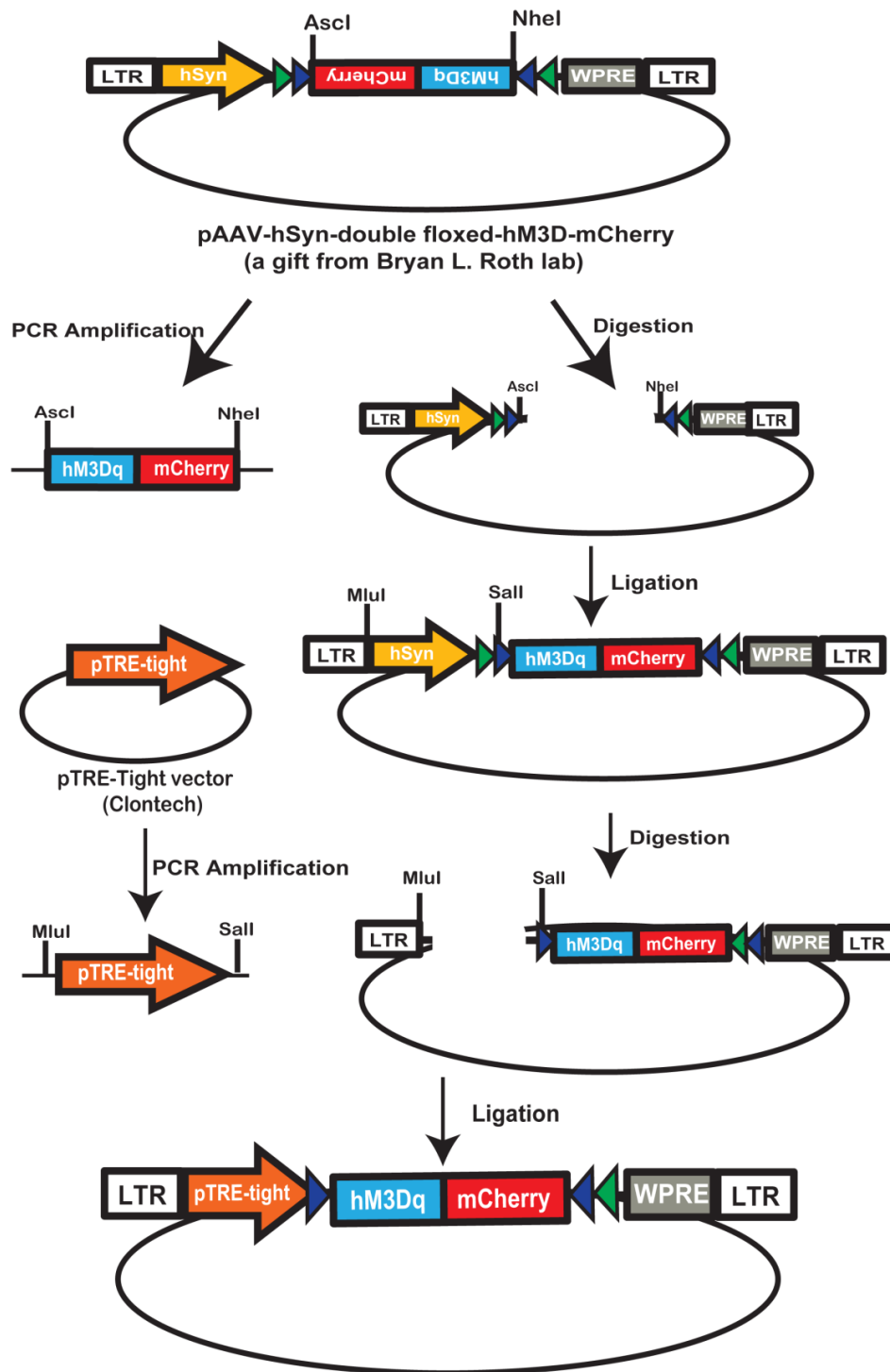


Figure 2-2 Restriction map and the original plasmid of pAAV-hSyn-DIO-hM3Dq-mCherry vector and PTRE-tight from clontech. hSyn, human synapsin promoter; hM3Dq, evolved M3-muscarinic receptor; PTRE-tight, a modified Tet response element; ITR, inverted terminal repeat; WPRE, woodchuck hepatitis posttranscriptional regulatory element.

For the generation of the pAAV-ITR- $P_{TRE-tight}$ -hM3Dq-mCherry-WPRE-pA-ITR construct, I started with the plasmid pAAV-hSyn-double flexed hM3Dq-mCHERRY (Krashes et al., 2011) (a gift from Bryan L. Roth; Addgene plasmid 44361), which contains an inverted hM3Dq-mCherry reading frame flanked by AscI and NheI sites. We PCR-amplified (forward primer hM3Dq-NheI-F: 5'-CGAAGGTTATGGCTAGCCTTACTTGTACAGCTCG-3'; reverse primer hM3Dq-AscI-R: 5'-CTTTATACGAAGTTATGGGCGCGCCACCATGAC-3') the hM3Dq-mCHERRY reading frame (including the Kozak and ATG initiation sequences CCATGG), and placed this fragment back in the NheI/AcsI digested parent plasmid, but now in the sense orientation between the lox sites to give "hSyn-double flexed hM3D-mCherry sense". I then removed the hSyn promoter from this construct by MluI and SalI digestion, and replaced the promoter with the TRE-tight ($P_{TRE-tight}$) promoter; this was PCR-amplified from the $P_{TRE-tight}$ plasmid (Clontech; forward primer, 5'-CTTCACACGCGTTTACTCCCTATCAGGATAGAG-3'; reverse primer, 5'-CAGCTGACTAGTCGACCCCCGGGTAC-3'), then digested with MluI and SalI. The final construct was pAAV-ITR- $P_{TRE-tight}$ -hM3Dq-mCHERRY-WPRE-pA-ITR.

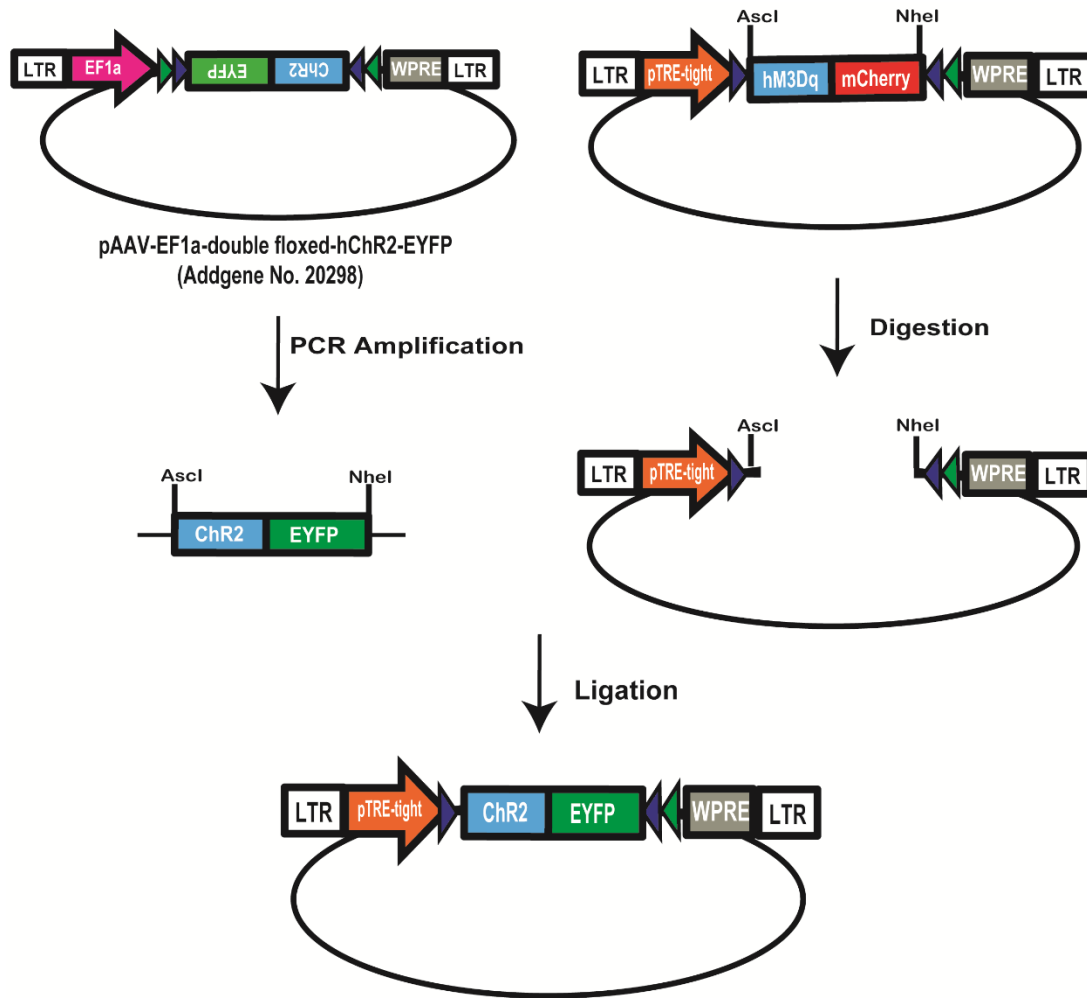


Figure 2-3 Restriction map and the original plasmid of pAAV-EF1a-double floxed-hChR2 (H134R)-EYFP vector and pAAV-ITR- P_{TRE-tight}-hM3Dq-mCherry-WPRE-pA-ITR construct. Human elongation factor-1 alpha; chR2, Channelrhodopsin-2; EYFP, enhanced yellow fluorescent protein; P_{TRE-tight}, a modified Tet response element; ITR, inverted terminal repeat; WPRE, woodchuck hepatitis posttranscriptional regulatory element.

Plasmid P_{TRE-tight}-TetO-eYFP-ChR2 was constructed as follows: the eYFP-ChR2 construct was amplified from the Addgene plasmid pAAV-EF1a-double floxed-hChR2 (H134R)-EYFP (catalogue No. 20298) using chR2-eYFP forward primer 5'-CGAAGTTATGGCTAGCCCTATTACTTGTACAGC-3' and reverse primer 5'-CGAAGTACCGCGCCACCATGGACTATGGC-3'. The

protocol used: 95 °C pre-cycle initialisation 5 min, 95 °C denaturation 30 s, 60 °C annealing 30 s, 72 °C elongation 1.5 min, 35 cycles, post-cycle 72 °C final elongation 5 min; Upstream NheI and downstream AscI restriction sites were incorporated into the eYFP-ChR PCR product. Then the PCR product was subcloned into pAAV- P_{TRE-tight}-hM3D-mCherry construct to replace the hM3D-mCherry reading frame.

2.2 AAV production and purification

2.2.1 AAV plasmid preparation

The AAV plasmids were as follows: pfΔ6 (containing relevant genes from adenovirus helper plasmid), pRVI (containing rep and cap sequences for AAV2), pH21 (containing rep and cap sequences for AAV1) (Klugmann et al., 2005). The plasmids were validated using restriction enzyme digestion.: HindIII for pfΔ6 (giving bands of 5.5kb, 3kb, 3kb, 2,3kb and 1.5kb), XbaI for pRVI (giving bands of 7.5kb and 3.8kb),and EcoRI for pH21 (giving bands of 4.5kb, 2.8kb and 0.2kb).

2.2.2 Transformation into HEK293 cells

AAVs could be purified by heparin binding (Klugmann et al., 2005; Murray et al., 2011). Human embryonic kidney 293 (HEK293) cells were plated out 24 hours before transfection. The transfection mixture used was: 62.5μg AAV plasmid (pAM-cfos-tTA, pAAV-P_{TRE-tight}-hM3D-mCherry), 125μg pFdelta6, 31.25μg pRVI 31.25μg pH21 1650μl 2.5 M CaCl₂, 1650μl 2.5 M CaCl₂, and 12ml H₂O. 16 hours after transfection, the medium was removed and replaced with 50ml of fresh DMEM. 60-65 hours after transfection, media were removed and discarded, cells were washed in 1xPBS, and after

washing, cells were collected in 50ml Nunc tissue culture tubes, and cells were pelleted at 800g for 5-10 min, supernatant were discarded and cell pellets were resuspended in 150mM NaCl, 20mM Tris pH 8.0. Sodium deoxycholate (Sigma #D5670) and benzonase endonuclease (Sigma #E1014) were added to a final concentration of 50U/ml. Cells were incubated in a 37 °C waterbath for 1hr. After incubating, cell debris was removed by centrifugation at 3000g for 15 min. The supernatant were stored at -20°C until use.

2.2.3 Heparin column purification

The supernatant was purified by a heparin column (Klugmann et al., 2005). The column was pre-equilibrated (1ml HiTrap Heparin columns, Sigma #5-4836) with 10ml 150mM NaCl, 20mM Tris pH 8.0. The pump was set at 25mm diameter, 1ml/min flow rate; the sample were loaded onto the column. Gradient elution was used increasing concentrations of sodium chloride: 200 mM (1 mL), 300 mM (1 mL), 400 mM (1.5 mL), 500 mM (3 mL), 1 M (1.5 mL) in the Tris 20mM (pH8). After purification, the vector was concentrated using AMICON ULTRA-4 (100000MWCO; Millipore; CatNo. UFC810024) at 2000g/2 min. The concentrator was refilled with 3.5ml of 0.9% NaCl for two times. The eluate was removed to a sterile tube; 250µl of 0.9% NaCl were added into the tube (Murray et al., 2011), then divided into 10 µL aliquots and stored at -80 °C.

2.2.4 Validation of AAV particles

Successful AAV production was verified by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). The gel was

prepared as follows: for the separator gel, water 2.2 mL, Tris-HCl (1.5 M, pH 8.8) 1.25 mL, SDS (10 %) 50 μ L, acrylamide 1.5 mL, ammonium persulfate (APS; 10 %) 50 μ L, TEMED 10 μ L; for the stacking gel:, water 1.6 mL, Tris-HCl (1.5 M, pH 6.8), SDS (10 %) 25 μ L, acrylamide 250 μ L, APS (10 %) 25 μ L, TEMED 10 μ L). Then 3 μ L and 7 μ L of purified virus were heated at 100 °C for 5 min with 2x Laemmli buffer, then run on the SDS-PAGE at 160 V for 1 h. Gels were stained with Coomassie blue and washed in water overnight. The expected coat protein weights were 97, 66, and 56 kDa. A typical gel is shown in Figure 2.4

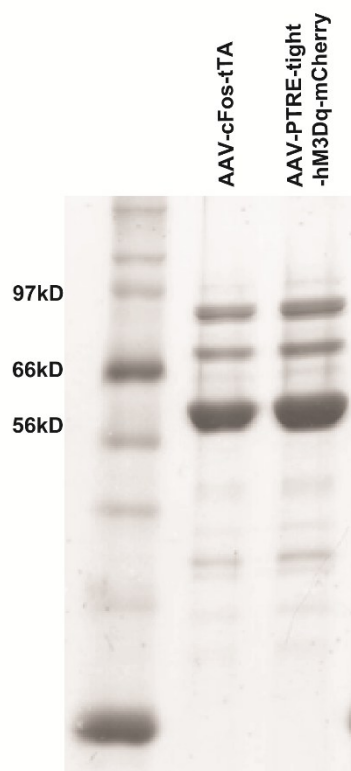


Figure 2-4 Picture of SDS gel of AAV-cFos-tTA and AAV- P_{TRE}-tight-hM3Dq-mCherry packaging by SDS-PAGE. The size of viral coat proteins is shown.

2.3 Mice

All animal experiments were performed in accordance with the United Kingdom Home Office Animal Procedures Act (1986) and had local ethical approval. All procedures were carried out under project licences PPL 70/7273 and 70/7617. I held a personal licence (I72F7785F). All the TetTag-DREADD experiments used adult male C57/Bl6 mice, 8-12 weeks old, purchased from Harlan UK. The *vgat^{lox/lox}* mice (Tong et al., 2008b) were purchased from JAX labs (stock NO. 012897; donated by Bradley Lowell). Mice were kept on a 12:12 light: dark cycle, at a maximum of four animals per cage, with free access to food and water. Mice fitted with Neurologger2A (see section 9) were singly housed.

2.4 AAV injection

Mice were anesthetized with 2% isoflurane in O₂ by inhalation and maintained on 1.5-2% isoflurane during the surgery. A digital mouse brain atlas was linked to the stereotaxic injection frame to guide and target of the area interested (Leica Angle Two System). Stereotaxic coordinates were based on Franklin and Paxinos's atlas of the mouse brain (Paxinos and Franklin, 2008). AAV injections were done with 0.5 μ l of two AAV plus 0.5 μ l 20% mannitol (total 1 μ l volume) to increase AAV spread (Mastakov et al., 2001). AAVs were injected bilaterally into the LPO (AP +0.2 mm, ML $\pm$ 0.75 mm, DV -5.7 mm relative to Bregma), or with one injection into MnPO (AP +0.4 mm, ML 0 mm, DV -4.5 mm relative to Bregma), or bilateral injections into the superior colliculi (AP -3.8 mm, ML $\pm$ 1.0 mm, DV -2.0 mm relative to Bregma) using a 10 μ l Hamilton syringe at a rate of 0.25 μ l/min controlled by an ultramicropump (World Precision Instruments).

2.5 EEG/EMG surgery

After the AAV injection procedure was completed, mice were still anesthetized with 1.5-2% isoflurane in O₂ at a flow rate by inhalation and maintained throughout surgery. The reference-ground EEG electrodes were implanted into the parietal bone (Bregma -1.5, mediolateral (ML) +1.5), the frontal bone (Bregma +1.5, ML -1.5) and the intraparietal bone located over the cerebellum (Lambda -2.0, ML 0.0) (Figure 2.5). For EMG, three teflon-insulated stainless steel wires (with the distal 3mm exposed) were embedded into the neck muscles behind the skull. The electrodes and EMG wires were secured to the skull with dental cement. After surgery, animals were removed into recovery cage with homoeothermic blanket. Post-operative analgesics and antibiotics were administered in the drinking water, which consisted of carprofen (100 ppm Rimadyl, Pfizer, UK) and enrofloxacin (100 ppm Baytril, Bayer, UK) respectively for three days. The EMG and EEG wires were later connected to Neurologger2A recording devices (see section 8.1).

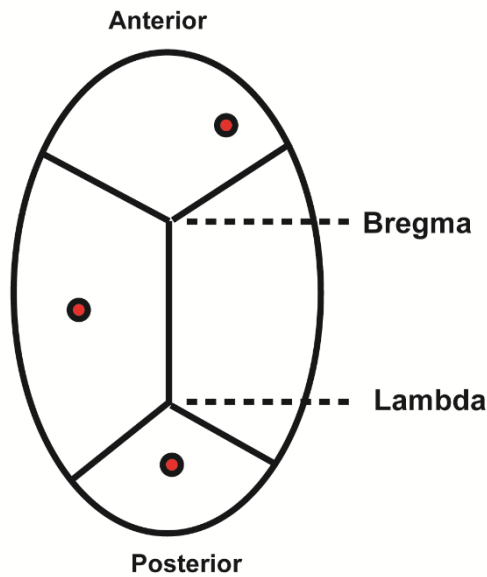


Figure 2-5 Cartoon of EEG recording electrodes (dots in the figure) on the mice skull. Recording electrodes were placed according to the Bregma and Lambda on the skull. The reference-ground electrode is placed into the parietal bone (Bregma -1.5, mediolateral (ML) +1.5). For recording electrodes, one electrode is placed into the frontal bone (Bregma +1.5, ML -1.5) and another electrode is located over the cerebellum (Lambda -2.0, ML 0.0).

2.6 Immunohistochemistry

Adult mice were anesthetized, transcardially perfused with 4% paraformaldehyde in PBS, pH 7.4. Brains were removed and 40- μ m-thick coronal sections sliced using a Leica VT1000S vibratome. Free-floating sections were washed in PBS three times for 10 minutes, permeabilized in PBS plus 0.4% Triton X-100 for 30 min, blocked by incubation in PBS plus 4% normal goat serum (NGS), 0.2% Triton X-100 for 1 h (all at room temperature) and subsequently incubated with a c-FOS monoclonal antibody

(1:10,000, Santa Cruz Biotechnology, CatNo.sc-253), and/or a mCHERRY monoclonal antibody (1:2000, Clontech CatNo.632543), or anti-GAD67 antibody (1:1,000; Millipore, MAB5406) or with GFP antiserum (1:1000, Molecular Probes, A6455). Primary antisera were diluted in PBS plus 2% NGS overnight at 4°C. Incubated slices were washed three times in PBS plus 1% NGS for 10 minutes at room temperature, incubated for 2 h at room temperature with a 1:1000 dilution of a Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1:1000, Molecular Probes®, CatNo.A11034) and Alexa Fluor® 594 goat anti-mouse IgG (H+L) (1:1000, Molecular Probes®, CatNo.A11005) in PBS plus 1% NGS, and subsequently washed three times in PBS for 10 min at room temperature. The sections were mounted on slides and coverslipped. Fluorescent images were collected on a Nikon Upright fluorescent microscope (Nikon, Tokyo, Japan). In each experiment, sections without primary antibody were included as a negative control. No positive elements were detected in these control sections.

c-Fos immunohistochemistry with diaminobenzidine (DAB) (A master student, Alessandro Morro, helped me with these DAB experiments, under my supervision)

We used DAB staining to better visualize nuclear c-fos expression over a relatively large area. 30 minutes after i.p. dexmedetomidine (50, 100, 400 µg/kg), or saline (0.9%) administration, experimental mice were anesthetized, perfused transcardially, and free-floating sections processed for immunohistochemistry with rabbit antibody to Fos (1:20,000, Ab-5, Calbiochem) after blocking endogenous peroxidase with 0.3% H₂O₂ in PBS. The primary

antiserum was localized using a variation of the avidin-biotin complex system (Vector Laboratories). In brief, sections were incubated for 120 minutes at room temperature in a solution of biotinylated goat antibody to rabbit IgG (PK-6101, Vector Laboratories) and then placed in the mixed avidin-biotin horseradish peroxidase complex solution (ABC Elite Kit, Vector Laboratories) for 60 minutes. The peroxidase complex was visualized by a 4 to 5-minute exposure to chromogen solution (0.05% 3, 30-diaminobenzidine tetrahydrochloride (wt/vol, Sigma-Aldrich), 0.4 mg/ml nickel ammonium sulphate to produce a blue-black product. The reaction was stopped by washing in distilled water and PBS. Sections were dehydrated and cover-slipped with quick mounting medium (Eukitt, Fluka Analytical).

2.7 Electrophysiology and single-cell qPCR (note: this work was done together with another PhD student, Zhiwen Ye; he did the electrophysiology, and I did the single-cell PCR assay)

For acute brain slice recordings of neurons in the lateral preoptic hypothalamus, 250 μ m-sections were cut with a Vibratome (Campden Instruments) in ice-cold slicing solution containing (in mM): 85 NaCl, 2.5 KCl, 1 CaCl₂, 4 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 75 sucrose, 25 glucose, pH 7.4 when bubbled with 95% O₂ and 5% CO₂. After incubating in slicing solution for 15-30 minutes at room temperature, slices were then transferred to oxygenated ACSF containing (in mM): NaCl 125, KCl 2.5, CaCl₂ 2, MgCl₂ 1, NaH₂PO₄ 1.25, NaHCO₃ 26, glucose 11. For whole-cell current clamp recordings, patch pipettes (4-6 M Ω) were backfilled with internal solution containing (in mM): 145 K-gluconate, 4 NaCl,

5 KCl, 0.5 CaCl₂, 5 EGTA, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, and 10 sucrose, pH 7.3, adjusted with KOH. Identification of hM3Dq/mCherry+ neurons was achieved by detection of red fluorescent under an upright microscope (Olympus BX51W1) equipped with mounted LED (M595L3, Thorlabs). ACSF containing 5 μ M CNO was perfused into the bath after stable whole cell recordings were acquired.

For single-cell qPCR, after achieving a whole-cell recording, Zhiwen Ye aspirated the content of the neuron into the recording pipette and expelled into single cell lysis/DNase I solution using the Single-Cell-to-CT Kit (Ambion). I then performed reverse transcription, cDNA pre-amplification and qPCR following the manufacturer's guidelines using the TaqMan® Gene Expression Assay system (Applied Biosystems, Foster City, USA). The primer reference numbers were: hprt: Mm01545399_m1; gad1: Mm04207432_g1; gad2: Mm00484623_m1; vglut1: Mm00812886_m1; vglut2: Mm00499876_m1.

2.8. Animal Behaviour

Behavioural experiments, except where specified otherwise, were performed during "lights-off".

2.8.1 EEG/EMG recording

EEG and EMG signals were recorded by a miniature 265 MB memory logging device attached to the head of a free-moving mouse. The Neurologger2A was originally designed by Vyssotski (Vyssotski et al., 2009).

The Neurologger2A have seven pins. The first two pin (pin 1 and 2) was soldered at the Neurologger2A and connected to reference electrode to create ground. Therefore, there left six pins, three for EEG and three for EMG. One of the three EEG inputs and one of the three EMG inputs serves as a reference.

The Neurologger2A was attached on the animal and EEG/EMG signals were recorded for 15 minutes. Saline or clozapine-N-oxide (CNO, 5mg/kg Co832, Sigma-Aldrich) was injected and signals were continued to record for 2 hours. An automatic protocol was used to score all recordings as REM sleep, NREM sleep or wakefulness.

Four data channels could be recorded at a sampling rate of 200 Hz and were low-pass filtered with a cut-off at 1 Hz (-3 db). The EEG data recorded by the Neurologger2A were downloaded to a computer and waveforms visualized using Spike2 software (Cambridge Electronic Design, Cambridge, UK) or MATLAB (MathWorks, Cambridge, UK).

2.8.2 Assay for sedation

100 µg/kg of dexmedetomidine (Tocris Bioscience, UK), dissolved in saline was delivered intraperitoneally and animals, fitted with Neurologger2A (Vyssotski et al., 2009), were placed immediately afterwards in the activity cage for 30 minutes to assess locomotor activity (Med Associates Activity Monitor Version 5 for mice).

2.8.3 Dexmedetomidine and CNO administration

For sleep deprivation experiments, animals were treated with a diet including doxycycline (40mg/kg) for one month. Sleep

deprivation started 2 days after removal of doxycycline. These experiments were performed during Zeitgeber time (ZT) 0-6. The control group was allowed normal sleep; the experimental group was sleep deprived for 4 hours by introducing novel objects or tapping lightly on the cages (Gerashchenko et al., 2008). To reduce the possibility of stress, we never touched the mice directly. Mice were then placed back into their home cages where they exhibited strong and sustained recovery NREM sleep. CNO (5 mg/kg) responses were examined four days from the recovery. At the end of the sleep recovery day, mice were put back on food with 1 g/kg doxycycline (or 1 g/l doxycycline in the drinking water), which was replaced with 40 mg/kg doxycycline the following day (Reijmers et al., 2007). CNO was dissolved in saline to a concentration of 0.5mg/ml. CNO (5mg/kg) were examined after three or four days from the recovery.

For dexmedetomidine experiments, after AAV injection, mice were raised on food with 40 mg/kg doxycycline (Harlan TD.120240 40 ppm Doxy Diet (2018, B)) for four weeks(Reijmers et al., 2007). For the behavioural experiments to examine dexmedetomidine-induced sedation, dexmedetomidine or saline i.p. injection took place 48 hours after removal of doxycycline. At the end of the sedation experiments, mice were then put back on the doxycycline diet (Harlan TD 09295 1000 ppm Doxy Diet (2018)). Four days after dexmedetomidine-induced sedation, clozapine-N-oxide (Co832, Sigma-Aldrich, dissolved in saline, 5 mg/kg) was injected i.p. into TetTag mice (eg. LPO-Tettag-hM3Dq and MnPO-Tettag-hM3Dq) fitted with Neurologger2A devices.

