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Zebrafish Sleep: From GeneZZZ to NeuronZZZ

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ABSTRACT

All animals have a fundamental and unavoidable requirement for rest, yet we still do not fully understand the processes that initiate, maintain, and regulate sleep. The larval zebrafish is an optically translucent, genetically tractable model organism that exhibits sleep states regulated by conserved sleep circuits, thereby offering a unique system for investigating the genetic and neural control of sleep. Recent studies using high throughput monitoring of larval sleep/wake behaviour have unearthed novel modulators involved in regulating arousal and have provided new mechanistic insights into the role of established sleep/wake modulators. In addition, the application of computational tools to large behavioural datasets has allowed for the identification of neuroactive compounds that alleviate sleep symptoms associated with genetic neurological disorders.

INTRODUCTION

Work over the past 15 years has demonstrated that sleep is evolutionarily conserved across the animal kingdom, indicating that sleep serves an essential, possibly universal, function [1*]. Moreover, the negative impact that sleep disruption has on immune, metabolic, cardiac and cognitive health demonstrates sleep's critical role in optimising daily behaviour and general physiological well-being. Many molecular and neuronal control systems are in place to regulate sleep's timing and duration; however, we are still discovering these systems and their rules. Two major discoveries have spearheaded advances in the field. First, the discovery that the human sleep disease, narcolepsy, is a dysfunction in hypocretin/orexin signalling demonstrated that small populations of peptidergic neurons can have profound consequences on human and animal sleep [2]. Second, the recognition that genetically tractable non-mammalian species exhibit sleep states has expanded the models available to sleep researchers and have facilitated screens for sleep mutants [3].

The study of sleep regulation in zebrafish larvae has taken up a unique model system niche, as they offer a sophisticated genetic toolkit coupled with the ability to monitor and manipulate the activity of conserved sleep/wake neurons *in vivo* (see Box 1). In this review, we discuss recent insights into sleep in this diurnal vertebrate and highlight novel methods that use the special properties of the zebrafish model.

MONITORING SLEEP AND AROUSAL IN LARVAL ZEBRAFISH

To enable relatively high-throughput monitoring of sleep/wake states in zebrafish larvae, the animals are tracked by videography in a 96-well plate format for several days on either a 24-hour light-dark cycle or on constant illumination (Figure 1a). Larval and adult zebrafish are diurnal and at night exhibit an increased number and duration of inactive bouts (Figure 1b), which have a typical duration around three minutes but can last for hours [4]. Quiescent bouts lasting at least one minute fulfill the major criteria used to behaviourally define sleep in non-mammalian systems, including an increased arousal threshold to multimodal sensory stimuli [5–7]. Larval sleep is also under circadian and homeostatic control—larvae exhibit both near-24 hour sleep/wake rhythms in constant conditions as well as rebound sleep following mechanical deprivation [5]. Since pharmacological and genetic interventions can selectively perturb components of waking levels and sleep bout structure, larval sleep and arousal are likely controlled by distinct mechanisms.

There are some limitations to using behavioural criteria as a proxy for sleep state. In particular, zebrafish sleep has not yet been further sub-divided, for example into rapid eye movement (REM) and non-REM (NREM) states as observed in mammals, birds, and recently, reptiles [8**], or into deeper and lighter stages of sleep, as suggested using systematic arousal threshold responses in *Drosophila* [9]. The use of more fine-grained analysis of locomotor structure, including detailed bout kinematics and head position [10] coupled with a more expansive probing of stimulus-evoked behaviour across the 24-hour light-dark cycle [11], may reveal dynamic changes in arousal states that could imply variable sleep stages in larvae. Leveraging the ability to functionally image whole-brain activity in zebrafish larvae will also give more insight into how behavioural readouts relate to brain state. In freely swimming larvae, locomotor activity across the day correlates with the activity of wake-promoting hypocretin neurons [12], but correlation of behaviour to activity of sleep-active neurons has not yet been achieved. Identifying neuronal correlates of sleep during drug-induced and natural sleep states in a fictive locomotion preparation may be especially useful in probing brain state switching, similar to that achieved for the neural control of locomotor modules during spontaneous and visually-evoked navigation [13*–15].

