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Circannual transitions in gene expression: Lessons from seasonal adaptations

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Abstract

Circannual timing is important for the coordination of seasonal activities, particularly promoting survival of individuals in adverse conditions through adaptive physiological and behavioral changes. This includes optimizing survival of offspring by coordinating reproductive efforts at appropriate times. Thus timing is very important for overall fitness. In this review, we provide several examples of circannually timed events, discussing the physiological changes that accompany these events, and some of the known genes and pathways underlying these changes. We then describe five candidate systems, including mammalian hibernation, that are potentially involved in circannual timing. Finally, we discuss several recent advances in molecular biology and animal husbandry that have made the use of non-model organisms for research more feasible, which will hopefully promote and encourage further advancement in the knowledge of circannual timing.

Keywords

Circannual timing; hibernation; migration; birdsong; thyroid hormone; VGF; retinoic acid; primary cilia; melatonin

1. Introduction

Circannual behavior is an adaptation to the physical world. Animals have no control over the physical environment so they have developed many different strategies to anticipate seasonal changes and prepare accordingly for survival and/or maximum fitness. One example of an adaptation to environmental stresses is diapause, a developmental arrest mechanism implemented by some invertebrate species, including the nematode *Caenorhabditis elegans* (reviewed in Carey et al., 2003) and some insects (discussed in chapter #). Here, we focus on circannual events utilized primarily by vertebrates. Examples of some of these events are provided in Figure 1. The molecular pathways underlying the timing of these circannual events are not well characterized, but active research on this subject has provided many advances in the field. Here, we will describe some well known examples of seasonal events, and provide some ideas about how they could be coordinated and controlled on a seasonal basis.

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2. Seasonal courtship and territorial behavior

Seasonally reproducing animals often display courtship or territorial behavior associated with their mating season. For instance, male songbirds sing complex courtship songs robustly in their breeding season both to attract mates and deter other males. Consequently, singing is a circannual behavior in various species, including canaries (*Serinus canaria*), song sparrows (*Melospiza melodia*), and many others. Songbirds have a system of nuclei (clusters of neurons in the central nervous system) that control song production and learning (Figure 2). The motor pathway controlling song production consists of projections from the interfacial nucleus of the nidopallium (Nif) to the nidopallial vocal nucleus HVC (used as a proper name). HVC projects to the robust nucleus of the archipallium (RA), which then projects to the midbrain and brainstem syringeal and respiratory motoneuronal pools that activate the muscles used for vocalizing (Brenowitz et al., 1997). In the anterior forebrain pathway, which plays an important role in song learning and development, HVC projects to striatal Area X, which then projects to the medial portion of the dorsal lateral nucleus (DLM) of the thalamus. DLM projects to the lateral magnocellular nucleus of the anterior nidopallium (IMAN), which then projects both back to Area X and also to RA to influence the song production pathway. Extensive seasonal plasticity has been reported in these song nuclei, with a significant increase in nuclei size during the breeding season when the singing behavior occurs (Nottebohm, 1981; Smith, 1996; Smith et al., 1997; Smith et al., 2004) (Figure 3a). This increase in size is due to neurite growth, increased synapse formation and neurogenesis (Tramontin and Brenowitz, 2000; Nottebohm, 2005).

Several parameters have an effect both on song behavior and the underlying neural mechanisms. Testosterone triggers singing behavior and an increase in song nuclei volume in female canaries, which do not usually sing or exhibit seasonal plasticity (Nottebohm, 1980). House sparrows (*Passer domesticus*) housed in constant conditions and administered melatonin mimicking long days comparable to their natural breeding season exhibited large song control nuclei. Conversely, sparrows administered short day melatonin durations exhibited small song nuclei (Cassone et al., 2008). Melatonin receptors are found in the song control system of house sparrows, including HVC, RA, and Area X (Whitfield-Rucker and Cassone, 1996), suggesting that melatonin signaling could influence seasonal song production.

On the molecular level, a recent study examined seasonal gene expression changes in HVC and RA in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) (Thompson et al., 2012). They identified over 300 differentially expressed genes, including cerebellin precursor protein 2 (CBLN2), which is important for synaptic plasticity and is upregulated during long days when the song nuclei are increased in volume, but down-regulated during short days. Similarly, brain-specific angiogenesis inhibitor 1-associated protein 2 (BAIAP2), important for positive neurite extension and axonogenesis, is upregulated at the beginning of exposure to long days, but is downregulated after long exposure to long days and in short days. In addition to plasticity-related genes, there was also differential expression of genes associated with apoptosis, angiogenesis, and proliferation, along with transcripts for growth factors and hormones. Mukai et al. (2009) used the same microarray methods in song sparrows to examine seasonal changes in gene expression in the hypothalamus in relation to

territorial aggression behavior, which included both singing and aggressive displays. They found differential expression of genes associated with thyroid hormone signaling, including glycoprotein hormones alpha chain precursor (*CGA*), mu-crystallin homolog (*CRYM*), and transthyretin precursor (*TTR*). *CGA* and *CRYM* were increased in spring, while *TTR* was increased in autumn. These changes occurred even in control animals and were therefore associated with season and not territorial behavior.

