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The evolution of asymmetric photosensitive structures in metazoans and the Nodal connection

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HIGHLIGHTS

- The epithalamus of vertebrates is an asymmetric organ displaying anatomical and molecular features underlying the presence of photosensitive structures

- Parapineal homology among vertebrates can be established using specific molecular markers and studying its biased connectivity to one of the habenular nucleus

- Asymmetric photosensitive systems can be found in non-vertebrate groups

- The ancestral role of Nodal signaling could be to break symmetry both in peripheral systems and brain structures

ABSTRACT

Asymmetries are observed in a great number of taxa in metazoans. More particularly, functional lateralization and neuroanatomical asymmetries within the central nervous system have been a matter of intense research for at least two hundred years. While asymmetries of some paired structures/organs (e.g. eyes, ears, kidneys, legs, arms) constitute random deviations from a pure bilateral symmetry, brain asymmetries such as those observed in the cortex and epithalamus are directional. This means that molecular and anatomical features located on one side of a given structure are observed in most individuals. For instance, in humans, the neuronal tract connecting the language areas is enlarged in the left hemisphere. When asymmetries are fixed, their molecular mechanisms can be studied using mutants displaying different phenotypes: left or right isomerism of the structure, reversed asymmetry or random asymmetry. Our understanding of asymmetry in the nervous system has been widely enriched thanks to the characterization of mutants affecting epithalamus asymmetry. Furthermore, two decades ago, pioneering studies revealed that a specific morphogen, Nodal, active only on one side of the embryo during development is an important molecule in asymmetry patterning. In this review, I have gathered important data bringing insight into the origin and evolution of epithalamus asymmetry and the role of Nodal in metazoans. After a short introduction on brain asymmetries (chapter I), I secondly focus on the molecular and anatomical characteristics of the epithalamus in vertebrates and explore some functional aspects such as its photosensitive ability related to the pineal complex (chapter II). Third, I discuss homology relationship of the parapineal organ among vertebrates (chapter III). Fourth, I discuss the possible origin of the epithalamus, presenting cells displaying photosensitive properties and/or asymmetry in the anterior part of the body in non vertebrates (chapter IV). Finally, I report Nodal signaling expression data and functional experiments performed in different metazoan groups (chapter V).

Keywords: Nodal signaling, epithalamus, photosensitive structures, evolution, morphology

I- Introduction

Do Brain asymmetries matter? A brief overview of functional brain asymmetries in vertebrates

Hemispheric specialization of the brain reflects lateralization of cognitive functions such as language, spatial orienting of attention and perception. This functional asymmetry is associated with anatomical asymmetry. For instance, language is associated to the asymmetry of the neuronal tract connecting the inferior frontal gyrus (Broca's area) to the superior temporal lobe (Wernicke's area), which is larger in the left hemisphere. Distinct anatomical features are also observed in the cortex such as a difference in width between the right and left frontal or occipital regions, resulting in tissue protrusion at the frontal or occipital pole, also referred to as the counterclockwise brain torque. Interestingly people with L/R reversal of thoracic and abdominal organs (a situation called *situs inversus totalis*) display a reversed (or clockwise) cerebral torque (Kennedy et al. 1999). This suggests that genetic mechanisms supervising asymmetries of the brain and of thoracic/abdominal organs might be closely related. In the absence of congenital heart defects or primary ciliary dyskinesia, complete reversal of organ and cerebral asymmetries does not compromise individual health. However, the abnormal distribution of visceral organs (a situation called heterotaxia) can lead to medical complications. Similarly an abnormal asymmetry pattern of the brain is observed in certain psychotic conditions such as schizophrenia (Oertel-Knöchel and Linden 2011); atypical cerebral dominance has been reported in the case of autism (Escalante-Mead et al. 2003), although the brain torque appears normal (Knaus et al. 2012); and migraines are associated with asymmetric placement of the pineal gland (Kaaro et al. 2008), which might be linked to an abnormal level of the melatonin precursor, serotonin, an important regulator of lateralization during embryogenesis (Fukumoto et al. 2005).

The epithalamus has been the focus of many studies aiming at understanding asymmetry in the vertebrate nervous system. The epithalamus is a diencephalic structure that emerges from the dorsal forebrain during embryogenesis. Although the epithalamus exists in a great diversity of species, its composition and asymmetry are quite different within vertebrates (described below) (Concha and Wilson 2001). The epithalamus is made of the pineal gland and the habenular nuclei. Transverse sections performed through the paired habenular nuclei display a clear left-right (L/R) difference in size, neuronal perikaryon clustering, expression pattern of molecular and neurochemical markers and subnuclear partitioning (Concha and Wilson 2001, Aizawa et al. 2011). The habenular nuclei are located on one of the major neural pathways of the limbic system known as the dorsal diencephalic conduction system. They constitute a relay station negatively regulating the activity of dopaminergic and serotoninergic neurons (Hikosaka 2010). Thus, the habenulae could represent a major therapeutic target in human psychotic disorders associated with monoamine metabolism dysregulation (reviewed in Aizawa et al. 2011; Hikosaka 2010).

Almost two decades of research on the zebrafish epithalamic asymmetries have unveiled the main functions and the genetic underpinnings of the epithalamus (reviewed in Halpern et al. 2003; Concha, et al. 2009; Roussigné et al. 2012). Recent studies have highlighted that behaviour and sensory processing are linked to habenular asymmetries. In zebrafish, dorsal habenula subdomains (lateral and medial) also display a L/R asymmetric pattern of projection to the interpeduncular nucleus (IPN) (Gamse et al. 2005; Aizawa et al. 2005), in addition to the L/R anatomical and molecular habenular asymmetries mentioned above. Experimental silencing of the neural tracts running from the lateral subregion of the dorsal habenula to the dorsal IPN modify the locomotion of zebrafish submitted to fear conditioning. Indeed, animals turn from flight to freezing behaviour when receiving the conditioned stimulus (Agetsuma et al. 2010). The regulation of these aversive memories might involve Cannabinoïd Type I Receptors (CB_1R) located in presynaptic terminals of the medial habenular neurons as it has been demonstrated in mice (Soria-Gomez et al. 2015). Anxiety has also been linked to changes in epithalamic asymmetry (Facchin et al. 2015). Regarding sensory processing, Dreosti et al (2014) showed that the functional properties of habenular neurons exhibit a lateralized pattern: neurons responding to olfactory stimuli are mainly located in the right habenula while those responding to light stimuli are in the left habenula.