NEUROCIRCUITRY OF SLEEP IN ZEBRAFISH

Classical and modern lesion studies in mammals highlighted numerous brain areas, including the basal forebrain, posterior hypothalamus, reticular formation, and hindbrain as critical for the regulation of arousal and sleep [16–18]. More recently, systematic searches for sleep/wake regulatory neurons using chemo- and optogenetic modulation of putative sleep/wake-inducing neurons are more precisely defining many key subpopulations as well as unravelling the complex dynamics at play among neurons that control brain state switching [19*,20]. Initial zebrafish studies focused on demonstrating the genetic, molecular,

pharmacological, and neuronal conservation of these mammalian-defined sleep-regulatory systems and are now beginning to reveal novel insights into sleep control in vertebrates.

Wake-promoting

Hypocretin

The arousal promoting peptide, Hypocretin/Orexin (Hcrt), is conserved in zebrafish [6,21–23] and is expressed by approximately 10 pairs of bilateral, glutamatergic neurons in the anterior hypothalamus at 5 days post fertilisation (dpf). Unlike mammals, which have two Hcrt receptors (HcrtR1 and HcrtR2), zebrafish possess a single Hcrt receptor (HcrtR) that is most similar to HcrtR1 [21]. Hcrt neurons send widespread projections to dopaminergic neurons in the diencephalon and norepinephrine (NE) neurons in the locus coeruleus (LC) in the larval brain. By adulthood Hcrt neurons project to other components of the ascending arousal system where HcrtR is also detected, including the serotonergic neurons of the dorsal raphe and the histaminergic neurons of the tuberomamillary nucleus (Figure 1c) [22].

Consistent with the neuroanatomical evidence, genetic studies indicate that Hcrt is wake-promoting in zebrafish. Genetic overexpression of Hcrt increases locomotor activity and decreases rest bout length [6] while also sensitizing larvae to changes in illumination (but not to other modes of stimulus-induced arousal) [11]. Similarly, optogenetic (with channelrhodopsin) and chemogenetic (with capscasin-sensitive TRPV1) activation of Hcrt neurons is sufficient to induce wakefulness and reduce sleep in a HcrtR-dependent way [7,24]. As in mammals, either genetic elimination of the Hcrt receptor or ablation of Hcrt neurons causes sleep and activity fragmentation in both adult and larval zebrafish [23,25].

Analysis of the neuronal targets of Hcrt neurons in zebrafish adds to our understanding of Hcrt neurons as integrators of multiple pathways involved in energy balance. For example, Hcrt neurons project to the pineal gland (as in mammals), which secretes the sleep-inducing molecule, melatonin. Perhaps this circuit component is important for maintaining sleep consolidation in addition to Hcrt's role in wake maintenance [26,27]. In mammals, Hcrt also has been implicated in regulating the interplay between arousal and feeding [28]. In zebrafish, Hcrt neurons project to and make functional connections with HcrtR-expressing gonadotropin releasing hormone 3 (GnRH3) neurons, which are involved in regulating energy status and sexual maturation in vertebrates [29]. In an HcrtR-dependent manner, Hcrt hyperpolarises and inhibits the firing of GnRH3 cells, thus providing the first evidence for a role of Hcrt signalling in feeding and sexual maturation in zebrafish [30].

Finally, transcriptional profiling of zebrafish Hcrt neurons has identified several conserved molecular components of these neurons' development and function. For example, the Hcrt-enriched transcription factor, Lhx9, is both necessary and sufficient for Hcrt neuron specification in mice and zebrafish, as genetic overexpression of *lhx9* promotes ectopic

differentiation of hypocretin neurons in zebrafish and mouse hypothalamus [31]. This is consistent with the genetic ablation of Lhx9 in mice, which results in hypersomnolence [32]. In another study, transcriptional profiling of isolated zebrafish Hcrt neurons identified the repolarising potassium channel Kcnh4a as enriched in these cells. *kcnh4a* mutant zebrafish are slightly hyperactive and have disrupted sleep/wake architecture at night, as would be expected if Kcnh4a intrinsically modulates the excitability of wake-promoting Hcrt neurons. This study expands the important roles of K+ channels in modulating sleep/wake circuitry, as has previously been shown in *Drosophila*, zebrafish, and mice [33**–35].