3. Migration

While seasonal courtship behavior specifically promotes successful reproductive behavior by attracting mates and defending resources, there are also several examples of circannually timed events that are important for individual survival in order to get to the next reproductive season. One example is migration. Migration refers to general animal movement, and is widely found in Kingdom Animalia, from mammals to arthropods (Drickamer et al., 2002; Dingle and Drake, 2007). Seasonal migration is an adaptive behavior where animals relocate prior to the onset of unfavorable conditions, such as cold temperatures and low food availability. For this section, we will focus on bird migration, one of the most well-known and well-studied examples. The arctic tern (*Sterna paradisaea*) is the most extreme case, with some individuals annually flying over 80,000 kilometers roundtrip between the Arctic and the Antarctic (Egevang et al., 2010).

Migration consists of two phases, a preparation phase and the actual migration phase. Both phases are seasonally timed according to photoperiod, a consistent and reliable seasonal cue (Drickamer et al., 2002). In the preparation phase, there are substantial behavioral, morphological, and physiological changes that occur, including hyperphagia (Odum, 1960), accumulation of fat reserves (Ramenofsky et al., 1999), muscle hypertrophy (Piersma, 1998), and changes in energy metabolism that specifically increase capacity for fatty acid utilization (Guglielmo et al., 2002; Guglielmo et al., 2002). Prior to migration, birds exhibit increased activity levels at the time of day they would normally migrate, termed migratory restlessness, which occurs even in captive birds kept in constant conditions (Drickamer et al., 2002).

During the migration phase, there is considerable species-specific variation, particularly in trip length, direction of travel, final destination, and frequency/duration of stopovers for refueling. Stopovers serve an important purpose in allowing the bird to replenish energy reserves lost during flight, and the frequency and duration of these stopovers has a great effect on total migration time (Schaub et al., 2008). Selection of stopover sites is important, because stopping at ideal areas with greater food availability can decrease the overall amount of time spent at the site. However, other factors can affect site selection as well, including the migrant's energetic condition, wind, and weather conditions (Moore et al., 2005). Some species fly over large bodies of water making it impossible to stop until reaching the other side (Lowery, 1946; Larkin et al., 1979). Therefore, stopovers must be precisely timed and coordinated to maximize fitness, both to accumulate enough energy for flight and to arrive at the final destination at the appropriate time.

In spite of the wealth of information available about migration physiology, there is little known about how migration is seasonally controlled at the molecular level, presumably due to the difficulty in studying such a dynamic phenotype in a laboratory setting. Variants in the circadian gene *Clock* have been implicated in the seasonal timing of breeding in barn swallows (*Hirundo rustica*) and are also predicted to be involved in migration (Caprioli et al., 2012). A microsatellite approach in European blackcaps (*Sylvia atricapilla*) found an association between migratory behavior and a microsatellite polymorphism within the 3'UTR of the *ADCYAP1* gene locus (Mueller et al., 2011). *ADCYAP1* encodes pituitary adenylate cyclase activating polypeptide 1 (PACAP) which stimulates adenylate cyclase and increases levels of cyclic adenosine monophosphate (cAMP). PACAP is involved in conveying photoperiod information via the retinohypothalamic tract (RHT), which directly connects the photosensitive ganglion cells of the retina to the suprachiasmatic nucleus (SCN) of the hypothalamus (location provided in Figure 4) (Hannibal et al., 1997). The SCN is considered the master circadian clock in mammals and birds, and is entrained to the external environment by light input from the RHT. Migration occurs on a circannual basis, but it is likely that the circadian system plays a role in this timing as well. Photoperiod varies according to the time of year, therefore the light signal received in the SCN would also vary seasonally.

The SCN also regulates the hormone melatonin, which is produced and released into the bloodstream by the pineal gland at night. Melatonin acts as both a clock and a calendar in the body, because melatonin is only present at night and the duration of melatonin is directly related to the length of the night, which varies seasonally. PACAP stimulated melatonin synthesis in pinealocytes (Simonneaux and Ouichou, 1993) and also modulated expression of clock genes in the pineal (Nagy and Csernus, 2007), and thus clearly plays a role in circadian and/or circannual timing. PACAP could be important for tracking photoperiod changes over seasons for coordination of seasonal activities in migrating birds and other animals with seasonal activity. Additionally, intracerebroventricular (ICV) injection of PACAP into chicks resulted in changes in energy metabolism, including increased lipid utilization (Tachibana et al., 2007), which indicates that PACAP could also be playing a role in migration preparation, facilitating a fuel switch to lipid metabolism.