Therefore, anatomical and functional asymmetries are essential for cognitive and sensorial processing in the vertebrate brain. In the following text I review molecular, anatomical and functional data related to the epithalamus in a wide range of vertebrate species. The epithalamus being a light sensing organ, I also explore photosensitive structures in non vertebrate groups asking how this asymmetric structure might have emerged and evolved.

II- The vertebrate epithalamus: molecular and morphological features

Together with the thalamus and the hypothalamus, the epithalamus is a posterior forebrain structure arising from the dorsal part of the diencephalon. As previously mentioned, it includes the habenular nuclei (or habenular complex) and a pineal body (or pineal complex). The pineal body grows from the diencephalic neuroepithelium as one or sometimes two finger-shaped evaginations. Although the morphology of the pineal organ is quite diverse among vertebrates, two conserved functions are associated to it: the production of the hormone melatonin and the ability to sense light, making the pineal organ an important regulator of circadian and seasonal rhythms. In light of these two functions, I will explore molecular and morphological features of the epithalamus across vertebrates.

Genes involved in melatonin synthesis and circadian rhythms

Melatonin is produced at night by the pineal neuroendocrine cells. It is responsible for synchronizing periodic activities (such as sleep) with environmental daylight periods. As a consequence, the duration of melatonin production depends on day length, varying in summer and winter. This variation is used as a seasonal clock by many animals. Note that in mammals, the light signals necessary to generate circadian rhythms are sensed by the eyes and sent to the pineal organ via the suprachiasmatic nucleus (SCN).

The precursor of melatonin is the essential dietary amino acid tryptophan. Four enzymatic reactions are required to synthesise melatonin. First, tryptophan is converted into 5-HTP (5-hydroxy-L-tryptophan) by the enzyme tryptophane 5-hydroxylase. Second, 5-HTP is decarboxylated by the 5-hydroxytryptophan decarboxylase to produce serotonin (5-HT). Third, serotonin is acetylated by the enzyme Aanat producing N-acetylserotonin. Finally, this last product is methylated by the enzyme Hiomt to generate melatonin.

Melatonin being the hormone of darkness, Aanat enzymatic activity increases at night. This increase has been related to two distinct mechanisms: a difference in Aanat protein degradation rate at night and day and cyclic transcriptional regulation. Cyclic transcription of Aanat is either autonomous, i.e. controled by an internal clock, or dependent on light signals (for review see Sapède and Cau 2013). The bHLH (basic Helix-Loop-Helix) PAS transcription factors baml/clock and the *orthodenticle homeobox 5* (*otx5*) gene product control the rhythmic expression of *aanat2* paralog both in zebrafish and gilthead sea bream through binding to respective DNA-binding sites (Gamse et al. 2002; Appelbaum and Gothilf 2006; Zilberman-Peled et al. 2007). However *aanat2* mRNA does not display cyclic accumulation in the pineal organ of turbot embryos (Vuilleumier et al. 2007) suggesting that the rhythmic availability of the *aanat* gene product uses different mechanisms among *actinopterygii*.

Genes involved in photoreceptor specification and eye formation

In addition to being a neuroendocrine complex, the pineal body is a light sensitive system working together with the paired lateral eyes - which also arise as evaginations from the diencephalon. Not surprisingly, the pineal organ expresses developmental genes important for photoreceptor specification such as those belonging to the *crx*/*otx* family and retina-specific proteins such as opsins. The pineal organ of all vertebrates studied so far has been shown to express one or two gene members of this family. *In situ* hybridization revealed that *otx5* mRNA is located in the pineal complex of teleosts, amphibians, sharks and lampreys (Vignali et al. 2000; Gamse et al. 2005; Dinet et al. 2006; Lagadec et al. 2015). Both *otx5* and *crx* (the cone rod homeobox gene) are expressed in the pineal complex of zebrafish (Gamse et al. 2005). In gnathostomes, previous phylogenetic analysis showed that *crx*/*otx5* forms a single orthology class and phylogenetic analysis in mammals pointed to absence of the *otx5* gene (Plouhinec et al. 2003). In the mouse, the pineal gland is characterized by expression of *crx* and *otx2* mRNAs. Animals carrying targeted inactivation of *otx2* lack a pineal gland (Nishida et al. 2003) while *crx* genetic ablation decreases the expression of pineal genes such as *aanat* (Furukawa et al. 1999). In zebrafish, *otx5* but not *crx* depletion leads to a reduction of circadian-regulated gene expression (Gamse et al. 2002). The different roles played by *otx5* in zebrafish and *crx* in mouse may reflect the partitioning of an ancestral function assigned originally to a unique *crx*/*otx* gene.

Besides *otx*/*crx* genes, the paired-box and homeobox-containing gene *Pax6* is an essential factor involved in metazoan eye formation (Gehring and Ikeo 1999). In vertebrates, Pax6 works together with the retina and the anterior neural fold homeobox *rx*/*rax* genes (Mathers et al. 1997). *Pax6* mRNA is expressed in the pineal organ of a wide range of vertebrates including shark, lamprey, *Xenopus*, chicken and mouse (Estivill-Torrus et al. 2001; Derobert et al. 2002; Lim and Golden 2002; Bandin et al. 2015;). Interestingly, Pax6 might also be involved in habenular morphogenesis by fine-tuning the expression of Shh in the mid-diencephalic organizer (Chatterjee et al. 2014). Furthermore, Pax6 patterns prosomere 2 (p2), the diencephalic segment from which the thalamus and

epithalamus are derived. More precisely, this study showed that molecular markers for habenular developing neurons such as *Irx1*, *Brn3a*/*Pou4f1*, *Nrp2* and *Etv1* are missing in the *Pax6* mutant mouse Pax6$^{Sey/Sey}$. *Xrx1* and *Rax* mRNAs are expressed in the pineal gland of *Xenopus* and rat respectively (Casarosa et al. 1997; Asbreuk et al. 2002). *Rax* knockout mice form neither optic cups nor eyes (Mathers et al. 1997). The relatively late ontogenic expression of *Rax* compared to *Pax6* and *otx/crx* in rodent pinealocytes led to the hypothesis that this gene might regulate the final differentiation and/or the maintenance of pineal cells, rather than early morphogenesis (Rath et al. 2013). Specific *Rax* deletion in the pineal gland should help decipher the role of Rax in pineal cells.