2.8.4 Locomotor activity

Mice were injected *i.p.* with 5mg/kg CNO. To allow time for CNO distribution, exploration in a novel environment was started from the 30 minutes after CNO administration. Behaviour was assessed for 30 minutes in an open field (40 cm x 40 cm x 30 cm) crossed by a grid of photobeams (VersaMax system, AccuScan Instruments). Counts were calculated of the number of photobeams broken during the trial in every five-minute. The software computes distance travelled, total time ambulatory and average velocity.

2.8.4 Measurement of body temperature

The body temperature of the mice was recorded using a miniature data logger (DSTnano; Star-Oddi, Gardabaer, Iceland) that was implanted seven days previously in the peritoneal cavity. The loggers were programmed to record every 30 minutes during 24-hour sleep recordings, every 5 minutes during sleep deprivation and recovery sleep, or every 2 minutes for experiments with dexmedetomidine or CNO injection. The loggers were recovered and the data downloaded at the end of the experiments. Ed Harding, another PhD student in the lab, helped me with these experiments.

2.9 Data Analysis

2.9.1 Sleep Scoring

For the EEG recording, two channels are recorded. Channel 1 is the cerebellar signal with reference subtracted while Channel 2 is the frontal signal with the reference subtracted. Before scoring, EEG

recordings are imported into Spike 7 software. EEG and EMG channels needed to be filtered. Frequencies below 0.5 Hz were filtered out of the EEG and frequencies between 5 and 45 Hz are picked out from the EMG channel.

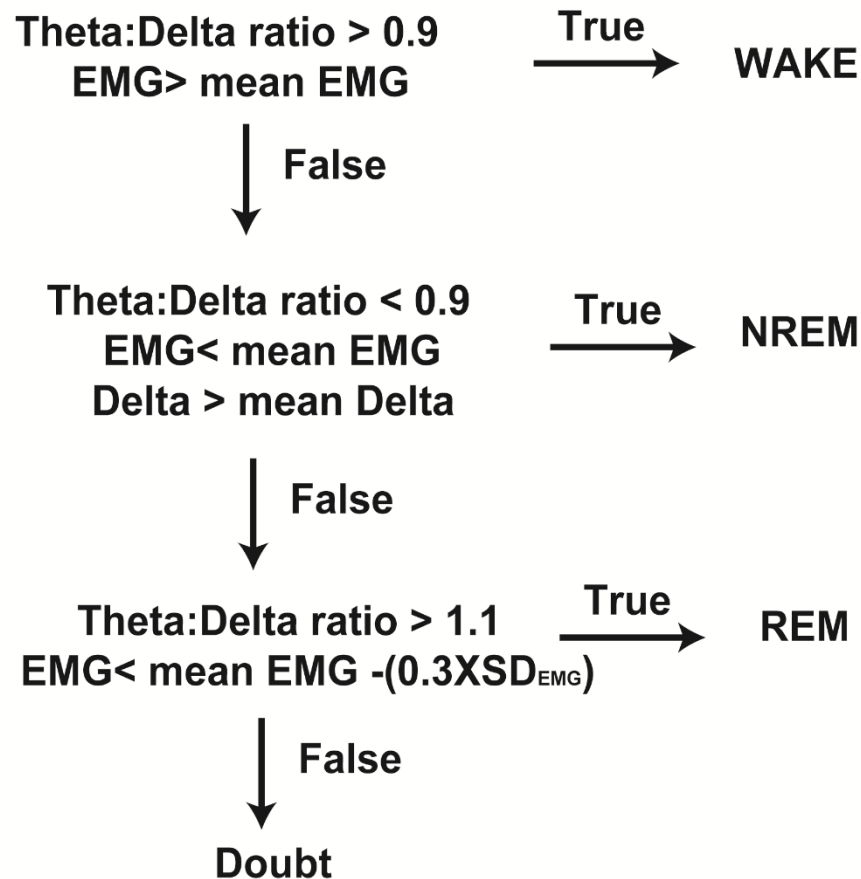


Figure 2-6 Logic rules used to determine sleep-wake stages during five second epoch. Wake should be normal theta to delta ratio and high EMG. NREM should be high delta and EMG lower than Wake. REM should be high theta and low EMG.

The Spike 7 can analysis the power spectrum and sleep scoring. The criteria of sleep scoring are explained in Figure 2.6. The score can be exported from Spike 7 to Microsoft Excel: percentage of NREM, REM and Wake in the certain time.

2.9.2 Statistical analyses

Prism6 and Origin were used for statistical analysis. Data collection and processing were randomized or performed in a counter-balanced manner. Normality was tested by the Shapiro-Wilk test. Equal variances were assessed by F-test. All data are represented as the mean $\pm$ s.e.m. For c-fos immunohistochemistry two-tailed unpaired t test was performed. For dexmedetomidine sedation and CNO experiments was ANOVA (with one or two factors and with or without repeated measured where appropriate) performed. Where F ratios were significant, statistical analyses were extended and bonferroni post hoc tests were used. $P < 0.05$ was considered significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). All the experiments were performed and analysed blind to treatment conditions.

Chapter 3 Neurons throughout the lateral preoptic (LPO) area and bed nucleus stria terminalis are activated during both recovery sleep and dexmedetomidine-induced sedation

3.1 Introduction

Immediate-early gene mapping, whereby when neurons are excited, they express the c-fos gene, has been used for many years to map the activity of neurons in numerous brain circuits (Hunt et al., 1987; Morgan et al., 1987; Wisden et al., 1990), including mapping those neurons active during sleep and sedation (Nelson et al., 2002; Scammell et al., 1998; Sherin et al., 1996). In the hypothalamus, neurons in the VLPO and MnPO display c-fos expression before or during sleep (Scammell et al., 1998; Sherin et al., 1996). Specifically, after 9-12 hours of sleep deprivation, c-fos expression in the VLPO is much higher in rats which were allowed recovery sleep for one hour than were culled immediately. High c-fos expression in the VLPO occurs during rebound sleep (Scammell et al., 1998; Sherin et al., 1996). Fos-positive neurons are also found in the MnPo during or before sleep (Gong et al., 2000; Gong et al., 2004). These neurons may build up their activity from “sleep pressure” to induce sleep. But it is unclear if activating GABAergic neurons in this area is sufficient for producing sleep rebound after sleep deprivation. Their roles in sleep-wake regulation of these neurons in this area are not well studied.

To understand where in the mouse brain the adrenergic α_2 receptor-induced sedative response is taking place (see Chapter 1, section 1.4), and if the natural sleep pathway is involved or not, I first confirmed c-FOS activation in the PO hypothalamic area. Previous work emphasized that the VLPO nucleus in the PO hypothalamus was specifically activated following dexmedetomidine administration (Nelson et al., 2003), and during normal and recovery sleep (Gong et al., 2004; Sherin et al., 1996).

Previously, Valentina Ferretti, Elly Steinberg and Cigdem Gelegen in our lab had established that when given at 100 $\mu\text{g}/\text{kg}$ *i.p.*, dexmedetomidine induced sedation and a sleep like state, as seen by strong delta power in the EEG, but no loss of righting reflex (this was only obtained at 400 $\mu\text{g}/\text{kg}$, dexmedetomidine) (Gelegen et al., 2014). I was particularly interested in the lower dose of dexmedetomidine because the sedation obtained with that dose was most similar to deep natural sleep, perhaps like that experienced during recovery sleep.

Note: Alessandro Morro, a Masters student in the lab, helped me under my supervision with some of the cell counting in this Chapter. All results in this chapter were published in Zhang et al., 2015.

Results

3.2 c-FOS activation in the PO and bed nucleus areas during recovery sleep and by dexmedetomidine administration

Before starting my main project (TetTagging, see later Chapters), I looked at c-FOS activation in the hypothalamic PO area following dexmedetomidine-induced sedation or during recover sleep after sleep deprivation. Please see Chapter 2.6 for details of the methods and procedures. C57Bl6 Mice (n=3) were injected with a sedative dose of dexmedetomidine (100 µg/kg) or control saline. Brains were taken either 30 mins or one hour after animals received dexmedetomidine or saline and analysed by immunocytochemistry looking at the expression of the endogenous c-fos gene. Brains were taken also two hours into recovery sleep after animal were sleep deprived for four hours.

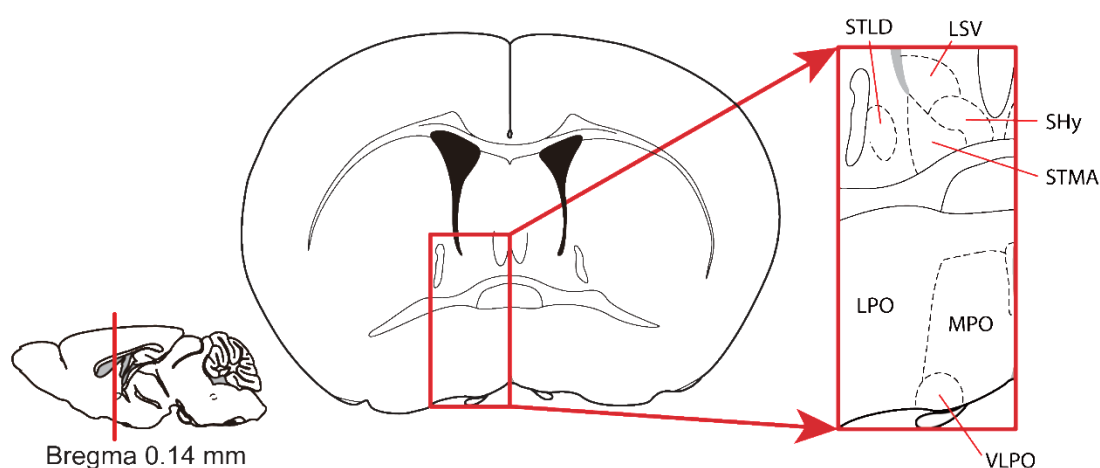


Figure 3-1 Schematic of the relevant preoptic hypothalamic and septal areas: left-hand drawing, midline-sagittal section, red line marks position of the section; middle drawing, coronal section, boxed area, magnified on the right, shows the relevant anatomical sites. Abbreviations: LPO, lateral preoptic area; LSV, lateral septum, ventral; MPO, medial preoptic area SHy, septo-hypothalamic nucleus; STLD, stria terminalis lateral dorsal; STMA, stria terminalis medial anterior; VLPO, ventral lateral preoptic area.

Previous work only described VLPO c-fos expression after dexmedetomidine injections (Lu et al., 2008). However, we found that following dexmedetomidine-induced sedation or recovery sleep after sleep deprivation that VLPO was not actually a particularly prominent area of c-fos induction: many c-fos-positive neurons were found in the wider PO area (LPO, MPO) and in a cluster of areas just dorsal of the preoptic region, in the BST (e.g. in the STMA and STLD), and in several other septal nuclei: the ventral lateral septum (LSV) and in the septo-hypothalamic nucleus (SHy) (Figs. 3.1, 3.2 and 3.3). These regions are also strongly c-fos-positive when rats were injected with the putative somnogen prostaglandin D2 (Scammell et al., 1998). So we found many c-fos-positive neurons in “unclassified” parts of the preoptic area.

After 30 minutes dexmedetomidine injection, the number of c-FOS cells reached a maximum. Then expression started to decrease to baseline levels before the sedation ended. Especially, the area surrounding the VLPO showed a high density for FOS-IR positive neurons during the first 30 minutes. The results, shown in Fig. 3.3, suggest that the entire PO hypothalamic area responds to dexmedetomidine.

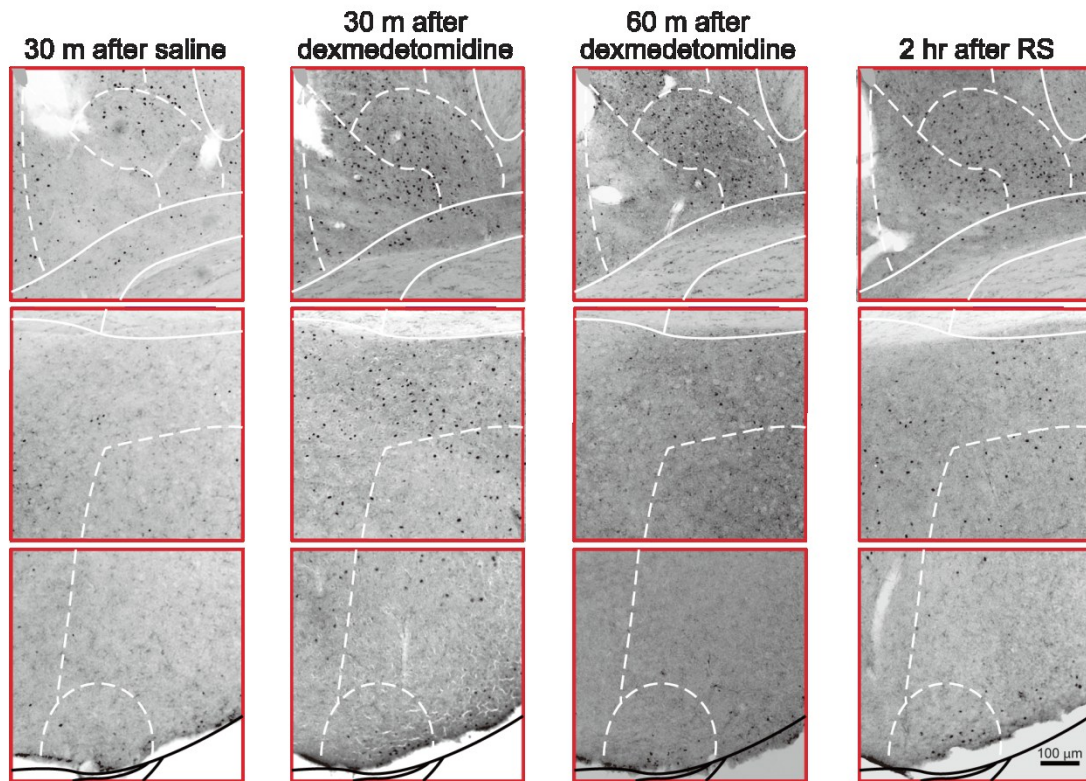


Figure 3-2 Dexmedetomidine-induced sedation and recovery sleep induced c-FOS expression in overlapping regions of the mouse hypothalamic preoptic area and septum. Photographs of nuclear c-fos protein (DAB staining method) 30 minutes after saline, 30 minutes and 1 hour after dexmedetomidine (100 µg/kg) injections or 2 hours into recovery sleep (RS).

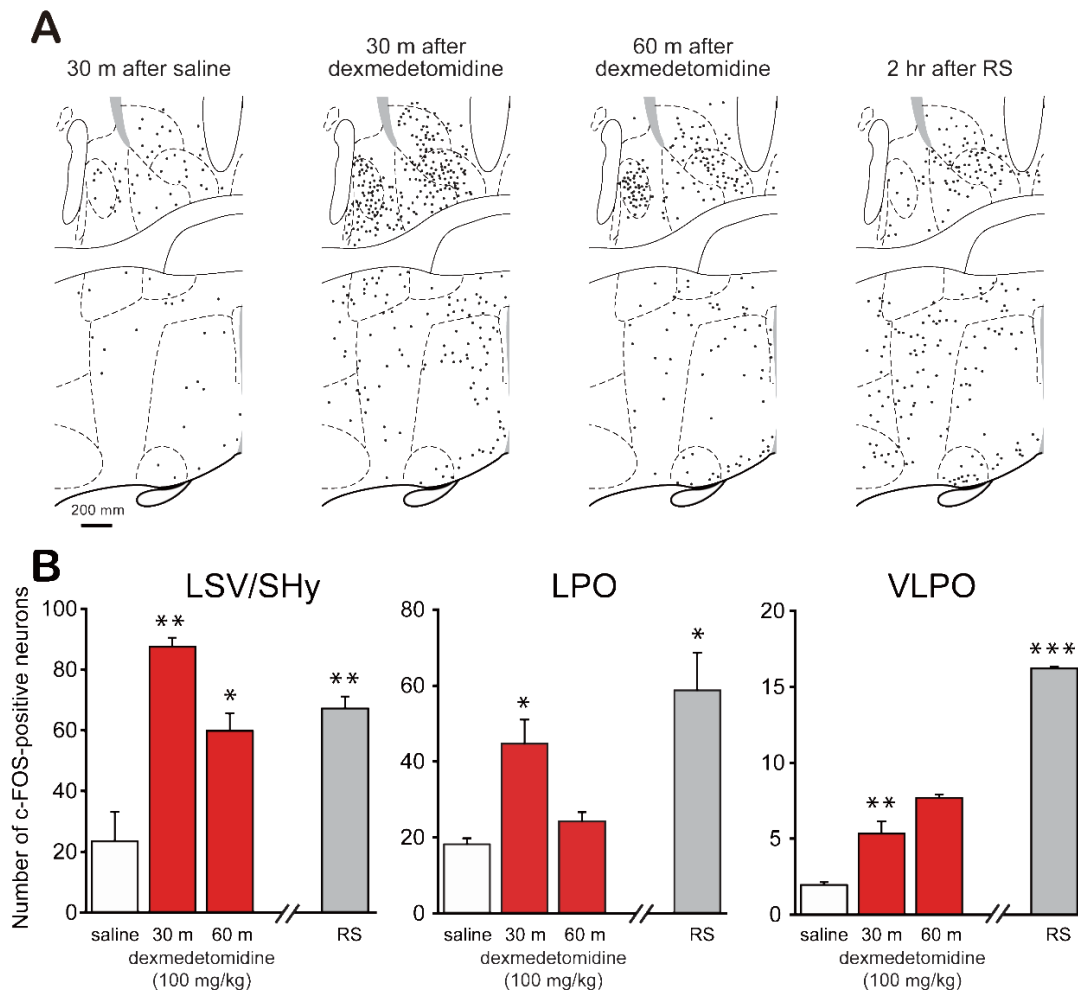


Figure 3-3 A: Line drawings of c-FOS protein expression in the boxed area at 30 minutes after saline injection and 30 or 60 minutes after dexmedetomidine (100 µg/kg) injections or 2 hours into recovery sleep after sleep deprivation; black dots represent c-FOS-positive cells; relative to its expression after a saline injection, the endogenous c-fos gene is induced widely in the area by sedative doses of dexmedetomidine or during recovery sleep. **B:** Number of c-FOS positive neurons in selected anatomical sites after saline (white) or dexmedetomidine (red) injections or recovery sleep (gray). Error bars represent the s.e.m. Asterisks represent significance relative to saline * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviations: LPO, lateral preoptic area; LSV, lateral septum, ventral; VLPO, ventral lateral preoptic area.

3.3 The majority of LPO neurons activated by dexmedetomidine are GABAergic

One hour after dexmedetomidine-induced sedation, mice were perfused and I then performed double-label immunocytochemistry for c-FOS and GAD67 (GAD1). As expected, given the high density of GABAergic neurons in the PO area, approximately 80% cFos-positive neurons in the lateral preoptic area were also labelled with GAD67 (Fig. 3.4). It suggests that a large majority of cFos-positive neurons may release GABA and the preoptic area may be recruited directly or indirectly by dexmedetomidine induced sedation.

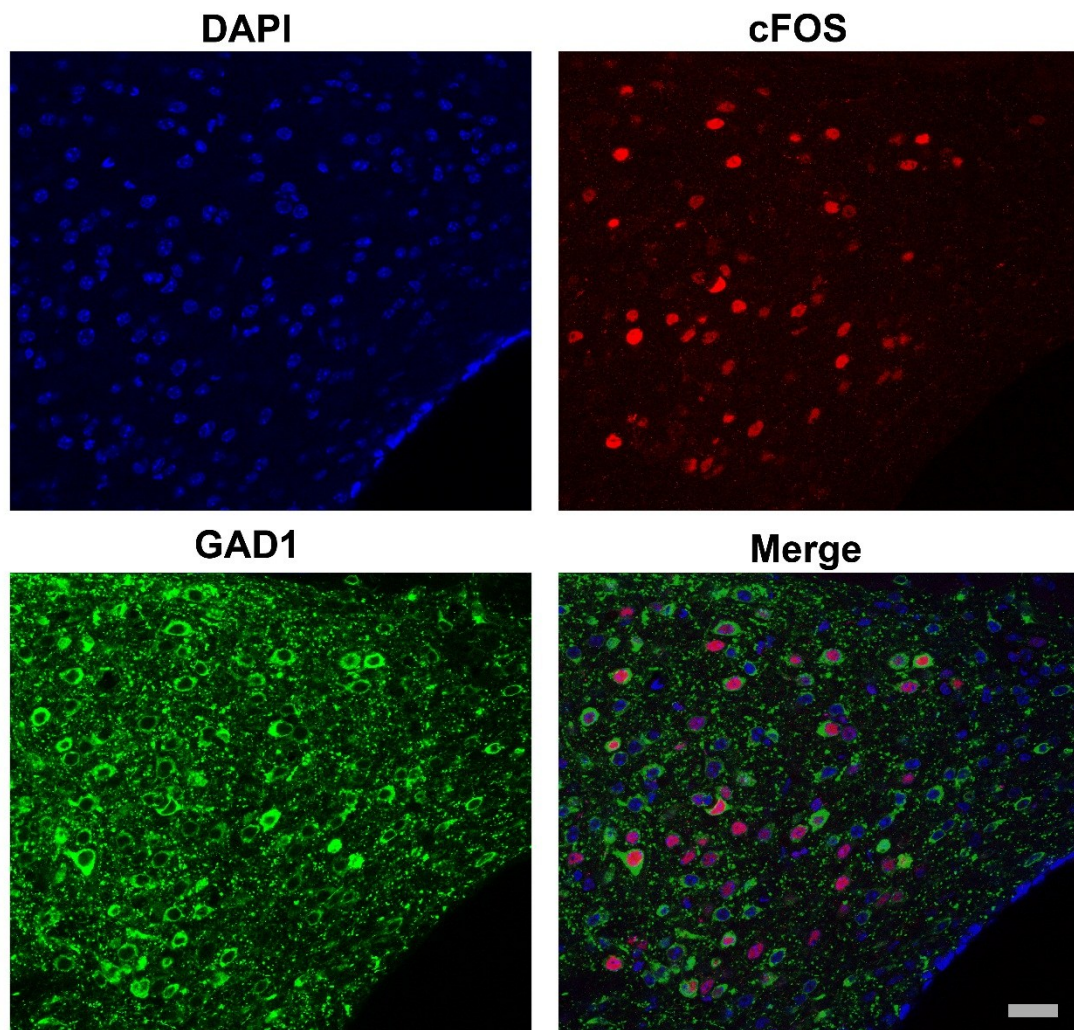


Figure 3-4 Representative images showing double labelled neurons in the ventrolateral preoptic area of mice at 1 hour after dexmedetomidine (100 $\mu\text{g/kg}$). c-Fos staining, red; glutamic acid decarboxylase 1 (GAD 1/GAD67) staining, green. Scale Bar: 40 μm

3.4 Discussion

Immediate early genes are genes whose expression is transiently induced following various electrical or chemical signals. These genes were originally discovered when quiescent fibroblasts were stimulated to proliferate, but then in the late 1980s, it was discovered that neurons also turn on these genes. A large number of immediate early genes are expressed in the brain, but one of

them, c-fos, has become a tool used to mark neural activity, although remarkably it is still not understood what precisely the c-FOS protein is doing in neurons. c-FOS expression became a useful marker because it is expressed in a low basal level in most neurons and it can rapidly be detected after a wide range of depolarizing stimuli (Hunt et al., 1987; Morgan et al., 1987; Wisden et al., 1990). Studies using c-Fos protein immunohistochemistry as a biomarker for neuronal activity have demonstrated that propofol, barbiturates, dexmedetomidine and chloral hydrate, and the volatile anaesthetics, isoflurane and halothane all increase the number of c-Fos positive neurons in the VLPO (Lu et al., 2008; Moore et al., 2012; Nelson et al., 2002; Nelson et al., 2003).

In this Chapter, using c-fos mapping I made links between recovery sleep and adrenergic sedation, as seen by similar patterns of c-fos staining in the hypothalamus. I found that the number of c-FOS-positive nuclei in the VLPO, but also LPO in general, and in the BNST during recovery sleep is more than five-fold increased compared with during wakefulness. I also found that these areas were similarly activated by a sedative dose of dexmedetomidine. Dexmedetomidine induces c-fos strongly in the mouse BNST, in particular, in the BSTLD. This region has the strongest c-fos response. Thus adrenergic α_2 receptor stimulation by dexmedetomidine must cause dis-inhibition of these neurons, either by inhibiting NA release from e.g. nucleus of the tractus solitarius (NTS) terminals coming into BSTLD, or, by *e.g.* directly inhibiting GABA interneurons in the BSTLD (α_2 receptors on terminals of GABA neurons). The BSTLD nucleus also becomes strongly c-fos-positive following application of the putative

somnogen prostaglandin D2 into the subarachnoid space anterior to the preoptic area of adult rats (Scammell et al., 1998). BNST is part of the central amygdala complex which regulates stress responses (e.g. blood pressure, heart rate), fear food intake, pain, and social behaviour (Ciriello et al., 2013). The BNST receives a strong nor-adrenergic input (Baldo et al., 2003). Projections from the preoptic area to the BNST may regulate physiological changes, such as respiration, blood pressure and body temperature. So maybe when the BSTLD is activated by dexmedetomidine, or during sleep deprivation it is responsible for the autonomic changes.

Chapter 4 The TetTag-DREADD system for recording neuronal populations activated by adrenergic $\alpha 2$ receptor-induced sedation or recovery sleep following sleep deprivation

4.1 Introduction

To look for neuronal populations whose activity would be sufficient for dexmedetomidine's actions and those involved in recovery sleep, we decided to genetically tag the actual neuronal populations activated by dexmedetomidine. Thus, the similar patterns of induced c-FOS during dexmedetomidine-induced sedation and during recovery sleep, indicated that much of the PO region (LPO and MnPO) was a suitable location for TetTag-DREADD mapping (Alexander et al., 2009; Garner et al., 2012; Reijmers and Mayford, 2009; Reijmers et al., 2007) to see if activating neurons in this location was sufficient for these behavioural states.

The hM₃D_q DREADD metabotropic receptors are uniquely activated by the ligand CNO (Armbruster et al., 2007). These receptors couple via Gq proteins to produce potassium channel closure and release of internal Ca²⁺. By combining this receptor with TetTagging, the hM₃D_q DREADD receptor gene is only turned on following periods of intense neural activity, and so records, or "tags" ensembles of neurons that have been activated *in vivo* by a stimulus (Garner et al., 2012).

The Tet-Tagging system has mainly used classical transgenic mice (Garner et al., 2012; Liu et al., 2012a). However, I set up the same system using AAV genomes that would allow more flexibility to selectively target small brain regions. Because of size constraints of the AAV genome, which can only be packaged if it is less than about 4.7 kb, I generated two AAV viruses (Figure 2.1, 2.2, 4.1), one which contained the *cfos-promoter-tet activator* transgene, and the other which contained the tet-operator promoter (P_{TRE-tight}) linked to an hM₃D_q-mCHERRY- receptor reading frame. Constructs of AAV-cFos-tTA and AAV-P_{TRE-tight}-hM₃D_q-mCherry were made followed the protocols (see in the Materials and Methods). Ilke Guntan, a master's student in the lab, helped me with some of the molecular biology steps, under my supervision. All results in this Chapter have been published in a more condensed format in (Zhang et al., 2015).