4. Hibernation

Hibernation, like migration, is another survival adaptation to periods of harsh conditions and low food availability. Obligate hibernation, the main focus of this section, is an annual period of heterothermy where mammals undergo a controlled reduction in body temperature, heart rate, and metabolic rate to conserve energy (Figure 5a) (Carey et al., 2003; Geiser, 2004). Hibernation is widespread in Class Mammalia suggesting that the ability to hibernate is an ancestral mammalian trait (Carey et al., 2003). The hibernation phenotype is variable across species, with the Arctic ground squirrel (*Urocitellus parryii*) on the extreme end of the spectrum with body temperatures recorded below 0°C (Barnes and Ritter, 1989).

During obligate hibernation, mammals go through periods of torpor, ranging from days to weeks, where body temperature is only a few degrees above the ambient temperature and metabolic rate is 2-4% of normal (Figure 5a) (Carey et al., 2003). These bouts of torpor are

interspersed with brief returns to normal body temperature and metabolic rate, called interbout arousals (IBAs) that typically last less than one day. The reason for these energy costly returns to normothermia is not known, but the IBAs occur regularly throughout the hibernation season, suggesting another specific timing mechanism is in place to coordinate them.

Hibernation of small mammals is more feasible to study in a lab environment than migration, so more is known about the molecular changes underlying the physiology of this phenotype. Several examples are described below.

4a. Satiety and feeding behavior

Obligate hibernation is circannually timed, with a period of preparation preceding the onset. During this preparation, mammals are hyperphagic (Barnes and Mrosovsky, 1974) and body weight increases dramatically due to accumulation of white adipose stores (Jameson Jr and Mead, 1964). Roche 454 sequencing of the white adipose tissue transcriptome in thirteen-lined ground squirrels revealed several genes in various tissues important in this preparation period. These include Acyl Co-A desaturase (*ACOD*) and adipocyte fatty acid binding protein (*FABP4*), which are involved in lipogenesis and are highly expressed in August when the squirrels are increasing their fuel stores in preparation for hibernation (Hampton et al., 2011). In October, there is an increase in leptin (*LEP*) in the white adipose tissue, which encodes for a satiety signal received by receptors in the hypothalamus (Figure 5b).

In the hypothalamic transcriptome of the same species, orexigenic hypocretin (*HCRT*), agouti-related protein (*AGRP*), and neuropeptide Y (*NPY*) are highly expressed during April when the ground squirrels have emerged from hibernation and are starting to increase their food consumption (Figure 5b) (Schwartz et al., 2013). Anorexigenic cocaine- and amphetamine-regulated protein prepropeptide (*CARTPT*) is low during April, but is highly expressed during October, when food consumption is declining (Figure 5b). *HCRT* remains elevated during October, while *AGRP* and *NPY* levels drop at this time (Figure 5b). This preparatory period is extremely important for survival during hibernation, because the accumulated white adipose stores are used as a fuel source for the entire hibernation season.

Food availability is a very strong entrainment stimulus during the active season in brown bears (*Ursus arctos*), a hibernating species that is metabolically suppressed, but only exhibits decreases in body temperature to 32–34°C, and thus is not considered an obligate hibernator (Ware et al., 2012). Bears shifted their activity patterns according to feeding times in spite of a natural photoperiod, exhibiting a behavioral flexibility that could be necessary to take advantage of unpredictable periods of food availability in order to gain the necessary energy reserves for hibernation (Ware et al., 2012). Additionally, similar to the previously mentioned findings in ground squirrel, leptin levels increased in brown bears prior to hibernation onset (Hissa et al., 1998).

4b. Fuel switch

Generally, hibernating mammals do not use food consumption as a source of energy, although there are some instances of food caching (Gillis et al., 2005). Some ground squirrel

species will not eat any food during hibernation, even if it is offered artificially in the lab (Torke and Twente, 1977). Instead, hibernators rely on a metabolic switch from carbohydrates to lipids by catabolizing their accumulated fat stores for energy.

The fuel switch is particularly important in the heart. In hibernation, the heart beats at a much slower rate during torpor bouts: about 5 beats per minute in the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) (Hampton et al., 2010). Northern blot analysis showed that two key genes involved in regulating metabolism were upregulated in the heart during hibernation, pancreatic triacylglycerol lipase (*PTL*), which frees fatty acids from triglycerides at low temperatures, and pyruvate dehydrogenase kinase isozyme 4 (*PDK4*), which prevents conversion of pyruvate to acetyl CoA, thus blocking glucose oxidation (Andrews et al., 1998). Protein analysis found that PDK4 and PTL protein were also upregulated in the heart during hibernation (Buck et al., 2002; Squire et al., 2003), along with succinyl CoA-transferase, the rate limiting enzyme in the catabolism of ketone bodies (Russeth et al., 2006). This fuel switch is evident in other tissues as well, including skeletal muscle, which is inactive during torpor but active during IBAs, and white adipose tissue, the primary fuel source for the body (Hampton et al., 2011).

