REVIEW

How can a binary switch within the pars tuberalis control seasonal timing of reproduction?

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Abstract

Life in seasonally changing environments is challenging. Biological systems have to not only respond directly to the environment, but also schedule life history events in anticipation of seasonal changes. The cellular and molecular basis of how these events are scheduled is unknown. Cellular decision-making processes in response to signals above certain thresholds regularly occur i.e. cellular fate determination, apoptosis and firing of action potentials. Binary *switches*, the result of cellular decision-making processes, are defined as a change in phenotype between two stable states. A recent study presents evidence of a binary *switch* operating in the pars tuberalis (PT) of the pituitary, seemingly timing seasonal reproduction in sheep. Though, how a binary switch would allow for anticipation of seasonal environmental changes, not just direct responsiveness, is unclear. The purpose of this review is to assess the evidence for a binary switching mechanism timing seasonal reproduction and to hypothesize how a binary switch would allow biological processes to be timed over weeks to years. I draw parallels with mechanisms used in development, cell fate determination and seasonal timing in plants. I propose that the adult PT is a plastic tissue, showing a seasonal cycle of cellular differentiation, and that the underlying processes are likely to be epigenetic. Therefore, considering the mechanisms behind adult cellular plasticity offers a framework to hypothesize how a long-term timer functions within the PT.

Key Words

- ▶ pars tuberalis
- ▶ binary switches
- ▶ seasonal
- ▶ timing

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Introduction

Anticipation and responsiveness to environmental signals is key to adapting to a changing environment. Organisms can schedule life history events in order to maximize fitness. For example, timing birth to the season of highest food availability. This is not solely a response to the prevailing environmental conditions but a scheduled/anticipated event, taking advantage of the predictability of seasonal cycles. These major physiological changes can take weeks to complete; thus, the organism must intrinsically know the time of year and anticipate upcoming conditions. Animals have evolved to use the

annual variation in day length (photoperiod), a highly predictive signal, together with endogenous long-term timers ('circannual clock'). Therefore, they keep track of seasonal time, even in constant conditions (i.e. the hibernacula) and activate a seasonal adaptive programme (Lincoln & Clarke 1994, Lincoln *et al.* 2005, 2006, Lincoln 2006, Wood & Loudon 2014, 2017, West & Wood 2018).

The cellular and molecular basis of how seasonal life history events are scheduled is unknown. Binary decisions are at the core of many different cellular processes, such as cellular fate determination, apoptosis and firing of action

potentials. These are all regarded as decision-making processes in response to signals above certain thresholds, which leads to all-or-nothing activation of downstream pathways. Thresholding of responses is an important way to properly space biological events and is seen during the accurately timed process of embryonic development (Ashe 2006, Chattwood *et al.* 2013). Developmental changes are characterized by binary switching of cellular phenotype from one stable state to another. A recent study presents evidence of a binary switch operating in the PT of the pituitary, seemingly timing seasonal reproduction (Wood *et al.* 2015). The purpose of this review is not to extensively review the seasonal field (for extensive reviews see: Dardente *et al.* 2014, Nakane & Yoshimura 2014, Wood & Loudon 2014, 2017), instead I will review the evidence for a binary switching mechanism in the PT. I shall draw parallels with mechanisms used in development, cell fate determination and seasonal timing in plants. Finally, I will hypothesize how a binary switch would allow biological processes to be timed over weeks to years.

The photoperiodic response

Nocturnal secretion of pineal melatonin acts as a critical transducer of photoperiod change, providing the brain with an internal hormonal representation of external photoperiod (Fig. 1, reviewed in Hazlerigg & Loudon 2008, Dardente *et al.* 2014, Nakane & Yoshimura 2014, Wood & Loudon 2014, 2017, West & Wood 2018). The PT is the only consistent site of melatonin binding in a wide range of seasonally breeding mammalian species (Morgan *et al.* 1994). It impinges on the hypothalamo–pituitary axis and governs both an anterograde (prolactin) and retrograde (TH) pathway to the pars distalis and hypothalamus, driving seasonal physiological adaptations (Wood & Loudon 2017, Korf 2018). Here, I focus on the better characterized photoperiodic thyroid hormone (TH) pathway relating to seasonal reproduction.

The PT-specific thyrotrophs contain receptors for melatonin (Klosen *et al.* 2002, Dardente *et al.* 2003, Johnston *et al.* 2006), which govern the local release of thyroid-stimulating hormone (TSH) (Dardente *et al.* 2010). The photoperiodic control of TSH production depends on upregulation of *TSH β* subunit by the transcriptional co-activator, *EYA3* on long photoperiods ('LP induction') (Dardente *et al.* 2010, Masumoto *et al.* 2010). PT TSH signals to hypothalamic tanycytes, which in turn modulate the seasonal biological availability of TH, via the expression of deiodinase enzymes (*DIOs*).