4.2 Two AAV viruses can co-transduce neurons *in vivo*

I first constructed the c-fos-tTA and P_{TRE-tight}-hM₃D_q-mCherry genes in AAV vector (See in Fig 2.1&2.2). After constructing the dual AAV TetTag system (Fig. 4.1A), I needed to check if two AAVs could independently co-transduce neurons. For ease of study, I tested this on two large brain regions, the caudate-putamen (Cpu) and the hippocampus. After AAV-cfos-tTA and AAV-P_{TRE-tight}-hM₃D_q-mCherry co-injection into the Cpu and hippocampus, mice were placed on a doxycycline-free diet for four weeks so that under chronic activation of the c-fos promoter, the resulting

accumulating tTA protein could freely activate the tetO-dependent hM3Dq-mCherry transgene.

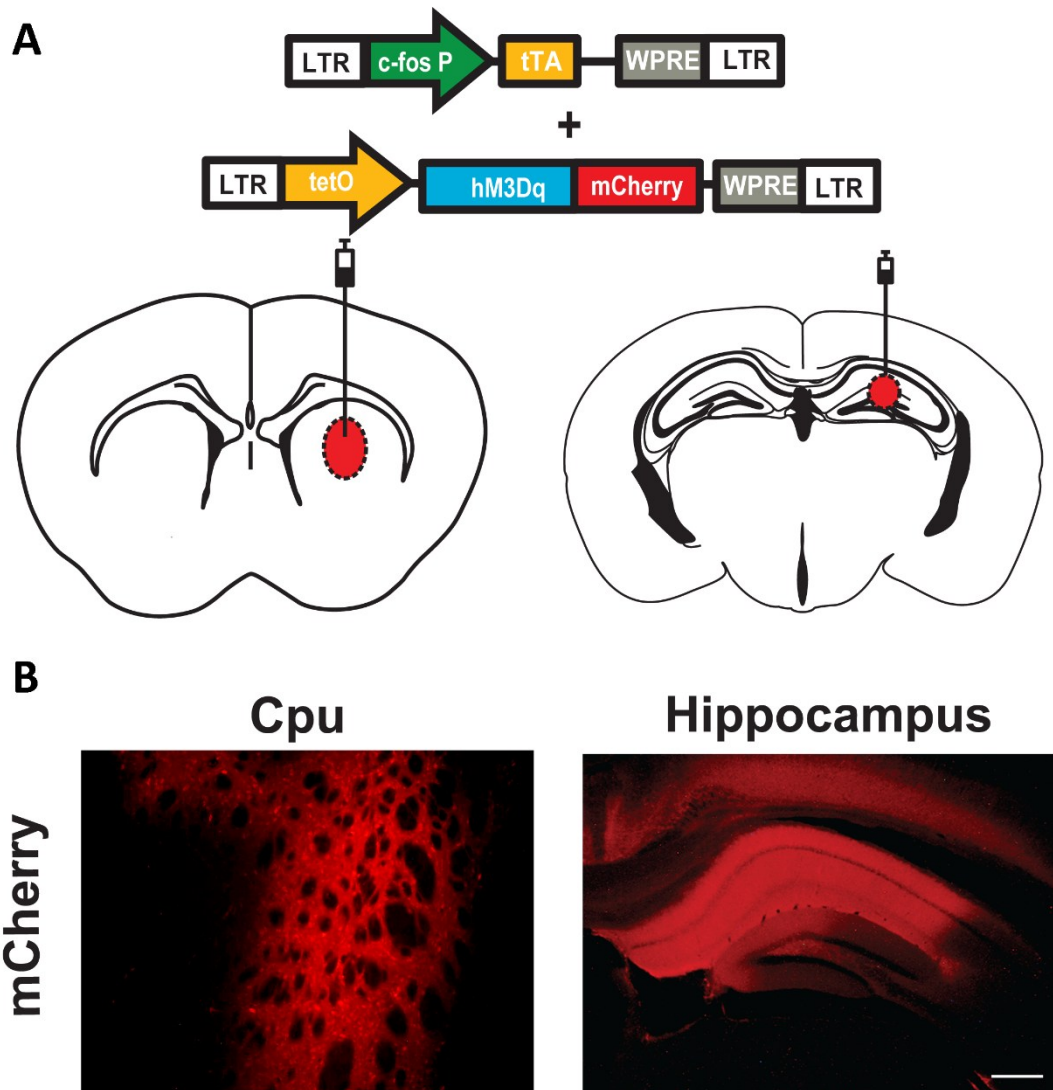


Figure 4-1 A: Schematic of AAV-cfos-tTA and AAV-P_{TRE-tight}-hM3Dq-mCherry co-injection into caudate putamen (Cpu) and hippocampus of C57bl6 mice. B: Immunostaining for virus-positive cells after stereotaxic bilateral injection of AAV-cfos-tTA and AAV-P_{TRE-tight}-hM3Dq-mCherry co-injection into caudate putamen (Cpu) and hippocampus in a coronal section. mCherry-positive cells can only be generated by double transduction with two AAVs. Scale bar 200µm

By using the mCherry as a marker of hM3Dq expression, I found that neurons could be co-transduced with the two AAV viruses with high efficiency (Fig. 4.1B).

4.3 Setting up the TetTag DREADD system in LPO and MnPO

Following on, from the previous Chapter showing endogenous c-FOS induction during recovery sleep or after dexmedetomidine-induced sedation, I wanted to test the c-fos-based TetTag system in the LPO and MnPO hypothalamic areas to test the sufficiency of re-activating these neurons following sleep deprivation or dexmedetomidine-induced sedation to produce sleep. I co-injected both the *AAV-cfos-promoter-tet activator* and *AAV-P_{TRE-tight}-hM₃D_q-mCHERRY* viruses bilaterally into the mouse LPO hypothalamus (“LPO-TetTag-hM3Dq” mice) and median preoptic (MnPO) hypothalamus (“MnPO-TetTag-hM3Dq” mice). In the virally injected animals, I looked at activity inducible hM3Dq-mCHERRY patterns in the LPO and MnPO (Figures 4.2 and 4.3). After the injections, I first maintained the mice for 4 weeks on a doxycycline diet to repress the effects of the cfos-promoter-tet activator transgene and thus keep background P_{TRE-tight}-mCHERRY-hM3D_q receptor gene expression to a minimum. After the first month, the mice were then given a doxycycline free diet for 48 hours. Then I looked at TetTag gene expression in these areas before and after mice were given the sedative dose of dexmedetomidine (100 µg/kg) or sleep deprived and then killed during recovery sleep (Fig 4.2).

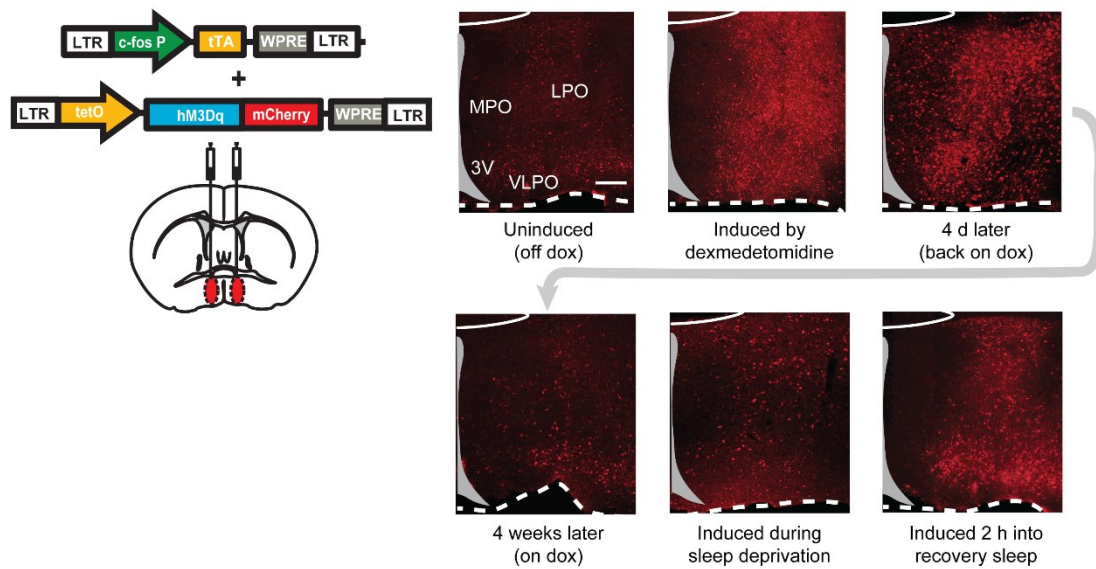


Figure 4-2 Time course of $P_{TRE-tight}$ -hM3Dq-mCHERRY transgene induction and decay for LPO-TetTag-hM3Dq mice. The images show coronal sections from one side of the brain stained for hM3Dq-mCHERRY expression (red), detected with mCHERRY antisera. The images were taken from animals killed at six time points: with doxycycline removed from the diet two days previously, just before dexmedetomidine-induced sedation; 2 hours after a sedative dose of dexmedetomidine; 4 days later on and back on doxycycline, 4 weeks after dexmedetomidine-induced sedation on a doxycycline diet; 4 hours after sleep deprivation; and 2 hours into recovery sleep following sleep deprivation. Induced hM3Dq-mCHERRY transgene expression was seen throughout the LPO area. Scale bar, 200 μ m.

LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq brains were taken 2 hours before, or 2 hours after animals received dexmedetomidine or a control saline injection, or 2 hours into recovery sleep. They were analysed for mCHERRY expression (Fig. 4.2 & Fig. 4.3). Before dexmedetomidine injection, but with no doxycycline present, there was a low but detectable level of $P_{TRE-tight}$ -hM3Dq-

mCHERRY transgene expression in scattered cells throughout the injected area in both the LPO-TetTag-hM3Dq mice and the MnPO-TetTag-hM3Dq mice (Fig. 4.2 and Fig. 4.3). This basal transgene expression was also present with doxycycline. In LPO-TetTag-hM3Dq brains, and in agreement with the induction of the endogenous c-fos gene (see previous Chapter), a wide area expressed hM3Dq-mCHERRY receptors following systemic dexmedetomidine administration, stretching from the bed nucleus stria terminalis/lateral septum and septal hypothalamic nuclei in the dorsal part of the region, the whole LPO area and through to the VLPO and extended VLPO area at the base (Fig. 4.2).

For recovery sleep, a similar LPO-P_{TRE-tight}-hM3Dq-mCHERRY transgene expression pattern was found; this expression started to already appear during the sleep deprivation period, and had become stronger two hours into recovery sleep (Fig. 4.2). For the MnPO-TetTag-hM3Dq animals, recovery sleep and dexmedetomidine-induced sedation both induced hM3Dq-mCHERRY transgene expression above basal levels in the MnPO area; however, there was a differential effect, because the gene induction was weaker following dexmedetomidine-induced sedation compared with that during recovery sleep (Fig. 4.3).

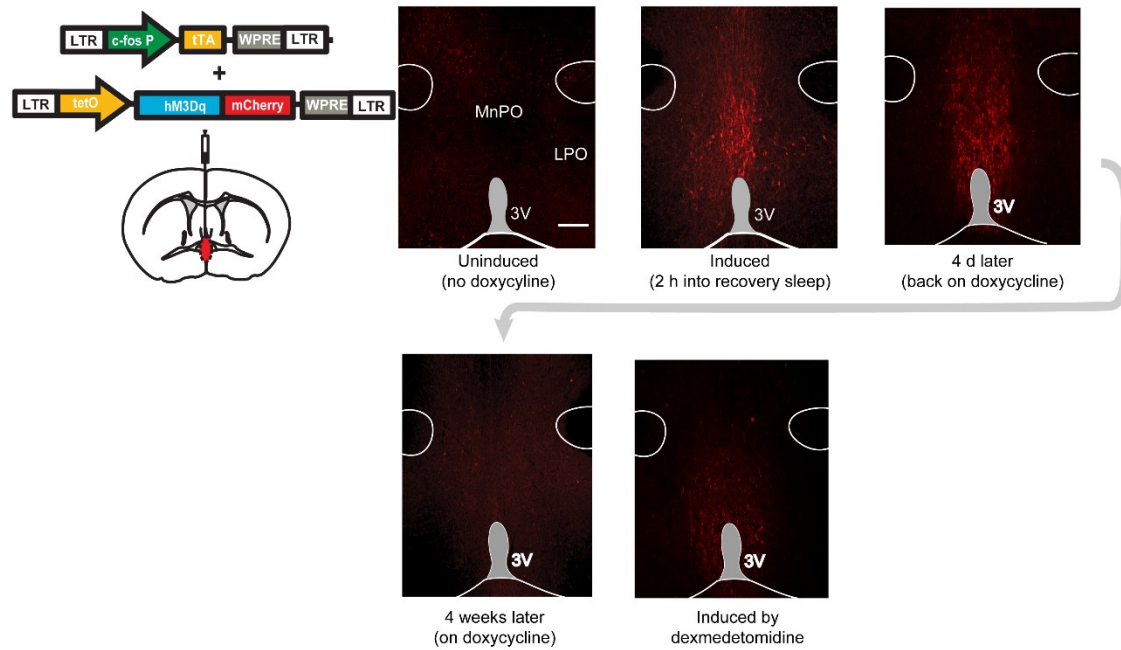


Figure 4-3 Induction of hM3Dq–mCHERRY transgene during recovery sleep in MnPO–TetTag–hM3Dq mice. The TetTag AAVs were injected at the midline into the MnPO area. The photographs show coronal sections stained for hM3Dq–mCHERRY expression (red). The left-hand photograph in the top row shows basal transgene expression with no doxycycline; the middle picture shows induced hM3Dq–mCHERRY transgene expression in the MnPO area 2 hours into recovery sleep; the third figure shows expression 4 days later with the mice back on doxycycline. The images below show the low expression 4 weeks later and the figure to the right shows the relatively low hM3Dq–mCHERRY transgene induction following dexmedetomidine sedation. Scale bar, 50 μ m; LPO, lateral preoptic area; MnPO, median preoptic area; 3V, third ventricle.

In separate experiments I determined that levels of the induced hM3Dq-mCHERRY receptor protein persisted for at least 4 days post-stimulus (e.g. dexmedetomidine-induced sedation or recovery sleep after sleep deprivation followed immediately by doxycycline in the diet – Fig. 4.2 and Fig. 4.3), but that during the 4-week

period following the first challenge, sleep-deprivation or dexmedetomidine-induced sedation, levels TetTag-hM3Dq-mCHERRY transgene expression had fallen back to baseline levels (Fig. 4.2 and Fig. 4.3).

4.4 CNO excites TetTagged neurons in the preoptic area

I tested whether CNO activated TetTagged neurons in the preoptic area *in vivo* by using c-Fos expression as the indicator of neuronal activity. LPO-TetTag-hM3Dq mice, which had previously been sedated with dexmedetomidine to induce transgene expression, were injected with CNO several days later and then culled 2 hours later for immunohistological analysis of c-Fos expression. CNO induced c-Fos signals in almost mCherry positive neurons in LPO (Figure 4.4A), suggesting that CNO activation of hM3Dq receptors elicits selective neuronal activation.

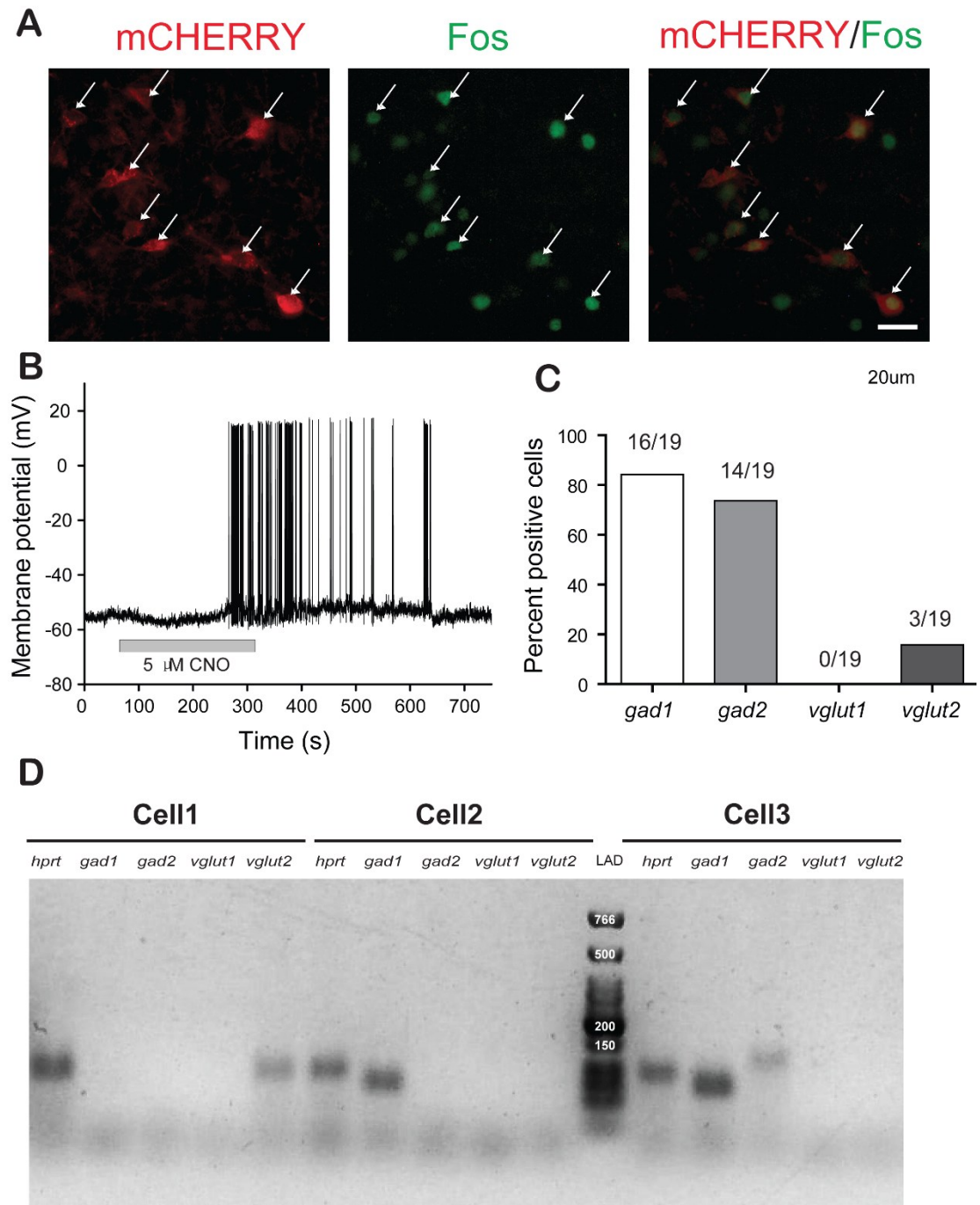


Figure 4-4 TetTagged neurons are excited by CNO and are predominantly GABAergic in LPO-TetTag-hM3Dq mice. (A) CNO induces nuclear *c-fos* expression (green) in hM3Dq-mCHERRY (red) expressing neurons in the LPO area of LPO-TetTag-hM3Dq mice. Double-label immunocytochemistry with antisera to mCHERRY and *c-fos*. The hM3Dq-mCHERRY expression was induced by a sedative dose (100 μ g/kg) of dexmedetomidine and then mice were injected with CNO and their brains taken 2 hours

afterwards. Arrows indicate examples of co-labelled cells. Scale bar: 20 μ m (B) A representative example of the effects of 5 μ M CNO on a mCHERRY-positive neuron. On average, neurons (n=8; 3 mice) were depolarized by 10.2 ± 2.1 mV. In the example shown, action potential firing was triggered. (Data provided by Zhiwen Ye) (C) These neurons were predominantly GABAergic. 84% were gad1 and/or gad2 positive, as assayed by single-cell qPCR. The remainder were glutamatergic (vglut2-positive). (D) Representative examples of the qPCR assay run out on a gel from three of the neurons. LAD, sizes shown is base pairs.

To confirm this directly, I repeated the experiments on a fresh batch of mice, except this time another graduate student in the lab, Zhiwen Ye, patch-clamped visually identified (mCHERRY-positive, primary fluorescence) Tet-Tagged neurons found in acute PO slices from LPO-TetTag-hM3Dq mice after dexmedetomidine-induced sedation. In neurons that were TetTagged, he found that CNO induced an excitatory response (8 out of 8 neurons in 3 animals), and could trigger action potential firing (Figure 4.4B). The average depolarization was 10.2 ± 2.1 mV in response to 5 μ M CNO. As expected, these results are consistent with the hM3Dq receptor coupling to excitatory mechanisms (Alexander et al., 2009). After recording from the TetTagged neurons, Zhiwen Ye sucked out the cytoplasm into the recording pipette, and I used single-cell qPCR on RNA/cDNA from individual cells (see 2.7 , Methods) to determine their phenotype: 84% were GABAergic (gad1 and/or gad2 expression); the remaining were glutamatergic (vglut2 expression) (Figure 4.4C & D).

4.5 Testing the sufficiency of TetTagged neurons in LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice for their ability to recapitulate recovery sleep after sleep deprivation and/or dexmedetomidine-induced sedation.

After confirming the inducibility, rough expression kinetics and the excitatory nature of the dual TetTag system, I performed experiments to test if neurons activated by dexmedetomidine-induced sedation or sleep deprivation following recovery sleep were sufficient to generate sleep. AAV-c-fos-tTA and AAV-P_{TRE-tight}-hM3Dq-mCherry were bilaterally co-injected into the LPO area in the mice brains. The experimental schemes are outlined in Fig 4.5, and the broad outline of the protocol is as follows: I set up two groups of mice as before: LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice. Mice were also implanted with electroencephalographic (EEG) and electromyographic (EMG) electrodes. I maintained the mice for 4 weeks on a doxycycline diet. After four weeks, doxycycline was then removed from the diet to allow potential inducibility of the hM3Dq gene, and 2 days later mice were given nearly five hours of sleep deprivation by presenting them with novel objects every hour, and then allowed recovery sleep and placed back on the doxycycline diet. Mice were injected with CNO (5mg/kg) three or four days later, and their EEG and motor activity recorded. Mice were then maintained for about a month on doxycycline, during which time the hM3Dq-mCherry receptor levels had returned to baseline. Doxycycline was removed, and two days later, mice were given a sedative dose of dexmedetomidine, their EEG and motor activity recorded, and placed back on doxycycline; three or four days later they were

given CNO. I now give the details of the results using this extended behavioural protocol.

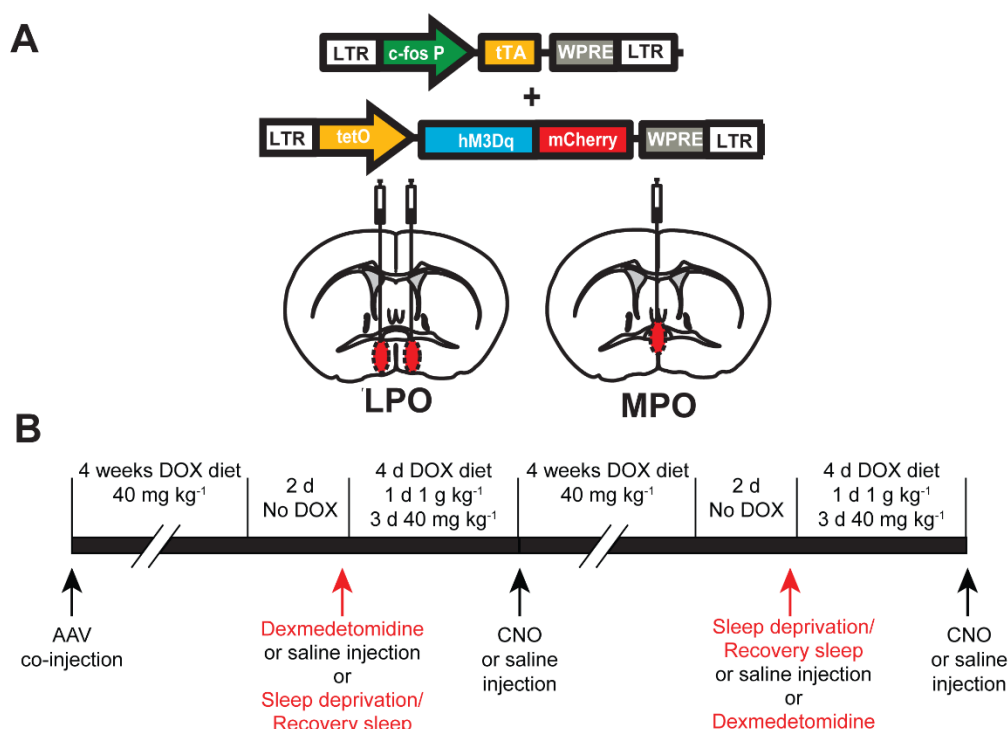


Figure 4-5 A, The AAV-c-fos-tTA was injected with AAV-P_{TRE}-tight-hM3Dq-mCherry into lateral preoptic area (LPO-TetTag-hM3Dq mice) and MnPO (MnPO-TetTag-hM3Dq mice). **B**, Experimental protocol. After injection, mice were habituated with doxycycline food. When off Dox 48 hours, after sleep deprivation, recovery sleep induces c-fos expression in the preoptic area. c-fos promoter drive the expression of tTA, which binds to TRE and drive the expression of hM3Dq-mCherry, labelling a subpopulation of activated cells in the preoptic area. Then mice were put back on DOX and tested 4 days later. Intraperitoneal injection of CNO (5mg/kg) to activate the cells labelled with hM3Dq/mcherry. Then mice were habituated with doxycycline food for further four weeks. When off Dox 48 hours, mice received dexmedetomidine injection and back on Dox. 4 days later, mice were intraperitoneal injected with CNO (5mg/kg) and behaviour assessed.

4.6 Sleep deprivation treatment

The sleep deprivation began at the onset of the “lights on” phase. Fig 4.6 shows that the proportion of NREM sleep in the first four hours is almost none. At the end of sleep deprivation, mice went into NREM sleep quickly. Similar results were obtained with both LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice.

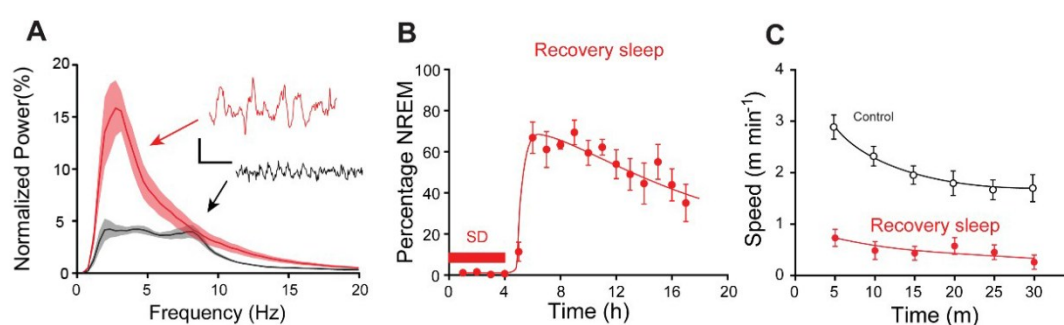


Figure 4-6 Sleep recording and locomotor activity after sleep deprivation of LPO-TetTag-hM3Dq mice. A: Fourier transform power spectra when the EEG/EMG signals were scored as either sleep (red) or wake (black) during recovery sleep. B: Percentage of time spent in NREM sleep per hour during sleep deprivation. During the first 5 hours, mice were sleep deprived. Then after 5 hours, mice went sleep quickly in their home cage. C: Speed in an open field 30 minutes after sleep deprivation. Error bars represent SEM. Two-way ANOVA ($F(1, 76) = 12.32, p < 0.0001$) with post-hoc Bonferroni t-test indicated a significant difference between animals received RS before and controls. 4.7 Specific re-activation of TetTagged LPO and BNST nuclei after sleep deprivation re-induces NREM sleep

After recovering from sleep deprivation, 4 days later, LPO-TetTag-hM3Dq mice were injected with CNO (5mg/kg *i.p.*) during the dark phase and their locomotor activity in the open field was analysed

(Fig. 4.7). Activity was measured 30 minutes post CNO injection. The control group went through the whole experimental protocol (Fig 4.5) but were allowed normal sleep instead of having sleep deprivation.