Further evidence of the fuel switch during hibernation comes from the blood. Analysis of hibernator serum indicated that levels of a fat-derived ketone, D- β -hydroxybutyrate (BHB) are elevated during torpor and is the preferential fuel source of the heart and brain during torpor and arousal, even in the presence of glucose (Andrews et al., 2009). BHB is also elevated during hibernation in the liver (Serkova et al., 2007), where it is produced. A key enzyme in the synthesis pathway of BHB, hydroxymethylglutaryl-CoA synthase 2 (*HMGCS2*), is also upregulated in the liver during hibernation (Epperson et al., 2004).

Seasonal molecular changes also occur in the brain to facilitate this metabolic fuel switch. Monocarboxylic acid transporter 1 (*MCT1*), which is also known as solute carrier family 16, member 1 (*SLC16A1*), is a ketone transporter that facilitates transport of BHB into the brain. This transporter is upregulated at the blood brain barrier during torpor in ground squirrels (Andrews et al., 2009). *SLC16A1* mRNA levels are also upregulated in the cerebral cortex and hypothalamus during hibernation (Schwartz et al., 2013).

4c. Central nervous system in hibernation

Like the heart, one area of the brain, the hypothalamus, remains active throughout the hibernation season (Kilduff et al., 1982; Kilduff et al., 1990; Bratincsak et al., 2007). This region of the brain is involved in many aspects important for hibernation and hibernation preparation, including food intake, circadian rhythms, sleep, and thermoregulation. The SCN of the hypothalamus, containing the circadian clock, is a likely candidate for the control of circannual hibernation timing. *c-fos* mRNA, used as a marker for neuronal activation, increases in the SCN during torpor and peaks during arousal (Bitting et al., 1994; Bratincsak et al., 2007), suggesting that it is involved in torpor bout timing. Broad hypothalamic lesions prevented successful hibernation (Satinoff, 1967) and focused lesions specific to the SCN altered hibernation timing in ground squirrels (Ruby et al., 1996), which suggests that the hypothalamus is important for both hibernation induction and maintenance. However, clock

gene rhythms (*per1*, *per2*, *bmal1*) are suppressed during hibernation (Revel et al., 2007), so it is unclear how or if the circadian clock is specifically involved in hibernation timing.

In contrast to the hypothalamus, the rest of the brain is relatively quiescent during hibernation. The cerebral cortex is the first area of the brain to lose activity as the animal goes into a torpor bout, followed by the brainstem and reticular formation, thalamus, and limbic system (Heller, 1979). They regain activity upon arousal in the opposite order. The cerebral cortex, hippocampus, and thalamus all exhibit extensive neurite and synaptic plasticity during hibernation, with synapses dissociating during torpor but reconnecting appropriately upon arousal to an IBA (Popov et al., 1992; Magariños et al., 2006; von der Ohe et al., 2006; von der Ohe et al., 2007) (Figure 3b). Synaptic dissociation could help conserve energy (Drew et al., 2001) and could also serve a neuroprotective purpose (Schwartz et al., 2013). Recent Illumina HiSeq sequencing of the cerebral cortex transcriptome indicates that the brain upregulates transcripts important for plasticity and remodeling prior to hibernation onset (Schwartz et al., 2013), suggesting that this synaptic plasticity is circannually timed as well.

5. Potential pathways involved in circannual timing

As mentioned previously, the hypothalamus controls many parameters important for these seasonal events, including feeding/satiety, circadian rhythms, reproduction, sleep, hormone release, and thermoregulation. Thus, it is likely that this brain region is involved in circannual timing, and there are several candidate systems potentially involved in coordinating this timing.

5a. Thyroid hormone signaling

Thyroid hormone signaling is affected by photoperiod and is involved in the coordination of seasonal reproduction (Nakao et al., 2008). Light associated with long days induces conversion of thyroxine (T4) to triiodothyronine (T3) in the mediobasal hypothalamus of Japanese quail (*Coturnix japonica*) by upregulation of *DIO2*, the type 2 iodothyronine deiodinase (Yoshimura et al., 2003). This increased T3 stimulates luteinizing hormone, which initiates expression of thyroid stimulating hormone *TSH* and *DIO2* in the pars tuberalis of the anterior pituitary gland (Figure 4) (Nakao et al., 2008). ICV injection of TSH into quail kept in short day conditions stimulated a long day-like growth of gonads and *DIO2* expression, triggering seasonal breeding (Nakao et al., 2008). Similarly in mammals, high *DIO2* expression is found in the blood vessels surrounding the arcuate nucleus of the hypothalamus and in the ependymal cells of the third ventricle of Siberian hamsters (*Phodopus sungorus*) maintained in long day conditions, which decreased after melatonin injections (Watanabe et al., 2004). Ependymal cells separate the brain from the cerebrospinal fluid filled ventricles, acting as a barrier and serving a role in transport (Bruni, 1998). *TSH* and *DIO2* were also shown to be upregulated in mice in response to increasing photoperiod (Ono et al., 2008). Therefore, it appears that thyroid hormone signaling plays an important role in seasonal timing in both mammals and birds. Similarly, thyroid hormone signaling also plays a role in the timing of amphibian metamorphosis, where individuals progress through specific developmental stages, discussed in chapter #.