Norepinephrine

Norephinephrine (NE) neurons of the locus coeruleus (LC) have been shown by pharmacological and optogenetic methods to be important regulators of arousal in both mammals and fish [36,37]. In contrast to mice, however, genetic knockout of the NE synthesising enzyme dopamine-beta-hydroxlase (Dbh) in zebrafish is viable, allowing for more detailed studies of NE signalling in mediating arousal. *dbh*$^{-/-}$ larvae have reduced activity and increased daytime sleep, but they are also more sensitive to mechanoacoustic stimuli, indicating that NE could also modulate arousal threshold [7]. However, dopamine is the biogenic precursor to NE and so this does not preclude the role of increased dopamine levels in modulating arousal threshold in *dbh*$^{-/-}$ larvae.

As in mammals, NE neurons of the locus coeruleus in zebrafish are stimulated by Hcrt (Figure 1c) [7,36]. Furthermore, Hcrt-induced arousal is strongly blunted in *dbh*$^{-/-}$ larvae, demonstrating clearly that NE itself is a critical output effector of wake-promoting Hcrt signals [7]. This work lays the groundwork for more detailed analyses of other circuit components downstream of Hcrt neurons.

Neuromedin U

The first genetic overexpression screen for sleep and arousal regulators in a vertebrate identified the neuropeptide Neuromedin U (Nmu) as a potent wake-promoting factor across the day:night cycle in zebrafish [38*]. Zebrafish *nmu* mutants show reduced locomotor activity and also have reduced body size as adults, indicating that, like Hcrt, Nmu may coordinate behaviour and metabolism. Genetic evidence indicates that Nmu mediates arousal through only one of three zebrafish Nmu receptors, NmuR2, while adult size is modulated through signalling via NmuR1. In mammals, Nmu was thought to exert its influence on arousal through stimulation of the glucocorticoid hypothalamic-pituitary-adrenal (HPA) stress axis, but zebrafish glucocorticoid receptor mutants respond normally to Nmu overexpression. Instead, a set of corticotropin releasing hormone (Crh) neurons in the brainstem respond to Nmu (Figure 1c), while pharmacological blockade of Crh receptor signalling blocks Nmu-mediated

arousal in larvae. This study therefore not only identified Nmu as a modulator of vertebrate sleep/ wake states, but also mapped this activity onto a set of neurons not previously implicated in vertebrate arousal. Whether a similar Nmu-regulated circuit exists in mammals has not yet been investigated.

Sleep-promoting

Melatonin

An understanding of melatonin's role in coordinating the circadian clock and the onset of sleep has always been complicated by the fact that its levels peak at night in both diurnal and nocturnal animals. In zebrafish, exogenous melatonin is a potent hypnotic, but its endogenous function was unclear until recently [5]. Zebrafish larvae that lack melatonin either due to a genetic lesion in the melatonin-synthesising enzyme (*aanat2*) or ablation of the melatonin-producing pineal gland have clock-independent reductions in sleep at night. However, both molecular whole-body and locomotor activity rhythms persist in both *aanat2* mutants and in animals that have their molecular clocks ablated in the pineal gland [39*,40]. Thus, melatonin is likely a specific sleep output signal downstream of the circadian clock. *aanat2*$^{-/-}$ animals are also more sensitive to the soporific effects of adenosine receptor agonists, thus providing an intriguing possible mechanism to link the circadian (via melatonin) and homeostatic (via adenosine) regulatory arms of sleep [39].