Genes involved in photoreception and phototransduction

In anurans, reptiles, lampreys and teleosts, the pineal gland is associated with an additional parietal structure called, depending on the species, the frontal organ, the parietal eye or the parapineal gland, respectively. Like the pineal gland, these structures all arise as a finger-shaped evagination of the diencephalic roof (Eakin 1973). They are asymmetrically connected to the left side of the habenular complex. These data arguing for homology relationships would be strengthened by molecular information (discussed below). Just as all vertebrates do not possess this additional parietal structure, others (crocodilian and hagfish) are devoid of a pineal gland (Jansen et al. 1930; Roth et al. 1980) which likely reflects environmental adaptation.

Located deeply in the brain, the mammalian pineal gland does not directly transduce photonic signals. Instead, light/dark information integrated by retinal photoreceptors are sent to this endocrine organ via norepinephrin releasing neurons from the suprachiasmatic nucleus. The resulting norepinephrin output on neuroendocrine cells of the pineal gland is the secretion of melatonin at night (Klein 2006).

In lampreys, anurans and reptiles, a pineal foramen allows the pineal complex or the frontal/parietal eye to be positioned beneath the skin. The vesicular part of the structure which lies just under the skin, carries a retina-like morphology that is not visible externally, contrary to the bilateral paired eyes. However the skin covering this small non mammalian eye-like organ displays a modified pigmented pattern compared to the rest of the skull epidermis. Furthermore, it contains pinealocytes that look like eye cellular photoreceptors and electrophysiological studies provided evidence that the pineal organ is directly sensitive to light (Koyanagi et al. 2004). In addition, several members of the light-sensitive opsin family are expressed in pinealocytes althought none of them exhibit an exclusive expression pattern in the pineal complex. Parapinopsin is an opsin phylogenetically related to the vertebrate visual pigment found in the parapineal of channel catfish (Blackshaw and Snyder 1997), the pineal complex of lampreys, fish and frogs (Koyanagi et al. 2004; Kawamo-Yamashita et al. 2007) and in the parietal eye of the green iguana (Wada et al. 2012). Pinopsin is another pineal opsin whose tissue localization has been demonstrated in birds and reptiles (Okano et al. 1994; Max et al. 1995; Fejer Z et al. 1997; Taniguchi et al. 2001). From a functional point of view, pinopsin which has been identified as a blue-sensitive pigment mediates a hyperpolarizing light response through the activation of gustducin G protein leading to a decrease of cGMP and the closing of CNG channels. This activity is reported for members of the c-opsin (ciliary opsin) subfamily generally present in c-PRCs (ciliary-photoreceptors). Interestingly, in the lizard parietal eye, this pinopsin has been found to colocalize with another c-opsin called parietopsin, whose light response is a depolarization by means of the G protein G_o (Su et al. 2006). This response is comparable to the one induced by invertebrate r-opsin (rhabdomeric opsin). Thus, in vertebrate non-mammalian species, two phototransduction pathways induced by two distinct c-opsins and using two distinct G-proteins operate in the same parietal eye photoreceptors: one leading to hyperpolarization and one leading to depolarization (Su et al. 2006).

Anatomical and molecular features of the habenular complex

As mentioned previously, the habenular complex is composed of two nuclei, each divided into sub-domains. These are named medial and lateral sub-nuclei in mammals, birds and reptiles and ventral and dorsal sub-nuclei in fish and amphibians. Based on a detailed analysis exploring neuron projections, molecular marker expression and habenular morphogenesis Amo et al. (2010) provided evidence that the zebrafish ventral habenular nucleus is homologous to the mammalian lateral habenular nucleus. Briefly, lateral/ventral habenulae specifically express Protocadherin 10 (*pcdh10*) and project to the median raphe but not the IPN (Amo et al. 2010). On the other hand, the zebrafish dorsal habenulae are characterized by the transcription of *brn3a*/*pou4f1* genes and the asymmetric expression pattern of two *potassium channel tetramerization domain* (*kctd*) containing gene family members: *kctd12.1/lov*, being mostly expressed on the left and *kcdt12.2/ron,* having a wider expression domain on the right. In addition, the dorsal habenula is also distinguishable from its ventral counterpart by its neuronal tracts projecting essentially to the IPN (Gamse et al. 2005; Aizawa et al. 2005). It is worth mentioning that the establishment of sub-domain homologies between species should also take into

account the morphogenetic movements taking place within the habenulae, such as those described in zebrafish (Amo et al. 2010). Indeed, they have reported that the developing ventral habenular territory migrates from a lateral to a more ventromedial position from the larval to adult stage.

Several zebrafish lines carrying mutations in different genetic components of the Wnt/β-catenin canonical signaling pathway, such as *tcf712* (effector of the Wnt pathway), *wls* (the transmembrane protein Wntless required for Wnt secretion) or *axin1* (protein belonging to the β-catenin degradation complex) provided evidence of a contribution of this cascade in habenulae formation. Ventral habenulae are missing in *tcf712* and *wls* mutants. In addition the dorsal habenula is reduced in size in *wls* mutants (Beretta et al. 2013; Kuan et al. 2015). The L/R asymmetric pattern of the habenulae is also altered in *tcf712* and *axin1* mutants (also known as *masterblind*, *mbl* mutants) (Carl et al. 2007; Hüsken et al. 2014). Kuan et al. (2015) hypothesized that habenular sub-domain formation and L/R patterning might be two independent time-separated targets of the Wnt pathway. These data are consistent with a well established role of Wnt signaling in diencephalon patterning (Lim and Golden 2007).

Fgf8 whose activity is concentrated in the dorsal diencephalon is also an important morphogenetic signal in habenulae morphogenesis. *Fgf8* hypomorphic mouse lines displayed reduced dorsal structures (thalamus and epithalamus) emanating from segment p2 and an analysis of cell proliferation revealed a decreased mitotic index in these regions compared to wild type mice (Martinez-Ferre and Martinez 2009). Progenitors of the epithalamus are derived from diencephalic p2 together with those of the thalamus as previously mentioned. While arising from a common pool of cells, postmitotic neuronal precursors from both structures carry distinct molecular signatures whose components are essential for maintaining the cell identity of thalamic neurons and for repressing the development of the epithalamus. Chatterjee et al. (2015) recently reported that deletion of the homeodomain transcription factor *Gbx2* in mouse led to an expanded expression territory of epithalamic markers, *Nrp2*, *Brn3a*/*Pou4f1*, *Robo3* and a loss of thalamic neurons.

