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Review Article

Review Article

Evo-devo applied to sleep research: an approach whose time has come

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Abstract

Sleep occurs in all animals but its amount, form, and timing vary considerably between species and between individuals. Currently, little is known about the basis for these differences, in part, because we lack a complete understanding of the brain circuitry controlling sleep–wake states and markers for the cell types which can identify similar circuits across phylogeny. Here, I explain the utility of an "Evo-devo" approach for comparative studies of sleep regulation and function as well as for sleep medicine. This approach focuses on the regulation of evolutionary ancient transcription factors which act as master controllers of cell-type specification. Studying these developmental transcription factor cascades can identify novel cell clusters which control sleep and wakefulness, reveal the mechanisms which control differences in sleep timing, amount, and expression, and identify the timepoint in evolution when different sleep–wake control neurons appeared. Spatial transcriptomic studies, which identify cell clusters based on transcription factor expression, will greatly aid this approach. Conserved developmental pathways regulate sleep in mice, *Drosophila*, and *C. elegans*. Members of the LIM Homeobox (*Lhx*) gene family control the specification of sleep and circadian neurons in the forebrain and hypothalamus. Increased *Lhx9* activity may account for increased orexin/hypocretin neurons and reduced sleep in Mexican cavefish. Other transcription factor families specify sleep–wake circuits in the brainstem, hypothalamus, and basal forebrain. The expression of transcription factors allows the generation of specific cell types for transplantation approaches. Furthermore, mutations in developmental transcription factors are linked to variation in sleep duration in humans, risk for restless legs syndrome, and sleep-disordered breathing. This paper is part of the "Genetic and other molecular underpinnings of sleep, sleep disorders, and circadian rhythms including translational approaches" collection.

Key words: evo-devo; Lim; homeobox; cavefish; specification

Statement of Significance

Sleep is a mysterious but essential behavior present in all animals. The amount, form, and timing of sleep varies considerably between species and between individuals. However, little is known about the basis for these differences, in part, because we lack a complete understanding of the brain circuitry controlling sleep–wake states and markers for cell types which can identify similar circuits across species. Studying evolutionary ancient transcription factors which act as master controllers of cell-type specification can identify novel cell clusters which control sleep and wakefulness; reveal the mechanisms which control differences in sleep timing, amount, and expression between species; identify the timepoint in evolution when different sleep–wake control neurons appeared; and provide a basis to understand sleep–wake abnormalities in developmental disorders.

Introduction

Sleep states have been described in every animal which has been closely examined. However, there are large variations in the amount, timing, and behavioral expression of sleep in different animals and humans, as well as variations in the ratio of different types of sleep [1–5]. In recent years, there has been increased interest in the evolution of sleep behavior but currently, there is no overarching framework which can help unify the neural mechanisms which underlie the differences and similarities of sleep between and within species.

"Evo-devo" is an area of biology which seeks to understand differences and similarities in the form and physiology of different species by studying the developmental processes which underlie the generation of different tissues. This approach focuses on the regulation of evolutionary ancient transcription factors which act as master regulators of cell-type specification

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during development in all animals to understand differences in anatomy and physiology [6, 7]. This approach proposes that morphological changes, and by extension changes in physiology and behavior, are mainly due to mutations in the cis-regulatory DNA sequences of these developmental transcription factors and of the target genes in the large networks that they control, typically encompassing tens or hundreds of genes [7]. In this review, I explain the utility of this approach for understanding sleep regulation and function as well as for sleep medicine (Figure 1).

Researchers have studied the brain circuitry which controls sleep and wakefulness for more than 100 years [8]. Early studies of the neuronal circuits which control sleep, beginning 50-60 years ago, focused heavily on a small number of aminergic and cholinergic neurotransmitter systems due to the development of histochemical and immunohistochemical stains which enabled visualization of their cell bodies in brain regions implicated in sleep–wake control, the availability of pharmacological tools acting on these systems, as well as their large cell bodies which facilitated unit recordings in vivo [9–11]. More recent experiments have focused on neurons that use the main inhibitory and excitatory neurotransmitters in the brain, GABA and glutamate, as essential components of sleep–wake circuitry [12, 13]. The new focus on ubiquitous GABAergic and glutamatergic systems, which make up 99% of the neurons in the brain [14], presents a problem in identifying the key neurons involved in sleep–wake regulation. In some cases, the expression of calcium-binding proteins or neuropeptides has been useful in identifying specific groups of subcortical GABAergic or glutamatergic neurons involved in regulating sleep–wake behavior and cortical electrical activity. However, this approach cannot be applied to most GABAergic and glutamatergic neurons. Furthermore, there are considerable variations in calcium-binding protein and neuropeptide content between species, during development, and in response to changes in neuronal activity. Thus, the use of these markers has limitations when attempting to compare sleep–wake circuitry between species. A potentially more fruitful approach to identify subpopulations of glutamatergic and GABAergic neurons, including sleep–wake control neurons, is to use transcription factors as markers [15, 16]. Recent RNA-seq and spatial transcriptomic approaches revealed that combinations of transcription factors, especially developmental transcription factors, are the most informative in discriminating distinct clusters of neurons and non-neuronal cells [16, 17].