Figure 1

Retrograde action of TSH on ependymal cells in the hypothalamus. Photoperiod is encoded by the nocturnal melatonin signal that is sculpted by day length, generating short-duration signals in response to long photoperiod (LP) conditions. The prime site of action is the pituitary pars tuberalis. LP activation of *TSH β* leads to an increase in *deiodinase 2* (*DIO2*) activity in adjacent ependymal cells (tanycytes), which express the TSH receptor. *DIO2* converts inactive T_4 to active T_3 . Rfamides (including *KISS-1*) and RF-related peptides serve as neuroendocrine intermediates in the regulation of reproduction across taxa and their regulation is altered in response to photoperiod (reviewed in Kriegsfeld 2006, Simonneaux *et al.* 2013). It has shown that T_3 regulates the expression of *RFRP* and *KISS-1* (Henson *et al.* 2013), potentially via TSH (Klosen *et al.* 2013) but the mechanism of T_3 action is uncertain. *RFRP* has been noted to have high expression in LP and a low expression in SP, regardless of breeding season. It has been suggested that *RFRP* subsequently acts either directly on GnRH neurons or indirectly via kisspeptin (kp) neurons or other interneurons in the arcuate nucleus (ARC) to synchronize reproduction with season in a species dependent manner (reviewed in Henningsen *et al.* 2016).

The *DIOs* convert T_4 to active T_3 (*DIO2*) and T_4 to inactive reverse T_3 (*DIO3*) (Dardente *et al.* 2014). Hypothalamic T_3 status controls central structures involved in seasonal metabolic physiology and reproduction (Murphy *et al.* 2012, Klosen *et al.* 2013, Bank *et al.* 2017). Reproductive effects are mediated through alterations in the GnRH pulse generator, potentially involving kisspeptin and *RFRP3* (Simonneaux *et al.* 2013, Hazlerigg & Simonneaux 2015, Beymer *et al.* 2016) (Fig. 2). The *EYA3*/*TSH*/*DIO*

Figure 2

Schematic representations of the annual photoperiod, short photoperiod (SP), long photoperiod (LP) and long photoperiod refractory (LP-R). Bars to represent the daily dark and light cycle on those photoperiods, and daily melatonin. Schematic of the clock gene, *CRY1*, in the ovine pars tuberalis on LP and LP refractory animals (LP-R). Adapted from data in [Lincoln et al. \(2005\)](#). *In-situ* hybridization of the ovine pars tuberalis on LP (4 weeks) and LP-R (LP 29 weeks) for *TSHβ* and *EYA3*, (adapted under the terms of the original Creative Commons licence from [Wood et al. \(2015\)](#)), and *DIO2* (adapted, with permission, from David Hazlerigg. Copyright 2018 D Hazlerigg).

photoperiodic switch is highly conserved amongst vertebrates ([Nakane & Yoshimura 2014](#)), but PT tissue is not conserved in lower vertebrates i.e. fish ([Lincoln et al. 2003](#), [Wood & Loudon 2017](#)).

Here, I focus on the PT-specific thyrotroph as point of initiation of the physiological change but also the site showing spontaneous reversion suggesting it is the site of endogenous long-term timekeeping (see below) ([Lincoln et al. 2003](#), 2006, [Wood & Loudon 2017](#)). However, I note that in mammals, photoperiodic information is transferred from mother to pup and that photoperiodic history dependence is seen in the hypothalamic tanycytes, not the PT ([Sáenz de Miera et al. 2017](#)). Therefore, both the PT and the hypothalamic tanycytes are critical sites of photoperiodic history, circannual cue integration and transmission to the endocrine and central nervous systems ([Lincoln et al. 2006](#), [Sáenz de Miera et al. 2014](#), [Wood & Loudon 2014](#), 2017, [Wood et al. 2015](#)).

At a PT tissue-level, *TSHβ* progressively increases in RNA expression over the first 4 weeks of LP ([Wood et al. 2015](#)) (Fig. 3A), but the increase in expression is already beginning 3 days after the switch to LP in sheep ([Dardente et al. 2010](#)) and the F344 rat ([Ross et al.](#)

[2011](#)). In fact, from the first long day increased *TSHβ* is observed in quail ([Nakao et al. 2008](#)), chicken ([Dunn et al. 2017](#)) and the bunting ([Majumdar et al. 2014](#)). This suggests a direct responsiveness and transcriptional activation and is referred to as 'LP induction', but it is important to remember this can only occur if the animal has experienced an appropriate duration of winter/short photoperiod signal and that the *TSHβ* signal is not maximal until 4 weeks of LP in sheep.