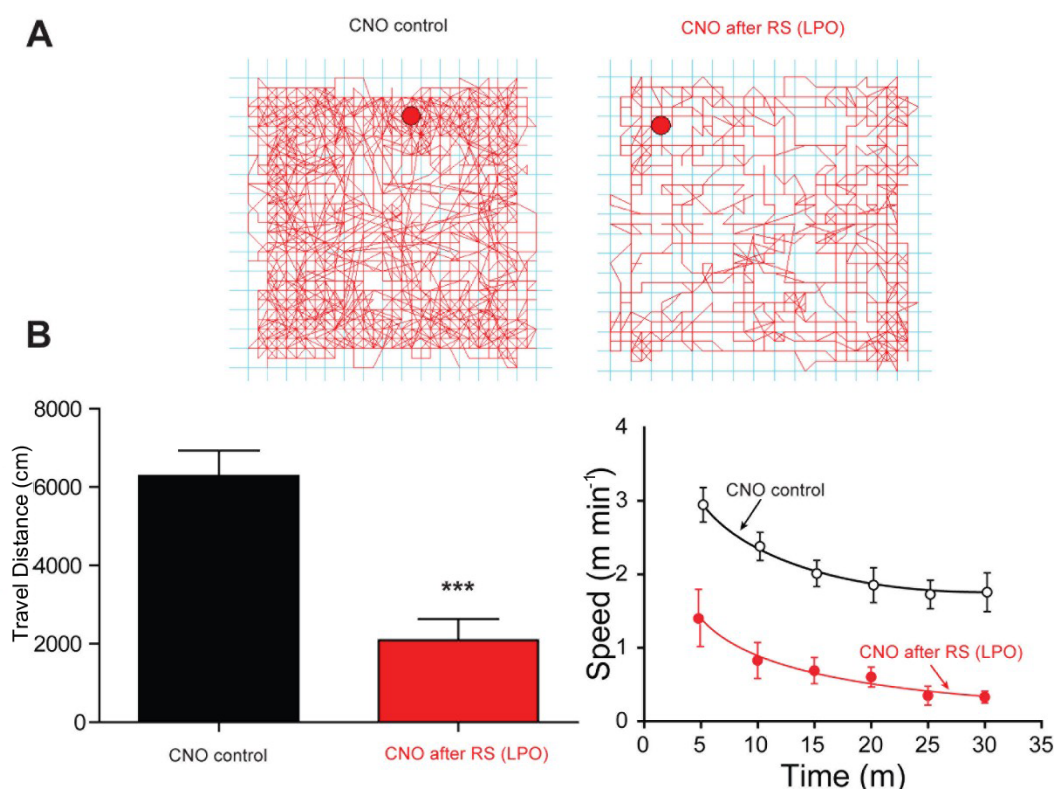


Figure 4-7 In LPO-TetTag-hM3Dq mice, CNO induces a cessation of movement/reduced movement in the sleep deprivation (SD) and recovery sleep (RS) group and does not affect the locomotor activity in the normal sleep mice. **A:** Representative 30-min locomotor tracks of a normal sleep-CNO mouse and a SD/RS-CNO mouse in response to injection of CNO. **B:** The figure on the left showed that CNO reduced the total travel distance of the RS-CNO mice (unpaired tTest, $P < 0.0001$ $n=7$). The figure on the right shows the travel distance every five minutes. After 30 minutes of CNO injection, mice barely moved even in the novel open field ($p < 0.0001$). Error bars represent SEM. Two-way ANOVA ($F(1, 84) = 117.8$, $p < 0.0001$) with post-hoc Bonferroni t-test indicated a significant difference between animals that received RS before and

controls. The ANOVA compared the time ($F_{5, 84} = 7.657$, $P < 0.0001$) and interaction between these two factors ($F_{5, 84} = 0.1512$, $P = 0.98$). Asterisks indicate significance ($p < 0.0001$). RS: recovery sleep

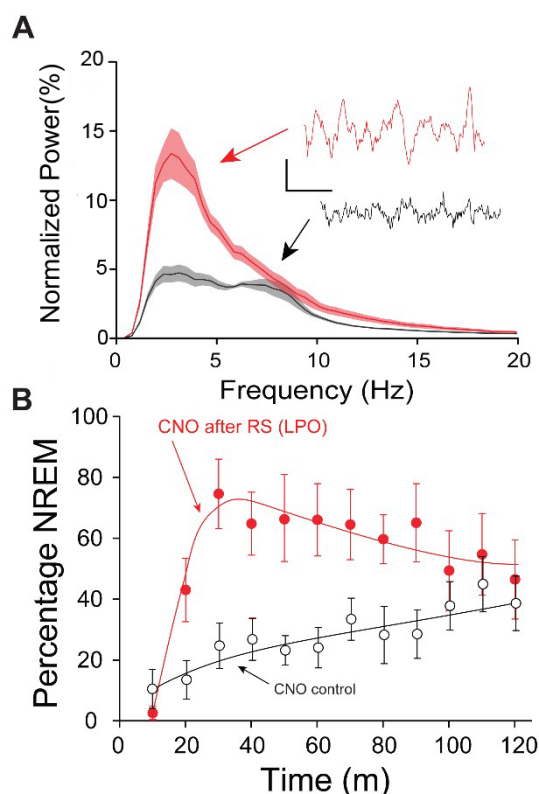


Figure 4-8 In LPO-TetTag-hM3Dq mice, CNO induces NREM sleep in the sleep deprivation group and does not affect sleep pattern in the control group. **A:** Fourier transform power spectra when the EEG/EMG signals were scored as either sleep (red) or wake (black) during CNO activation. **B:** Percentage of time spent in NREM sleep after CNO injection in the mice home cages. Group data showing that CNO induced NREM sleep of the sleep-deprivation-CNO mice ($n=7$). During the two hours after CNO injection, the RS-CNO mice spent double time in NREM sleep compare to the CNO control mice. Error bars represent SEM. Two-way ANOVA ($F(1, 180) = 41.91$, $p < 0.0001$) with post-hoc Bonferroni t-test indicated a significant difference between animals that received RS before and controls from 20mins to 70mins after CNO injection. The ANOVA compared

the time ($F_{1, 180} = 2.975$, $P < 0.0001$) and interaction between these two factors ($F_{11, 180} = 2.492$, $P = 0.0062$).

Giving CNO to LPO-TetTag-hM3Dq mice which previously had received sleep deprivation made them sedated and move rather little in the open field. A more than three-fold decrease in movement was induced thirty minutes after CNO injection (Fig. 4.7) and LPO-TetTAG-hM3Dq mice went into sustained NREM sleep (Fig. 4.8B).

The proportion of time spent in NREM sleep was significantly different in control LPO-TetTag-hM3Dq mice (see Fig4.8B). Normally, the duration of a single NREM period is about 4-5 minutes in mice. However, the LPO-TetTag-hM3Dq mice after CNO injection could have 20 minutes NREM duration. By contrast, giving CNO to LPO-TetTAG-hM3Dq mice that had previously had only normal sleep did not induce significant behavioural change or NREM sleep (Fig. 4.7& 4.8).

4.8 Specific re-activation of TetTagged LPO and BNST nuclei after a sedative injection of dexmedetomidine re-induces NREM sleep

After the sleep deprivation and CNO reactivation experiments, I then maintained the same groups of LPO-TetTag-hM3Dq mice on doxycycline for a further four weeks to allow any dexmedetomidine-induced CNO receptors to decay to basal levels. After four weeks, following the experiment protocol explained in Fig 4.5, mice were then given an injection of a sedative dose of dexmedetomidine (100 $\mu\text{g/kg}$) (Fig. 4.9).

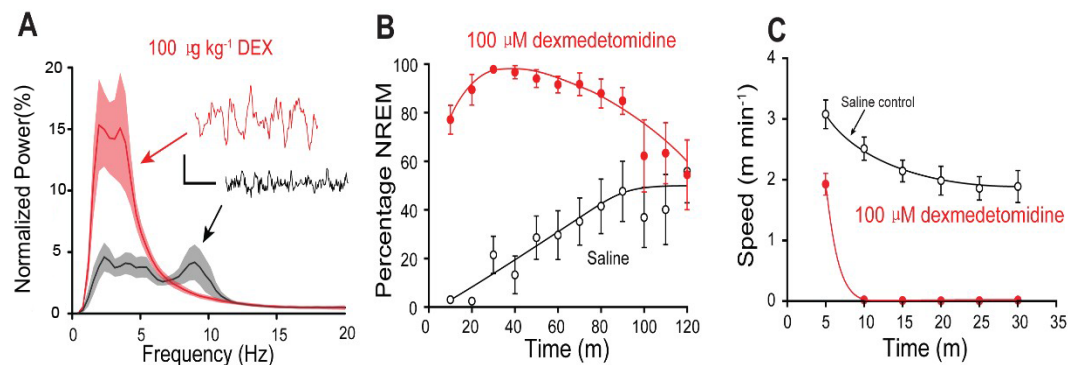


Figure 4-9 EEG recording and locomotor activity after dexmedetomidine-induced sedation of LPO-TetTag-hM3Dq mice. A: Fourier transform power spectra when the EEG/EMG signals were scored as either sleep (red) or wake (black) during dexmedetomidine-induced sedation. **B:** Percentage of time spent in NREM sleep per ten minutes after dexmedetomidine injection into LPO-TetTag-hM3Dq mice. Compared to the saline injection, mice with DEX injection sedated within ten minutes. Then after nearly 90 minutes, mice recovered. Two-way ANOVA ($F(1, 40) = 79.86$, $p < 0.0001$) with post-hoc Bonferroni t-test indicated a significant difference between animals that had received dexmedetomidine injection and controls from 10 mins to 90 mins after dexmedetomidine injection. Error bars represent SEM. **C:** Group data showing that DEX reduced the travel distance ($n=7$). After five minutes of DEX injection, mice stopped moving in the open field. Two-way ANOVA ($F(1, 84) = 21.21$, $p < 0.0001$) with post-hoc Bonferroni t-test indicated a significant difference between animals that had received dexmedetomidine injection compared with saline-injected controls.

Fig 4.9C shows that the LPO-TetTag-hM3Dq mice stopped moving 5 minutes after dexmedetomidine injection. Approximately five minutes after dexmedetomidine injection, the EEG of LPO-TetTag-

hM3Dq mice exhibited prominent and sustained NREM which lasted ~90 minutes relative to the mice given only saline (Fig. 4.9A, B). Mice were then put back on the doxycycline diet for 4 days to repress the induction of further TetTag-hM3Dq receptors, and after this injected *i.p.* with CNO or saline and their EEG and behavioural responses recorded. In the locomotor behaviour test, activity was measured 30 minutes after CNO *i.p.* injection. Their locomotion in an open field was strongly reduced.

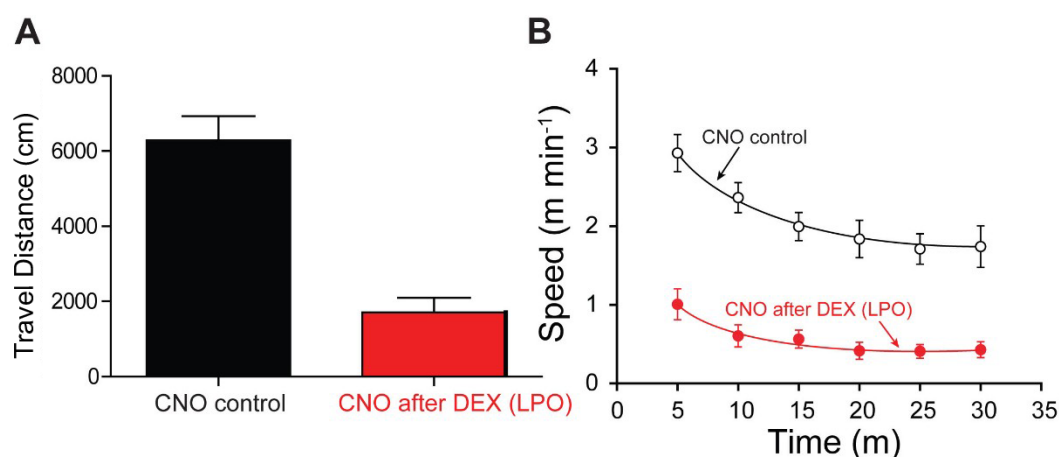


Figure 4-10 In LPO-TetTag-hM3Dq mice, CNO induces sedation mice previously injected with dexmedetomidine and does not affect the locomotor activity in the saline-injected mice. **A:** Group data showing that CNO reduced the total travel distance of the CNO-after DEX mice (unpaired tTest $P < 0.0001$ $n=7$). **B:** The software collected the travel distance every five minutes. After 30 minutes of CNO injection, mice barely moved even in the novel open field. Error bars represent SEM. Two-way ANOVA ($F(1, 65) = 41.05$, $p < 0.0001$) with post-hoc Bonferroni t-test indicated a significant difference between animals that had received dexmedetomidine before and controls. The ANOVA compared the time ($F(5, 65) = 7.996$, $P < 0.0001$) and interaction between these two factors ($F(5, 65) = 1.078$, $P = 0.3791$). $p < 0.0005$

After CNO injection, within the first hour, the total time in NREM sleep of LPO-TetTag-hM3Dq mice was significantly increased compared with the control mice (Fig. 4.11). These results indicated that activation of these groups of neuronal activity resulted in increased time spent in NREM sleep accompanied by a decrease in time spent in Wake time.

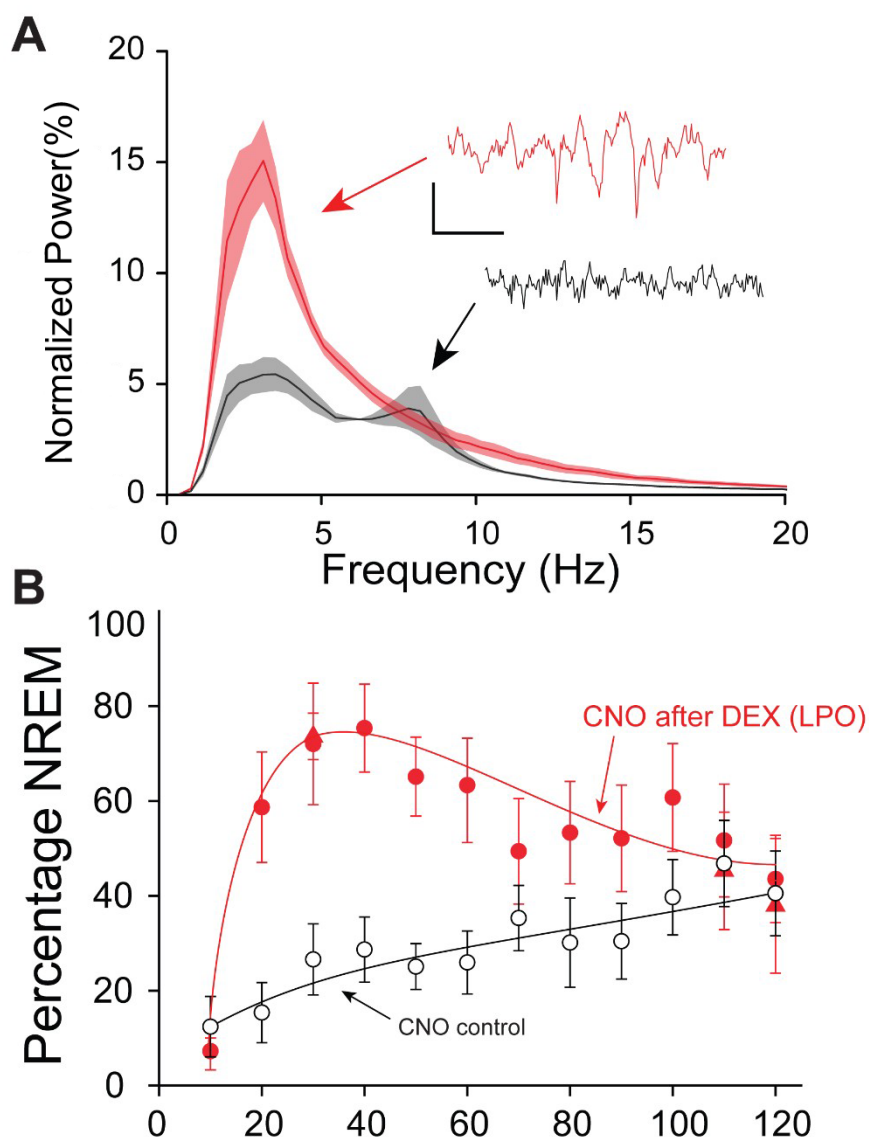


Figure 4-11 In LPO-TetTag-hM3Dq mice CNO induces NREM sleep in the dexmedetomidine-injected mice and does not affect sleep pattern in the saline-injection mice. **A:** Fourier transform power spectra when the EEG/EMG signals were scored as either sleep (red) or wake (black) after CNO injection. **B:** Percentage of time

spent in NREM sleep after CNO injection in the home cages. Group data showing that CNO induced NREM sleep of the DEX-CNO mice (n=7). During the two hours after CNO injection, the DEX-CNO mice spent more than double time in NREM sleep compare to the Saline-CNO mice. Error bars represent SEM. Two-way ANOVA ($F(1, 168) = 45.899, p < 0.0001$) with post-hoc Bonferroni t-test indicated a significant difference between animals received that had DEX before and controls from 20mins to 60mins after CNO injection. The ANOVA compared the time ($F(1, 168) = 2.836, P = 0.002$) and interaction between these two factors ($F(11, 168) = 2.721, P = 0.0029$).

Thus dexmedetomidine seems to induce sedation by selectively activating similar ensembles of neurons in the preoptic hypothalamus that are activated by sleep deprivation.

4.9 Summary of TetTag transgene expression patterns in LPO-TetTag-hM3Dq mice

Some LPO-TetTag-hM3Dq mice that had been sedated with dexmedetomidine did not show any subsequent CNO-induced behaviour at either the EEG or behavioural levels relative to that observed with saline injection. I was interested to know where and if the TetTagging had worked in these animals, and in addition I wanted to confirm the correct injections in the other mice. Thus after completion of the behavioural testing, I re-induced the TetTag expression by giving the animals another injection of dexmedetomidine and then two hours later killed the mice for histological analysis. As previously described in the first part of this Chapter, I found a wide area of the lateral preoptic hypothalamic region expressed hM3Dq-mCherry receptors

following systemic dexmedetomidine administration, stretching from BNST in the dorsal part of the region through to the VLPO area (Fig. 4.12). The boxed sections in animals I-L (Fig. 4.12) were mice where hM3Dq-mCHERRY transgene was induced but there was no behavioural or EEG signs of sedation.

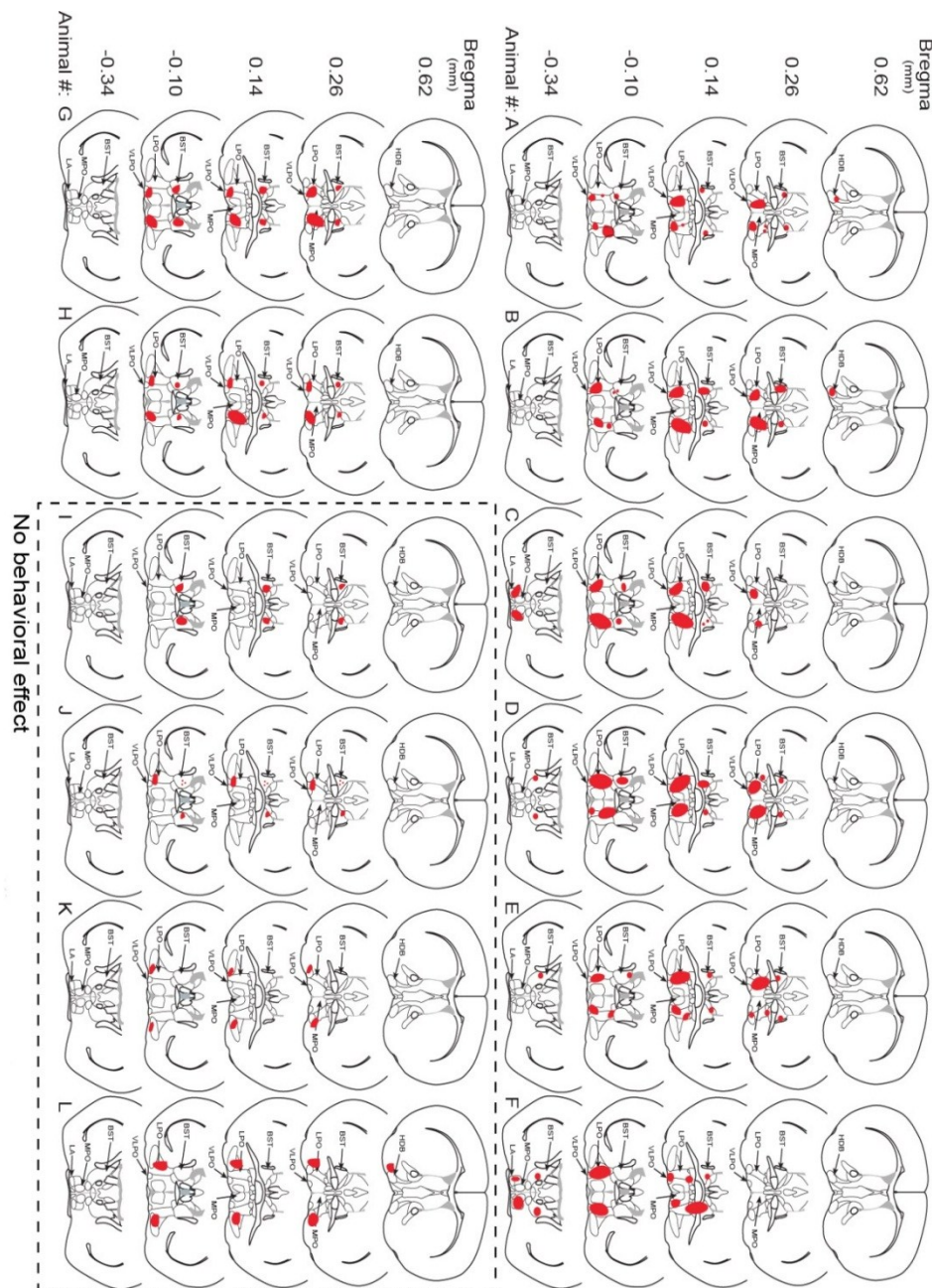


Figure 4-12 LPO TetTag-hM3Dq-mCherry expression patterns: Induction of hM3Dq-mCHERRY expression 2 hours following dexmedetomidine-induced sedation where AAVs were bilaterally

injected into the LPO and surrounding areas. Each letter, A through to L, summarizes the expression in individual mice as seen by serial coronal sectioning through the AAV injection sites. Red indicates hM3Dq-mCHERRY expression. Abbreviations: BST, bed nucleus stria terminalis; LPO, lateral preoptic area; MPO, medial preoptic area; VLPO, ventral lateral preoptic area.

Although the hM3Dq-mCHERRY gene was clearly induced by dexmedetomidine treatment in these animals, the transgene expression sites were on the lateral margin of the LPO area or even further out laterally (Fig. 4.12; animals I, J, K and L). Thus activating these lateral TetTagged neurons with CNO was not sufficient to induce sleep. One animal, (mouse I), had induced hM3Dq receptor only in the BNST areas, but also exhibited no CNO-induced behaviour, so BNST stimulation alone was not sufficient to recapitulate dexmedetomidine-induced sedation.

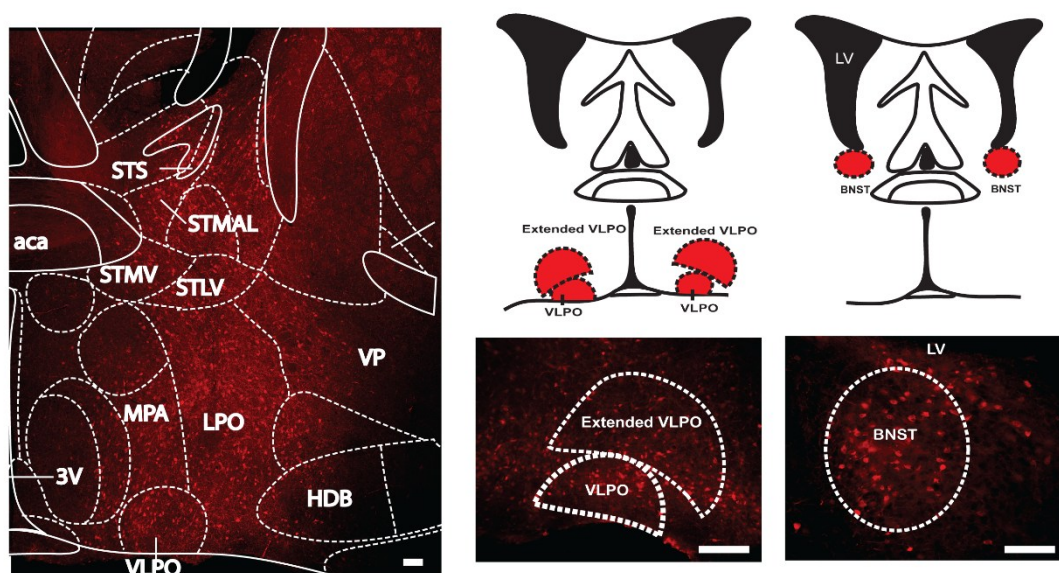


Figure 4-13 Representative coronal sections throughout the anteroposterior axis in the bilaterally common area of LPO-TetTag-hM3Dq mice. The common injection sites in clustered BNST and VLPO around the injection site. Both Scale bar: 100µm Abbreviations: 3V: 3rd ventricle; aca: anterior commissure, ant;

BNST: Bed nucleus of the stria terminalis; HDB: horizlimb diagonal band; LPO, lateral preoptic area; LSV, lateral septum, ventral; LV: lateral ventricle MPA: medial preoptic area; STLV: ben nu st lat div ventral; STMAL, stria terminalis medial anterior; STMV: bed nu st, med, div, ventral; VLPO, ventral lateral preoptic area; VP: ventral pallidum.

Of the animals that did responds to CNO injections with sleep, a typical TetTag transgene expression pattern is shown in Fig. 4.13. The virus expression pattern is similar to the c-Fos activation after dexmedetomidine administration (see Chapter 3.2 & 3.3). The whole region contained neural activity-induced hM3Dq receptors, whereby presumably these neurons have been dis-inhibited by dexmedetomidine.

4.10 Specific activation of MnPO after sleep deprivation re-induces a behavioural state resembling NREM sleep

For MnPO-TetTag-hM3Dq mice, I performed the same series of experiments as for the LPO-TetTag-hM3Dq mice described in the above sections: four weeks on doxycycline, two days off doxycycline, sleep deprivation and recovery sleep and back on doxycycline, CNO testing four days later, then a one month period on doxycycline, then two days off doxycycline, then dexmedetomidine-induced sedation followed by CNO testing four days later.