Large-scale studies in *Drosophila melanogaster*, *C. elegans*, and vertebrate nervous systems have concluded that developmental transcription factors distinguish neuron types most effectively and can distinguish almost all unique populations of neuronal cells [16–18]. Transcription factors controlling the development of different tissues were uncovered through analysis of development mutants. Classic studies by William Bateson had described animals that displayed extra, missing, or altered parts. He termed developmental variants in which one body part was transformed into the likeness of another "homeotic" from the Greek word homeos meaning similar [6]. Later studies showed that homeotic mutants in the fruit fly, *D. melanogaster*, were due to changes in single genes which contained a specific DNA binding domain named the homeobox [19, 20]. Homeotic genes with this DNA binding domain were called homeobox genes or *Hox* genes for short. Many of the homeobox-containing genes are found in clusters throughout the genome, indicating an ancient system for the control of development [21, 22]. Subsequently, homologs of the homeobox genes were identified in vertebrates as duplications of the Drosophila gene clusters. The homeobox gene superclass

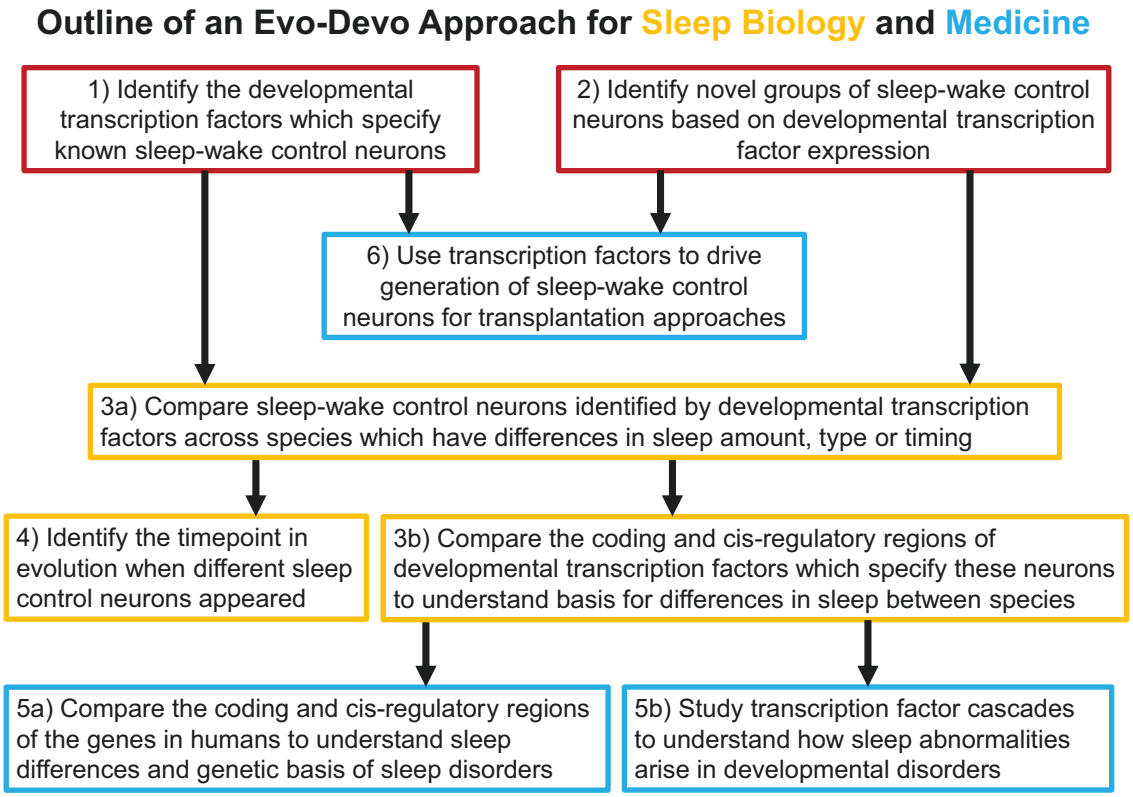

Figure 1. Roadmap and flowchart for the use of an evo-devo approach in sleep research and sleep medicine.

predates the evolutionary split between animals, plants, and fungi but many homeobox genes appear unique to animals [23]. The functional diversification of homeobox genes by gene duplication and divergence has been shown to be an important mechanism in the evolution of distinct body plans and organs in bilaterian metazoans [24]. In this review, I examine how using homeobox and other developmental transcription factors as markers can benefit sleep research and medicine (Figure 1).

Using developmental transcription factors for identification purposes has already proven useful in uncovering novel subtypes of cortical GABAergic interneurons [25], as well as subtypes of globus pallidus GABAergic projection neurons [26, 27]. Only recently has the study of these transcription factors been applied to understand evolutionary differences in sleep–wake circuitry and behavior [28, 29] as well as to identify novel sleep circuits and their ontogeny, as described in subsequent sections. Linking clusters of neurons identified by expression of developmental transcription factors to sleep behavior can be done using fate-mapping i.e. mark these neurons during development and follow their identity in adults or by using strains of animals which express transgenes expressed under the control of the promoter regions for these transcription factors [30]. Many of these developmental transcription factors also remain active in adults [21, 30, 31]. Thus, neurons expressing these transcription factors can also be identified in adults by staining or other techniques which identify the protein or mRNA. One major advantage of this approach is that homeobox genes and other developmental transcription factors are highly conserved during evolution [21, 22, 32, 33], allowing identification of functionally related neuronal or glial cell types across widely divergent species and allowing correlations with behavioral phenotypes.