The tissue response of *TSHβ* in the PT is seemingly consistent with progressive changes in sheep LH and FSH under natural photoperiods ([Billings et al. 2002](#), [Lomet et al. 2018](#)) and square wave transitions ([Bittman et al. 1983](#)), leading to the downstream reproductive changes. However, the correlations between in *DIO/TSH* and LH/FSH in hamsters are not so clear, especially when considering refractory animals (see below), potentially implicating the tanycytes as important modulators of the refractory response in hamsters ([Milesi et al. 2017](#)). Though, a directly correlative approach to causality is not necessarily appropriate when dealing with autoregulatory feedback loops across tissues. Therefore, I will focus on how these gradual tissue-level responses may be

Figure 3

(A) RNA-seq normalized counts per million displaying the amount of *TSHβ* (purple) and *CHGA* (green) transcript in the first 4 weeks of LP; SP, short photoperiod; LPD1, 1st day of long photoperiod; LPD7, 7th day of long photoperiod; LPD28, 28th day of long photoperiod. Data from Wood *et al.* (2015). (B) *In-situ* hybridization quantification for *TSHβ* (purple) after 4 weeks (LPW4), 16 weeks (LPW16) and 29 weeks (LPW29) of long photoperiod. Data from Wood *et al.* (2015). (C) Schematics showing the difference in a single-cell binary response, which can occur at a variable length of time from the LP switch and a tissue progressive response, slowly accumulating over time in LP. (D) The model proposes that an endogenous timer switches *TSHβ* expression in the PT thyrotroph cells, driving TSH and hypothalamic thyroid hormone metabolism independently of photoperiod. Individual PT thyrotroph cells are either in a long (*TSHβ*+) or short (*CHGA*+) photoperiod state, and the relative proportion of these binary-state cells determines the phase of the circannual cycle and the subsequent reproductive physiology. Data from Wood *et al.* (2015). Adapted under the terms of the original Creative Commons licence from Dardente (2015).

generated in the PT to suggest how this tissue can act as an endogenous timer.

Endogenous timekeeping: scheduling and anticipation of events

The amount of light taken from a single day is indistinguishable from a matched point in autumn or spring; thus, for photoperiodic information to be meaningful, it must be registered within the context of recent preceding photoperiods (Butler *et al.* 2010, Sáenz de Miera *et al.* 2017, West & Wood 2018). Therefore, life history trajectories and seasonal programmes are set depending on the prior photoperiodic history. Importantly, the observed acute inductive effects of LP

are only able to occur if the appropriate prior history has been experienced (reviewed in: West & Wood 2018). This demonstrates that the photoperiodic response is not a direct/immediate response to any photoperiod change but is a scheduled/anticipated responsiveness to ensure physiological changes occur at the appropriate time of year.

Changes in seasonal physiology can persist in constant conditions, and even in the absence of a pineal gland (Woodfill *et al.* 1994, Sáenz de Miera *et al.* 2014). These rhythms are no longer entrained to the solar year but proceed according to the prior photoperiodic history. In short-lived species, these rhythms only revert once and usually lead to the animal being locked on a reproductive phenotype (refractory). Long-lived species

show a persistence of rhythms for many years in constant conditions (circannual rhythms) (Gwinner 1986). In either case, there is endogenous timing resulting in spontaneous changes in seasonal physiology.

To understand the mechanisms animals use to anticipate seasonal environmental changes and respond appropriately, we must consider where in the photoperiodic TH pathway the endogenous timer acts. By placing sheep into constant LP, we can reveal the circannual rhythm and assess the known photoperiodic circuits (Fig. 2). Here, both the melatonin signal and the local circadian clockwork in the PT does not change, continuing to reflect the prevailing photoperiod (Lincoln *et al.* 2002, 2005) but *EYA3/TSH/DIO* do spontaneously revert in constant conditions, tracking the cycles of seasonal physiology of the animal (Sáenz de Miera *et al.* 2013, Wood *et al.* 2015). *DIO* and *TSH β* reversion have also been demonstrated in European hamsters (Sáenz de Miera *et al.* 2014).

PT *TSH β* progressively declines on constant LP (Sáenz de Miera *et al.* 2013, Wood *et al.* 2015), tracking the circannual cycle of reproductive physiology, referred to as the 'LP refractory' state (Figs 2 and 3B). This decline is also observed under natural photoperiod between May (~14.5 h light) and August (~14.5 h light), representing only an hour and a half decline from solstitial photoperiod i.e. still a LP (Lomet *et al.* 2018). Importantly, *TSH β* is not the only PT-expressed gene to change after constant LP (Wood *et al.* 2015, Lomet *et al.* 2018), but it serves as a good marker for the LP physiological state and currently provides the best causal link to seasonal reproductive changes (Hanon *et al.* 2008).