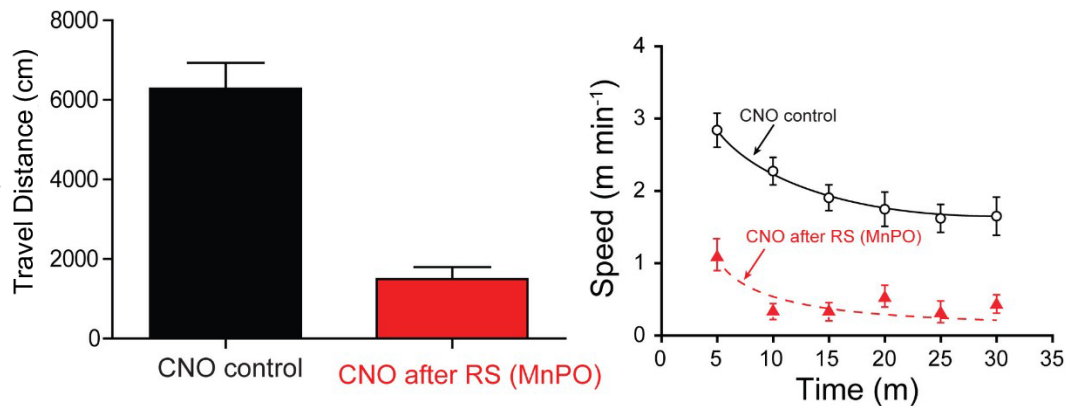


Figure 4-14 For MnPO-TetTag-hM3Dq mice, CNO induces sedation after sleep deprivation but does not affect the locomotor activity in MnPO-TetTag-hM3Dq mice which had been allowed normal sleep. Group data showing that CNO reduced the total travel distance of the sleep-deprivation-CNO mice (unpaired tTest $P < 0.001$ $n=7$). 30 minutes after CNO injection, mice barely moved even in the novel open field. Error bars represent SEM. Two-way ANOVA ($F(1, 84) = 192.67$, $p < 0.0001$) with post-hoc Bonferroni t-test indicated a significant difference between animals that had received RS before and saline-injected controls. The ANOVA compared the time ($F(5, 84) = 7.629$, $P < 0.0001$) and interaction between these two factors ($F(5, 84) = 1.290$, $P = 0.2777$).

MnPO-TetTag-hM3Dq mice which had sleep deprivation previously looked sedated and moved rather little in the open field (Fig. 4.14). To investigate the effects of re-inducing the sleep active neurons in the MnPO, after CNO injection, the EEG was recorded. I found that 30 minutes after CNO injection, most of the MnPO-TetTag-hM3Dq mice entered sustained NREM sleep sustained delta power for over two hours following CNO administration. The proportion of time spent in NREM sleep was significantly different from control mice (see Fig4.15). Thus similar to the results

obtained for LPO, it seems re-activated TetTagged neurons in MnPO were sufficient to initiate and sustain recovery sleep following sleep deprivation.

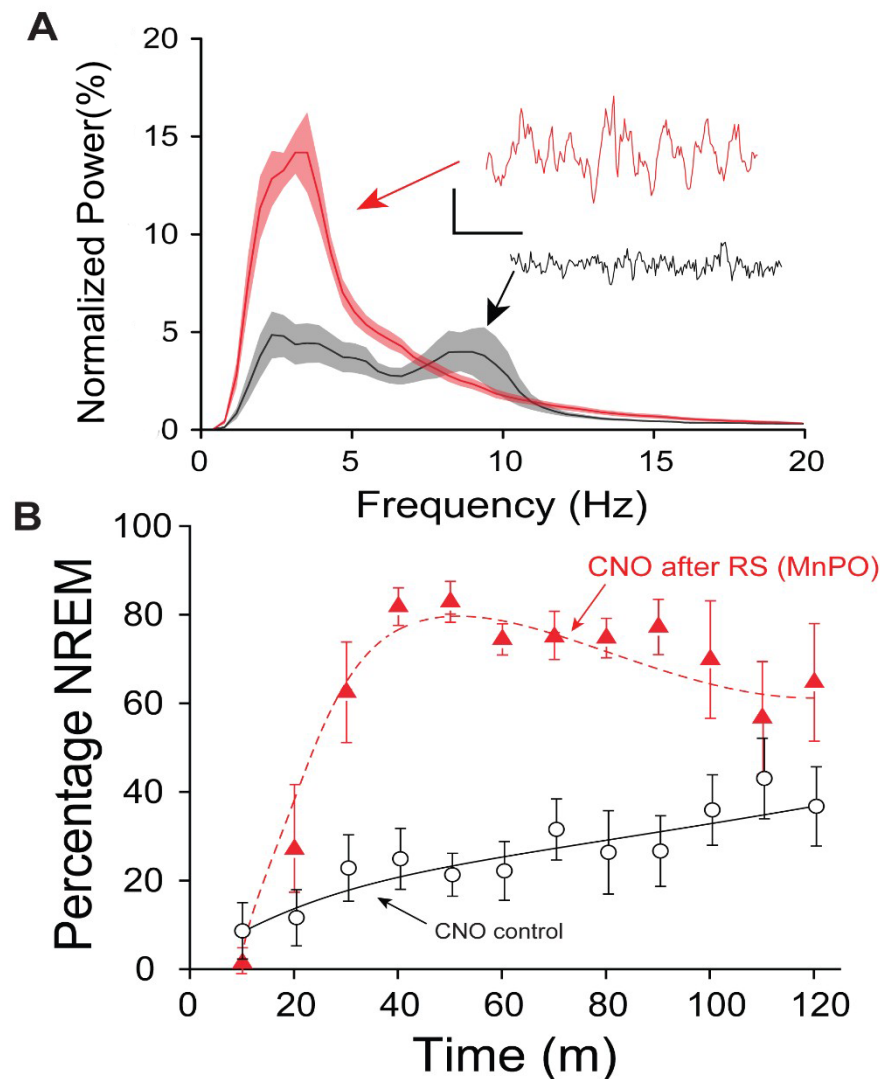


Figure 4-15 For MnPO-TetTag-hM3Dq mice CNO induces NREM sleep in the SD-CNO mice but does not affect sleep pattern in the normal sleep-CNO mice. **A:** Fourier transform power spectra when the EEG/EMG signals were scored as either sleep (red) or wake (black) after CNO injection. **B:** Percentage of time spent in NREM sleep after CNO injection in the mice home cages. Group data showing that CNO induced NREM sleep of the sleep-deprivation-

CNO mice (n=7). During the two hours after CNO injection, the SD-CNO mice spent more than double time in NREM sleep compare to the Normal sleep-CNO mice. Error bars represent SEM. Two-way ANOVA ($F(1, 168) = 98.42, p < 0.0001$) with post-hoc Bonferroni t-test indicated a significant difference between animals that had received RS before and controls from 30mins to 100mins after CNO injection. The ANOVA compared the time ($F(1, 168) = 6.664, P < 0.0001$) and interaction between these two factors ($F(11, 168) = 4.779, P < 0.0001$).

4.11 MnPO is only partially involved in dexmedetomidine-induced sedation

After a one month recovery period following the sleep deprivation and CNO reactivation experiments described in the previous section, the group of MnPO-TetTag-hM3Dq mice were injected with a sedative dose of dexmedetomidine and then allowed to recover for 4 days. Mice were then injected with CNO during the dark phase and their locomotor activity in the open field (Fig. 4.16) and EEG (Fig. 4.17) was analysed. The control group of MnPO-TetTag-hM3Dq mice went through the whole experimental protocol but received saline injections instead of dexmedetomidine injections.

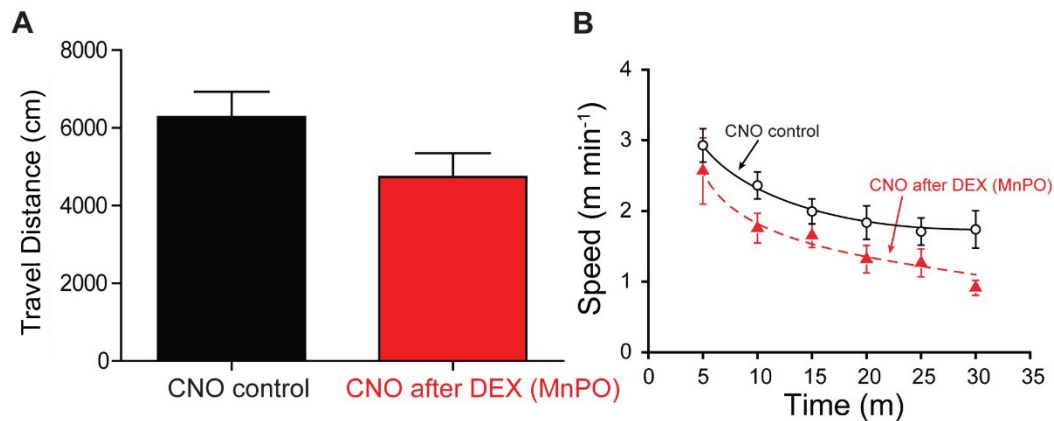


Figure 4-16 MnPO-TetTag-hM3Dq mice: CNO injections did not make significant difference of the locomotor activity between the DEX-CNO mice and the Saline-CNO mice. A: Group data showing that the total travel distance of the DEX-CNO mice did not reduce compared to the Saline-CNO mice (unpaired tTest $P > 0.05$ $n=7$) $P=0.11$. B: 30 minutes after CNO injection, mice moved as much as saline-CNO mice. Error bars represent SEM. Two-way ANOVA ($F(1, 55) = 2.971$, $p=0.0673$) with post-hoc Bonferroni t-test indicated a significant difference between animals that had received dexmedetomidine before and saline-injected controls. The ANOVA compared the time ($F(5, 55) = 9.675$, $P < 0.0001$) and interaction between these two factors ($F(5, 55) = 0.2975$, $P = 0.9126$).

In contrast to the results with LPO-TetTag-hM3Dq mice, CNO injection to the MnPO-TetTag-hM3Dq mice that were previously dexmedetomidine-sedated did not induce significant behavioural change (Fig. 4.16). However, a high delta/theta ratio in the EEG (Fig. 4.17) was produced. Thus there was a notable disconnect between EEG (looks like NREM) and behaviour (mice still moving). This suggests that the lateral preoptic hypothalamic area, but not the MnPO, contains neurons sufficient for adrenergic α_2 receptor-induced sedation.

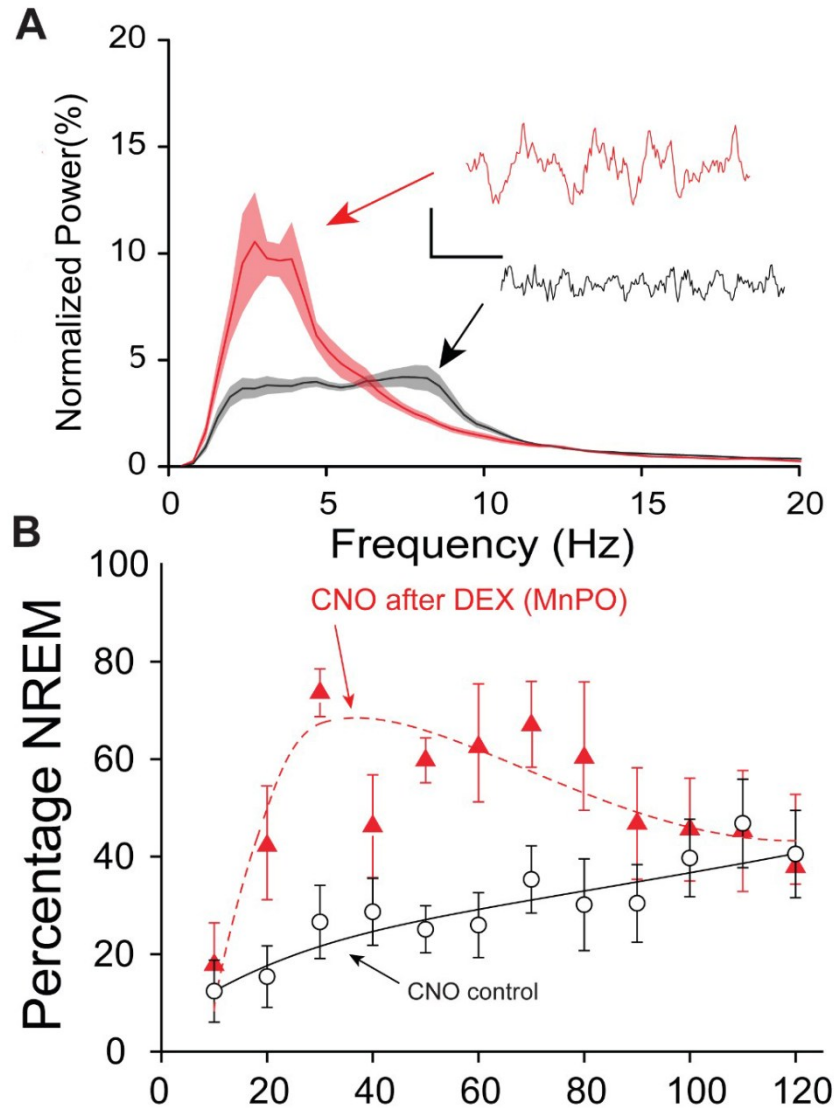


Figure 4-17 CNO induce NREM sleep in the DEX-injection mice based on EEG recording. A: Fourier transform power spectra when the EEG/EMG signals were scored as either sleep (red) or wake (black) after CNO injection. B: Percentage of time spent in NREM sleep after CNO injection in the home cages. Group data showing that CNO induced NREM sleep of the DEX-CNO mice (n=7). During the two hours after CNO injection, the DEX-CNO mice spent more time in NREM sleep compare to the CNO control mice. Error bars represent SEM. Two-way ANOVA ($F(1, 143) = 19.06, p = 0.0276$) with post-hoc Bonferroni t-test indicated a significant difference between animals that had received dexmedetomidine before and saline-injected controls from 50mins to 70mins after CNO injection.

4.12 Summary of TetTag transgene expression patterns in MnPO-TetTag-hM3Dq mice

After the series of behavioural experiments was completed on the MnPO-TetTag-hM3Dq mice, I reinduced the TetTag expression by sleep depriving the mice and allowing them to enter recovery sleep and killed the mice after this sleep to validate the specificity and efficiency of transgene expression. The viral hM3Dq-mCherry expression pattern was quite restricted: mCherry fluorescence was primarily detected in the MnPo area, with no expression in neighbouring regions (Fig. 4.18). Considering all the MnPO-TetTag-hM3Dq mice, in Figure 4.19, the boxed sections, J-L, were from mice where the hM3Dq-mCHERRY transgene was induced following sleep-deprivation and recovery sleep, but there was no behavioural or EEG signs of NREM sleep following CNO administration. In animals K & L, for example, the intended midline injection of AAV into MnPO had missed, and resulted in unilateral hM3Dq-mCHERRY induction in the LPO area; but activating these receptors with CNO was insufficient to trigger sleep behaviour.

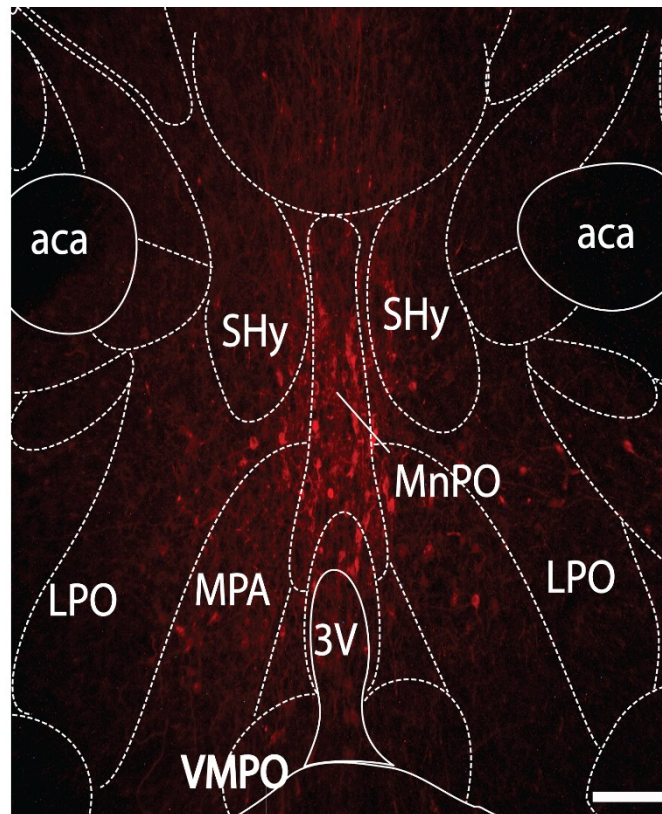


Figure 4-18 Transgene expression in MnPO-TetTag-hM3Dq mice after sleep deprivation and recovery sleep. A representative example of coronal sections throughout the MnPO area. Targeted region of the MnPo (AP=0.4mm, ML= 0 mm, DV= -4.5 mm from Bregma) Scale bar: 100µm Abbreviations: 3V: 3rd ventricle; aca: anterior commissure, ant; LPO, lateral preoptic area; MnPO, median preoptic area; MPA: medial preoptic area; SHy: septohypothalamic nucleus; VMPO, ventmed preoptic area.

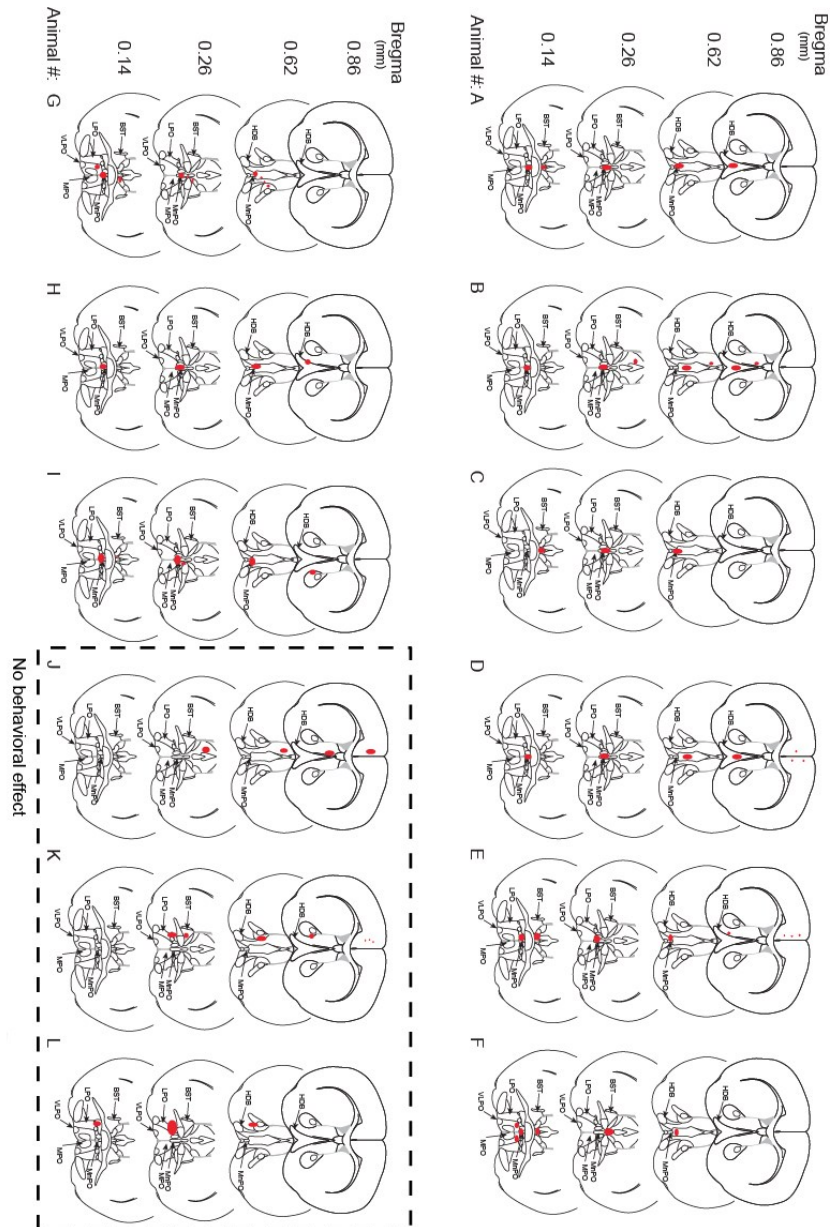


Figure 4-19 MnPO-TetTag-hM3Dq expression patterns: Induction of hM3Dq-mCHERRY expression 2 hours into recovery sleep after sleep deprivation (animals A-F) or two hours following dexmedetomidine-induced sedation (animals G-I) where AAVs were midline-injected into MnPO and surrounding areas. Each letter, A through to I, summarizes the expression in individual mice as seen by serial coronal sectioning through the AAV injection site. Red indicates induced hM3Dq-mCHERRY expression. Abbreviations: BST, bed nucleus stria terminalis; LPO, lateral

preoptic area; MPO, medial preoptic area; VLPO, ventral lateral preoptic area.

4.13 Controls for the TetTag-hM3Dq method

After confirming the effectiveness of the pharmacogenetic approach in the preoptic areas, I performed similar experiments in the superior colliculus (Fig. 4.20), which I considered a brain region unlikely to be involved in either dexmedetomidine-induced sedation or recovery (NREM) sleep after sleep deprivation. These experiments were aimed at checking the specificity of the behaviour and EEG effects obtained in the LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice.

AAV-c-fos-tTA and AAV-P_{TRE-tight}-hM3Dq-mCherry were bilaterally injected into the superior colliculus (SC) area in adult mice to generate SC-TetTag-hm3Dq mice.

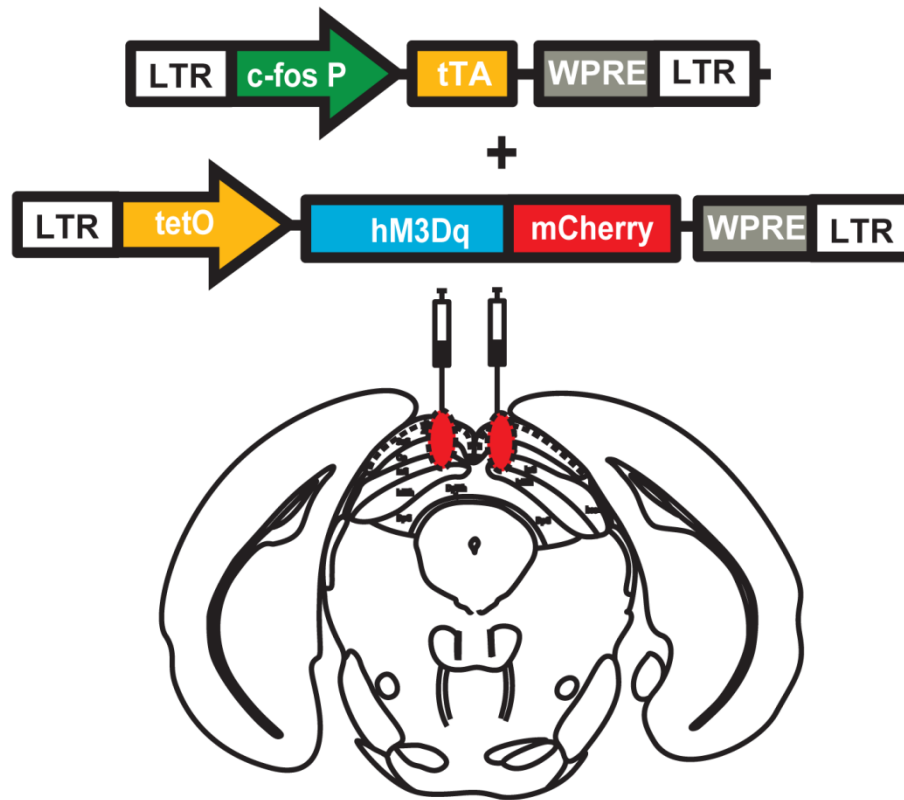


Figure 4-20 Schematic shows AAV-c-fos-tTA and AAV-P_{TRE}-tight-hM3Dq-mCherry were co-injected bilaterally into the superior colliculi (SC) to generate SC-TetTag-hM3Dq mice.

After the AAV injections, mice were then given the full experimental procedure: doxycycline repression for one month, doxycycline removal, then sleep deprivation and recovery sleep treatments followed by three or four days recovery and then CNO injection.

As expected, giving CNO to the SC-TetTag-hM3Dq mice that were previously sleep deprived did not show any clear differences in their EEGs (Fig. 4.21).

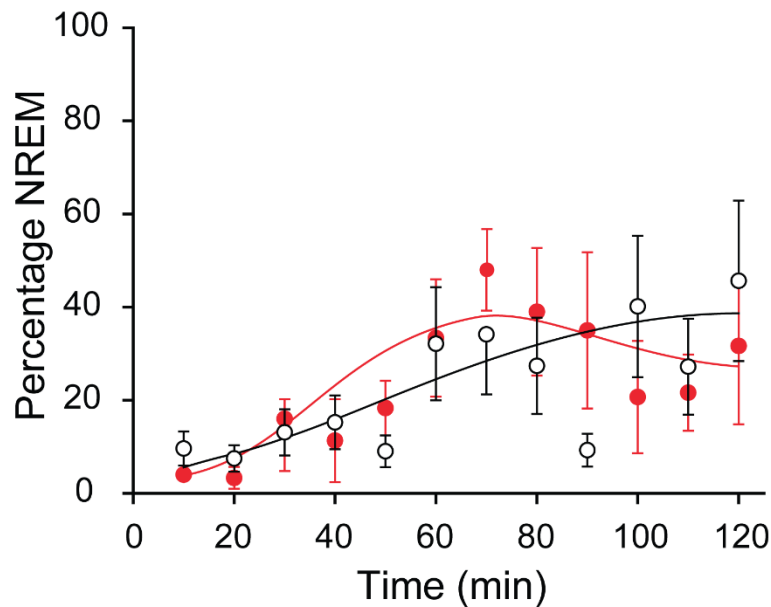


Figure 4-21 SC-TetTag-hM3Dq mice: CNO did not induce significant NREM sleep in the SD-CNO mice compared to Normal sleep-CNO mice. Percentage of time spent in NREM sleep after CNO injection in the home cages. Group data showing that CNO induced NREM sleep of the SD-CNO mice (n=6). During the two hours after CNO injection, the SD-CNO mice did not spend more time in NREM sleep compared to the normal sleep-CNO mice. Error bars represent SEM. Two-way ANOVA ($F(1, 108) = 8.347$, $p = 0.786$) with post-hoc Bonferroni t-test indicated no significant difference between animals that had received RS before and controls.

No behavioural was either found: the mice kept moving and there was little hM3Dq-mCHERRY receptor expression seen in the colliculi, suggesting the hypothalamic effect is specific.

Similarly, giving CNO to the SC-TetTag-hM3Dq mice that were previously dexmedetomidine-sedated did not show any clear difference in their EEGs compared with that mice produced by saline injections only. (Fig. 4.22) No behavioural change was found and again there was little mCHERRY expression seen in the colliculi (data not shown)

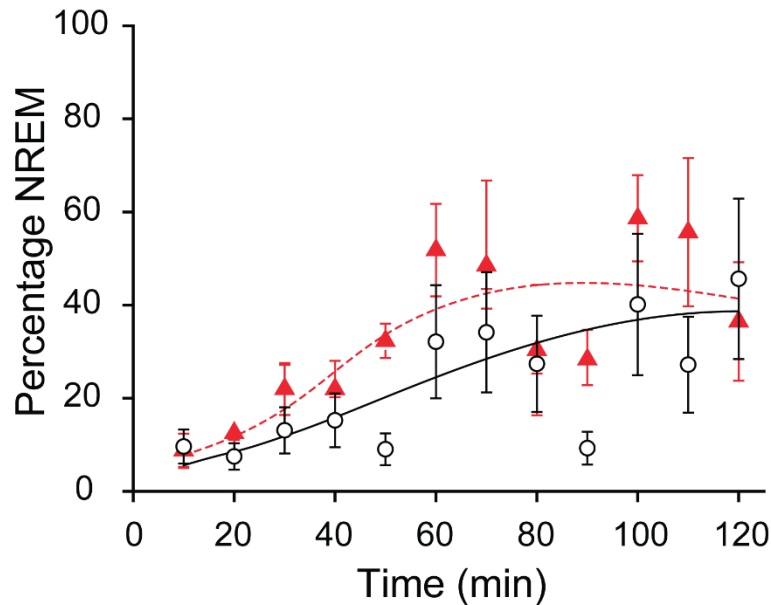


Figure 4-22 SC-TetTag-hM3Dq mice: CNO did not induce significant NREM sleep in the DEX-injection mice compared to saline-injection mice. Percentage of time spent in NREM sleep after CNO injection in the home cages. Group data showing that CNO induced NREM sleep of the DEX-CNO mice (n=6). During the two hours after CNO injection, the DEX-CNO mice did not spend more time in NREM sleep compared to the Saline-CNO mice. Error bars represent SEM. Two-way ANOVA ($F(1, 108) = 0.1354$, $p = 0.7136$) with post-hoc Bonferroni t-test indicated no significant difference between animals that had received DEX before and controls.