While L/R asymmetry between the two habenular nuclei is conserved in vertebrates (for review see Concha and Wilson 2001), the laterality direction of these asymmetries diverges. In other words the morphological, molecular and histological characters found for instance in the left habenula of one species are not necessarily present in the left habenula of other species. This aspect is particularly well exemplified by the size of the two habenulae. In some vertebrate groups such as reptiles, frogs, coelacanths, teleosts and cartilaginous fish, the left habenula is bigger than the right. In contrast lungfish, lampreys and hagfish display a larger right habenula compared to the left (Nieuwenhuys et al. 1998). Note that this grouping is based on a selected number of species. One cannot exclude the existence of variability within species belonging to the same class or order. Aizawa et al. (2007) attempted to understand the cellular mechanism responsible for the difference in size between the two sub-domains of the zebrafish dorsal habenula. Using BrdU pulse labelling, they examined neuron birthdates and provided evidence of asymmetric neurogenesis whereby early-born neurons mostly populate the left lateral dorsal sub-domain first. In contrast late-born neurons mostly colonize the right medial dorsal subdomain (Aizawa et al. 2007). For a compilation of molecular and anatomical data of the epithalamus see the supplementary table.

III- The mystery of parapineal homology among vertebrates

In mammals the pineal is a relatively complex organ made of three parts: the proximal part coming into close contact with the cerebrospinal fluid of the third ventricle, the distal part exhibiting a variable position with respect to the skull (or sometimes lying under the skull in a so-called superficial position) and the more or less long intermediate parenchyma linking the proximal and distal tissues. According to the size and the presence/absence of each portion, an attempt of classification has been proposed by Vollrath (1979) reflecting the great variability of pineal morphology in mammals. In primates, the pineal organ consists of a single bulk of tissue neighboring the third ventricle. In the syrian hamster, the intermediate elongate part is almost missing making the pineal a structure composed of two-halves: the superficial and the deep pineal gland. In contrast, in guinea-pigs, the intermediate part linking the proximal and distal gland is continuous. Whether the so-called proximal gland found in the mammalian pineal organ could represent a parapineal ganglion as defined in lampreys, amphibians, reptiles and teleosts would need molecular analysis and a careful study of its connectivity, in particular to the left habenula.

Indeed, the homology between the parietal eye of reptiles and the parapineal nucleus of lamprey and teleosts is based on a common target for their projections: the left habenula. Because this biased connection is

not observed for the frontal organ in amphibians, its homology with the parapineal has been discussed (Concha and Wilson 2001). But here again molecular analysis such as the detection of parapineal markers (*gfi1*, *tbx2a*, *sox1a*, *otx5,* Dufourcq et al. 2004; Gamse et al. 2005; Snelson et al. 2008; Clanton et al. 2013) during the embryonic development of the structure would help to clarify this point. In addition it is worth mentioning that the biased projection of the parapineal tract to the left habenula cannot be considered as a strict morphological criteria to identify the parapineal. The right habenula of the rainbow trout (*Oncorhynchus mykiss*) for example also receives a small set of fibers from the parapineal (Yanez et al. 1996).

In vertebrates the pineal complex therefore displays a great diversity in terms of morphology growing one or two derivatives (the pineal and parapineal nuclei) from which a retina-like sensory epithelium able to generate a chromatic response can be formed. The question of the presence/absence of a parapineal nucleus and the target of its efferent fibers in vertebrates is of importance. Indeed, as shown in zebrafish, this nucleus plays a key role in the establishment of habenular L/R asymmetry (Gamse et al. 2003, Regan et al. 2009). Its migration from the midline towards the left during zebrafish embryogenesis depends on a left-sided activity of Nodal signaling in the diencephalon (Figure 1). Indeed, if this molecular activity is missing or bilateral, the migration is randomized and switches from directional asymmetry (95% left; 5% right) to anti-symmetry (50% left; 50% right) (Concha et al. 2000; Concha et al. 2003; Gamse et al. 2003). The parapineal localization (either on the left, either on the right) is slightly more stochastic in the channel catfish (12% left; 88% right, n=9; Blackshow and Snyder 1997). It would be interesting to know whether the Nodal pathway is active in the channel catfish diencephalon and if so, whether its laterality follows that of the parapineal. A positive answer to this question, i.e. Nodal activity found predominantly on the right, would indicate that a mechanism of laterality controlled by Nodal is not a peculiarity of zebrafish but could be extended to other teleost groups. The real parapineal nature of the parapinopsin-expressing structure identified in the channel catfish (*Ictalurus punctatus*) should however be clarified first, using for example the above-mentioned markers. Furthermore, probe design for these markers should be possible with the recent publication of the catfish genome (Liu et al. 2016). Finally, it is important to note that, as described in zebrafish, a small proportion of medaka embryos display a right-sided location of the parapineal (Ishikawa et al. 2015).

The great diversity of shape and position of the parapineal organ across vertebrates and its putative homologous structure in some species suggests that besides the parapineal-dependent mechanism operating in zebrafish to break epithalamus asymmetry, additional mechanisms might exist. This hypothesis is supported by the fact that some species such as cartilaginous fish lack parapineal nuclei while exhibiting strong habenular asymmetry (Concha and Wilson 2001; Butler and Hodos 2005; Lagadec et al. 2015). In addition our recent study reported that molecular and anatomical habenular asymmetry is observed in the lamprey brain before the onset of parapineal morphogenesis (Lagadec et al. 2015). Up until recently, it was hypothesised that epithalamic asymmetry may have been stochastic at the base of the vertebrate lineage, based on the fact that the left-sided expression of Nodal in the diencephalon had not been described in any other vertebrate taxa except teleosts (Concha et al. 2009). We know now from results scored both in jawless vertebrates (*Petromyzon marinus* & *Lampetra planeri*) and in a cartilaginous fish (*Scyliorhinus canicula*) that brain laterality is not random in basal vertebrates and that its coupling with Nodal signaling did not appear in osteichythes but was in fact settled 580 million years ago (Lagadec et al. 2015, Figure 1). At the metazoan scale, Nodal signalling was first described to control peripheral asymmetries but brain asymmetries have not been investigated so far. The different roles of the Nodal pathway in breaking symmetry will be discussed in the last section of this review.

IV- The origin of the epithalamus: photosensitive structures in non-vertebrate metazoans

An asymmetric non-visual photoreceptive structure located in the anterior part of the body is not a strict feature of the vertebrate brain. Urochordates, cephalochordates and annelids are all provided with photoreceptors or associated neurons asymmetrically arranged in an anterior module (Table 1). Here, I present what we know about these ancestral photoreceptive structures in order to bring light on the potential origin of the vertebrate epithalamus.