LP induction appears to be well synchronized between individual animals, whereas the LP refractory decline (when animals are kept on constant LP) is variable amongst individual animals. This is unsurprising given the lack of a synchronising signal from a photoperiodic change. During LP induction at a cellular level, there may be greater synchronization potentially leading to a more consistent output. If the cells are synchronized, two possibilities exist for how this gradual tissue response is generated during LP induction: (1) all the thyrotrophs within the tissue progressively increase the expression of *TSH β* from the time of the LP switch. Or (2) individual thyrotrophs 'switch on' *TSH β* expression at different rates once exposed to LP. For option 2, variation in threshold of responses in individual cells would lead to the gradual accumulation in the number of *TSH β* -expressing cells after LP induction. These options represent the difference between a whole tissue response and a single-cell response

that accumulates into a tissue-level output (Fig. 3C). But what evidence is there that there are individual cell switches in response to photoperiod?

Binary switch within the PT

Mathematical modelling of the photoperiodic response predicted a switching of cellular phenotype in the PT in response to photoperiod (Ebenhöh & Hazlerigg 2013) but without identification of a cellular marker of short photoperiod (SP – 'winter'), this could not be tested. A large transcriptomic dataset on the sheep PT, allowed the identification of the SP marker, chromogranin A (*CHGA*) (Wood *et al.* 2015). *CHGA*, a hormone packaging molecule, strongly co-localizes to the secretory granules in PT-specific thyrotrophs in SP and displays the progressive tissue-level response, contrasting to the LP signal *TSH β* (Fig. 3A) (Wood *et al.* 2015). Unlike *TSH β* , the role for *CHGA* in a seasonal context is unknown, but the increase in expression corresponds to a decrease in rough endoplasmic reticulum (RER) and a decrease in secretion from these cells (Wood *et al.* 2015). However, prolactin was the hormone output monitored in this study, due to castrate male being used; therefore, it cannot be directly linked to reproductive outputs (Wood *et al.* 2015).

Using *CHGA* as a marker for the 'winter' state and *TSH β* protein expression as a marker for the 'summer' state, the distribution of individual PT thyrotroph cells were mapped at 4-week LP, SP and 29-week LP (LP refractory). This revealed that virtually no PT-specific thyrotrophs (defined by α GSU expression) co-express both *CHGA* and *TSH β* (0.01% – 2 cells out of 17,000) (Wood *et al.* 2015), indicating that PT thyrotrophs can only exist in one of two binary states, winter-like (*CHGA*+ve) or summer-like (*TSH β* +ve). Importantly, this switching occurred at 29-week LP (LP refractory), reverting to the winter-like state, demonstrating that it occurs as an endogenously timed process (Wood *et al.* 2015) (Fig. 3B and D). The possibility that these observations were due to two different cell populations i.e. LP and SP specialized subsets of PT-specific thyrotrophs was considered. Cell counts revealed that in SP 4 weeks nearly all (91.6%) PT-specific thyrotrophs expressed *CHGA* and in LP 4 weeks 66.6% of PT-specific thyrotrophs expressed *TSH β* (approx. 5% still express *CHGA* and the remainder only expressing α GSU); therefore, the most likely explanation is that within a population of PT-specific thyrotrophs, cells switch from one phenotype to another (Wood *et al.* 2015). To add to that conclusion we and others have demonstrated

that cell division in the PT is very low (<0.2%) and not within the PT-specific thyrotroph population (Migaud *et al.* 2011, Hazlerigg *et al.* 2013, Wood *et al.* 2015). Also the RNA expression of α GSU did not change, nor did the expression of established cell cycle genes (Wood *et al.* 2015). We cannot completely rule out that these observations are due to the presence of two different cell populations without cell fate mapping approaches, which are currently not tractable in seasonal model species. These observations have only been made in sheep, in this one study, and unfortunately, an individual cell level characterization has not been made at different points of the seasonal cycle.

Nevertheless, a binary switch has good explanatory value for the progressive tissue-level changes observed and subsequent cycles of physiology that occur and may provide a framework to understand seasonal timekeeping. To hypothesize how a binary switch could operate to time biological processes over weeks to years we would need to know: (1) What initiates the binary switch (LP induction), (2) How the cycle is endogenously generated (LP refractory), (3) What is the basis of individual cell variability in response and (4) What is the role of input history (prior photoperiod).

Initiating the binary switch: a seasonal differentiation process?

The PT has a distinct developmental origin from the rest of the pituitary gland, and it emerges from the rostral tip region of Rathke's pouch. The bHLH transcription factor hairy enhancer of split (HES1) is a PT-specific differentiating factor (Akimoto *et al.* 2010, Aizawa *et al.* 2016). All glycoprotein hormones comprise a common α subunit (α GSU) and a specific β subunit (β FSH/ β LH/ β TSH). In embryological development, α GSU is the first subunit gene expressed in the pituitary (Stoeckel *et al.* 1993, Kameda *et al.* 1998, Kita *et al.* 2007, Raetzman *et al.* 2007, Akimoto *et al.* 2010, Inoue *et al.* 2013). CHGA (with α GSU) appears just prior to TSH β in embryonic development of the mouse pars distalis (Akimoto *et al.* 2010) and is an early embryonic signal in zebrafish (Xie *et al.* 2008) and the chicken PT (Kameda *et al.* 1998). In the main pituitary upon differentiation to specific endocrine cell types, expression of the β subunit is gained in adult endocrine cells. In this context, adult PT-specific thyrotrophs are unique since they persist as α GSU+ precursors, and only mature into α GSU/TSH β expressing thyrotrophs when exposed to LP. Therefore, by definition, the PT-specific