Studies of developmental transcription factors in sleep and circadian biology—focus on the Lim homeobox gene family

Animal homeodomain genes can be divided into 10 distinct classes [23, 33]. Of these 10 classes, the homeobox gene family which has been studied the most in sleep and circadian biology is the LIM homeobox (*Lhx*) transcription factor family. The *Lhx* genes are unique to the animal lineage and have patterning roles during embryonic development in flies, nematodes, and vertebrates, with a conserved role in specifying neuronal identity [22, 33]. The LIM domain is a zinc finger protein motif that is named for the founding genes of the class: *lin-11*, islet (*isl*)-1, and mitosis entry checkpoint (*mec*)-3 [34–36]. The *Lhx* proteins contain two tandem LIM domains N-terminal to their homeodomain allowing dimerization with LIM domains in other proteins of the same subfamily. *Lhx* genes are divided into six subgroups based on homology: *Lhx1/5/lin-11*, *Lhx2/9/Apterous*, *Lhx3/4*, *Lhx6/8*, *Islet*, and LIM homeobox (*Lmx*)/*Lim-6* [33, 37]. During development, cells express different combinations of *Lhx* genes and produce a LIM code that defines cell fate [22, 37]. In both vertebrates and *Drosophila*, motoneuron subtype identification is determined by a combinatorial code of *Lhx* genes and similar codes appear to be present in a variety of central nervous system neurons [22].

Several members of the LIM homeobox family have been implicated in the specification of GABAergic, cholinergic, and orexin/hypocretin neurons involved in sleep–wake control as well as their activity in adults (Table 1). Multiple developmental transcription factors (*Lhx2*, *Six3*, and *Six6*) are broadly expressed in the anterior hypothalamus and needed for the specification of different nuclei from hypothalamic neuroepithelium including the master circadian clock located in the suprachiasmatic nucleus (SCN) [41, 42]. However, *Lhx1* has a more restricted role in controlling the terminal differentiation of neurons in the SCN and regulating their function [42–44], although it is also expressed in other hypothalamic and extra-hypothalamic brain regions. Deletion of *Lhx1* during development in mice results in the loss of neuropeptides in the SCN, dampens clock gene expression and impairs the synchrony of circadian oscillator neurons [42, 44]. Furthermore, *Lhx1* regulates light control of sleep timing [44] and heat resistance of the SCN [43]. Phase-shifting light pulses acutely reduce *Lhx1* expression and its target genes which are involved in SCN coupling [44]. Taken together, these findings reveal *Lhx1* as a key transcription factor involved in SCN development and circadian rhythmicity which could potentially be used to identify master circadian regulator neurons in other species either alone, or in combination with other developmental transcription factors. Study of *Lhx1* expression in the developing anterior ventral hypothalamus in different species may be useful in understanding changes in the organization of circadian regulation. Presumably, *Lhx1* would not be expressed in this brain region in fish species where a clear SCN master clock has not been identified [65]. Instead, in fish, circadian rhythms are controlled by the pineal gland. Another Lhx family member, *Lhx9* is needed for early development of the mammalian pineal gland [59], whereas a different family member *Lhx4* is strongly regulated by circadian phase and plays an important role in nocturnal melatonin synthesis in rats and humans [60]. A different *Lhx* family member, *Isl1* plays a similar role in pigs [61].

Another subfamily of *Lhx* genes which control the development of sleep–wake control neurons is the *Lhx6/Lhx8* subfamily. In the forebrain, *Lhx6* and *Lhx8* are expressed early in the medial ganglionic eminence region which generates many forebrain GABAergic and cholinergic neurons [25]. Expression of *Lhx8* is required for the development of forebrain cholinergic neurons [38, 39], including the basal forebrain cholinergic neurons which regulate sleep homeostasis and cortical fast electrical activity [8]. Whether *Lhx8* regulates the activity of basal forebrain cholinergic neurons and their role in sleep homeostasis in adults has not yet been tested. The related transcription factor *Lhx6* is expressed in medial ganglionic eminence neurons which will later use GABA as a neurotransmitter, although it does not control the expression of the genes required for GABAergic neurotransmission [66]. Many GABAergic projection neurons in the basal forebrain and globus pallidus express *Lhx6* [26], as do cortical and striatal parvalbumin and somatostatin-positive interneurons [66]. In preliminary studies, chemogenetic activation of basal forebrain *Lhx6* neurons promotes wakefulness [40]. *Lhx6* can also be expressed in a subset of cholinergic forebrain neurons which express GAD65 but not GAD67 [67] and thus may define a unique population of dual phenotype cholinergic/GABAergic neurons which have been identified in the basal forebrain and striatum [40, 67, 68]. *Lhx6* is not only expressed in the telencephalon but also present in a restricted number of neurons in the diencephalon. The use of *Lhx6* and a diencephalon-specific developmental transcription factor as markers allowed the identification of a novel group of sleep-active neurons in the hypothalamus and zona incerta [45]. Expression of *Lhx6* is required for the survival of these neurons [46]. Several subtypes of diencephalic Lhx6+ neurons are distinguished by the expression of the transcription factors *Dlx1/2*, *Nkx2-2*, and *Nkx2-1* [46]. *Nkx2-2* specifically regulates the development of sleep pressure-sensitive cells in the zona incerta [46]. *Lhx6*+ zona incerta neurons directly inhibit wake-active hypocretin/orexin neurons and GABAergic neurons in the lateral