Thyroid hormone signaling also appears to play a role in hibernation. Levels of thyroxine-binding globulin (*TBH*) are increased during hibernation in the liver of the golden-mantled ground squirrel (*Callospermophilus lateralis*) (Epperson and Martin, 2002). *TBH* binds both T3 and T4. In the hypothalamus of the thirteen-lined ground squirrel, thyrotrophin releasing hormone (*TRH*) expression peaks in October during the pre-hibernation period (Schwartz et al., 2013). Additionally, *DIO2* expression peaks in April after completion of hibernation, a time when the squirrels are becoming reproductively active. *DIO2* expression is very low in every other time period observed. *CGA*, the alpha subunit of TSH, increases greater than 4 fold in April compared to any other collection point. *CRYM*, a thyroid hormone binding protein, also shows seasonal differential expression with decreased expression in October compared to all other points. *TTR* shows minimal expression in the hypothalamus, but is differentially expressed in cerebral cortex, with increased expression in the hibernation collection points compared to outside hibernation. As mentioned previously, *CGA*, *CRYM*, and *TTR* are all differentially expressed in song sparrow hypothalamus according to season as well.

5b. VGF signaling

VGF (non-acronymic) is a protein involved in energy balance and reproduction (Jethwa and Ebling, 2008). It is processed into several small peptides by prohormone convertases and is expressed throughout the brain, although it is most abundant in the hypothalamus. *VGF* mRNA is induced in the arcuate nucleus of the hypothalamus (Figure 4) in Siberian and Syrian hamsters (*Mesocricetus auratus*) switched from a long day photoperiod to short days (Barrett et al., 2005). *VGF* knockout mice are lean and hypermetabolic, and resisted obesity even with genetic predisposition (Hahm et al., 2002). These mice exhibited cold-intolerance, decreased fat storage in both white and brown adipose tissue, upregulation of brown adipose uncoupling proteins 1 and 2 (*UCPI-2*), and an increase in the overall amount of mitochondria and in the density of mitochondrial cristae (Watson et al., 2009).

VGF also could play a role in synaptic plasticity. Long term depression (LTD) could not be induced in hippocampal slices taken from *VGF* knockout mice, although there were no deficits in long term potentiation (LTP) (Bozdagi et al., 2008). LTD and LTP are activity-dependent changes in synaptic efficacy resulting from synaptic plasticity--actual changes in the synaptic structure. LTD weakens synaptic strength, while LTP enhances it. The absence of *VGF* resulted in the inability to weaken synaptic strength, suggesting that *VGF* could be important for the reduction of synaptic connectivity seen in the song control system after the breeding season in songbirds, and also in torpor bouts of hibernating mammals.

Hibernation is characterized by changes in energy metabolism and thermoregulation and also involves coordinated synaptic plasticity, making *VGF* a candidate for involvement in the control of this phenotype. In the thirteen-lined ground squirrel brain, *VGF* expression is increased in October in both the cerebral cortex and the hypothalamus (Schwartz et al., 2013). This time point is characterized by decreased food consumption, increased white adipose stores, and onset of shallow torpor bouts. *VGF* in the hypothalamus could be playing a role in the seasonal switch from the hyperphagic behavior of summer and early fall

to the hypophagic behavior seen in hibernation. The fact that the same expression pattern is seen in the cerebral cortex suggests that it could also be playing a role in synaptic plasticity.

5c. Modulation of neuronal cilia

The primary cilium is a cellular organelle found in almost all eukaryotic cells, including neurons (Louvi and Grove, 2011). Each cell has only one primary cilium, a protrusion of the cell membrane covering a cytoskeletal axoneme core consisting of nine microtubule pairs which provides structural support to the cilium (Figure 6). The axoneme is anchored by a ciliary basal body, which is a modified centriole. There is a transition zone at the base of the primary cilium for selected protein entry via intraflagellar transport. The exact function of neuronal primary cilia is unclear, but these organelles sense and receive signals from the environment and relay that information to the cell (Adams, 2010).

Deficits in primary cilia are the cause of several disorders, deemed ciliopathies, including Bardet-Biedl syndrome (BBS) and Alstrom syndrome (ALMS), both characterized by obesity (Badano et al., 2006). BBS is associated with mutations in at least 11 genes (*BBS1-11*) (Mutch and Clément, 2006). *BBS1* has been implicated in fat storage in *Caenorhabditis elegans* (Mak et al., 2006). Loss of primary cilia in the brain using conditional alleles of two ciliogenic genes (*Tg737* and *Kif3a*) induced hyperphagia and obesity in adult mice (Davenport et al., 2007). This phenotype was induced with the targeted deletion of primary cilia exclusively on POMC-expressing neurons in the hypothalamus, suggesting that satiety circuits are disrupted. Further, knockout of *BBS2*, *BBS4*, or *BBS6* in mice induces leptin resistance, indicating that BBS proteins are necessary for reception of this satiety signal (Seo et al., 2009).