thyrotroph appears to undergo a seasonal cycle of differentiation/maturation on exposure to LP, essentially undergoing a seasonal recapitulation of a developmental state, to drive seasonal physiological changes.

Differentiation dramatically changes a cell's size, shape, metabolic activity and responsiveness to signals. Therefore, changes in gene expression and morphology are used to define differentiation. Within the sheep PT, two large scale transcriptomic analysis clearly illustrate that genes related to development, cytoskeletal remodelling and tissue plasticity are enriched on LP (Wood *et al.* 2015, Lomet *et al.* 2018). PT thyrotrophs increase in size, increase RER, gain a secretory phenotype and reorganize into networks on LP, presumably to coordinate the secretion of TSH (Wood *et al.* 2015) (Fig. 4A). Differentiation can occur without cell division (O'Neill & Stockdale 1972, Yang *et al.* 2007), which may be consistent with the lack of evidence for cell division in the PT thyrotroph (Migaud *et al.* 2011, Hazlerigg *et al.* 2013, Wood *et al.* 2015). This ability to differentiate without division has been suggested to allow for faster responses to signals (Yang *et al.* 2007). In the context of the PT, it is possible that over winter PT thyrotrophs are 'primed' to respond to an LP signal, explaining the rapid production of TSH β during LP induction. Therefore, I propose that the binary switch is initiated by a seasonal differentiation of PT thyrotrophs to a mature TSH-secreting state.

A seasonal cycle of differentiation would require a differentiation signal/factor that is expressed on LP. A more detailed analysis of published data on the first, seventh and 28th day of LP, shows 24 genes changing within the PT on transition to LP were related cellular differentiation and cell cycle (Wood *et al.* 2015). *KLF11*, *TRIM2* and *EYA3* have a pattern of expression that would be consistent with a transient LP differentiation signal and a progressive accumulation of TSH β -positive cells (Fig. 4B). *KLF11* has roles in the differentiation of adipocytes to brown fat (Loft *et al.* 2015) and the differentiation of pancreatic progenitor cells (Teo *et al.* 2018). *TRIM2* regulates neuronal differentiation (Khazaei *et al.* 2011) and epidermal and hair follicle differentiation (Joost *et al.* 2016). Due to the difficulty in the dissection of the PT, it is possible that some median eminence or pars nervosa was in the sample; therefore, it remains to be shown if either *TRIM2* or *KLF11* have a PT-specific expression. *EYA3* does have specific PT expression (Fig. 2A), and, has an extensively documented role in retinal, pituitary and muscle development and differentiation (Xu *et al.* 1997, Jemc & Rebay 2007, Kozmik *et al.* 2007, Kumar 2009, Gordon *et al.* 2012, Tadjuidje & Hegde 2013). In the context of seasonal

Figure 4

(A) Seasonal remodelling of the *pars tuberalis*. Diagrams representing the tissue-level changes are below. SP and the LP-R state are characterized by a FS cell network, separating individual PT-specific thyrotrophs. LP is characterized by larger PT thyrotrophs remodelled into an integrated network. Adapted from: Wood *et al.* (2015). (B) RNA-seq normalized counts per million displaying the amount of *TRIM2*, *KLF11* and *EYA3* transcript in the first 4 weeks of LP; SP, short photoperiod; LPD1, 1st day of long photoperiod; LPD7, 7th day of long photoperiod; LPD28, 28th day of long photoperiod. * denotes FDR less than 0.01; **0.001; ***0.0001; ****<0.00001. Data from Wood *et al.* (2015). (C) A model for the binary switch. Demonstrating the differences between differentiation, de-differentiation, transdifferentiation and death and replacement. (D) RNA-seq normalized counts per million displaying the amount of *EZH2*, *SUV39H2* and *JADE3* transcript in the first 4 weeks of LP; SP, short photoperiod; LPD1, 1st day of long photoperiod; LPD7, 7th day of long photoperiod; LPD28, 28th day of long photoperiod. * denotes FDR less than 0.01; **0.001; ***0.0001; ****<0.00001. Adapted under the terms of the original Creative Commons licence from Wood *et al.* (2015).

biology, *EYA3* is emphasized to be a clock-dependent transcriptional co-activator of TSH β (Dardente *et al.* 2010, Masumoto *et al.* 2010, Wood & Loudon 2014), not a cellular differentiation signal. However, after 4 weeks of LP in sheep only 38% of TSH β -expressing cells also express *EYA3* (Wood *et al.* 2015). Another study recently noted a lack of an *Eya3*-Tsh β relationship (Hazlerigg *et al.* 2018). This suggests that *EYA3* is not the sustaining signal for TSH β expression. Finally, the TSH receptor is required to sustain TSH β expression (Ono *et al.* 2008), suggesting positive feedback of TSH and transience of *EYA3*. A role for *EYA3* as a seasonal differentiation signal is feasible, but remains to be demonstrated (Fig. 4C).