Urochordates

Located at the anterior end of the neural tube, the sensory vesicle of ascidian species contains two sensory organs involved in phototactic and geotactic responses, respectively the ocellus and the otolith. Initially aligned on the midline, the ocellus and the otolith pigment cells move to the right side by the end of tail bud stage (Nicol and Meinertzhagen 1991). Interestingly, *Pax6* mRNA is expressed in the anterior brain vesicle and along

the posterior neural tube (tail bud stage) but this expression is lost at the larval stage when the sensory vesicle is formed (Glardon S et al. 1997; Mazet et al. 2003). Furthermore, *Ci-rx*, the *Ciona intestinalis* orthologue of rx, is essential for ocellus formation (D'Aniello et al. 2006) and two molecules involved in the phototransduction cascade, *Ci-arrestin* and *Ci-opsin1*, colocalize in the ocellus photoreceptors (Kusakabe et al. 2001; D'Aniello et al. 2006). Finally *Ci-Otx* is specifically transcribed in cells of the anterior neural structure where it orchestrates anterior neural patterning (Wada et al. 1996; Hudson and Lemaire 2001; Oonuma et al. 2014), as it does in many other bilaterians. A recent study revealed that the *Ci-Otx* gene product also directs posterior neural tissue specification (Roure et al. 2014), indicating that a co-option took place in ascidians for Otx. Whether the ocellus is homologous to the vertebrate eye or the vertebrate pineal complex is still under debate. The lack of genes encoding components of the circadian machinery or encoding the enzymes responsible for melatonin synthesis in the *Ciona intestinalis* genome (Dehal et al. 2002) is one of the points that precludes the resolution of this issue.

Cephalochordates

The cephalochordate amphioxus displays an overall asymmetry of the oropharyngeal area and four unpaired photoreceptive modules; two composed of c-PRCs which constitute the frontal/unique eye and the unpaired lamellar body located on the dorsal anterior neural tube; and two carrying r-PRCs which constitute a dorsal column made of Joseph cells and the Hesse organ formed by several dorsal pigmented ocelli. Based on the expression of *Pax6* mRNA in the cerebral vesicle which gives rise to both the frontal eye and the lamellar organ, an early study proposed that the amphioxus frontal organ is homologous to the paired vertebrate eyes and the amphioxus lamellar body is homologous to the vertebrate pineal organ (Glardon et al. 1998). The presence of *rx* mRNA in the anterior part of the cerebral vesicle and the expression of c-opsin in frontal eye photoreceptors tend to confirm the homology with the vertebrate eyes. However, the absence of expression of *Otx* mRNA and *rx* gene product in the lamellar organ is in opposition with the proposed homology of the amphioxus lamellar body with the pineal gland (Vopalensky et al. 2012). Lastly, among the two known AANAT families (the structurally distinct vertebrate AANAT group and non-vertebrate AANAT group), seven members of the non-vertebrate AANAT family have been found in the amphioxus genome (Pavlicek et al. 2010). To date though, no data exist concerning their distribution in the lamellar organ. Thurston Lacalli reported that the lamellar body is more elaborated in the larvae compared to the adult (Lacalli 2008) which might be in relation with the role of this structure in diel vertical migration (DVM), a typical larval behaviour consisting in migration into the water column from the mesopelagic zone to the water surface at night. The lamellar body also controls circadian rhythms (Lacalli et al. 1994) while the frontal eye could be a shadow detector operating when the animal stands in the epipelagic zone (Lacalli 2004; Stokes and Holland 1995). Taken together, these data collected from both lancelets and tunicates may indicate that an epithalamic region did exist in the chordate ancestor. The existence of this brain compartment outside chordates can be discussed as follows.

Annelids

The so-called DVM is a general circadian behaviour also accomplished by marine zooplankton. Interestingly, in the trochophore larva of the annelid *Platynereis dumerili*, ciliary swimming orchestrating the DVM is regulated by melatonin levels. Cells expressing *hiomt*, the melatonin synthesis marker, are located at the dorsal side of the episphere (Tosches et al. 2014). These cells have been previously demonstrated to be ciliary photoreceptors (c-PRCs) based on: i/the presence of the first identified invertebrate c-opsin *(c-opsin-1)*, ii/ the organization of their membrane foldings (as cilia rather than microvilli) and iii/ the expression of *rx* mRNA and *baml*, a key component of the circadian clock (Arendt et al. 2004). These c-PRCs constitute a paired structure located on each side of the midline of the dorsal part of the episphere, while an asymmetric serotoninergic cell is observed adjacent to c-PRCs (Tosches 2012; Figure 2). Moreover, both this asymmetric cell and the lineage leading to c-PRCs express *Tbx2/3* (Tosches 2012), the ortholog of vertebrate *Tbx2b* which has been demonstrated to be critical for pineal complex specification in zebrafish (Snelson et al. 2008). This set of molecular and morphological features displayed by the *Platynereis* c-PRCs corroborates the hypothesis proposed by Tosches and Arendt that the vertebrate pineal might have evolved from the cellular compartment of the annelid larval brain (Tosches and Arendt 2013).

The possible homology between these c-PRCs in annelids and the vertebrate epithalamus should however be taken with caution. Indeed, the epithalamus comprises the pineal complex and the habenular complex while the proposed comparison only concerns the pineal complex. In addition the vertebrate forebrain and the anterior portion of the protostome episphere, the apical plate, are quite different in structure, making comparisons tricky. However as in other portions of the body, gene expression territories such as those of *six3*

and *rx* are found both in vertebrate embryo forebrains and in protostome larvae apical plates. These transcription factors are important for the patterning of the apical plate. In vertebrates, the *six3* gene product is expressed in the diencephalon (Braun et al. 2003), from which the epithalamus is derived. Furthermore, *rx* mRNA is involved in the specification of c-PRCs (see section II). These genes are also expressed in the developing pineal. Based on these observations Tosches and Arendt proposed that the c-PRCs of the pineal (which is different from the entire epithalamus) might represent a legacy of the protostome apical plate (note that this legacy might also comprise the retina and the anterior hypothalamus, Tosches and Arendt 2013). Thus, instead of defining a structure that may be the epithalamus in protostomes, it is more reasonable to see a set of cells with multiple abilities (endocrine secretion, seasonal and/or circadian rhythm control, photodetection) that evolved as more intricate structures such as the pineal complex, retina and anterior hypothalamus. In addition, a clear structural homology will only be resolved after a complete analysis of these cPRCs (and the tissue that surrounds them) in a large number of metazoan groups that span the evolutionary distance between annelids and chordates. This analysis would help to infer whether the structure was present or not in their last common ancestor.