Table 1. Developmental transcription factors which regulate the specification and adult function of sleep–wake control neurons in defined regions of the brain

Brain region/neurotransmitter system	Functional role	Developmental transcription factor(s)	References
Basal forebrain cholinergic neurons	Cortical activation and sleep homeostasis	*Lhx8*, *Islet 1*	[38, 39]
Basal forebrain GABAergic neurons (subset)	Wake promotion	*Npas1*, *Lhx6*	[30, 40]
Suprachiasmatic nucleus of the hypothalamus	Circadian rhythms	*Lhx1*, *Six3*	[41–44]
Zona incerta GABAergic neurons (subset)	Sleep promotion	*Lhx6*	[45–47]
Lateral hypothalamus orexin/hypocretin neurons	Wake promotion sleep–wake stabilization REM suppression	*Lhx9*, *Peg3*	[28, 48–50]
Midbrain reticular formation/ventrolateral periaqueductal gray GABAergic neurons	REM sleep control	*Gata*, *Nkx2-2*, *Skor2*	[51]
Pontine tegmentum glutamatergic/cholinergic neurons close to the superior cerebellar peduncle and parabrachial nucleus	Inhibit REM sleep and promote NREM sleep	*Atoh1/Math1*	[52]
Brainstem serotonergic neurons	Wake active, REM suppression	*Lmx1b*, *Pet1*, *Nkx2-2*	[53, 54]
Locus coeruleus noradrenaline neurons	Arousal, REM suppression, control of sleep spindles	*Phox2a*	[55]
Parabrachial glutamatergic neurons (subset)	Arousal?	*Lmx1b*	[56]
Medullary neurons/astrocyes	Breathing, sleep homeostasis	*Phox2b*	[57, 58]
Pineal gland	Melatonin synthesis	*Lhx9*, *Lhx4*, *Isl1*	[59–61]
C. elegans ALA neuron	Sleep promotion	*Lhx3*, *Chx10*, *Phox2*	[62]
C. elegans RIS neurons	Sleep promotion	*Lim-6* (*Lmx* subfamily of *Lhx* genes), Aptf-1 (Ortholog of *Tfap-2* in *Drosophila* and *Tfap-2beta* in humans)	[63, 64]

hypothalamus [45]. *Lhx6*+ neurons in the ventral zona incerta are also activated during paradoxical (REM) sleep rebound [47] and deletion of *Lhx6* from the diencephalon decreases both NREM and REM sleep [45]. Thus, zona incerta *Lhx6*+ neurons promote sleep, especially REM sleep, via inhibition of wake-active populations in the hypothalamus and brainstem.

Lhx1 and *Lhx6* are not the only LIM homeobox genes expressed in the mammalian hypothalamus. Another *Lhx* family member, *Lhx9*, plays an important, evolutionary conserved role in directing the specification of a subset of orexin/hypocretin neurons [48, 49] which degenerate in the sleep disorder, narcolepsy [69, 70]. Transcriptional profiling using a hypocretin translating ribosome affinity purification (bacTRAP) line identified *Lhx9* as a highly expressed transcript in hypocretin neurons, a finding which was confirmed with immunohistochemical staining [48]. Constitutive knockout of *Lhx9* in mice led to a ~30%-40% reduction in the number of hypocretin neurons and hypocretin fibers in target regions while hypothalamic anatomy otherwise appeared normal and there were no changes in the numbers of neighboring dopamine or melanin-concentrating hormone neurons [48]. Electrophysiological and behavioral profiling revealed that *Lhx9* knockout mice had a 20% reduction of wakefulness across 24-hour recordings but no cataplexy or REM sleep changes, consistent with a modest loss of hypocretin/orexins. Interestingly, most hypocretin neurons expressed detectable levels of *Lhx9* mRNA although Lhx9 protein was only detectable in a subset of neurons. *Lhx9* did not regulate hypocretin promoter activity in vitro or hypocretin expression in vivo and overexpression of *Lhx9* in adults did not rescue the hypersomnolent phenotype indicating that *Lhx9*'s role is primarily in specifying a subset of these neurons. *Lhx9* is also expressed in hypocretin neurons in zebrafish and is necessary and sufficient to specify hypocretin neurons in this species [49]. In contrast to the findings described above, in a different study, *Lhx9* directly induced hypocretin expression, and two potential binding sites for Lhx9 were identified in the hypocretin promoter [49]. Furthermore, these authors found that *Lhx9* was sufficient to specify hypocretin neurons in the developing mouse hypothalamus [49]. Overexpression at embryonic day 10.5 led to increased numbers of hypocretin neurons in the lateral hypothalamus at postnatal day 6. Collectively, although there are some important unresolved differences, these two studies confirm that *Lhx9* is a key regulator of the specification of hypocretin/orexin neurons in two species, zebrafish and mice, although additional factors are likely required, especially in mice. Interestingly, while the mouse hypothalamus contains thousands of hypocretin/orexin neurons, adult zebrafish only contain around 40 hypocretin neurons. It would be interesting to determine if altered regulation of *Lhx9* contributes to these differences in numbers and differences in sleep–wake regulation. Other developmental transcription factors involved in specifying hypocretin/orexin in mice and zebrafish have recently been reported, including the transcription factors *Peg3*, *Ahr*, *Nr2f2*, *Six6*, *Prrx1*, and *Nkx6-2* [50]. Similar to *Lhx9*, downregulation of *Peg3* results in reduced hypocretin (and MCH) numbers in mouse hypothalamus and abolishes them in zebrafish. Studies of the promoter regions of these genes across species are likely to be informative in understanding changes in the hypocretin/orexin system and sleep, as described in the next section.