An endogenous cycle of binary switching: seasonal de-differentiation or death?

If PT thyrotrophs do differentiate to a mature TSH-producing cell type then an explanation for the reversion to a α GSU-positive cell that is phenotypically characteristic of an immature endocrine cell during the LP refractory state and over a natural seasonal cycle is required. I suggest there are three possibilities: (1) A subsequent reversion to an immature/progenitor state (de-differentiation); (2) Trans-differentiation from a different mature cell type or (3) A cycle of death and replacement (Fig. 4C).

Traditionally, cell fate determination has been viewed as a process of progressively increasing restriction of cellular fate potential on the path to a fully mature differentiated cell. Recent studies have highlighted possibility that cells do not assume fixed final differentiated phenotypes in adult tissues. This plasticity of differentiated cells is likely to be central to survival through rapid response to injury or environmental changes (Paksa & Rajagopal 2017). The reversion of a PT-specific thyrotroph to a progenitor or SP (winter-like) state via de-differentiation is potentially the most parsimonious explanation in the PT (Fig. 4C). There is precedence for this, pancreatic β -cells in type 1 diabetes, de-differentiate (Talchai *et al.* 2012, Cinti *et al.* 2016) and insulin therapy re-differentiates these cells (Wang *et al.* 2014). Therefore, adult endocrine cells can undergo cycles of differentiation and de-differentiation.

Trans-differentiation (lineage reprogramming) suggests that a mature cell becomes a different cell type. For example, mature δ cells in the pancreas and hepatocytes in the liver converting to β -cells and biliary epithelial cells, respectively. The most common example of trans-differentiation is limb regeneration in amphibians (reviewed in: Shen *et al.* 2004, Cai *et al.* 2007). These examples indicate that by changing the environment of the cell, there can be a reprogramming of fate (Paksa & Rajagopal 2017). However, the difference between de-differentiation and trans-differentiation becomes less clear when the majority of evidence points to differentiated cell, de-differentiating to a progenitor or stem cell phenotype, and then re-differentiating to a different mature cell type (Cai *et al.* 2007). In the context of the PT, and the strictest definition of trans-differentiation, the presence of an infiltrating (or resident) cell type that switches fate would be required. One study observed the presence of dividing CD45-positive cells with a microglia phenotype in the PT (Hazlerigg *et al.* 2013), there was no seasonal variation in the numbers of cells observed, but these could represent a candidate cell for trans-differentiation. *SRRM4* shows transcriptional changes in the PT on LP (Wood *et al.* 2015) and is known to drive neuroendocrine trans-differentiation of prostate adenocarcinoma cells (Li *et al.* 2017), potentially providing a candidate gene, but it is difficult to draw conclusions from data on a mixed cell population.

Finally, a cycle of death and replacement (from a stem cell population) generating a circannual rhythm (seasonal histogenesis) was proposed before the observation of a binary switch (Hazlerigg & Lincoln 2011). Seasonal histogenesis could provide a mechanism to explain loss and replacement of TSH-positive cells within the PT. But there have been few studies of cell turnover in the

PT, and these do not present a consistent picture as to the extent to which cell division varies with seasonal state (Migaud *et al.* 2011, Hazlerigg *et al.* 2013, Wood *et al.* 2015). In the context of a cycle of de-differentiation the capacity to gain proliferative function is a characteristic of Schwann cells, kidney cells and Sertoli cells following insult (Shen *et al.* 2004, Cai *et al.* 2007). Therefore, de-differentiation and histogenesis are not mutually exclusive explanations. Seasonal histogenesis remains to be adequately tested in the PT and would require assessment of cell death and division.

I have provided a potential framework to address how the seasonal cycle of binary switching may operate in the PT but I have not offered an explanation for how this would allow for long-term timekeeping. I propose that individual cell variation in the threshold of responsiveness and variation in the subsequent reversion could generate a timer process.