QRFP

QRFP is a member of the highly conserved RFamide family of neuropeptides and has been implicated in feeding and locomotor activity in rodents, with hyperlocomotion induced upon peptide injection in mice [41]. In zebrafish, expression is localised to a small number of glutamatergic neurons in the hypothalamus adjacent to the Hcrt neurons (Figure 1c), with widespread projections into the hypothalamus and midbrain [42]. In contrast to the rodent studies, genetic overexpression of QFRP in zebrafish larvae specifically promotes daytime sleep and decreases activity independently of the circadian clock. Conversely, zebrafish harbouring mutations in either *qrfp* or double mutants that lack the QRFP receptors Grp103a and Grp103b are more active than wild type siblings. This may represent another example in which nocturnal and diurnal animals have conflicting responses to behaviourally relevant signals, although a developmental role for QRFP in rodent bone formation has limited genetic analysis of behaviour [43].

SLEEP, DISEASE, AND PREDICTIVE PHARMACOLOGY

Many neuropsychiatric and neurodevelopmental disorders, including depression, schizophrenia, Alzheimer's disease, and autism, are associated with sleep disturbances

[44,45], but it remains unclear in most cases to what extent sleep disruption contributes to disease severity and progression. Several studies have now identified sleep and arousal endophenotypes in zebrafish genetic models of disease. For example, a zebrafish model of the most common cause of mental retardation, Fragile-X syndrome, shows modest hyperlocomotion, especially during light/dark transitions [46]. Imaging studies revealed that *fmr1* mutant larvae have increased branching of cholinergic and glutamatergic mechanosensory and motor neurons, although it is not clear if this phenotype causes the hyperactivity. Dravet syndrome, a form of paediatric epilepsy caused by mutations in the voltage-gated sodium channel gene *SCN1A*, has also been modelled in zebrafish by *scn1Lab* mutations. Mutant *scn1Lab* zebrafish larvae exhibit spontaneous seizures, evident both behaviourally and neurologically. Two small molecule screens found the anti-histamine, clemizole, and the serotonin agonist, fenfluramine, as attenuators of seizure, although this may be due to generalized sedation rather than a selective rescue of epilepsy [47,48].

The complexity of larval zebrafish sleep/wake behavioural dynamics coupled with the diversity of response profiles (behavioural fingerprints) found in previous screens of more than 5000 small molecules affords the possibility of making informed predictions of drug-genotype interactions by comparing mutant behavioural fingerprints to the complete drug panel [34]. This form of 'predictive pharmacology' unbiasedly identified estrogenic compounds as selective, non-sedating rescuers of the night-time hyperactivity in zebrafish mutants harbouring mutations in the autism-risk gene, *cntnap2*. Given the sexual dimorphism in autism susceptibility, and the proposal of the female protective hypothesis [49], using zebrafish imaging techniques to identify estrogen/autism risk gene sensitive circuits responsible for night-selective arousal may provide insight into how sleep circuits are disrupted in autism [50**].

CONCLUDING REMARKS

Studies into the genetic and neural components involved in regulating evolutionarily conserved behaviours such as sleep have profited from advances in zebrafish genetic and imaging tools. Validation of conserved network modules involved in regulating sleep and wake in zebrafish has paved the way for genetic and neuropeptide screens that have identified novel modulators of vertebrate sleep that are likely to be relevant in mammals. Moving forward, studies that combine *in vivo* neuronal imaging with advanced locomotor kinematic analysis of the larval zebrafish will provide detailed insights into zebrafish brain states and the regulatory neurocircuitry involved. Finally, the analysis of sleep/wake stages in genetic models of disease can be combined with targeted pharmacological screens for behavioural modulators to elucidate links between drug targets, neuronal circuits, and behaviour.

Figure Legend

Figure 1 a) Videography for monitoring activity of up to 96 larvae simultaneously. Larvae are placed into individual wells of a multi-well plate and illuminated by white and infrared lights to allow tracking of larvae over a 14:10 light:dark schedule. b) Representative activity (i) and sleep (ii) plots for an individual 6-7dpf larval zebrafish. c) Summary of neuronal nuclei known to modulate sleep/wake in larval zebrafish. Many interconnections have been validated by anatomical studies, while the Hcrt to LC connection was validated by functional imaging [7].