V- Nodal signaling and the control of asymmetries at the metazoan level

How morphological asymmetries are established within bilaterians is an intensively studied issue in the field of developmental and cell biology. Whether these asymmetries affect external structures or internal organs, they have been demonstrated to depend on a wide range of cellular, subcellular and molecular mechanisms that can act simultaneously or independently within a given species. In vertebrate model organisms (mainly zebrafish, mouse, *Xenopus*) and also in rabbit, the chiral rotation of cilia within the node produced a leftward flow of extracellular morphogen implying that structural molecules involved in ciliogenesis and cilia motility are of crucial importance to generate L/R asymmetries (reviewed in Vandenberg and Levin 2013; Coutelis et al 2014). Cells at the Hensen's node in chicken are devoid of monocilia and L/R asymmetry is rather yielded by a leftward displacement of cells around the node as demonstrated by time-lapse analysis (Gros et al. 2009). In *Xenopus*, the restricted location of ion pumps in one blastomere of 4-cell stage embryos generates a membrane potential difference leading to an asymmetric (right-sided) accumulation of serotonin (reviewed in Vandenberg and Levin 2013). In protostomes and more particularly in snails, spiral cleavage of blastomeres observed at very early stage of development instructs the direction of shell coiling (Shibazaki et al. 2004; Kuroda et al. 2009) whereas in drosophila, the type I actin-based motor protein Myosin ID is driving the dextral 360° rotation of male genitalia as this spinning becomes inverted in MyoID mutant (Speder et al. 2006).

Morphological asymmetries can then be viewed as the translation of a chirality observed at the cellular level relying on actin and microtubule dynamics and/or bioelectrical, molecular, biophysical gradients set up at a very early cleavage stage. This view constitutes a more integrated mechanism connecting events taking place at the cell, tissue and organ level. For a more detailed consideration of the mechanistic bases of asymmetry, refer to the recent review written by Vandenberg and Levin 2013, to the special Genesis issue (Halpern et al. 2014) or to Coutelis et al. (2014).

Besides the fact that morphological asymmetries can reflect intracellular asymmetries, asymmetric activity of *Nodal-Lefty-Pitx2* cassette during embryogenesis is a general mechanism involved in the set up of morphological asymmetries. Initially described in the lateral plate mesoderm of vertebrate embryos, the TGFβ superfamily members Nodal, Lefty and the transcription factor Pitx2 are the key components of this conserved cascade with nodal inducing Lefty, Pitx2 and itself as a positive feedback loop. More precisely, Nodal binds to activin and activin-like receptors leading to the phosphorylation of Smad transcription factors responsible for the transcription of the target genes (Capdevila et al. 2000). It is now clear that the Nodal pathway, likely reflecting the downstream activity of the intracellular mechanisms described above, acts as a symmetry breaker in metazoans. Furthermore, in some species, the Nodal pathway is also involved in asymmetry directionality. Which of these roles constitutes Nodal's ancestral role is an important question.

Directional and random asymmetries

One compelling feature of asymmetries is that they can be either directional or randomized within a population. For example, the nematode *C. Elegans* possesses two bilateral pairs of neurons (gustatory ASE and olfactory AWC) displaying asymmetries in the expression of chemoreceptors of the receptor-type guanylyl cyclase (gyc-5 and gyc-7) or G-protein-coupled receptors (srsx-3 and str-2). For ASE neurons the direction of asymmetry is fixed: left ASE (ASEL) expresses gyc-7 while right ASE (ASER) expresses gyc-5. In contrast, the asymmetry of AWC neurons displays an anti-symmetric pattern of GPCR expression: one neuron expresses srsx-

3 (AWC^{OFF}) and the neuron on the opposite side expresses str-2 (AWC^{ON}). The choice of srsx-3 expression on one side or the other is randomized within the population. Recently Cochella et al (2014) revealed that directional asymmetry and anti-symmetry are coupled by a genetic link; loss of expression of the zinc finger transcription factor die-1 abrogated the asymmetries of both ASE and AWC neurons. Thus, when one describes the asymmetry of a structure, the directional or randomized aspect of this asymmetry should be considered.

The Nodal pathway is conserved in metazoans

First described in chicken (Levin et al 1995), the left sided activity of the Nodal pathway was then reported in a wide range of chordate embryos such as amphioxus (Yu et al. 2002), ascidian (Morokuma et al. 2002), zebrafish (Long et al. 2003), *Xenopus* (Lustig et al. 1996), mouse (Collignon et al. 1996; Lowe et al. 1996) and more recently in catshak and marine lamprey (Lagadec et al 2015). In echinoderms and hemichordates, the Nodal pathway appears to be active on the right side of the embryo (discussed below) (Duboc et al. 2005; Wlizla 2011). For a long time the Nodal pathway was believed to be an innovation of deuterostomes but we know now that it is also present in snails, as well as several other lophotrochozoans (Grande and Patel 2009; Grande et al. 2014) and also cnidarians (Watanabe et al. 2014). Thus, the Nodal pathway is highly conserved in metazoans. Its absence in ecdyzosoan species such as *D. melanogaster* or *C. elegans* might reflect a secondary loss.

The role of the Nodal pathway in urochordates, cephalochordates and echinoderms

In *Ciona intestinalis*, Nodal signaling controls ocellus asymmetry on the right side. When Nodal signaling is abrogated, the pigment cell stays on the midline and photoreceptors are duplicated on the left side (Yoshida and Saiga. 2011; Figure 2). Similarly, right isomerism of the oropharyngeal region together with the restoration of a symmetric somitogenesis are observed in amphioxus after Nodal abrogation (Soukup et al. 2015; Bertrand et al. 2015; Figure 2). Experiments achieved in sea urchin illustrate the fact that Nodal signaling can either be an instructor of asymmetry (symmetry breaker) or an instructor of asymmetry directionality. In the first case, the phenotype produced upon Nodal abrogation is a restoration of symmetry (Figure 2) as observed in amphioxus and *Ciona*. In the other, the asymmetry is still present upon abrogation but the handedness of this asymmetry becomes randomized (i.e. the left-sided features can be seen on the right and vice versa).