Involvement of the neuronal primary cilia in satiety and feeding behavior indicates that they could be very important in regulating several circannually timed events. Both migration and hibernation require periods of hyperphagia and weight gain, along with long periods of hypophagia. In the hypothalamus of the thirteen-lined ground squirrel, several BBS transcripts are differentially expressed across the hibernation season, including *BBS1*, which is elevated during April and October, and *BBS2*, *BBS4*, *BBS5*, and *BBS9*, which are all elevated during hibernation (both torpor and IBA) (Schwartz et al., 2013). Additionally, intraflagellar transcripts *IFT80*, *IFT81*, and *IFT88* are also upregulated during torpor in the ground squirrel hypothalamus. Ground squirrels will not eat during interbout arousals even if food is provided artificially in the lab (Torke and Twente, 1977), most likely due to strong satiety signals that are in place to prevent foraging during periods of time when food is scarce and conditions are unfavorable. The expression of BBS and IFT transcripts during hibernation could be playing a role in orchestrating this satiety through modulation of neuronal cilia.

5d. Retinoic acid signaling

Retinoic acid is a derivative of retinol (vitamin A) which has a wide variety of effects on development, differentiation, and homeostasis (Chambon, 1996). Retinoic acid has two receptor families, RXR and RAR, each with three subtypes (α , β , γ). Retinoic acid signaling is affected by photoperiod and could play an important role in circannual timing.

Lengthening photoperiod was shown to enhance retinoic acid signaling in mouse hypothalamus (Shearer et al., 2010). Cellular retinoic acid binding protein 1 (*CRBP1*) is downregulated during short photoperiods (Barrett et al., 2006). Retinoic acid receptors (*RXR*, *RAR*), *CRBP1*, and *CRBP2* are upregulated in the arcuate nucleus of the hypothalamus in Siberian hamsters when they are switched from short to long photoperiods (Ross et al., 2005). *RXR* appears to be specifically associated with changes in body weight (Ross et al., 2004).

Retinoic acid signaling is also important for synaptic plasticity. Retinol deficiency resulted in a loss of LTP and LTD in mouse hippocampus, which was reversed by direct application of retinol or retinoic acid (Misner et al., 2001). Deficiency of *RAR β* eliminates LTP and LTD in mice hippocampus, while *RXR γ* deficiency results in a loss of LTD but not LTP (Chiang et al., 1998), similar to the *VGF* knockout mice discussed above. Retinoic acid and *RAR α* have also been shown to play a role in enhancing synaptic scaling, a method of homeostatic plasticity employed by individual neurons to stabilize their firing rates (Aoto et al., 2008). Synaptic plasticity is a characteristic of several circannual rhythms discussed here, including hibernation and seasonal birdsong.

In the thirteen-lined ground squirrel hypothalamus, several retinoic acid signaling-related genes are differentially expressed across the hibernation season (Schwartz et al., 2013). *RXR α* , and *RXR γ* are both elevated during the pre-hibernation October collection point. *CRBP1* is elevated in April compared to all other collection points. Stimulated by retinoic acid gene 6 homolog (*STRA6*) is also elevated in April, while the gene encoding for retinoic acid induced 1 (*RAI1*) is upregulated in both April and October. There are also several transcripts associated with retinol, the precursor to retinoic acid, including retinol binding protein 1 (*RBPI*), and retinol dehydrogenase 11 (*RDH11*). *RBPI* is involved in the transport of retinol and is elevated in April. *RDH11* is elevated during hibernation, and interestingly is involved in adaptation to dark in the retina (Kasus-Jacobi et al., 2005), suggesting a potential role in transducing photoperiod.

5e. Melatonin and clock genes

Melatonin is produced and released by the pineal gland at night and is inhibited during the day by light via the SCN, which receives a direct photic input from the retina by the RHT. This photoperiodic input entrains the circadian clock to the surrounding environment. A suite of clock genes in the SCN (*Per*, *Cry*, *Bmal1*, *Clk*, *Rev-Erba*, *RORA*, *DEC1*) form autoregulatory feedback loops that generate the 24 hour expression patterns that drive circadian rhythms in all cells (Figure 7) (Kawamoto et al., 2004; Ko and Takahashi, 2006). Because the nightly duration of melatonin is representative of the length of the night, melatonin is an excellent molecular indicator for time of year. Additionally, light input to the SCN also varies according to seasonal time, so the circadian clock could also be playing a role in interpreting circannual time.

Melatonin receptors are found throughout the brain, but the most concentrated areas are the pars tuberalis and parts of the hypothalamus (Morgan et al., 1994), supporting the role of melatonin in seasonal behavior. Melatonin receptors are also found in the song control system of songbirds (Whitfield-Rucker and Cassone, 1996), and as mentioned previously,

exogenous melatonin can stimulate or inhibit singing behavior and the associated neural changes (Cassone et al., 2008). Untreated pinealectomized pied flycatchers (*Ficedula hypoleuca pallas*) failed to orient in the correct direction for migration, but pinealectomized flycatchers that had received nightly melatonin injections exhibited the correct migratory orientation (Schneider et al., 1994). Binding of radiolabeled melatonin was decreased in the pars tuberalis of hibernating ground squirrels (Stanton et al., 1991), most likely due to a decrease in receptor number, suggesting that the melatonin system is seasonally regulated.