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BOX1: Zebrafish Toolkit:

Although the zebrafish was established as a model for the study of vertebrate development nearly 40 years ago, only in the past 15 years has it gained widespread use to study the genetic and neural basis of behaviour. This rise in popularity is based on three cornerstones that provide a unique toolkit that is well suited for the study of sleep regulation:

1. *High fecundity and rapid external development of free-swimming larvae.* Larvae exhibit overt sleep stages and other complex behaviours as early as 4dpf [2,3]. During these early larval stages, small molecules added to the water are readily absorbed and can access the brain, which lacks a fully developed blood-brain barrier [4]. The large number of offspring generated and ease of pharmacological manipulation has provided a platform for performing high-throughput screens for both drug and gene discovery [5,6].
2. *A fully sequenced genome and conserved genetics.* Up to 80% of disease-associated human genes have a clear ortholog in zebrafish, and most genes that have been implicated in mammalian sleep are conserved in fish [7]. Genome editing techniques, like Crispr/Cas9, facilitate targeted mutant generation, while transgenic techniques enable labelling and controlling neurons [8].
3. *Optical translucency of the larval zebrafish brain.* The small number of neurons (about 100,000) in the 6dpf larva facilitates *in vivo* whole brain functional imaging and targeted neuronal manipulations [9]. Structures of particular relevance for the study of sleep are the hypothalamus and brainstem, which are well conserved between fish and humans, including many subpopulations that are relevant for sleep regulation.

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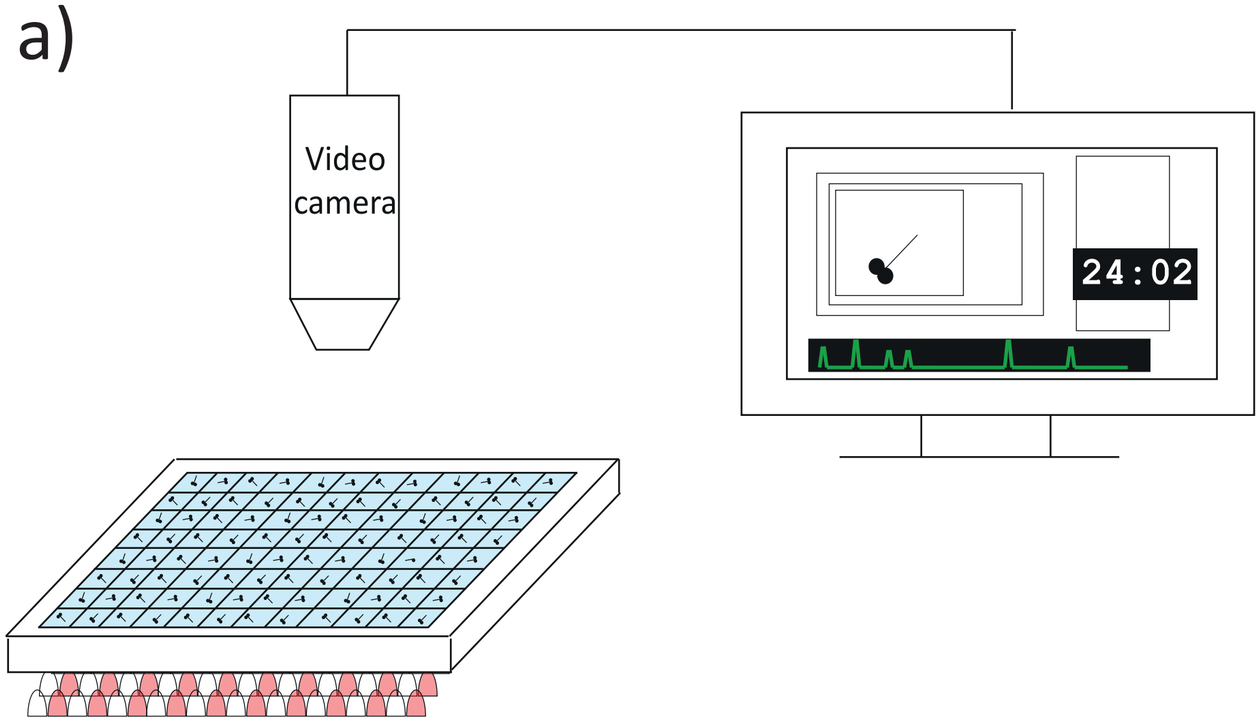
a)