LIM homeobox genes have also been implicated in the control of sleep in invertebrates. In *C. elegans*, several different states of inactivity, most notably the developmental state Lethargus, have properties which allow them to be considered analogous to mammalian sleep [63, 71]. Sleep in *C. elegans* is induced by two sleep-active neurons, RIS and ALA [71]. *Lim-6*, a member of the *Lmx* subfamily of LIM homeodomain genes [33], specifies GABAergic neurotransmission in the RIS neuron and determines

RIS-dependent sleep activity through the expression of the AP-2 class developmental transcription factor *Aptf-1*, which in turn specifies the expression of FLP-11 neuropeptides [63]. At sleep onset, RIS depolarizes and releases FLP-11 to induce a systemic sleep state. In *Drosophila*, neuronal-specific knockdown of the AP-2 ortholog, Tfap-2, abolished nighttime sleep and affected neuronal development, whereas conditional knockdown in the adult also produced a milder sleep phenotype [64]. Mutation of the human ortholog, Tfap-2beta, causes Char disease, a severe neurodevelopmental disorder which may be associated with sleep disruption [64]. Thus, these findings suggest an evolutionarily conserved role of LIM homeobox and AP-2 family transcription factors in sleep regulation and sleep disruption in *C. elegans*, *Drosophila,* and humans.

Differentiation of the ALA neuron is coordinated by the combined action of LIM homeodomain protein *Lhx3* (*Ceh-14*) and two members of the Paired-like homeodomain family, *Chx10* and *Phox2* [62]. *Lhx3* is expressed only in the ALA neuron and is required for ALA-specific gene expression throughout development. These examples illustrate commonalities to the developmental programs which control sleep in vertebrates. Furthermore, they represent an independent line of evidence, beyond pharmacological and genetic similarities, for rest in invertebrates being analogous to sleep in vertebrates [71]. Given the conserved role of *Lhx* genes in specifying GABAergic neurons, it is tempting to speculate that mutations or duplications of *Lhx* genes or their cis-regulatory regions may have been involved in the generation of novel neural cell types regulating sleep and wakefulness during evolution.

The most primitive species where a sleep-like state has been described are Cnidarians such as the upside-down Jellyfish and Hydra, organisms which use neural nets and do not have a well-defined nervous system [72, 73]. Four families of Lim homeobox genes are present in the Cnidarian lineage [33] but it has not yet been investigated whether they play a role in sleep regulation.

The sections above describe the identification of the transcription factors which specify several previously known populations of sleep–wake control neurons. However, the identification of sleep-promoting *Lhx6+* neurons in the zona incerta illustrates the potential to use developmental transcription factors to identify novel subpopulations. Other examples of this are findings in the brainstem which identified a population of excitatory glutamatergic neurons in the pons which regulate REM and non-REM sleep based on the expression of the pro-neuron transcription factor gene *Atoh1* (Atonal homolog 1, *Math1*) [52] and delineation of a midbrain GABAergic population involved in REM sleep control which expresses the developmental transcription factors *Gata2*, *Nkx2-2*, and *Skor2* [51]. In the parabrachial region of the dorsolateral pons, the LIM homeobox gene *Lmx1b* identifies a population of glutamatergic neurons with ascending projections to the cerebral cortex and may be involved in arousal [56]. In the basal forebrain, a key node of the ventral part of the ascending reticular activating system, we have recently identified a novel population of GABAergic wake-promoting neurons which express the developmental transcription factor, neuronal PAS domain 1 (*Npas1*) [30]. The related transcription factor, *Npas2*, is a major component of the molecular circadian clock in cells outside the SCN and regulates NREM sleep oscillations and sleep homeostasis [74, 75].

In addition to identifying novel subpopulation of neurons, several studies have identified developmental transcription factors underlying the generation of previously known brainstem neurons involved in sleep–wake control (Table 1). Paired-like homeobox proteins *Phox2a* and *Phox2b* are critical for the development of brainstem noradrenergic cell groups, with *Phox2a* having a specific role in the development of the locus coeruleus [55]. *Phox2b* is expressed more widely in the brainstem and specifies neurons and astrocytes in the medulla involved in respiration and sleep homeostasis. Mutations in *Phox2b* cause sleep-disordered breathing [57, 58]. Brainstem serotonin neurons are specified by the combined action of the transcription factors Lim homeobox 1b (*Lmx1b*), *Nkx2-2*, and plasmacytoma expressed transcription factor 1 (*Pet1*) [53]. *Lmx1b* is also expressed in adults and regulates serotonin synthesis [54]. Much remains to be learned about the specification of different classes of glutamatergic neurons in the brainstem reticular formation involved in the control of ascending arousal, REM sleep, and muscle atonia. The transcription factors which specify brainstem cholinergic neurons involved in REM sleep control also remain to be uncovered.