4.14 Discussion

The TetTag method demonstrated that both adrenergic α_2 agonists and sleep deprivation induce NREM sleep by locally exciting neurons in the PO area of the hypothalamus (Zhang et al., 2015).

The biochemical mechanism for how recovery sleep is initiated and maintained remains a mystery. Candidate sleep homeostat molecules, which accumulate proportionally to the amount of sleep deprivation, and act locally in the preoptic area, include PGD2 and

adenosine (Brown et al., 2012). For the mechanisms initiating sleep, one view is that discrete hypothalamic preoptic areas, such as VLPO and MnPO control sleep, and GABAergic neurons in these nuclei fire before sleep onset. VLPO was functionally identified because it contained more FOS-positive neurons during sleep (Sherin et al., 1996). Rats with VLPO lesions have 50% less NREM sleep (Lu et al., 2000). VLPO projection neurons contain GABA and galanin, and are suggested to inhibit the aminergic arousal neurons, e.g. histamine in the TMN and adrenergic neurons in the LC. The literature tends to emphasize VLPO and the associated flip-flop model. However, I found, both by endogenous c-fos staining and TetTagging, that many more neurons than those in VLPO become excited during sleep-deprivation and recovery sleep or following dexmedetomidine administration. Nevertheless, the TetTagging approach shows the sufficiency of the LPO and MnPO areas in initiating and maintaining sleep, fitting with the classic experiments by Sherman and Clemente (Clemente and Serman, 1963), whereby electrical stimulation of the PO area in cats induced behavioural sleep.

By artificially reactivating LPO/BNST neurons initially activated by a systemic low-dose of dexmedetomidine, I found that such neurons are sufficient to induce sedation (NREM sleep). One explanation for these results could be that adrenergic α_2 agonists preferentially inhibit local GABAergic interneurons in say LPO. These, in turn, would then inhibit sleep-active GABAergic projection neurons less. Thus, following dexmedetomidine administration, the projection neurons would then fire more by dis-inhibition to induce sedation. However, Dr. Valentina Ferretti

in our lab found that a knockdown of *adra2a* transcripts in the LPO area did not alter the sedative effects of 100 µg/kg dexmedetomidine (Fig.4.23; Zhang et al., 2015).

Alternatively dexmedetomidine could activate inhibitory $\alpha 2A$ receptors on the terminals of inhibitory afferents coming into the PO area, for example GABA inputs, or NA inputs from nuclei other than the LC (we showed that *adra2a* knockdown in the LC did not affect dexmedetomidine-induced sedation – Zhang et al., 2015). The reduced local release of GABA or NA into the PO hypothalamus would then allow dis-inhibition and excitation of the sleep-promoting neurons.

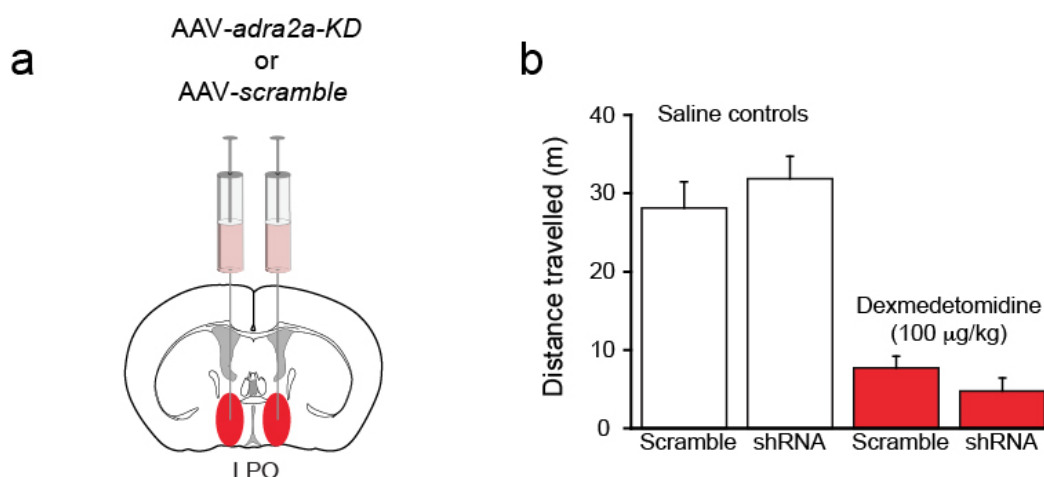


Figure 4-23 Knock down of adrenergic $\alpha 2A$ receptor transcripts in the preoptic area (LPO) of the hypothalamus had no effect on dexmedetomidine-induced sedation. (a) Schematic illustrating bilateral injection of AAVs expressing either dsRED-mir30-sh*adra2a* or dsRED-mir30-shscramble transgenes into the LPO of adult mice. (b) The total distance travelled in 15 minutes by saline-injected (white bars) or dexmedetomidine-injected (100 µg/kg) (red bars) mice was not significantly different (n=6 scramble, n=7 shRNA; P=0.19) for mice with AAVs expressing either the knock-

down or scramble transgenes. (Courtesy of Dr. Valentina Ferretti; Zhang et al., 2015)

Dexmedetomidine and sleep deprivation also induced TetTag-hM3Dq expression in another PO nucleus, MnPO. Although reactivation of MnPO with CNO did fully recapitulate recovery sleep after sleep deprivation, it only partially recapitulated dexmedetomidine-induced sedation. There was an interesting disconnect between a strong increase in the EEG delta/theta ratio, and animal movement – the mice were not sleeping – but perhaps “sleep walking”.

In addition, the natural sleep rhythm over 24 hours was not a sufficiently strong stimulus to induce the “TetTag-hM3Dq neuronal ensembles”, at least under our experimental protocol: their formation apparently required the stronger drivers of dexmedetomidine or recovery sleep following sleep deprivation. In basal conditions CNO did not induce a change in the EEG compared with dexmedetomidine sedation and recovery sleep, and animals did not behaviourally enter sleep. Indeed, in mice with hM3Dq receptors selectively but continuously expressed in GABAergic neurons in the PO hypothalamic area (Saito et al., 2013), systemic CNO produced only a small increase in NREM sleep, and only during the day, but not the night.

Although I have shown that neurons in the *same region* in the same animals are activated by sleep deprivation or dexmedetomidine, I have not shown that these are the *same neurons*. It seems likely that they would be, but a more

sophisticated version of the TetTagging method would be required to show this. Mayford (Mayford, 2014) has recently proposed such a strategy, using AND NOT gates, which I outline as follows.

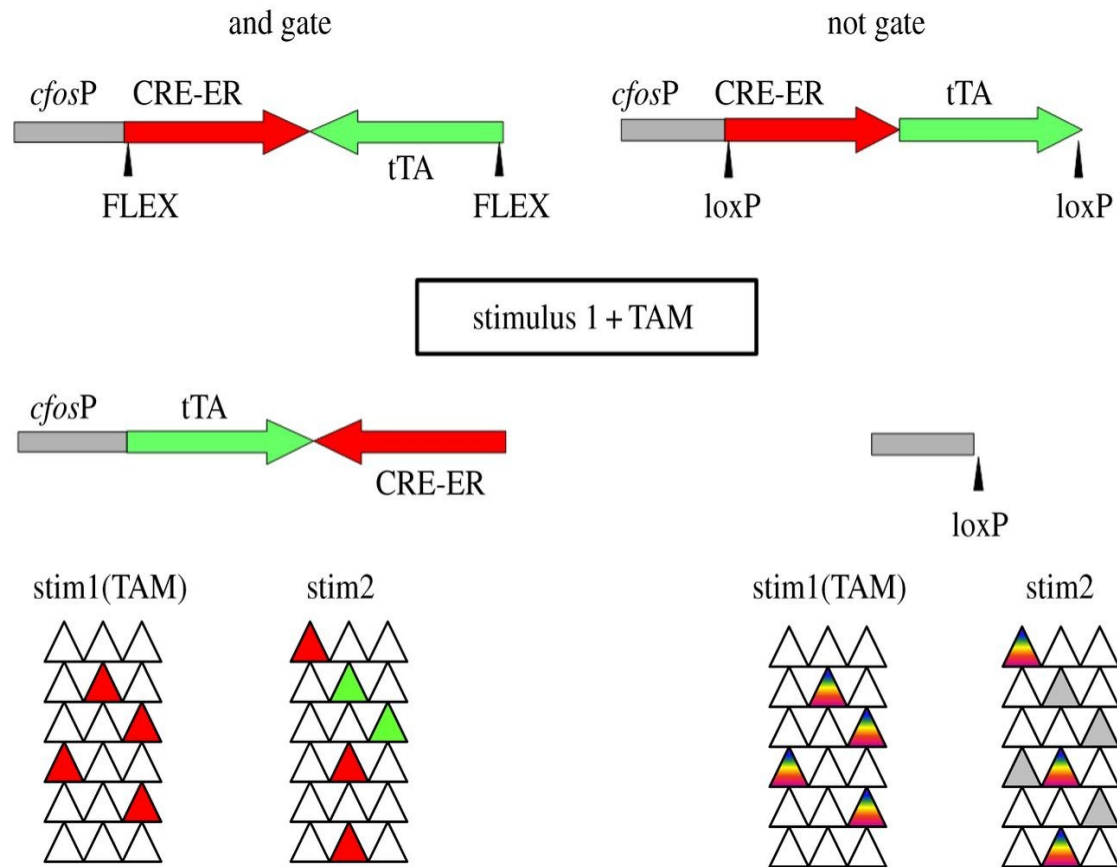


Figure 4-24 Possible method to differentiate neuron ensembles based on genetic switches. Two cellular arrangements are proposed which are an ‘and gate’ and a ‘not gate’ mark neurons active at two time points or only at one point, respectively. Tam, tamoxifen. Taken from (Mayford, 2014).

The ‘AND gate’ genetic switch consists of the cFos promoter and reverse-oriented CRE-ERT2 with tTA in opposed directions (See in Fig 4.24 Left). For functional activity the Cre also requires tamoxifen to be present, and contains an ER binding site for this steroid. In theory, during the first simulation, such as dexmedetomidine-induced sedation, neurons which are activated

will induce Cre recombinase and trigger inversion of the flanked sequences in response. Then the construct will become cFos-tTA, which is the same as conventional TetTagging. This can drive the expression of $P_{TRE-tight}$ -hM3Dq-mCherry, and thus the neurons associating with dexmedetomidine-induced sedation will be labelled with hM3Dq-mCherry. After CNO injection, the mice will be sedated. However, during the second stimulus (recovery sleep after sleep deprivation), and if this requires the same neurons as for dexmedetomidine-induced sedation, the activation of neurons in response to sleep recovery pathway will be detected by c-Fos promoter and it will again activate by TetTagging the hM3Dq-mCherry receptor, and the subsequent CNO injection will again activate sleep. But if dexmedetomidine-induced sedation and sleep recovery do not share the same groups of the neurons, the hM3Dq-mCherry cannot be expressed and no behaviour induced after CNO injection.

The same principle applies for the 'not gate' construct. During the first stimulation (dexmedetomidine-induced sedation), neurons which are activated will induce both Cre recombinase and tTA. The tTA will activate $P_{TRE-tight}$ -hM3Dq-mCherry and tag the neurons, but at the same time Cre will delete the Cre-tTA construct (Figure 4.24), so that there can only be one round of tTA induction from these cells. So if the same neurons are involved for recovery sleep induction and dexmedetomidine-induced sedation, CNO will induce one round of sleep but not a second. In contrast, if dexmedetomidine-induced sedation and sleep recovery do not share the same groups of the neurons, the hM3Dq-mCherry would

express during the second stimulus, and mice should go into NREM sleep after CNO injection.

Chapter 5 $\alpha 2$ adrenergic receptor-induced sedation requires GABAergic neurons for rapid onset but not its maintenance

5.1 Introduction

Based on my results in Chapter 4, dexmedetomidine induced sedation may be accomplished by activating the sleep-active neurons in the LPO area, subsequently inhibiting the cortical-activating areas. In Chapter 3, I showed that the majority of c-FOS expression in LPO during dexmedetomidine-induced sedation was in GABAergic interneurons (see Chapter 3, Fig. 3.4). There were also some FOS-positive cells which did not contain GAD67. Given the mix of cell types where the hM3Dq-mCHERRY receptor was induced in the LPO hypothalamus, in this chapter I test directly if dexmedetomidine-induced sedation actually requires GABAergic neurons, by deleting the vesicular GABA transporter gene, *vgat*, from LPO neurons.

5.2 Results

I deleted the vesicular GABA transporter (*vgat*) gene from the LPO hypothalamic area by injecting AAV- Cre-2A-Venus bilaterally into the LPO of mice homozygous for a floxed *vgat* gene (Tong et al., 2008a), *vgat*^{lox/lox}, to give LPO- Δ *vgat* mice (Fig. 5.1). For controls, *vgat*^{lox/lox} mice were injected with AAV-GFP to generate LPO-GFP mice.

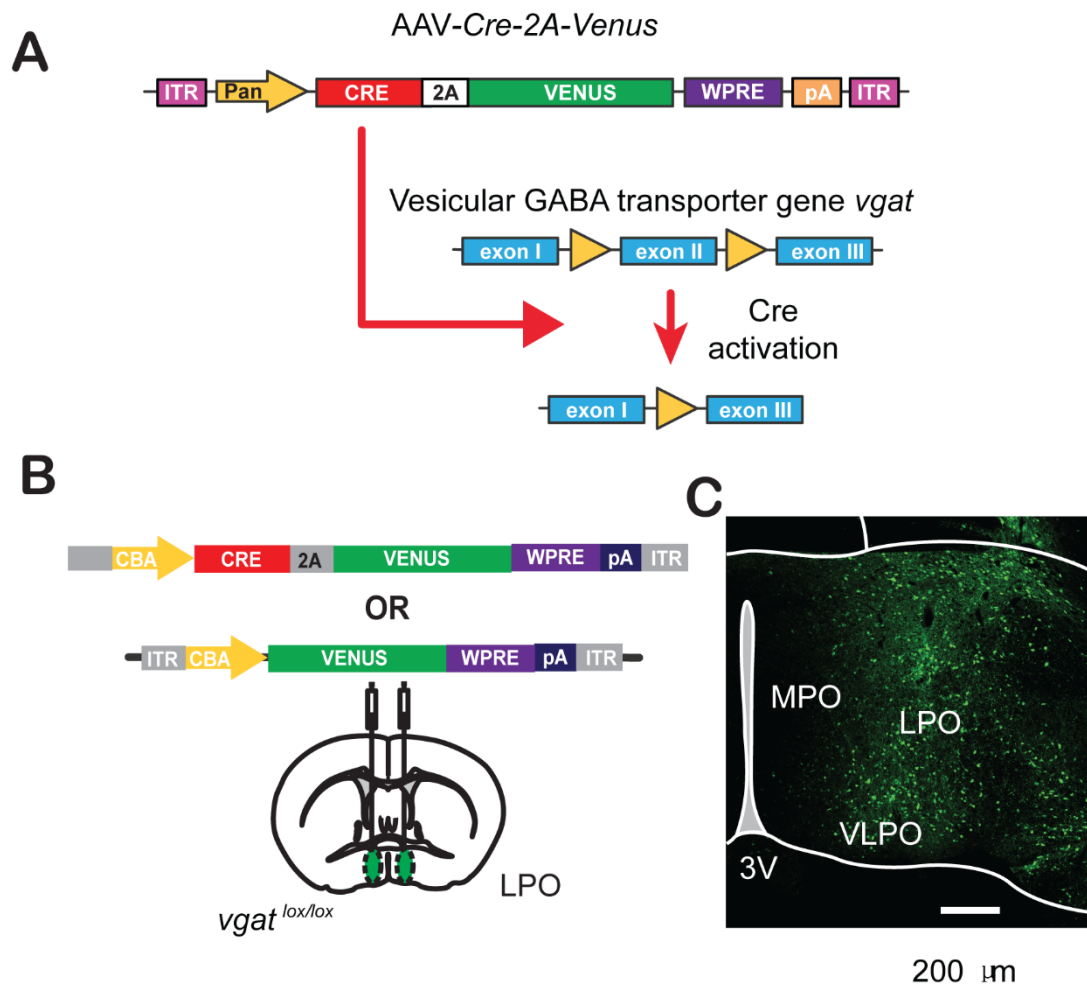


Figure 5-1 A, Schematic shows site-specific recombinase system. LoxP sites are placed before and after exon 2 of the *vgat* gene without compromising gene expression (Tong et al., 2008). Cre recombinase deletes exon 2 and ablates expression of function VGAT protein. B, Schematic shows AAV-Cre-2A-Venus or AAV-GFP bilaterally injected into the LPO hypothalamus area of *vGAT* flox/flox mice. CBA, chicken β -actin promoter. C, the image shows the extent of Cre-2A-Venus expression, as detected by staining with EGFP antisera

5.3 $\alpha 2$ adrenergic receptor-induced sedation requires GABAergic neurons for rapid onset

After virus injection, mice were allowed to recover for 1 month. Then mice were injected with saline to check their baseline activity.

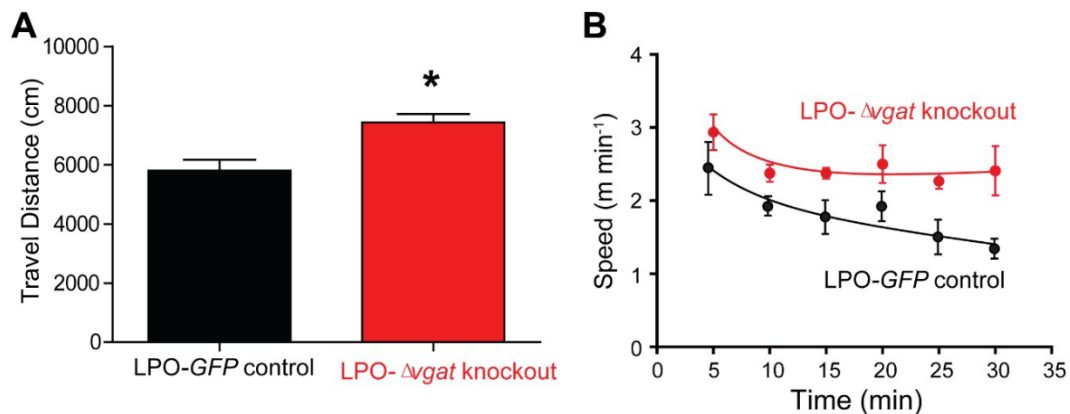


Figure 5-2 The total travel distance of the LPO- Δ vgat mice was significantly increased compared to the LPO-GFP control mice (n=6 unpaired tTest). $P < 0.005$. LPO- Δ vgat mice also seem to run faster, although this is not significant. There is no significant difference in the first 20 minutes. Error bars represent SEM. Two-way ANOVA ($F(1, 66) = 2.1956$, $p = 0.2743$) with post-hoc Bonferroni t-test indicated no significant difference between LPO- Δ vgat mice and LPO-GFP control mice. .

For the baseline activity, there was a small but significant increase in distance travelled between LPO- Δ vgat and LPO-GFP mice (Fig. 5.2), and LPO- Δ vgat mice also travelled faster, although this was not significant. (Fig. 5.2). Thus preventing GABA release from the LPO area made mice slightly hyperactive.

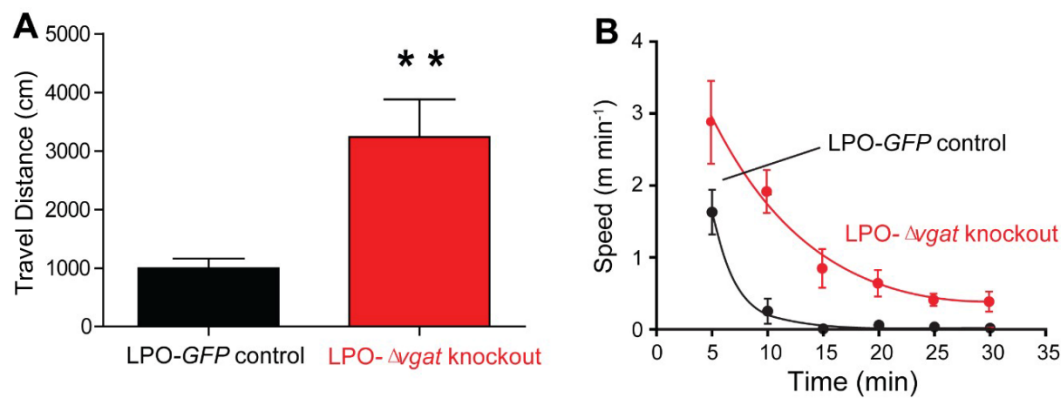


Figure 5-3 LPO-Δvgat mice remain active for a longer time after dexmedetomidine injection compared with LPO-GFP mice. In the open field assay LPO-Δvgat mice still travel significantly further and faster compared to the LPO-GFP control mice (n=6) after dexmedetomidine (100 μg/kg) injection (unpaired tTest $P < 0.001$). The speed of LPO-GFP mice was significantly less (two-way ANOVA, $F(1, 66) = 219.554$ $P < 0.0001$) than in LPO-Δvgat mice (n=8) from five minutes to fifteen minutes after dexmedetomidine injection. Error bars represent SEM.

When given sedative doses of dexmedetomidine (100 μg/kg), and placed into a novel cage, GFP-expressing control mice with an intact vGAT gene became sedated as normal and soon ceased movements (Fig. 5.3). Some five minutes after injection, control LPO-GFP mice showed the expected large increase in delta power in their EEG compared with that produced by saline injection (Fig. 5.4A), they had sustained NREM lasting for ~90 minutes (Fig. 5.4B), and within about 10 minutes after injection they had ceased moving (Fig. 5.3).

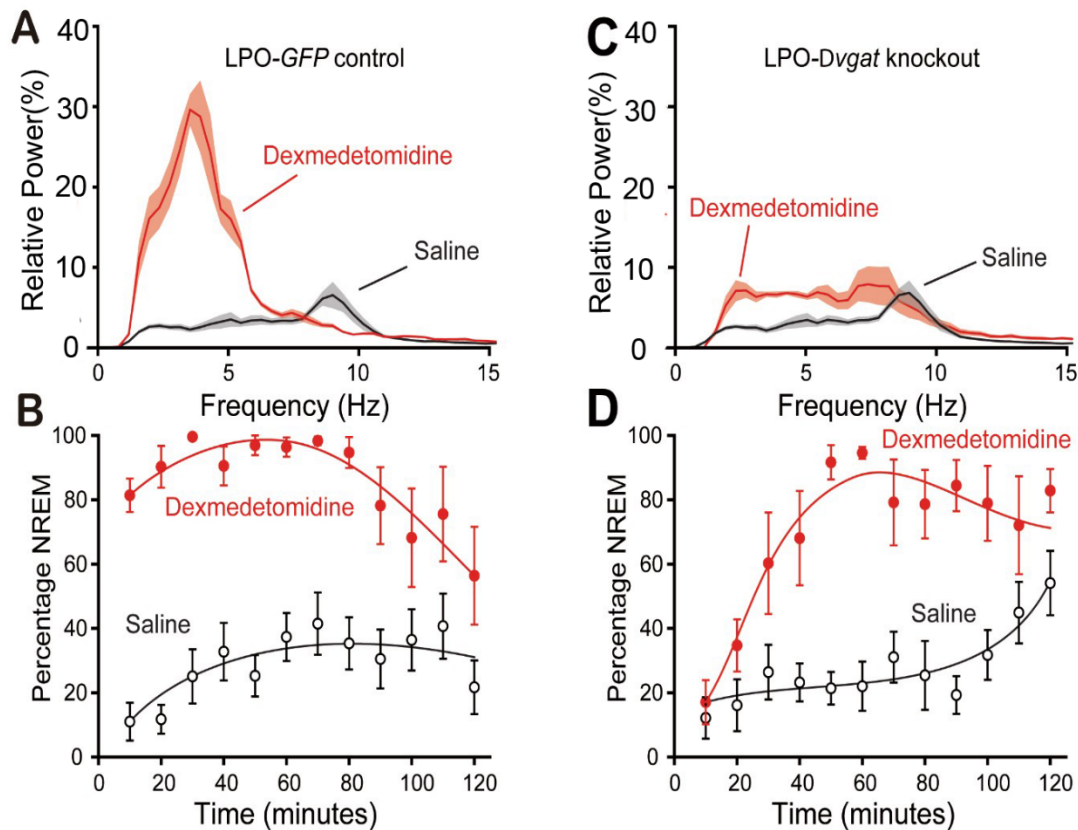


Figure 5-4 Selective knockout of the GABA vesicular transporter gene (vgat) in the PO hypothalamic area (LPO- Δ vgat mice) slows the transition to dexmedetomidine-induced sleep. (A) EEG power spectra ten minutes after dexmedetomidine (100 μ g/kg – red line) or saline (black) injection in control mice (n=8) expressing AAV-GFP in the LPO (LPO-GFP mice; n=6). Lighter shaded envelopes indicate the s.e.m. (B) Two-way ANOVA ($F(1, 144) = 252.95$, $p < 0.0001$) with post-hoc Bonferroni t-test indicated that the percentage of time scored as NREM sleep after dexmedetomidine (100 μ g/kg; filled circles, n=6) was significantly greater than in saline injected (open circles, n=8) LPO-GFP control mice from 10 minutes to 90 minutes. The ANOVA compared the time ($F_{11, 144} = 2.166$, $P = 0.0193$) and interaction between these two factors ($F_{11, 144} = 1.735$, $P = 0.0713$). (C) EEG power spectra ten minutes after dexmedetomidine (100 μ g/kg – red line; n=6) or saline (black) injection in mice (n=8) expressing AAV-Cre-2A-Venus in the LPO (LPO- Δ vgat mice). (D) Two-way ANOVA ($F(1, 144) = 129.82$, $p < 0.0001$) with post-hoc Bonferroni t-test indicated that the

percentage of time scored as NREM sleep after dexmedetomidine (100 µg/kg; filled circles, n=6) was significantly greater (two-way than in saline (n=8) in AAV-Cre-2A-Venus mice from 40 minutes to 90 minutes. The ANOVA compared the time ($F_{11, 144} = 5.32$, $P < 0.0001$) and interaction between these two factors ($F_{11, 144} = 2.588$, $P = 0.006$).