Circadian clock genes could also be playing a role in seasonal timing. Clock genes were constantly expressed throughout hibernation in European hamsters (*Cricetus cricetus* L.) but their 24 hour rhythm was eliminated (Revel et al., 2007). Specifically, *Per1* was elevated during hibernation to daytime levels of euthermic hamsters, while *Per2* was expressed at approximately nighttime levels. *Bmal1* expression was intermediate between day and night levels. In the thirteen-lined ground squirrel hypothalamus, two clock genes were differentially expressed: *BMAL1* (*ARNTL*) and *DEC1* (*BHLHE40*). *BMAL1* exhibits low expression in April compared to every other collection point, while *DEC1* is elevated during torpor (Schwartz et al., 2013).

6. Recent advances in circannual timing research

Advances in molecular biology have allowed substantial progress in seasonal timing research, particularly in hibernation. Use of two dimensional gel electrophoresis and mass spectrometry has uncovered changes in the proteome of hibernating thirteen-lined ground squirrels (Russeth et al., 2006; Epperson et al., 2010a; Epperson et al., 2010b). An *in vivo* approach employing proton magnetic resonance spectroscopy (^1H MRS) was used to measure brain metabolites during hibernation in ground squirrels (Henry et al., 2007). Next-generation sequencing, including Roche 454 (Hampton et al., 2011) and more recently Illumina HiSeq 2000 (Schwartz et al., 2013), has uncovered transcriptional changes during hibernation in several tissues of the thirteen-lined ground squirrel. Additionally, microarrays have been recently utilized to analyze seasonal gene expression changes in song control regions in sparrows (Mukai et al., 2009; Thompson et al., 2012).

Further advancing research in circannual timing, the Broad Institute of MIT and Harvard (<http://www.broad.mit.edu>) has sequenced the genomes of four hibernating mammals over the last decade, including *Ictidomys* (formerly *Spermophilus*) *tridecemlineatus* (thirteen-lined ground squirrel), *Myotis lucifugus* (little brown bat), *Eptesicus fuscus* (big brown bat), and *Erinaceus europaeus* (common hedgehog). There are few genomic resources available for migrating bird species, although recently a genome-wide single nucleotide polymorphism set was developed for the barnacle goose (*Branta leucopsis*) (Jonker et al., 2012). The genome of the zebra finch (*Taeniopygia guttata*) has been sequenced as well (Warren et al., 2010), and while this is not a seasonal breeding/singing species, it does exhibit plasticity associated with song learning in the brain during juvenile development (Brenowitz and Beecher, 2005), which could provide clues about the pathways underlying seasonal plasticity in songbird brains.

In addition to recent technological advancements and approaches making it easier to examine molecular changes in non-model organisms, there have also been advances in genetic manipulations of non-model organisms used in seasonal studies. Recently, we published a study using adenovirus to overexpress the metabolic hormone *FGF21* at several different points during the year in thirteen-lined ground squirrels (Nelson et al., 2013). This transgenic approach sets the stage for similar studies in non-model organisms where generating a knockout animal or obtaining a specific cell line has been difficult or impossible.

Finally, advancements in husbandry resources have made keeping non-model experimental animals in the laboratory a less risky venture. For instance, a wealth of information exists on caring for a captive colony of thirteen-lined ground squirrels, along with information on wild capture and captive breeding (Vaughan et al., 2006; Merriman et al., 2012). Recently published husbandry practices for bats are also available (Giannini, 2011). Similarly, husbandry information exists for passerine birds (Order Passeriformes), which includes many migrating species and seasonal singers (Bateson and Feenders, 2010). These resources provide an important foundation to help scientists focus on using these valuable species to address research questions without having to devote as much time to establishing protocols for animal care and colony maintenance.

7. Conclusions: Future directions and Potential Applications

As the previous section details, advances in molecular tools, particularly for non-model organisms, will greatly improve and enhance our ability to study seasonal behaviors and the circannual timing underlying them. The value of non-model organisms to the study of seasonal phenotypes needs to be better understood and even heralded, particularly because these organisms can find answers to questions ill-suited to traditional model organisms (Bolker, 2012). Taking advantage of unique phenotypes like hibernation could greatly benefit several areas related to human health (for example (Klein et al., 2010; Mulier et al., 2011)). Uncovering some of the genes and pathways underlying the induction and maintenance of hibernation in particular could benefit research in obesity, neuroprotection, cardiovascular disease, muscle disuse atrophy, sleep, and seasonal disorders like SAD. Similar benefits could arise from studying the molecular genetics of migrating birds or seasonally singing birds. In the words of Jessica Bolker: There's more to life than rats and flies (Bolker, 2012).