Further analysis of developmental transcription factors is likely to uncover additional novel subpopulations of sleep–wake control neurons, as well as reveal the developmental and evolutionary origin of other known subpopulations. The developmental origin of some important sleep–wake related regions remains to be revealed. In particular, we do not yet know the transcription factors which control the specification of descending REM muscle atonia neurons in the dorsolateral pons which are lost in REM sleep behavior disorder or the midbrain reticular formation neurons which are the origin of the ascending reticular activating system and are damaged in disorders of consciousness. Similarly, we do not understand the specification of sleep-promoting parafacial or melanin-concentrating hormone neurons or sleep-active GABAergic neurons in the ventrolateral and median preoptic hypothalamic regions, although RNA-seq analysis of gene expression across the hypothalamus provides some clues [85].

The first evo-devo approach applied to sleep regulation: alterations of sleep duration in cavefish

As described in the section above, progress is being made regarding our understanding of the specification of sleep and circadian neurons by developmental transcription factors, especially those in the LIM homeobox family. Knowledge of the developmental transcription factors which specify different types of neurons is a prerequisite for an evo-devo approach which attempts to explain differences in sleep behavior within and between species. A beautiful example of such a comparative approach in the sleep–wake field is provided by studies of *Astyanax mexicanus*, a model organism for studying evolutionary variations in anatomy and physiology [28, 29, 86]. This teleost fish exists as two populations (morphotypes), surface fish that inhabit rivers, and multiple cave populations with convergent evolution of sleep loss. Sleep is dramatically reduced in adult cavefish compared to surface counterparts. What could account for this difference in sleep behavior? Hypocretin/orexin neurons are a well-known wake-promoting cell group in the hypothalamus which degenerate in the sleep disorder narcolepsy [69, 70]. The number of hypocretin/orexin (HCRT)-positive hypothalamic neurons is increased significantly in cavefish, and HCRT is upregulated at both the transcript and protein levels by as much as 3-fold [28, 29]. Pharmacological or genetic inhibition of HCRT signaling increases sleep in cavefish, suggesting enhanced HCRT signaling underlies the evolution of sleep loss [29]. Furthermore, ablation of the lateral line or starvation increases sleep in cavefish but not surface fish and similarly inhibits hypocretin expression only in cavefish [29]. Thus, several lines of evidence suggest that increased hypocretin activity

promotes sleep loss in cavefish. However, a fascinating recent study shows that hypocretin signaling is not absolutely required for sleep–wake control in fish [87], since several fish species of the Botiidae family have mutations in the hypocretin gene and/or the type 2 hypocretin receptor, which render them nonfunctional [87]. Interestingly, loss of hypocretin signaling in fish does not lead to behavioral arrests or cataplexy-like events [87], as observed in narcolepsy in mammals.

What could account for increased hypocretin activity which drives sleep loss in cavefish? As described in the previous section, the Lim homeobox transcription factor, *Lhx9*, regulates the specification of hypocretin/orexin neurons in zebrafish and in mice [48, 49]. Retaux *et al.* confirmed that *Lhx9* is expressed in regions of the brain which go on to generate hypocretin neurons and used morpholino knockdown approaches to test a causal role in the specification of hypocretin neurons in *A. mexicanus* [28]. Three different knockdowns of *Lhx9* reduced the numbers of hypocretin neurons in the hypothalamus whereas embryos injected with *Lhx9* mRNA had more hypocretin neurons. Alie *et al.* went on to investigate whether altered regulation of *Lhx9* could account for increased numbers of hypocretin/neurons in cavefish, when compared to surface fish [28]. Indeed, they found that *Lhx9* expression is turned on earlier and over a larger domain in cavefish compared to surface fish. Importantly, cavefish with knockdown of *Lhx9* had similar amounts of locomotor activity, a proxy for sleep–wake activity, as surface fish [28]. Collectively these two studies show that, as predicted by the evo-devo approach, variations in sleep circuitry and behavior can be accounted for by differential regulation of a master developmental transcription factor, *Lhx9*. Interestingly, increased activity of hypocretin neurons in aged mice results in sleep loss [88], suggesting a conserved role of hypocretin neurons in regulating sleep duration across different species. Further studies of *Lhx9* expression in the hypothalamus and the cis-regulatory regions of *Lhx9* across species would be interesting to test to what extent Lhx9 and hypocretin activity can account for differences in sleep duration more generally.