During sea urchin larval development, the left coelomic pouch gives rise to a structure called the rudiment. Interestingly, Nodal signaling is active on the right side in sea urchin embryos which thus represents an inversion with regard to the above-mentioned chordate embryos (Duboc et al. 2005). However, note that this right-sided expression of Nodal has been hypothesized to reflect an inverted position of the dorsal-ventral axis in echinoderms, as reported for protostomes. In this case, Nodal signalling would in fact have a left-sided activity as in others chordates (Duboc et al. 2005, Blum et al. 2014). Inhibition of Nodal signaling after the onset of sea urchin gastrulation induces the duplication of the rudiment on the right side. This suggests that Nodal activity on the right might repress cellular mechanisms that are operating on the left side to produce the formation of the rudiment (Duboc et al. 2005, Figure 2). In addition, this paper showed that there are several other asymmetric Nodal expression territories in the sea uchin embryo: the right sided endomesodermal cells at the tip of the archenteron and, a couple of hours later, the right ectoderm. Blocking the expression or making it bilateral in endomesodermal cells leads to randomization of both Nodal expression in the ectoderm and in the rudiment location. Therefore, it seems that the role played by Nodal in the endomesodermal cells is not to break symmetry in the ectoderm but to give the direction of asymmetry (Bessodes et al. 2012). These studies highlight the importance of determining the spatio-temporal expression of Nodal during embryogenesis. Indeed, designing experiments with a defined timing of abrogation should enable a complete interpretation of Nodal function.

The role of the Nodal pathway in the mouse

Mouse mutants in which Nodal is bilaterally expressed in the lateral plate mesoderm (*HNF-3β*, *iv*, *fused toes*, *kif3b* mutants) display randomization of embryonic turning and heart tube looping (Lowe et al. 1996; Collignon et al. 1996; Heymer et al. 1997; Nonaka et al. 1998). Whereas right-sided expression of Nodal (*inv* mutants) is characterized by *situs inversus* (Lowe et al. 1996, Collignon et al. 1996). These data indicate that the handedness of Nodal expression tends to go with the direction of axial rotation. However many other mouse mutants where components of the Nodal pathway are disrupted (*type IIB activin receptor*, *lefty-1*, *pitx2*, *fgf8* mutants) display visceral or thoracic isomerisms (Oh and Li 1997; Meno et al. 1998; Kitamura et al. 1999; Lin et al. 1999; Meyers et al. 1999) suggesting that Nodal signaling function is not restricted to the control of directionality but can also instruct symmetry breaking in mammals. The complete list of mouse mutants displaying laterality defects is presented in Bisgrove et al. 2003. Determining both the timing of Nodal pathway activity and

the precise epistatic relationship between the different genes involved in L/R asymmetry might be the key point to decipher the role of Nodal in asymmetry breaking versus laterality control.

The role of the Nodal pathway in the brain of teleosts, sharks and lampreys

In zebrafish, asymmetry of the epithalamus is characterized by leftward migration of the parapineal during embryogenesis. Leftward (versus rightward) migration occurs in 95% of wild type animals. This left sided migration is coupled to activity of Nodal signaling on the left hand side of the diencephalon both in zebrafish and medaka (Concha et al. 2000; Signore et al. 2009, Figure 1). In zebrafish mutants where Nodal is either bilaterally expressed or switched off in the diencephalon, the migration of the parapineal gland is randomized arguing that Nodal signaling also acts as a directional instructor in this process (Concha et al. 2000). However, a more recent study reported that Nodal activity in the zebrafish diencephalon also controls subtle asymmetric neurogenesis between the two habenulae, independently of parapineal migration (Roussigné et al. 2009, Figure 1). As previously exposed, the catshark exhibits important habenular asymmetries while not possessing a parapineal nucleus. In lamprey, habenular asymmetries are observed before the onset of parapineal morphogenesis. Nodal signaling, active on the left half of the dorsal diencephalon, as in zebrafish embryos, is a strong symmetry breaker of the epithalamus in these species as its repression leads to right isomerism - the left habenula adopting right habenula identity (Lagadec et al. 2015, Figure 1).

Finally, an asymmetric and late expression of *pitx2* has been observed in the habenula of flounder fish which display external asymmetry in the position of the eye after metamorphosis. *Pitx2* expression is reported in the left habenula either in dextral (both eyes localized on the right side) or sinistral (both eyes located on the left) species (Hashimoto et al. 2007). Chemical treatment leading to absence or bilateral expression of *pitx2* induces a subtle influence on eye asymmetry with 6% of animals displaying reversed eye sideness and 6% presenting symmetrical eyes (Suzuki et al. 2009) meaning that the central mechanism leading to asymmetric eye migration in flatfish remains to be discovered.

The Nodal pathway is inactive in the brain of birds, mammals and amphibians

Since the expression of *Nodal* gene product in the left diencephalon has been observed only in cyclostomes, cartilaginous fish and teleosts, its absence in tetrapods constitutes an interesting issue (Figure 1). One possibility is that this “brain” expression has been missed because it might be extremely transient in birds, mammals and amphibians or because the sampling of tetrapod embryos tested for Nodal expression in the brain is insufficient to conclude. A more complete sampling might include turtles (for example *Pelodiscus sinensis*, *Chelonia mydas* or *Emys orbicularis*), lizards/snakes (*Anolis carolinensis*/*Natrix natrix*) and crocodilians (*Alligator mississippiensis* or *Crocodylus porosus*) whose embryos have been already used for *in situ* hybridization. The second possibility is the existence of a specific enhancer for the expression in the left diencephalon within the *Nodal* locus as reported for the left lateral plate mesoderm (ASE enhancer, Adachi et al. 1999). A third possibility is epigenetic regulation of the Nodal locus. Recently, Arai et al. (2015) provided evidence that an ERE sequence located between –3.0 to –0.4 kb upstream of the *Nodal* gene +1 (transcriptional start) site is silenced by several PRC2-mediated histone methylations. Further studies have unveiled that a serotonin-controled epigenetic mechanism represses the activity of Nodal signaling on the right side of *Xenopus* embryos, a step necessary for the correct situs of visceral organs (Carneiro et al. 2011). The mechanism implies histone deacetylations in the intronic region of *Xnr-1*. Analysis of the expression of Nodal in the diencephalon of zebrafish mutants for histone methyltransferase/deacetylase and/or proteins of the polycomb complexe would reveal whether chromatin modification can indeed drive the asymmetric expression of Nodal in the forebrain.

The role of Nodal signalling in breaking symmetry in protostomes

Snails

Functional studies of Nodal signaling are scarce in protostomes. The *Nodal* gene seems to exist only in lophotrochozoa/spiralia genomes (Grande and Patel 2009; Grande et al. 2014). Grande and Patel first found that the location of *Nodal* and *Pitx* expression at the trochophore stage of snails is linked to the chirality of their shell (Figure 2). Indeed, these genes are expressed on the right in dextral snail species (clockwise turning shell) and on the left in sinistral species (counterclockwise turning shell) (Grande and Patel 2009). In addition, Nodal acts as a symmetry breaker in snails as its abrogation leads to the loss of shell coiling. Interestingly, besides the ectodermal right-sided or left-sided expression of Nodal near the shell gland, this study reported an asymmetric “cephalic” expression of Nodal in both species. Might this asymmetric Nodal expression in the snail head hint at an asymmetric structure in the brain? If so, is this structure composed of photosensitive/sensory cells as

observed in annelids and in chordates? These questions constitute a basis for future exciting projects dealing with the evolution of anterior photosensitive structures in metazoans.