The role of individual variability in accurate timing

Endocrine cells have acute, medium and long-term outputs, and these must be regulated across different time scales and in response to diverse environmental signals, whilst achieving accurate control of hormone expression. At first glance, the idea of individual cell variability to environmental signals may seem to be contrary to accuracy and control of hormone expression. However, stochastic heterogeneity in single-cell response can lead to tissue progressive responses. Release of prolactin from the pituitary, for example, is based on heterogeneous patterns of gene expression at a single-cell level but a well-regulated average level of gene expression is maintained (Featherstone *et al.* 2011, Harper *et al.* 2011). Prolactin expression follows a binary 'on-off' process, with activity of individual cells being unpredictable, but the overall activity of a group of cells being predictable (Hey *et al.* 2015, Featherstone *et al.* 2016). The conclusions from these study were that heterogeneous single-cell responses allowed the tissue to be more functionally adaptive to the environment and result in more robust tissue-level responses, avoiding inappropriate amplification of signals through feedback mechanisms (Featherstone *et al.* 2011, 2012, 2016, Harper *et al.* 2011, Hey *et al.* 2015).

During development, pluripotent cells differentiate and become restricted to specific lineages. Often, this depends on positional information and morphogenic gradients, the clear banding (position-effect variegation) seen in the *Drosophila* embryo is a good example, but there

are also examples of position-independent patterning (Kay & Thompson 2009). Position-independent patterning is characterized by cells switching or differentiating from one phenotype to another in an apparently stochastic manner, with variability in timing, across the tissue. Position-independent patterning makes it appear as if two cell types are randomly 'sprinkled' throughout a tissue, and for that reason, it is referred to as, salt and pepper differentiation. Re-patterning is also a feature of this process, as seen in the PT (Fig. 4A). Examples of this include the early mouse embryo, and the R8 photoreceptor neurons in *Drosophila* that differentiate into rhodopsin 5-sensitive or rhodopsin 6-sensitive subtypes through stochastic expression of specific transcription factors determining cell (Eldar & Elowitz 2010, Jukam *et al.* 2013). This stochasticity is presumed to add robustness and accuracy to timing of differentiation during development (Kay & Thompson 2009, Eldar & Elowitz 2010, Jukam & Desplan 2010, Jukam *et al.* 2013). Importantly, many terminal differentiation processes show variability in the time from sensing the initial inducing signal to the final commitment to their new fate, this allows cells to defer commitment, which prevents responses to transient signals. In the context of the PT, differences in sensitivity to melatonin and variability in the rate of commitment, may be a mechanism to allow a long-term timer process.

Input history: how is individual variability encoded?

Within the PT we expect individual variability in the responsiveness to LP, whereby, PT thyrotrophs differentiate into a mature TSH-secreting state (LP induction), but, also in the reversion to winter phenotype (LP refractory). Individual variability may arise through and be modulated by (1) input history to the cell affecting the threshold sensitivity of that cell, and, (2) individual differences in sensitivity and therefore, the rate of commitment.

Vernalization is the process of preparation for flowering, which depends on cold temperatures being experienced for a suitable length of time to permit flowering on rewarming (spring). It is characterized by a progressive binary switching of cells, in a salt and pepper fashion (see above) based on individual variation in responsiveness. Cells progressively switch over weeks of cold exposure, acting as a long-term timer, and only giving a full flowering response when the majority of cells have flipped in state (Angel *et al.* 2011, Song *et al.* 2012). Importantly, the input history, in this case low temperature,

is key to 'priming' cells to respond to a spring signal. This priming is achieved by a long-term epigenetic repressive process is initiated at the beginning of winter. Prolonged cold in the winter leads to the accumulation of repressive marks mediated by polycomb complex proteins acting on the flowering control locus (FLC), which usually blocks the activation of flowering locus T (FT). The longer the exposure to cold, the greater the number of cells entering an FLC-repressed state, therefore upon rewarming FT is activated and flowering can occur, with the extent of flowering based on the number cells in the FLC-repressed state (Angel *et al.* 2011, Andrés & Coupland 2012, Song *et al.* 2013). Therefore, plants have a seasonal timer based on binary switching, regulated by chromatin accessibility, which is dependent on duration of cold exposure.

As stated earlier, on natural photoperiod in sheep, *TSH β* begins to decline whilst still on LP (Lomet *et al.* 2018); similar results have been seen in Siberian hamsters but only assessed at the *DIO2* level (Petri *et al.* 2016). Comparing the gonadal responses of quail and starlings, quail faithfully track the natural photoperiod, but starlings begin to regress their gonads whilst on LP (Dawson 2015). This suggests that LP induction not only leads to a simultaneous activation but also an initiation of long-term repressive process leading to the LP refractory state (Dawson 2015). If this is the case, we need to consider how cells are primed to respond to an LP signal and how they are eventually 'shut down'. I propose two possibilities: (1) Chromatin is maintained in a permissive or primed state and the thyrotrophs can always respond to an LP signal if the transcription factors are present, or, (2) chromatin accessibility is limited to prevent early responses to LP signals and/or to shut down responses after a prolonged period in LP.