Implications for genetic studies of sleep–wake behavior and sleep medicine

Several genome-wide association studies in humans have implicated developmental transcription factors in sleep–wake control and sleep disorders (Table 2). Sleep duration is associated with variations near paired homeobox 8 (*Pax8*) [2, 81] and with DNA methylation levels of *Pax8* in hypersomnia patients [82]. Sleep duration and short sleep are also associated with polymorphisms of forkhead box P2 (*Foxp2*) [2], whereas SATB homeobox 2 (*Satb2*), is suggested to contribute to the risk of insomnia and sleep disturbances in Glass syndrome [81]. Mutations of Foxp2 cause developmental disorders associated with speech disruption but these patients also commonly exhibit sleep disturbances [76]. Intronic variants in the homeobox gene *Meis1* are implicated in a 50% increase in risk for restless legs syndrome (RLS) [78]. Polymorphisms of *Meis1* are also implicated in risk for insomnia [79] and for periodic limb movements (PLM) which disrupt sleep [80]. *Meis1* is expressed in the developing spinal cord as well as the cerebellum, forebrain, and substantia nigra. Which of these regions mediate Meis1's role in sleep–wake control and in the pathogenesis of RLS and PLM is still unclear, but one study reported that the risk allele of the lead single-nucleotide polymorphism in the *Meis1* locus for RLS reduces enhancer activity in the *Meis1* expression domain of the mouse embryonic ganglionic eminences which generate forebrain GABAergic and cholinergic neurons [89]. The transcription factor ladybird homeobox corepressor 1 (*Lbxcor1*), is also implicated in risk for RLS, possibly related to its role in the development of spinal cord sensory pathways [78]. In the brainstem, *Phox2b* is involved in the specification of neurons and astrocytes in the medulla which regulate respiration and sleep [58]. Polyalanine expansion and frameshift mutations of *Phox2b* lead to congenital central hypoventilation syndrome, which includes sleep-apnea as one of its major symptoms [57, 83]. In mice, deletion of the homeobox transcription factor, Goosecoid-like (*Gscl*), one of the genes deleted in DiGeorge syndrome or 22q11 deletion syndrome, reduced time spent in REM sleep, reduced theta power and increased arousability from REM sleep, apparently without altering the anatomy of the interpeduncular nucleus where it is expressed [77].

Genome-wide association studies in humans have identified polymorphisms in genes which affect sleep duration but collectively these polymorphisms only account for a small part of the variance. The strongest association for sleep duration is at the *Pax8* locus but the effect only accounts for around 2-3 minutes of sleep duration [90]. According to the evo-devo framework, it would be of interest in the future to determine whether alterations in the cis-regulatory regions of master developmental

Table 2. Developmental transcription factors linked to sleep and developmental disorders with sleep abnormalities

Transcription factor	Functional effect of disruption and disorder	References
Forkhead box protein P2 (*Foxp2*)	Change in sleep duration/insomnia in developmental disorders with speech disruption	[76]
Goosecoid-like homeobox (*Gscl*)	REM sleep abnormalities in DiGeorge or 22q11 deletion syndrome	[77]
Ladybird homeobox corepressor (*Lbxcor1*)	Restless legs disorder	[78]
Myeloid ecotropic viral integration site 1 homolog (*Meis1*)	Restless legs disorder, insomnia, periodic limb movements during sleep	[78–80]
Paired homeobox 8 (*Pax8*)	Change in sleep duration/hypersomnia	[2, 81, 82]
Paired-like homeobox 2B (*Phox2b*)	Sleep-disordered breathing	[58, 83]
POU class 3 factor 2 (*POU3F2*)	Autism spectrum disorder	[84]
Special AT-Rich sequence-binding homeobox 2 (*Satb2*)	Insomnia and sleep disturbance in Glass syndrome	[81]
Transcription factor activator protein-2 beta (*Tfap-2beta*)	Sleep disturbances in Char disease	[64]

transcription factors might account for more of the variance in sleep physiology by affecting the development of sleep–wake circuits, particularly since these factors regulate the expression of a large number of target genes. In *Drosophila*, a genome-wide association study uncovered single-nucleotide polymorphisms which accounted for 55% of the variance in sleep and the majority (85%) were located in noncoding regions [91], as predicted by the evo-devo framework proposed here. Genes involved in developmental processes were a common theme for all sleep traits and roughly half of the genes associated with sleep traits were expressed during development [91]. In particular, a network of 114 genes was identified which interact genetically or physically with the epidermal growth factor receptor pathway involved in the development of the nervous system [91]. Other studies in Drosophila and mice have linked the wnt signaling pathway which is active during central nervous system development [92], the nuclear factor-binding κ light chain in B cells (NF-κB) family of developmental transcription factors [93] and the developmental transcription factor, *Pdm3* [84], in developmental changes in sleep and alterations in sleep amount, timing or architecture. Detailed, state-of-the-art experiments in Drosophila revealed the mechanism by which the POU family transcription factor *Pdm3* acts to promote sleep during development by regulating the innervation of a sleep-promoting region, CCX, by inhibitory input from wake-promoting dopamine neurons, through regulating the expression of a synaptogenesis gene, Msp300, in CCX neurons [84]. The human homolog of pdm3, *POU6F2*, has been associated with subtypes of autism spectrum disorders [84]. Another gene, *insomniac* (*inc*), an adaptor for the autism-associated gene Cul3 ubiquitin ligase, acts in a defined period of neuronal of *Drosophila* development to impact sleep in adults [94]. Collectively, these findings suggest that developmental alterations can impact adult sleep phenotypes and be a potential cause for adult sleep disorders [84, 94].