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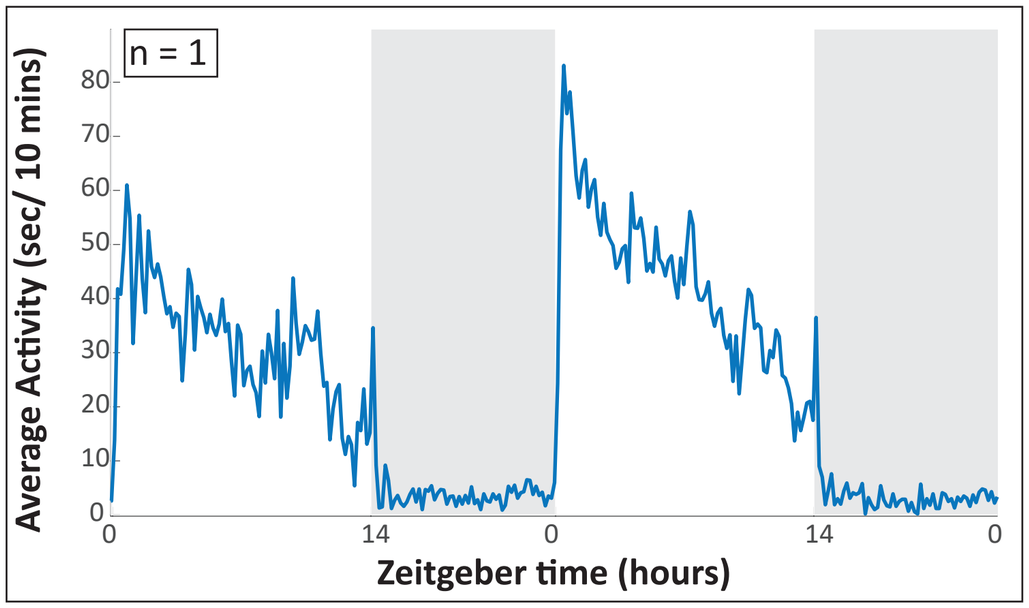