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Figure 1.

Circannually timed events. Representative examples and approximate annual timing of different seasonal events are provided along with an example species. Light/dark cycles illustrated above the months are representative of the annual changes in photoperiod in the northern United States of America. Reproduction represents both mating behavior and gestation.

Figure 2.

Birdsong song control system. Projections of the motor pathway, which controls song production, are in green. Projections of the anterior forebrain pathway, which controls song learning and development, are shown in blue. Model is based on (Tramontin and Brenowitz, 2000; Nottebohm, 2005). DLM: dorsal lateral nucleus, HVC: used as proper name, IMAN: lateral magnocellular nucleus of the anterior nidopallium, Nif: interfacial nucleus of the nidopallium, RA: robust nucleus of the arcopallium.

a. Song control system

b. Hibernation

Figure 3.

Seasonal plasticity in the brain. a. Illustration of the representative changes in song nuclei volume in the brains of songbirds from spring, when courtship singing occurs, to winter, when singing behavior ceases. Model is based on data from Smith et al. (1996). b.

Illustration of the plasticity and remodeling that occurs during hibernation in the ground squirrel. Dendrites regress and synapses dissociate during torpor, but reconnect upon return to IBA. Model is based on data from several sources (Popov et al., 1992; Magariños et al., 2006; von der Ohe et al., 2006; von der Ohe et al., 2007). HVC: used as proper name, RA: robust nucleus of the arcopallium, IBA: interbout arousal.

Figure 4.

Hypothalamic and pituitary anatomy. Sagittal view of mammalian hypothalamic and pituitary anatomy. The mediobasal hypothalamus is encompassed by red dashed lines. The pars tuberalis, part of the anterior pituitary, is shaded in gray. AHA: anterior hypothalamic area; ARC: arcuate nucleus; DMN: dorsomedial nucleus; ME: median eminence; MN: mammillary nuclei; OC: optic chiasm; POA: preoptic area; PHA: posterior hypothalamic area; PVN: paraventricular nucleus; SCN: suprachiasmatic nucleus; SO: supraoptic nucleus; VMN: ventromedial nucleus

Figure 5.

Food consumption and satiety signaling in hibernation. a. Body temperature of a seasonal hibernator. The solid black line displays a representative trace of annual core body temperature taken from a transmitter implanted in a thirteen-lined ground squirrel. The red dashed line represents ambient temperature in the environmental chamber. During hibernation (November 1-March 31), the ambient temperature is lowered to 5°C, food is removed, and the animals are kept in constant darkness. During this time squirrels cycle through bouts of torpor and interbout arousals (IBAs), examples of which are labeled. IBAs represent returns to euthermia despite low ambient temperature. b. Food consumption and differential expression of orexigenic (HCRT, AGRP/NPY) and anorexigenic (CARTPT, LEP) transcripts over the hibernation season. Food consumption (g/day) is represented by the black line. Each point represents average daily consumption over a week ($n=11$). During the hibernation period, food is removed, so no consumption is recorded. Error bars are standard error of the mean. Expression values of orexigenic and anorexigenic genes are shown as the percentage of the maximum value. HCRT (blue), AGRP/NPY (green), and CARTPT (orange), are taken from Illumina HiSeq 2000 transcriptome sequencing of the thirteen-lined ground squirrel hypothalamus during October, Hibernation, and April ($n=3$ for each point) (Schwartz et al., 2013). LEP (yellow) is from the same method of sequencing of the white adipose tissue ($n=2$ for each point). Torpor and IBA were analyzed separately, but there was no difference between them for all four genes, so they were combined into one point (Hibernation). AGRP: agouti-related protein; CARTPT: cocaine and amphetamine

regulated transcript prepropeptide; HCRT: hypocretin (orexin) neuropeptide precursor; IBA: Interbout arousal; LEP: leptin; NPY: neuropeptide Y

Figure 6.
Primary cilium structure. Model based on (Adams, 2010; Louvi and Grove, 2011)

Figure 7.

Mammalian circadian clock genes. Clk and Bmal1 dimerize and drive the expression of DEC1, Rev-erba, RORA, Cry, and Per, along with clock controlled genes that drive rhythmic processes of the body. Per and Cry then dimerize and feed back to inhibit Clk and Bmal1. DEC1 and Rev-erba inhibit transcription of Bmal1, while RORA drives transcription of Bmal1. The rhythmicity of these autoregulatory feedback loops drive the circadian rhythms in all cells. The master circadian clock is located in the suprachiasmatic nucleus of the hypothalamus and is entrained to the environmental photoperiod through a direct input from the retina. Model is based on (Kawamoto et al., 2004; Ko and Takahashi, 2006). Bmal1: brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like; Clk: Clock; Cry: Cryptochrome; DEC1: differentially expressed in chondrocytes 1; Per: Period; Rev-erba: nuclear receptor subfamily 1; group D; member 1; RORA: retinoic acid receptor-related orphan receptor alpha.