By contrast, 10 minutes after *i.p.* injection of dexmedetomidine into LPO-Δvgat mice, there was only a small shift of the EEG to delta frequencies (Fig. 5.4 C). This absence of effect was striking (compare Fig. 5.4 A&B). However, about 30 minutes after injection, the percentage of NREM in the LPO-Δvgat mice was significantly higher than saline injected animals (Fig. 5.4D), and the mice became sedated (Fig. 5.3), such that after 30 minutes they were as sedated as dexmedetomidine-injected LPO-GFP mice (Fig. 5.3 & 5.4). Compared with control mice, they remained active for a longer time. However, both groups of mice (control and Cre injected) showed delta in their EEG.

After the behavioural experiments were completed, the mice were killed and the gene transfer efficacy was determined by immunohistochemical analysis using a GFP antibody (Fig. 5.1C). On the sections, the presence of GFP indicated the potential deletion of the vgat gene. Abundant expression was observed in much of the PO area. The virus expression pattern was similar to the mCherry expression seen in the Tet Tagging experiments after dexmedetomidine administration (see Chapter 4).

5.4 Discussion

I found that GABAergic neurons in the LPO area are required for rapid-onset dexmedetomidine-induced sedation and the associated changes in the EEG. However, the LPO- Δ vgat mice did eventually become sedated. One possibility is that probably any co-released transmitters, such as galanin, are also required for dexmedetomidine induced-sedation. Or it may be that I failed to knock out all the vgat-positive neurons in the area. Last but not least, dexmedetomidine-induced sedation is a complex behavioural process, which may involve other brain regions at the same time, for example the recently identified parabrachial nucleus in the brainstem (see Figure 1.4 in Chapter 1; Anaclet et al., 2014)

To better understand the role of GABAergic neurons of the PO hypothalamic area in the dexmedetomidine-induced sedation, we need to be selective in identifying the cells activated during TetTagging. For this, we would need to implement cell type-selective TetTagging with Cre-inducible responder genes. AAV- $P_{TRE-tight}$ -double flexed-hM3Dq-mCherry could be produced (Fig. 5.5). Then we could co-inject AAV-c-fos-tTA and AAV- $P_{TRE-tight}$ - double flexed- hM3Dq-mCherry, we performed the similar protocol in Fig4.5B into, for example, vgat-Cre or galanin-Cre mice. Then TetTagging would be restricted to selected cell types. This might give better insight into mechanisms of dexmedetomidine-induced sedation or recovery sleep. By using a range of Cre driver mice, we can dissect subtypes of neuron responsible for sedation and recovery sleep response.

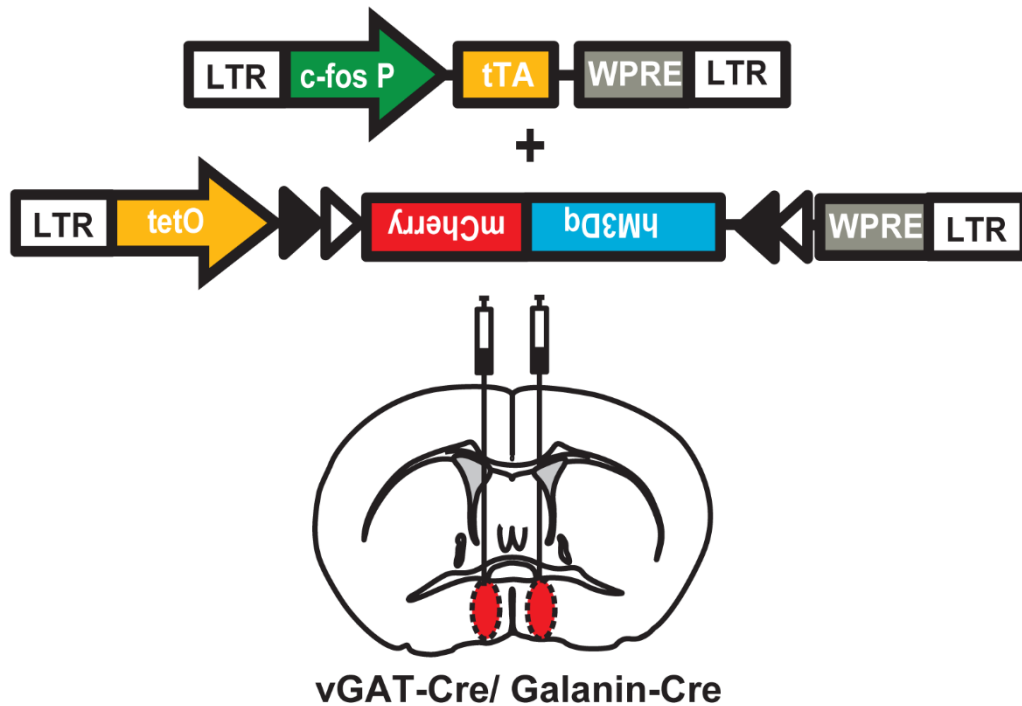


Figure 5-5 A possible future experiment: The AAV-c-fos-tTA and AAV-P_{TRE-tight}-double flexed- hM3Dq-mCherry viruses could be co-injected into the LPO area of Cre driver mice e.g. galanin-Cre mouse. In this experiment the hM3Dq reading frame is inverted relative to the tetO promoter in a Cre-dependent flex switch. Thus the tTA protein will only be able to drive hM3Dq receptor expression in Cre expressing neurons.

Chapter 6 Using Tet-tagging to map the projections from the LPO after dexmedetomidine induced-sedation

6.1 Summary

Having functionally identified PO hypothalamic neurons as being sufficient for dexmedetomidine induced sedation and recovery sleep after sleep deprivation (Chapter 4), the next logical step is to analyse where these neurons project. For example, do TetTagged LPO neurons directly innervate wake-active neurons such as histamine neurons and noradrenergic neurons? I decided to use TetTagging to mark just those axons whose cell bodies were depolarized during dexmedetomidine induced sedation. To tackle this, I used anterograde circuit mapping with Channel Rhodopsin fused to YFP. The actual protein is: ChR2H134R-EYFP. The H134R mutation enhances light-evoked ChR currents and reduces spike-frequency adaptation (Nagel et al., 2005; Pulver et al., 2009). The ChR-YFP protein is a superb substrate for anterograde axonal transport (Petreanu et al., 2007; Tye and Deisseroth, 2012), and then activating the terminals and projection fields with blue light might reveal the transmitters and something about the animal behaviour. In this Chapter, I first constructed a $P_{TRE-tight}$ -ChR2-eYFP gene and packaged it into AAV capsids. By co-injecting AAV- $P_{TRE-tight}$ -ChR2-eYFP and AAV-cfos-tTA into LPO, I then obtained some preliminary (pilot) TetTagging data about the projections of the activated circuitry following dexmedetomidine-induced

sedation.

Results

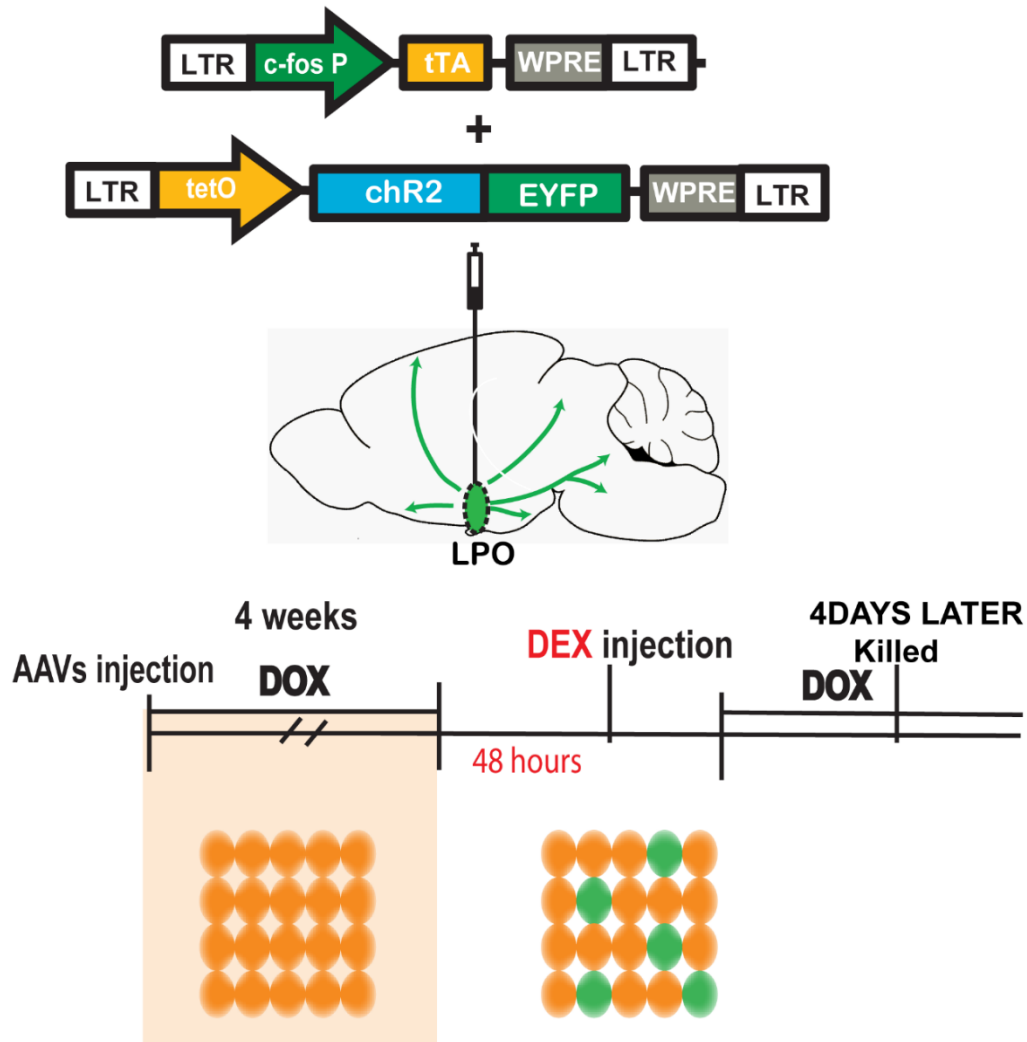


Figure 6-1 Mapping activated neural connections with LPO-TetTag-ChR-YFP mice. A, The AAV-c-fos-tTA was co-injected bilaterally with AAV-P_{TRE-tight}-ChR2-eYFP into the LPO area. B, Experimental protocol. After injection, mice were habituated with doxycycline food. The orange circles represent the neurons in the preoptic area. When off Dox 48 hours, after dexmedetomidine injection, dexmedetomidine injection i.p. induces c-fos expression in the preoptic area; the c-fos promoter drive the expression of tTA, which binds to tetO and drive the expression of ChR2-eYFP, labelling a subpopulation of activated cells and their projecting

axons (Green) in the preoptic area. Then mice were put back on DOX and culled for 4 days later.

6.2 The TetTagged projections from the LPO area after dexmedetomidine induced-sedation

I first assembled the $P_{TRE-tight}$ -ChR2EYFP construct by modifying the $P_{TRE-tight}$ -hM3Dq-mCherry gene (see Chapter 2, section 2.1 and Figure 2.3) and then packaged it into AAV capsids (see Chapter 2). I co-injected AAV-c-fos-tTA and AAV- $P_{TRE-tight}$ -ChR2-eYFP bilaterally in the LPO to generate LPO-TetTag-ChR-YFP mice. The mice were maintained on doxycycline in their diets. After four weeks post viral injection, doxycycline was removed from the diet, and two days later, were injected with the sedative dose of dexmedetomidine, 100 μ g/kg, and then put back on a doxycycline diet and allowed to recover for four days. I then killed the mice by perfusion, and serially sectioned the brains. I checked the projections of neurons in the LPO by immunostaining with GFP antibody.

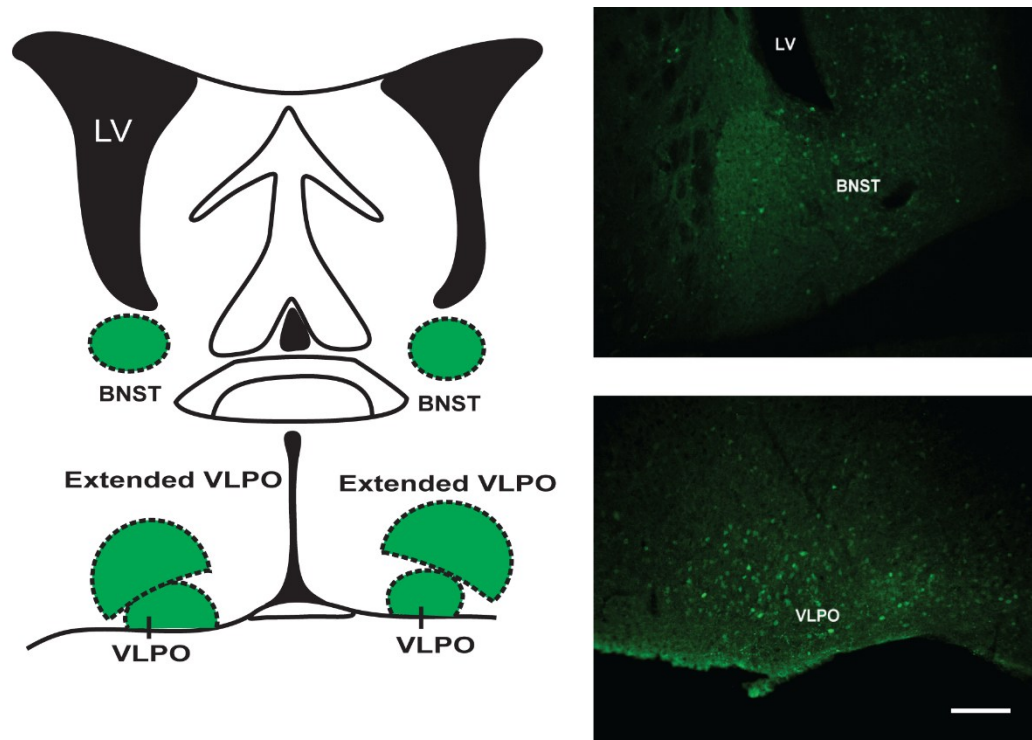


Figure 6-2 LPO TetTag expression patterns in an LPO-TetTag-ChR-YFP mouse following dexmedetomidine-induced sedation. Green areas (left image) mark the densest induction of TetTag-ChR-YFP expression. ChR-YFP-positive cells bodies are found in the BNST and VLPO ad areas dorsal to VLPO (here termed “extended VLPO”). BNST, bed nucleus of the stria terminalis; LV, lateral ventricle; VLPO – ventrolateral preoptic area. Scale bar: 200µm

TetTag ChR2-eYFP expression in cell soma was widely detected in the LPO / BNST and I selected three mice in which ChR2-eYFP expression were confirmed to be restricted within the LPO area and BNST (Fig. 6.2). I then looked by immunostaining for the presence of ChR-YFP positive fibres (axons) in the rest of the brain. The overall summary of ChR-YFP staining throughout the brain is shown in Figure 6.3. Overall there was little staining, except for a few prominent symmetrically stained fibre tracts and projection sites. The principal projection sites were M1 motor cortex (Fig. 6.3

and 6.4), the tuberomammillary area (Fig. 6.3 and 6.5), the region of the locus ceruleus (Fig. 6.3 and 6.5), and the periaqueductal grey matter (Fig. 6.3 and 6.6).

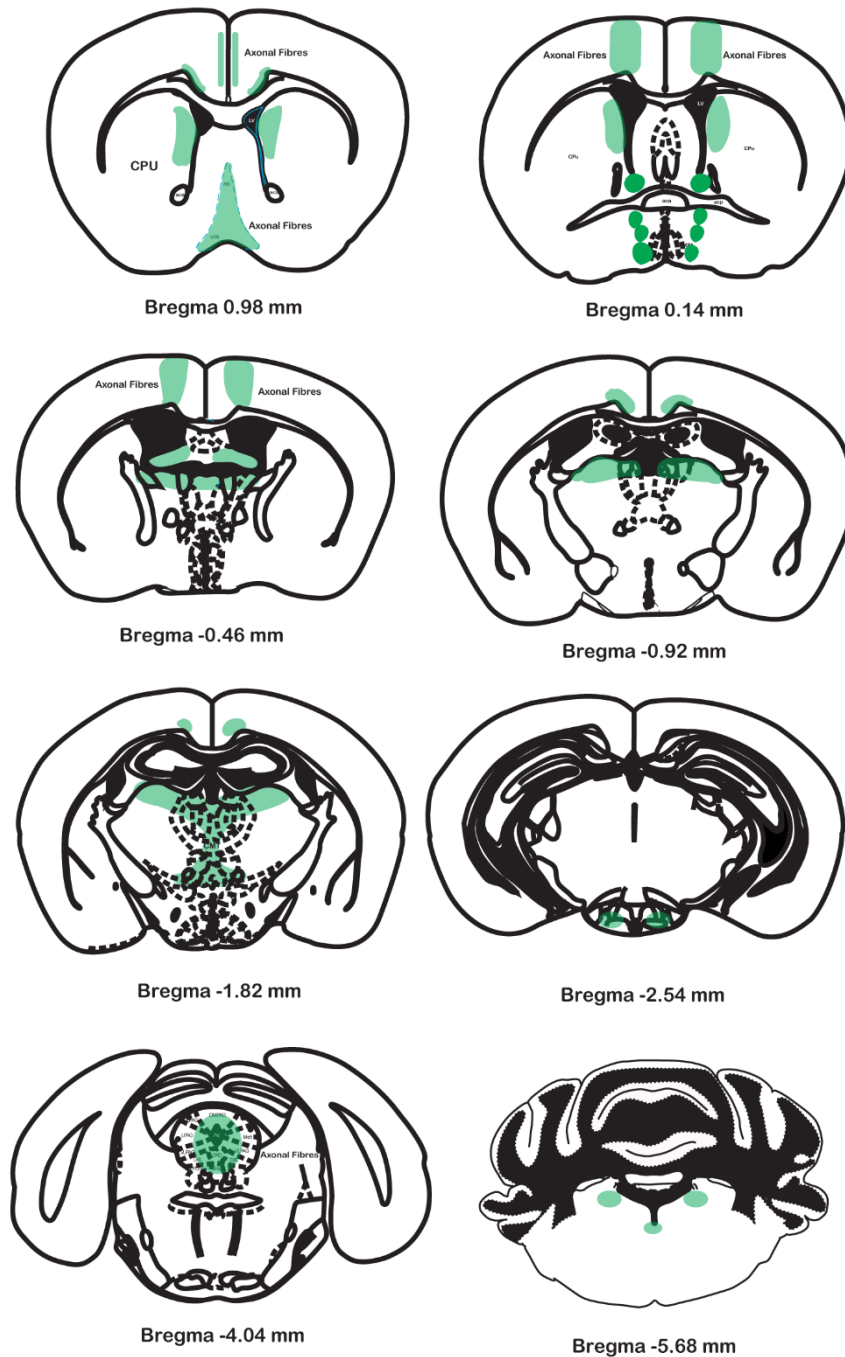


Figure 6-3 Schematic summary of ChR-YFP-positive fibres (light green) in LPO-TetTag-ChR-YFP mice following dexmedetomidine induce sedation. Groups of TetTagged activated cells in the injected area (LPO region) are indicated (green).

A striking and novel projection was a densely stained fibre bundle which seemed to ascend through the MnPO region (Fig. 6.4) and terminate bilaterally in the primary motor cortex (Fig. 6.4). The fibres seemed to enter through the outer layer of cortex and descend down to layers 5/6 (Fig. 6.3&6.4).

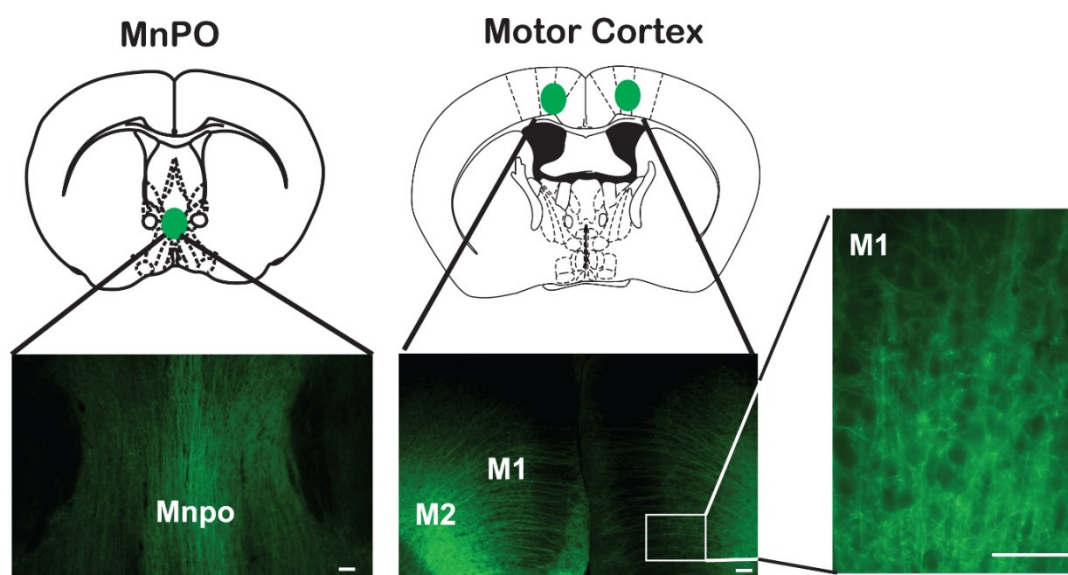


Figure 6-4 Following dexmedetomidine-induced sedation of LPO-TetTag-ChR-YFP mice, TetTagged ChR2-positive axonal fibres were found in the MnPO hypothalamus and motor cortex (M1). The right hand picture shows a dense plexus of TetTagged LPO fibres terminating in the deep cortical layers. Slices were stained with anti-GFP antibody. MnPO: median preoptic area. Scale bars: 200µm

The projecting fibres in the LPO-TetTag-ChR-YFP mice also projected to well-known arousal regions, including the tuberomamillary nucleus (Fig. 6.5) (TMN), locus coeruleus (LC) (Fig. 6.5) and the periaqueductal grey matter (PAG) (Fig. 6.6). For the LC, it was not clear if the fibres terminated in the LC itself or in a closely neighbouring area. We would need to co-stain with

tyrosine hydroxylase to mark out the LC neurons, but I ran out of time to do this experiment.

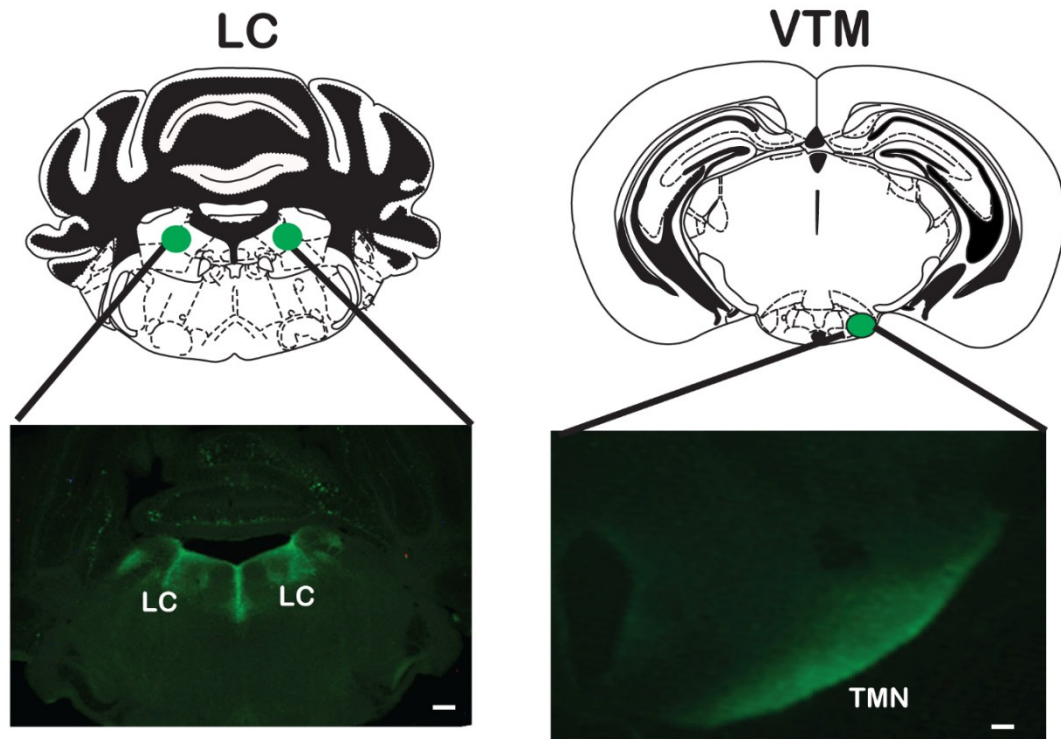


Figure 6-5 Following dexmedetomidine-induced sedation of LPO-TetTag-ChR-YFP mice, TetTaged ChR2-postive axonal fibres were found in the locus coeruleus and tuberomamillary nucleus. Slices were stained with anti-GFP antibody. LC: locus coeruleus VTM: ventral tuberomamillary nucleus Scale bar: 200 μ m

Periaqueductal gray (PAG)

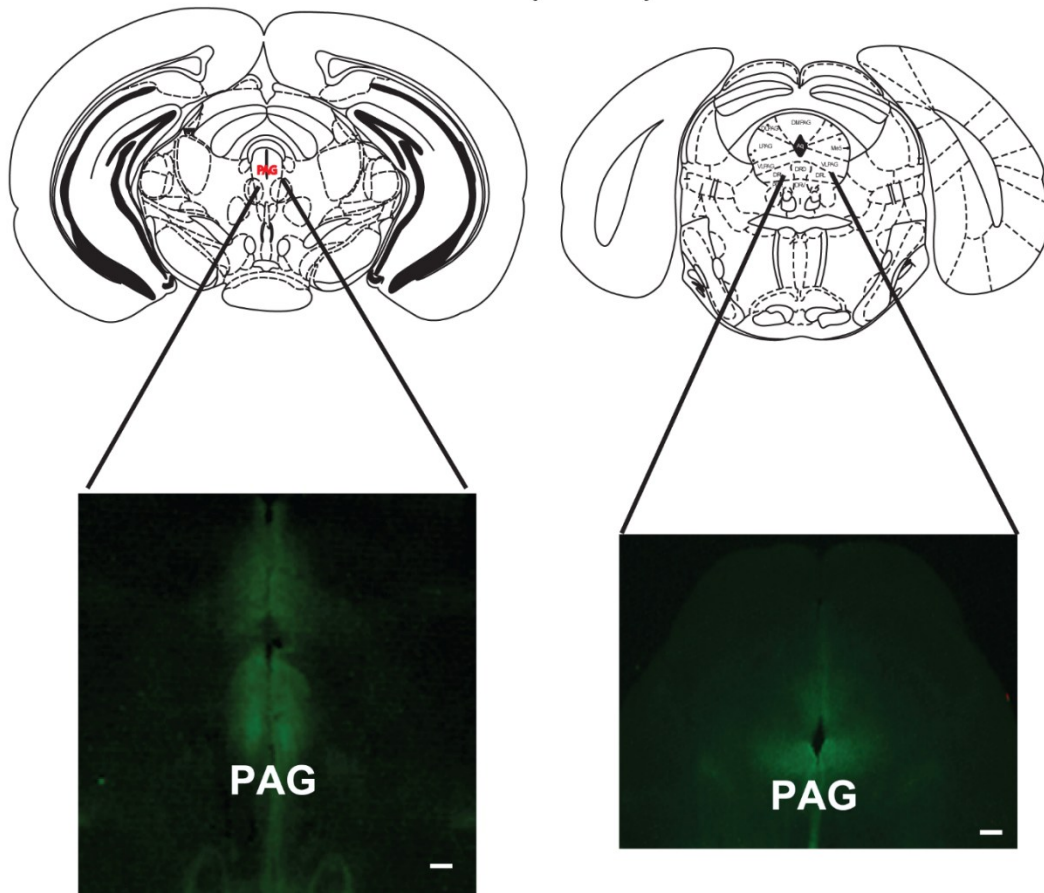


Figure 6-6 Following dexmedetomidine-induced sedation of LPO-TetTag-ChR-YFP mice, TetTagged ChR2-positive axonal fibres were found in the periaqueductal grey matter. Slices were stained with anti-GFP antibody. PAG: periaqueductal grey. Scale bar: 200 μ m

6.3 Discussion

In this Chapter I used TetTag-anterograde tracing with the ChR-YFP protein induced under the control of the c-fos promoter to determine the projecting axons of neurons which had been activated (disinhibited) by dexmedetomidine (Fig. 6.3). This

approach revealed interesting connections from disinhibited neurons in the LPO hypothalamic area to the rest of the brain. I have identified both new and established connections using this variant of TetTagging. In preliminary experiments, I found that dexmedetomidine-induced sedation produces only a few prominently labelled fibre tracts from the PO area (Fig. 6.3). One of these is a projection to the deep layers of the motor cortex M1. Strikingly, no other neocortical area receives TetTagged fibres. This seems to be a previously undescribed projection from the PO hypothalamus, and may be responsible for the inhibition of animal movement during sedation. It is highly likely to be GABAergic, but this requires verification. Future experiments could use optogenetics *in vitro* and *in vivo* in the motor cortex to test this idea and characterise the post-synaptic cortical neurons to see if we can elicit a behavioural response – perhaps motor freezing.