Possibility 1 is based on cell fate of the adult intestine. Here, the positioning of the cell in the crypt determines the cell fate and the cells exist as multipotent stem cells, with a broadly permissive chromatin state, meaning that there are multiple possible pathways of differentiation depending on the transcription factors present. Therefore, chromatin state is not the determining factor for cell fate, merely permissive to either fate depending on the transcription factors available (Paksa & Rajagopal 2017). If in the PT thyrotroph, the chromatin was maintained in a permissive state, as in the intestine, then you would expect all PT thyrotrophs to respond at the same time to any LP signal regardless of the circannual phase. This seems unlikely as there is a known requirement for a winter in order to respond appropriately to an LP signal and the observations of a progressive tissue-level response do not support this.

Possibility 2 is based on a vernalization model where there would be preparation or priming of PT thyrotrophs to respond to LP over winter, and/or, the initiation of a longer-term repressive process on LP leading to the disappearance of TSH β cells. The targets of this proposed limited chromatin accessibility could be *EYA3* or regulators of *EYA3*. Priming to over winter suggests a prolonged winter would synchronize all cells to respond immediately to LP. Given the earlier discussions of the importance of a robust, non-spurious response, it seems possible that even when primed, these cells will have different sensitivity thresholds and therefore individual variance in responsiveness, as demonstrated in cell fate determination in development (Eldar & Elowitz 2010). Differences in individual cell sensitivity to signals has also been shown in *Dictyostelium* (slime mould), here, 'cells exhibit different intrinsic response biases or discrete transcriptional activation thresholds to signals' resulting in salt and pepper differentiation (Chattwood *et al.* 2013).

Whether timing is conferred by cyclical changes in chromatin accessibility remains to be demonstrated, and it is possible that, upstream regulators of specific transcription factors, not the known key circuits, are targets of this cyclical chromatin remodelling. Evidence for seasonal changes in epigenetic state within the PT are limited to the identification of differentially regulated transcripts of known epigenetic modulators (Wood *et al.* 2015, Lomet *et al.* 2018). EZH2, a member of the PRC2 complex that lays down the repressive H3K27me3 mark during development, is upregulated in the PT in LP (Wood *et al.* 2015, Lomet *et al.* 2018) (Fig. 4D). EZH2 is required for proper differentiation of a mature lung secretory cell population during development (Snitow *et al.* 2015), potentially indicating a role for EZH2 in a seasonal cycle of differentiation through changes in chromatin state. Nineteen other chromatin/histone modifiers were also identified as differentially expressed in the PT on LP, these include the histone methyltransferase; *SUV39H2* (also identified by Lomet *et al.* 2018), and, histone acetyltransferase; *JADE3* (Fig. 4D) (Wood *et al.* 2015). Furthermore, through its phosphatase activity, *EYA3* promotes DNA repair through post-translational modification of H2A.X to allow recruitment of DNA repair factors (Cook *et al.* 2009). Characterization of the chromatin state in the PT remains to be investigated in order to test these hypotheses.

As a final point, the role for the tanycytes in the 3rd ventricle (ependymal zone) should not be overlooked. As discussed earlier, the mothers photoperiodic history programmed the trajectory of the offspring's reproductive development and sensitivity to photoperiodic signals,

through changes in sensitivity to TSH in the tanycytes not the PT (Sáenz de Miera *et al.* 2017). In sheep, the reproductive switch-off at the end of the winter breeding season can be blocked by thyroidectomy (THX) with the greatest transcriptional effects in the ependymal zone (Lomet *et al.* 2018). The hypothalamic region is a known stem cell niche and seasonal remodelling has been observed in the median eminence (ME) of both the quail and sheep (Yamamura *et al.* 2004, Wood *et al.* 2015). Therefore, the hypotheses presented here, may also be relevant for timing circuits in the ependymal zone. SHH, a known developmental morphogen, is expressed by the tanycytes in a seasonal manner, suggesting seasonal differentiation either in the ME and/or morphogenic signals from the ME that may signal to the PT. This highlights the potential importance of the interaction between tanycytes and the PT in the generation of a seasonal rhythm. Mathematical modelling to consider sensitivity, cellular plasticity and PT/tanycyte crosstalk in long-term timing is required.

Conclusions

The phase of the seasonal cycle is defined by a binary switching of cellular phenotype of the PT-specific thyrotroph to TSH β -positive and the proportion of cells in that state. Therefore, it is hypothesized that the basis of seasonal rhythm generation is a cellular population-based timer within the PT. A cellular population based timer is used to time seasonal flowering in plants. I propose that the adult PT is a plastic tissue, showing a seasonal cycle of differentiation, and that the underlying processes are likely to be epigenetic. Considering the mechanisms behind adult cellular plasticity offers a framework to hypothesise how a long-term timer functions within the PT. Finally, the PT may offer a unique tissue to explore cellular plasticity in an adult mammal, as no injury or disease state is required to initiate this process.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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