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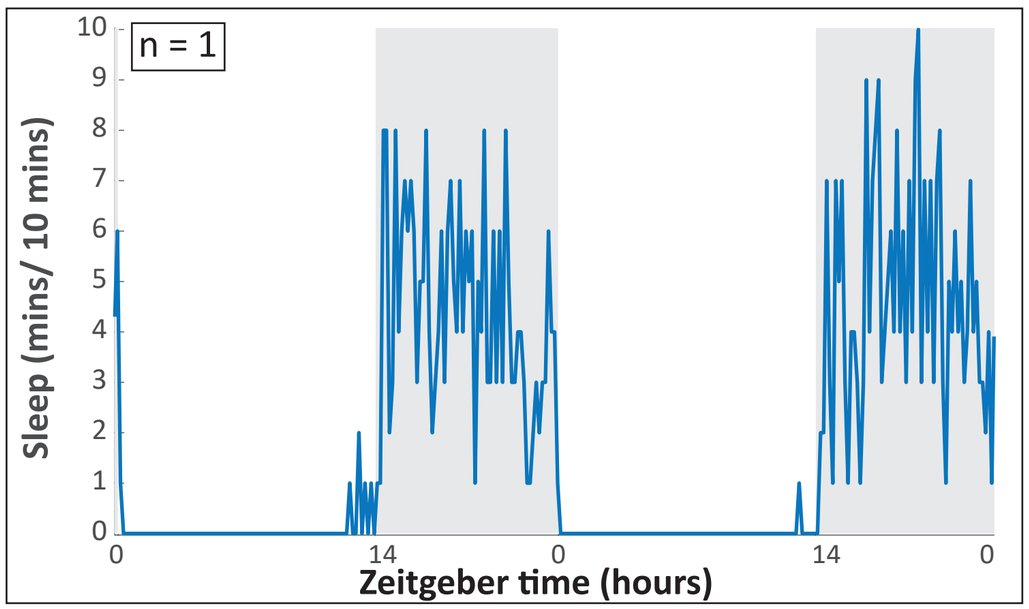
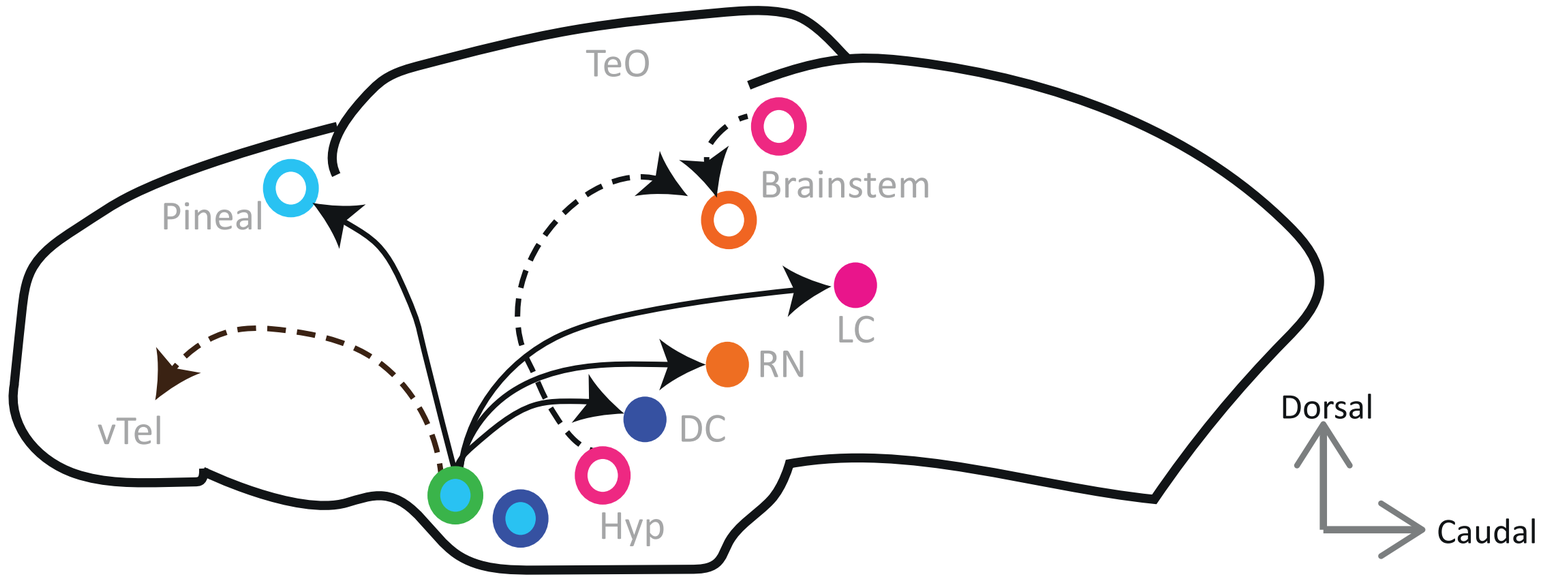


c)



Neurotranmitters

- Norepinephrine
- Glutamate
- Serotonin
- Dopamine
- GABA

Neuropeptides/neurohormones

Wake-promoting

- Hcrt
- Nmu
- Crh

Sleep-promoting

- QFFP
- Melatonin

→ Validated connectivity

⇢ Proposed connectivity

Neuroanatomical areas:

Hyp - Hypothalamus
LC - Locus Coeruleus
vTel- ventral Telencephalon
TeO - Optic Tectum
RN - Raphe Nucleus
DC - Dopaminergic Diencepahlic Cluster