I also found the TetTagged ChR-YFP fibres in some arousal regions: the TMN, the LC and the periaqueductal grey (PAG). This fits with the known projections of the PO area to these sites (Saito et al., 2013; Saper et al., 2010). To see whether the activity of dexmedetomidine-induced neurons modulates the activity of these arousal projection sites, patch-clamp recordings can be used. For example, Saito and his colleagues revealed that using light to stimulate 10 Hz firing frequencies in PO neurons expressing ChR2 decreased the discharge rate of orexin neurons that these PO projections target (Saito et al., 2013). The TMN contains neurons which produce the wakefulness-promoting neurotransmitter, histamine. The “flip–flop switch” model states that the VLPO and TMN inhibit each other to stabilize the sleep-wakefulness

transition (see Chapter 1). Stimulation of PO inputs to the TMN can trigger the inhibitory postsynaptic potentials which can be abolished by GABA_A antagonists (Yang and Hatton, 1994). Similarly, several studies have found that the GABAergic neurons of the VLPO send inhibitory projection to the locus coeruleus (LC) and LC send the inhibitory signal back to the VLPO (Osaka and Matsumura, 1994; Sherin et al., 1998). During NREM sleep and REM sleep, there is increased inhibitory neurotransmitters release to the arousal region. Thus during dexmedetomidine induced-sedation, neurons in the LPO may also release GABA and galanin to the LC. The PAG contains wake-active dopaminergic neuron (Lu et al., 2006). The PAG may also inhibit glycinergic projections to regulate the sleep-wake state (Lu et al., 2006).

Chapter 7 Dexmedetomidine sedation and recovery sleep induce hypothermia that is differentially recapitulated by reactivating ensembles in LPO or MnPO

7.1 Introduction

Certain preoptic sleep-promoting neurons are temperature-sensitive (Bachtell et al., 2003; McKittrick, 2000; Szymusiak et al., 2007). Warming the preoptic area locally triggers sleep onset and maintains NREM sleep (Szymusiak et al., 2007). It is not known if the raised temperature activates the same neurons that participate in thermoregulatory circuitry, as well as sleep, but body temperature does tend to drop by one or two degrees during sleep. In addition, dexmedetomidine induces a sharp decrease in body temperature during sedation (Hunter et al., 1997). Using Tet-Tagging, I wanted to investigate if there is an overlap between sleep-promoting neurons and temperature-regulating neurons during recovery sleep and sedation. So in this Chapter I examine if the neural circuitries in the LPO and MnPO areas are sufficient to trigger sedative or recovery sleep-induced hypothermia. I TetTagged two new cohorts of mice, one with AAV injections into LPO and one with AAV injections into MnPO. I then determined the effects of dexmedetomidine sedation and recovery sleep on body temperature in both groups and determined if, in the TetTagged mice, CNO could recapitulate the effects.

The results in this Chapter have been published in Zhang et al., 2015. Ed Harding, another PhD student in the lab, helped me set up the initial experiments with the temperature loggers. The LPO-Tettag-hM3Dq and MnPO-Tettag-hM3Dq mice are described in Chapter4.

Results

7.2 Characteristics of natural NREM sleep and circadian body temperature of virally-injected C57BL/6 mice

I carried out experiments using a miniature data logger (DSTnano; Star-Oddi, Gardabaer, Iceland) that was implanted seven days previously in the peritoneal cavity of LPO-TetTag-hM3Dq and MnPo-TetTag-hM3Dq mice. Before any treatments, I checked the efficiency of the temperature loggers. We measured the body temperature throughout 24-hour sleep-wake cycle. The loggers were programmed to record every 30 minutes during 24-hour sleep recordings. The body temperature of the mice had a clear variation over 24 hours and tended to drop by one or two degree during sleep (Fig. 7.1B). The body temperature of the mice was higher during the dark period when they were most active (Fig. 7.1B); as for the sedation experiments reported in the Chapter4, all investigations of temperature were done during the dark period.

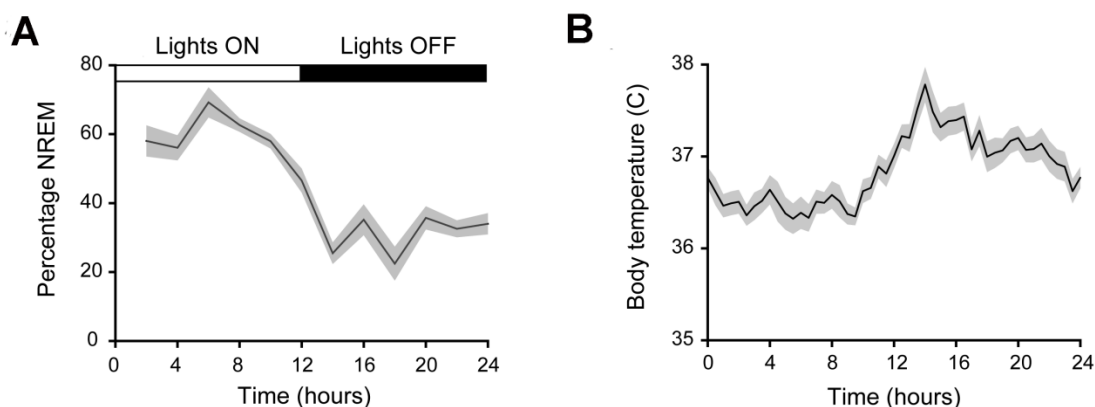


Figure 7-1 Characteristics of natural NREM sleep and circadian body temperature of LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice in their home cage. (A) Percentage of NREM sleep throughout the 24-hour sleep-wake cycle. (B) Body temperature throughout the normal 24-hour sleep-wake cycle. The envelopes represent the s.e.m. All of these data are for LPO-TetTag-hM3Dq (n=10) and MnPO-TetTag-hM3Dq mice (n=10) combined, because these were indistinguishable. All data are from 12 hours light: 12 hours dark cycles. Lighter shaded envelopes indicate the s.e.m.

Next I checked whether saline or CNO injection changes the body temperature of LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice that had not yet been subject to overt stimuli such as sleep deprivation and recovery sleep or dexmedetomidine-induced sedation. The temperature loggers were programmed to record every 2 minutes for experiments with saline or CNO injection. I found that neither saline nor CNO injections caused a change in body temperature (Fig. 7.2).

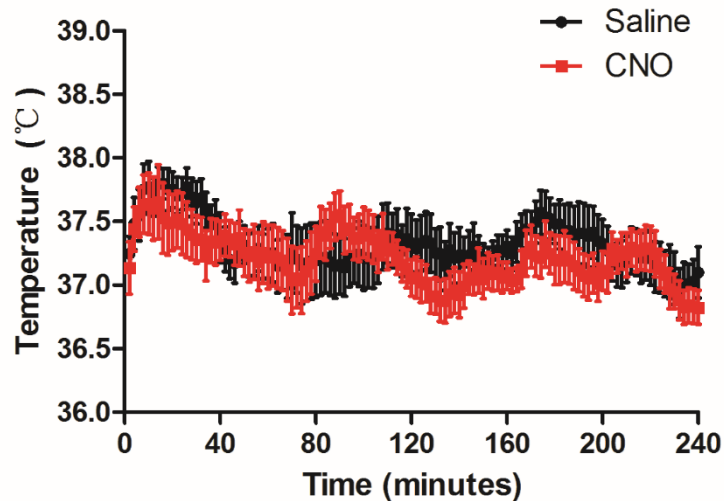


Figure 7-2 Changes in body temperature after saline or CNO injection into LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice. CNO (n=4) (red) or saline (N=4) (black) was *i.p.* injected. The temperature logger collected the data for four hours. There is no significant difference between these two groups. The error bar indicates the s.e.m.

7.3 Dexmedetomidine sedation and recovery sleep after sleep deprivation induce hypothermia that is differentially recapitulated by reactivating ensembles in LPO or MnPO

I next started to use TetTagging to examine if the neural circuitries in the LPO and MnPO areas were sufficient to trigger sedative or recovery sleep-induced hypothermia. I used the similar protocol as described in Chapter 4 (see in Fig4.5).

First, LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice were sleep deprived for four hours and allowed recovery sleep. The temperature loggers were programmed to record every 5 minutes during sleep deprivation and recovery sleep. At the end of sleep deprivation, mice were allowed recovery sleep in their home cage

and went into NREM quickly (e.g. Fig.4.5). Sleep deprivation elevated their body temperature to about 38°C, but during the first few hours of recovery sleep this temperature fell to that occurring during natural NREM sleep, about 36.5°C (Figs. 7.1 and 7.3A). Three or four days later, mice were given CNO. In both the LPO- and MnPO-TetTag-hM3Dq groups, CNO treatment produced a substantial drop in body temperature (Fig. 7.3B&C), comparable to, but somewhat larger than that seen during recovery sleep (Fig. 7.3A). Thus, as we found for the effects on delta power and immobility, both LPO and MnPO can contribute equal and parallel effects in producing hypothermia in recovery sleep after sleep deprivation.

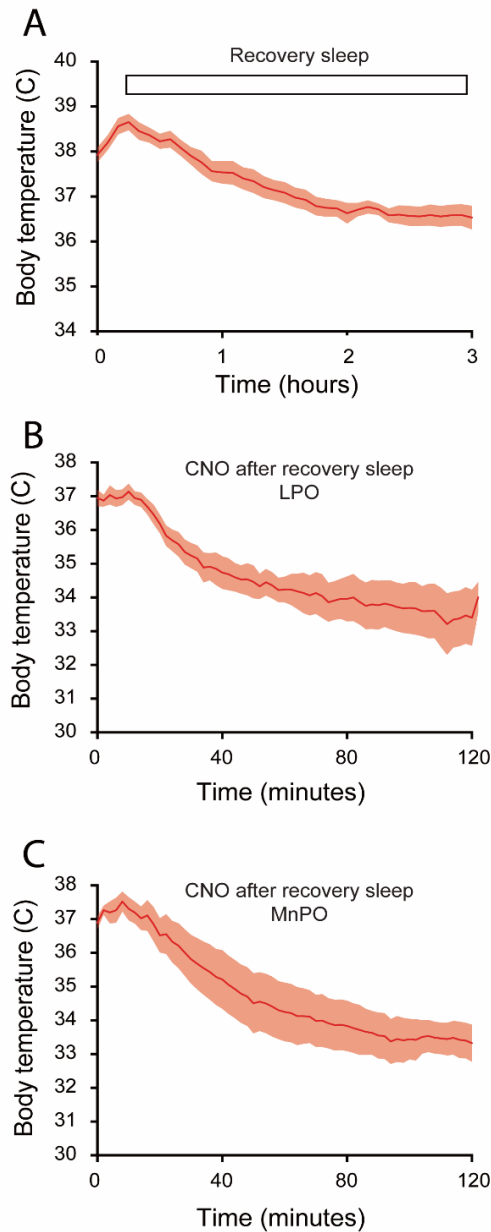


Figure 7-3 Hypothermia is recapitulated by reactivation of genetically tagged neuronal ensembles in LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice following recovery sleep. Each panel shows changes in body temperature following: (A) recovery sleep (n=10), (B) CNO reactivation after recovery sleep for LPO-TetTag-hM3Dq mice (n=6), and (C) CNO reactivation after recovery sleep for MnPO-TetTag-hM3Dq mice (n=5). The data in panels (A) is for LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice combined, because these were indistinguishable. Lighter shaded envelopes indicate the s.e.m.

A parallel group of LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice were subjected to dexmedetomidine-induced sedation. Two days after doxycycline removal, the LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice were given the sedative dose of 100 µg/kg dexmedetomidine. This caused a strong hypothermia (Fig. 7.4A), consistent with previous reports (Hunter et al., 1997; MacDonald et al., 1991). The body temperature fell to about 25°C within two hours. The mice were then placed back onto a doxycycline diet for four days, and after this period they were injected with CNO *i.p.* In the LPO-TetTag-hM3Dq animals, CNO reactivation of the dexmedetomidine-induced hypothalamic ensembles largely recapitulated the temperature drop (Fig. 7.4B). The body temperature dropped nearly seven degrees within two hours. There was, however, little effect in MnPO-TetTag-hM3Dq mice (Fig. 7.4C). The body temperature dropped around three degrees in two hours, which is also bigger than the normal circadian changes.

Thus following dexmedetomidine sedation, activated neuronal ensembles in LPO, but not MnPO, are responsible for the hypothermia produced by this drug.

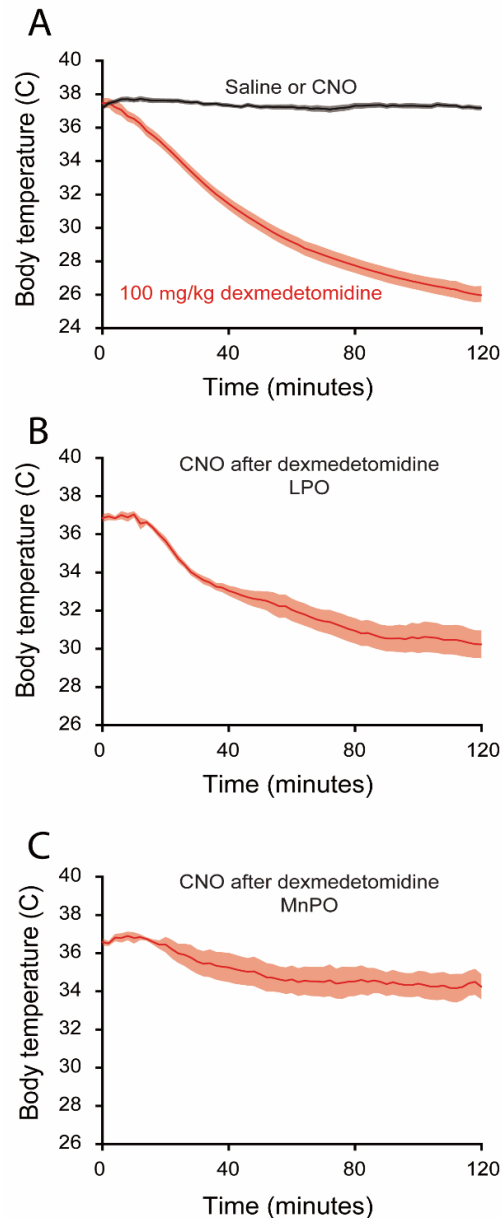


Figure 7-4 Hypothermia is recapitulated by reactivation of genetically tagged neuronal ensembles in LPO-TetTag-hM3Dq but not MnPO-TetTag-hM3Dq mice following dexmedetomidine-induced sedation. Each panel shows changes in body temperature following: (A) dexmedetomidine sedation (n=10) (red) or saline (n=20), (B) CNO reactivation after dexmedetomidine sedation for LPO-TetTag-hM3Dq mice (n=5), (C) CNO reactivation after dexmedetomidine sedation for MnPO-TetTag-hM3Dq mice (n=5), The data in panels A) is for LPO-TetTag-hM3Dq and MnPO-TetTag-hM3Dq mice combined, because these were indistinguishable. Lighter shaded envelopes indicate the s.e.m.

7.4 Discussion

The thermoregulatory centre of human and other mammal animals is located in the PO area of the hypothalamus (Szymusiak et al., 2007). The preoptic area receives the input from the peripheral thermosensory neurons, and also controls the local brain temperature as a central thermosensor. Sleep-active and temperature-sensitive neurons are widespread in the PO area (Alam et al., 2014; Szymusiak et al., 2007; Takahashi et al., 2009).

As expected, I found that the body temperature of the mice changed over the 24 hour period and also after sleep deprivation. Sleep deprivation increases c-Fos expression in neurons of the PO area; these activated neurons might regulate temperature changes, but there is no direct proof of this, only the correlation with c-fos activation (Nelson et al., 2002; Peterfi et al., 2004). The numbers of cFos-positive neurons increases in the LPO and MnPO both during cold and warm stimuli in rats (Wang et al., 2014), and these neurons are probably involved in thermoregulation in response to different stimuli.

For the TetTagging experiments, there was an interesting difference between LPO and MnPO in regard to temperature regulation. By artificially reactivating the neurons initially activated by a systemic low-dose of dexmedetomidine, I found that LPO neurons are sufficient to recapitulate the strong hypothermia. I also found that reactivating the same or similar groups of neurons in the LPO also produced a drop in body temperature after sleep deprivation. The hypothermia effect was also found in the MnPO: whereas MnPO could recapitulate the temperature decrease seen in recovery sleep,

no ensembles that regulate temperature were activated in MnPO by dexmedetomidine sedation.

Are sleep and hypothermia interdependent? As has been previously suggested, temperature changes in animals and humans correlate with changes in EEG frequencies. The drop in temperature actually may be the reason for sleep, for example to allow metabolic recovery (McGinty and Szymusiak, 1990). After the end of the daily torpor-associated hypothermia, there is an increased NREM sleep in the Djungarian hamster (Palchykova et al., 2002). The increase in NREM sleep is positively correlated with torpor duration (Deboer and Tobler, 2003). Alternatively, the reduced brain temperature might help maintain sleep. In future it will be interesting to disentangle the effects of sleep onset and body temperature decrease. Using Cre-driver lines so that we can stimulate subsets of Cre-activated neurons, for example Galanin-Cre, future experiments could determine if the same PO hypothalamic neurons command both sleep onset and the body temperature decrease. The type of TetTagging experiments using the AND and NOT gates (Mayford – and see Chapter 4 Discussion, Fig 4.24) could also be undertaken to distinguish if the same neurons control both sleep onset and the temperature decrease.

Chapter 8 Conclusions and some further questions

8.1 Conclusions

In this thesis, I explored whether the deep sedation imposed by dexmedetomidine, and the deep recovery sleep from sleep deprivation require similar neural circuitry and were actually the same state. For this, I used a TetTag pharmacogenetic analysis, which has been highly useful in understanding memory formation in the hippocampus (Garner et al., 2012). I first confirmed that dexmedetomidine-induced sedation and recovery sleep induced the expression of the native c-fos gene in the PO area (Chapter 3). I then set up the c-fos based TetTagging system in a dual AAV system (Chapter 2 methods and Chapter 4). Using this system, I genetically recorded activated neuronal ensembles in the PO hypothalamic area following sleep deprivation or dexmedetomidine-induced sedation in the same animals, and then allowed these “tagged ensembles” to be selectively reactivated later using the DREADD (hM3Dq) system and its ligand CNO (Alexander et al., 2009; Garner et al., 2012) (Chapter 4). In both cases reactivating the “ensemble” produced NREM sleep and hypothermia (Chapter 4 and Chapter 7) (Zhang et al., 2015). This demonstrates that this PO circuitry is sufficient to sense and respond to the stimuli that trigger deep sleep and the associated hypothermia, and that drug-induced sedation and recovery sleep appear to be the same state (Zhang et al., 2015). The process seems to require GABAergic neurons for its initial induction, because

LPO-Δvgat mice were not initially sedated by dexmedetomidine, although eventually they succumbed (Chapter 5). If I had more time, I would have developed the TetTag-ChR-YFP approach to functionally map the TetTagged connections from the LPO area activated following sedation (Chapter 6). In particular, one intriguing Tet-ChR-YFP projection, likely GABAergic, seemed to project selectively to the motor cortex.

8.2 Examples of further questions that could be addressed with TetTagging

In this section, I outline various extensions of the TetTag method which could be used to expand our knowledge of the circuitry involved in sleep and sedation.

8.2.1 Identification of the inputs to the sleep-active or sedative-active PO neurons

Valentina Ferretti in our lab showed that a knockdown of *adra2a* transcripts in the LPO did not alter the sedative effects of 100 µg/kg dexmedetomidine (Fig4.23 Zhang et al., 2015). The explanation for our results could be that adrenergic $\alpha 2$ agonists preferentially inhibit local GABAergic interneurons. These, in turn, would then inhibit sleep-active GABAergic projection neurons less. Alternatively dexmedetomidine could activate inhibitory $\alpha 2A$ receptors on the terminals of inhibitory afferents coming into the PO area, for example GABA inputs or NA inputs from nuclei other than the LC. The reduced local release of GABA or NA into the PO

hypothalamus would then allow dis-inhibition and excitation of the sleep-promoting neurons.

To look at inputs to the sleep-active or sedative-active PO neurons, we could do selective retrograde mono-trans-synaptic tracing of TetTagged PO neurons with rabies virus (Miyamichi et al., 2011; Wickersham et al., 2007). This approach requires a transgene with a triplet of proteins that results in only TetTagged neurons being able to synthesize and package new rabies virus. This will result in rabies-mCherryG virus moving across synapses to label neurons upstream of the TetTagged (activated) PO cells. Identifying these upstream cells would help us find the inputs whose changed activity is likely to cause the dis-inhibition or excitation of the PO neurons. Such upstream cells could be local interneurons or some GABAergic projections from other nuclei.

8.2.2 Identification of the projections of PO neurons which are activated during recovery sleep or drug-induced sedation

To look at the outputs from the activated PO neurons, we could use TetTagging with ChR-EYFP to anterograde map their projection fields as outlined in Chapter 6. We could employ serial two-photon tomography to map out these TetTag-induced ChR-EYFP projections throughout the brain (Ragan et al., 2012). Serial two-photon tomography tracings of TetTag ChR-EYFP fibres will reveal if the neurons that send a projection to, for example the motor cortex, also project to hypothalamic areas regulating body temperature. These terminals could be stimulated *in vitro* and *in*

in vivo with blue light to learn more about their transmitters and the behaviours they control.

8.2.3 Identification of the receptors or channels required on sleep-active PO neurons to produce and maintain recovery sleep or drug-induced sedation

In Chapter 5, I found that GABA was needed for the induction of sedation, but not its maintenance. Perhaps galanin or another co-messenger is required for maintenance of sleep? Thus we need to be selective in identifying the cells activated during TetTagging and knock out those neurons to test the hypothesis.

We could use TetTagging in the PO hypothalamus during recovery sleep or $\alpha 2$ -induced sedation to induce destabilized-Cre, which is Cre with a peptide tag that causes rapid degradation of the protein (Sando et al., 2013); this degradation can be prevented by *i.p.* injection of the antibiotic trimethoprim, which rapidly crosses the blood brain barrier to stabilize and activate the Cre and allow it to ablate candidate (floxed) genes (Sando et al., 2013). The TetTagging–DD-Cre procedure will take place in various strains of floxed-allele mice, based on expression profiles of candidate channels/receptors in the PO area. We will determine how these mice with receptors selectively deleted from sleep-active neurons subsequently respond to $\alpha 2$ agonists or sleep deprivation. We could also see if the receptors/channels that were deleted are required for subsequent natural sleep.

8.2.4 Is there feedback from the neocortex to the hypothalamus signalling the need to sleep?

Sleep maybe especially needed for regenerative processes in the neocortex (Tononi and Cirelli, 2014; Vyazovskiy and Harris, 2013). As wakefulness progresses, the neocortex may tire and signal the PO hypothalamus that sleep should be activated. Intriguingly, subsets of neocortical interneurons express cFOS during recovery sleep after sleep deprivation; homeostatic sleep pressure is a primary driver for this (Dittrich et al., 2015). These interneurons, and their upstream partners, may form an uncharacterized feedback circuit. TetTagging, starting with the hM3Dq receptor, and activation with CNO, and anterograde mapping with TetTag-ChR-YFP would allow an exploration of their role.

8.2.5 Why do we wake up?

Another important issue is to understand why sleep-active neurons stop firing so that wakefulness takes over. Waking has a circadian influence, but it could also reflect the dissipation of the ill-defined “sleep drive”. In fact, the classical distinction between homeostatic and circadian sleep drives (see Chapter 1, section 1.3, for a description) may be false, since most neurons could have local clocks. Are sleep-active neurons using local clocks that measure something that inhibits or activates them? Recently, our laboratory demonstrated a local circadian clock in the brain, controlled by the clock protein BMAL1, which regulated histamine synthesis (Yu et al., 2014). Could BMAL1 in sleep-active neurons be involved in timing the duration of their firing? Using *bmal1^{lox/lox}* mice we could

use recovery sleep to induce TetTag-destabilized-Cre(Sando et al., 2013) in sleep-active PO neurons. This system would allow the selective ablation of the BMAL1 gene in sleep-active neurons. Would the abolition of this putative local clock affect how long these animals sleep after subsequent sleep deprivation or sedative administration?

8.2.6. Do all types of sedatives work through the PO area to induce sedation?

In this thesis I investigated one class of sedative, $\alpha 2$ adrenergic drugs. However, there are many more types of sedative drugs acting through different receptor classes. We have the allosteric potentiators of GABA-A receptors, such as benzodiazepines, zolpidem, etomidate and propofol. Benzodiazepines and zolpidem reduce the time to sleep onset, but tend to reduce NREM delta power, whereas etomidate and propofol induce powerful NREM, but at light doses they are sedatives rather than anaesthetics(Baker et al., 2014; Franks, 2008; Winsky-Sommerer, 2009). Then there are, for example, histamine H1 receptor antagonists, and orexin receptor antagonists. Do all of these sleep inducing drugs act through a final common pathway? TetTagging as applied in Chapter 4 could answer this. Indeed, in some preliminary experiments performed as I was writing up this thesis, I have already found using LPO-TetAtg-hM3Dq mice that etomidate can induce sedation (NREM sleep) by acting through LPO. I could induce sedation in LPO-TetAtg-hM3Dq mice with etomidate, and then later recapitulate this sedation with CNO injection.

8.2.7 What is the relative importance of the PO area and the recently identified parabrachial region for sleep induction and maintenance?

TetTagging could be deployed to explore the relative importance of other sleep-active neurons and nuclei (see Fig 1.4, in Chapter 1). Clearly we need to understand how they all link together.

8.3 Closing words

The above questions are just examples of the many possible applications of TetTagging to the study of neural circuitry regulating sleep and sedation. I would like to close this thesis as I started Chapter 1, by reflecting that it is still amazing that we do not yet know the reason for sleep, yet we have to go to sleep every day, and if we go without sleep the pressure builds until we have to sleep. The kind of TetTagging experiments described in this thesis could eventually reveal something about the physiological drive to sleep and may even eventually explain why we sleep.

Investigating the pathways activated by sedative drugs could lead to improved experimental drug design for clinical treatment of sleep disorders.

Chapter 9 Reference

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