Study of the regulation of cis-regulatory regions of transcription factors involved in the specification of sleep–wake circuits seems likely to provide insights into the normal trajectory of sleep amount and form during development [95, 96] as well as alterations of sleep and wakefulness in neuropsychiatric disorders with a developmental component. For instance, considerable work has focused on the involvement of forebrain cortical and striatal GABAergic interneurons derived from the medial and caudal ganglionic eminences (MGE/CGE) in the pathophysiology of neuropsychiatric disorders. However, the MGE and CGE are also the origin of GABAergic projection neurons involved in sleep–wake control in the globus pallidus and basal forebrain. Thus, alterations of progenitor neurons might lead to altered numbers, location, or activity of MGE or CGE-derived neurons which regulate sleep–wake activity and cortical activation, as well as alterations in cortical and striatal interneurons. In fact, genes expressed in the subpallial regions which generate forebrain interneurons and projection neurons in the striatum, globus pallidus, and basal forebrain are enriched in GWAS studies of self-reported sleep duration [2, 90].

Degeneration of sleep–wake neurons has been identified in a variety of disorders. Loss of orexin/hypocretin neuron in the lateral hypothalamus is the cause of most human cases of narcolepsy [69, 70] whereas degeneration of subcoeruleus/sublateral dorsal nucleus muscle atonia neurons in the dorsal pons results in REM sleep behavior disorder [97]. During aging, the numbers of sleep-promoting GABAergic/galaninergic neurons in the ventrolateral preoptic area [98] as well as arousal-promoting cholinergic and GABAergic neurons in the basal forebrain are reduced [99] and they are further reduced in dementia patients [98]. Thus, strategies to replace these lost neurons may prove beneficial. Transplantation of brain embryonic progenitor cells has been successfully tested in preclinical models of epilepsy and Parkinson's disease [100, 101]. Transplantation and overexpression approaches have also shown promise in rodent narcolepsy models [102]. Knowledge of the transcription factor cascades which control the development of orexin/hypocretin neurons could allow more refined approaches to enhance the numbers of viable orexin/hypocretin neurons, for instance, using overexpression of Lhx9 [48, 49]. Currently, the transcription factors which control the specification of muscle atonia neurons in the dorsal pons or ventrolateral preoptic area sleep-promoting neurons are unknown, but when identified they could prove useful in developing transplantation approaches to treat REM sleep behavior disorder or sleep fragmentation in dementia respectively. Many developmental transcription factors are also active in adults [31]. Thus, modulation of their activity might represent a novel pharmacological target to complement existing approaches to modulate sleep and arousal [103].

Limitations of this approach

Comparative approaches to compare the circuitry controlling sleep–wake behavior in different brain regions across species to understand differences in sleep amount timing and expression are only possible once the transcription factors controlling the specification of specific sleep–wake circuits have been elucidated in one species. As described above, some progress has been made for specific circuits in the basal forebrain, hypothalamus, and brainstem. Nonetheless, we are far from a complete understanding. In particular, the specification of major sleep–wake neurons in the ventrolateral preoptic area, median preoptic area, dorsolateral pons, and brainstem reticular formation is largely unknown. However, with the advent of whole-brain cellular atlases of the mouse brain, based on RNA-seq and spatial transcriptomics approaches [14, 16], it is likely that this gap can be filled very soon.

Another potential limitation of the approach described here is that transcription factors are used at different times and places throughout the development of the nervous system and most cell types can only be defined through the coordinated action of several transcription factors. How many transcription factors might be needed to define neural identity? This question has recently been addressed. In *C. elegans*, the combined expression of homeodomain proteins can be used to identify all of its neurons [18]. While some neurons can be identified based on the expression of a single homeodomain gene, on average, the combined expression of four distinct homeodomain genes is needed [18]. Thus, the need to direct expression based on the coordinated action of several different promoter regions places some limitations in designing viral vectors which express proteins which can be used for gain or loss of function approaches to test the function of the target cell type in sleep–wake control. However, molecular biology is moving at a rapid pace, and it seems likely that this limitation will be overcome soon. In fact, progress has already been made in this area, allowing targeting of neurons based on double or triple intersectional targeting [104] or multiplexing CRISPR to generate unlimited genetic switches [105]. A related issue is that the cis-regulatory regions of master developmental transcription factor genes tend to be very large and composed of multiple elements [7]. However, it seems likely that smaller fragments may be sufficient to direct expression in particular neuronal classes, especially when combined with other promoters/enahncers.

Conclusions

The central recommendation of this perspective is for sleep researchers to identify the transcription factor cascades which specify the identity of sleep–wake regulatory neurons and glia and the cis-regulatory DNA regions which control developmental transcription factor expression (Figure 1). Studying the developmental transcription factor cascades which control the specification of neurons and glia has the potential to identify novel circuits which control sleep and wakefulness, reveal the mechanisms which control differences in sleep timing, amount, and expression in different species and identify the timepoint in evolution when different sleep–wake control neurons appeared. Study of these developmental transcription factors will be facilitated by large-scale studies which propose a similar approach to define every unique cell type in mouse brains [14–16] and will allow identification of novel cell types involved in sleep–wake control where no other unique markers are available, particularly in ancient regions such as the brainstem reticular formation, hypothalamus, and basal forebrain. Identification of these transcription factor cascades may lead to novel therapeutic approaches for sleep and circadian disorders by creating specific types of sleep–wake neurons to replace those which are lost in degenerative disorders or by manipulating the activity of those transcription factor cascades which remain active in adults.

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