Annelids

In the annelid *Platynereis dumerilii*, the molecular mechanism steering the asymmetric positioning of the serotoninergic neuron associated to the paired c-PRCs (Tosches 2012, Figure 2) has not been investigated so far. The *Nodal* gene doesn't seem to be present in the *Platynereis* genome leaving many questions related to asymmetric morphogenesis of the lophotrochozoa brain unanswered. Recently, Grande et al (2014) uncovered *Nodal* and *Pitx* orthologs in the genomes of rotifer, nemertean, mollusc, brachiopod species and annelids *Capitella teleta* and *Pomatoceros lamarckii* highlighting a wide distribution within spiralian genomes. In the same study, analysis of *Nodal* and *Pitx* mRNA expression patterns revealed a right- but not exclusive sided expression in brachiopod embryos *(Terebratalia transversa)*. *Nodal* and *Pitx* genes were also found in the chaetognath *Flaccisagitta enflata* but not in *Xenoturbella* and acoelomorph genomes.

The role of Nodal signalling in breaking symmetry in cnidarians

Finally, nodal works as a symmetry breaker in the freshwater polyp (cnidarian) *Hydra* (Figure 2). Nodal is expressed as a spot at the budding zone on the main body axis where it induces Pitx and the formation of a secondary polyp leading to an early axial asymmetry. When Nodal is experimentally down-regulated, this asymmetric budding does not occur while an ectopic expression of Nodal around the main axis induces an expansion of bud formation (Watanabe et al. 2014).

VI- Conclusion

Here, I gave molecular and morphological guides to explore homology relationships of the epithalamus in different vertebrate species. Furthermore, I reviewed our current knowledge on asymmetric photosensitive structures in non-vertebrate metazoans, asking whether these could constitute ancestral structures from which the vertebrate epithalamus evolved. The case of annelids is significant as an asymmetric serotoninergic neuron is found associated to c-PRCs expressing markers also present in the vertebrate pineal complex. The presence of asymmetric and cephalic expression of Nodal in gastropod embryos might be an indication of the existence of an asymmetric photosensitive structure. Advanced topographic cell analysis in snails could bring further insight into this issue. In addition, while *Lefty* and *Pitx2* expressions are generally used as a readout of the Nodal transduction cascade in vertebrates, most of the invertebrate studies reported Pitx but not Lefty expression which might indicate that the pathway could work differently in these two groups. The search of homologous genes in each non-vertebrate species together with their territory of expression might help to address this question. Finally, the compilation of data on the Nodal signaling pathway indicates that it is more widely distributed in metazoans than expected. Importantly, it appears that Nodal's ancestral role would be to break symmetry both in peripheral systems and brain structures, rather than direct asymmetry.

FIGURES

Figure 1: The role of Nodal as a symmetry breaker in metazoans (part I)

Schematic representations of Nodal expression territories in vertebrate embryos (dorsal view). At the basis of the vertebrate lineage, Nodal displays two asymmetric domains: the left lateral plate mesoderm (LPM) and the left diencephalon (D). The left diencephalon gives rise to the epithalamus, an asymmetric structure composed of the habenulae and the pineal complex (pineal + parapineal). The parapineal is connected to the left habenula in lampreys and teleosts but is absent in catshark. In teleosts, the parapineal controls asymmetries as right isomerism is observed i/ when the parapineal stays on the midline (Regan et al. 2009), ii/ in zebrafish mutants with unspecified parapineal cells (Snelson et al. 2008) and iii/ in embryos upon parapineal laser ablation (Gamse et al. 2003). Nodal abrogation in catshark and lampreys leads to right isomerism (Lagadec et al. 2015), suggesting that a Nodal-dependent symmetry breaking mechanism was present in the vertebrate ancestor. Note that in teleosts Nodal abrogation also controls a subtle asymmetry during neurogenesis of a subset of neurons (Roussigné et al. 2009). So far, the expression of Nodal in the left diencephalon has not been reported in tetrapods.

Figure 2: The role of Nodal as a symmetry breaker in metazoans (part II)
Illustrations of asymmetric structures in non vertebrate metazoans and schematic depictions of phenotypes produced after Nodal abrogation. In *Hydra*, the main body axis grows an asymmetric polyp. Mollusca have a coiling shell (clockwise or counterclockwise rotation). Amphioxus display a staggered arrangement of somites and an overall asymmetry of the oropharyngeal region (not illustrated). The ocellus is positioned on the right side of the sensory vesicle in *Ciona*. The sea urchin possesses an unpaired structure, the rudiment, located on the left side at larval stage. For all these animals, Nodal is asymmetrically expressed and its abrogation restores symmetry (Grande and Patel 2009; Yoshida and Saiga 2011; Watanabe et al. 2014; Bertrand et al. 2015; Soukup et al; 2015;). In echinoderms and hemichordates, the Nodal pathway is active on the right side of the embryo (Duboc et al; 2005, Wlizla 2011). In the polychaete *Platynereis dumerilii*, an asymmetric serotoninergic neuron is associated to cPRCs but no expression of Nodal has been reported in this species (Tosches 2012). A, anterior; P, posterior; L, left; R, right; 5-HT, serotonin; cPRCs, ciliary photoreceptors

TABLE
Table 1: Molecular guide for photosensitive structures outside vertebrates that could account for the origin of the vertebrate epithalamus. References can be found in the text together with species used in the study. Expression patterns generally refer to those described in embryonic structures.

CONFLICT OF INTEREST
The author has declared no conflict of interest

ABBREVIATIONS
5-HT: serotonin
5-HTP: 5-hydroxy-L-tryptophan
Aanat: arylalkylamine N-acetyltransferase
bHLH: basic Helix-Loop-Helix
c-PRCs: ciliary photoreceptors
DVM: diel vertical migration
ERE: epigenetic regulatory element
Hiomt: hydroxyindole O-methyltransferase
IPN: interpedoncular nucleus
p2: prosomere 2
r-PRCs: rhabdomeric photoreceptors
SCN: suprachiasmatic nucleus

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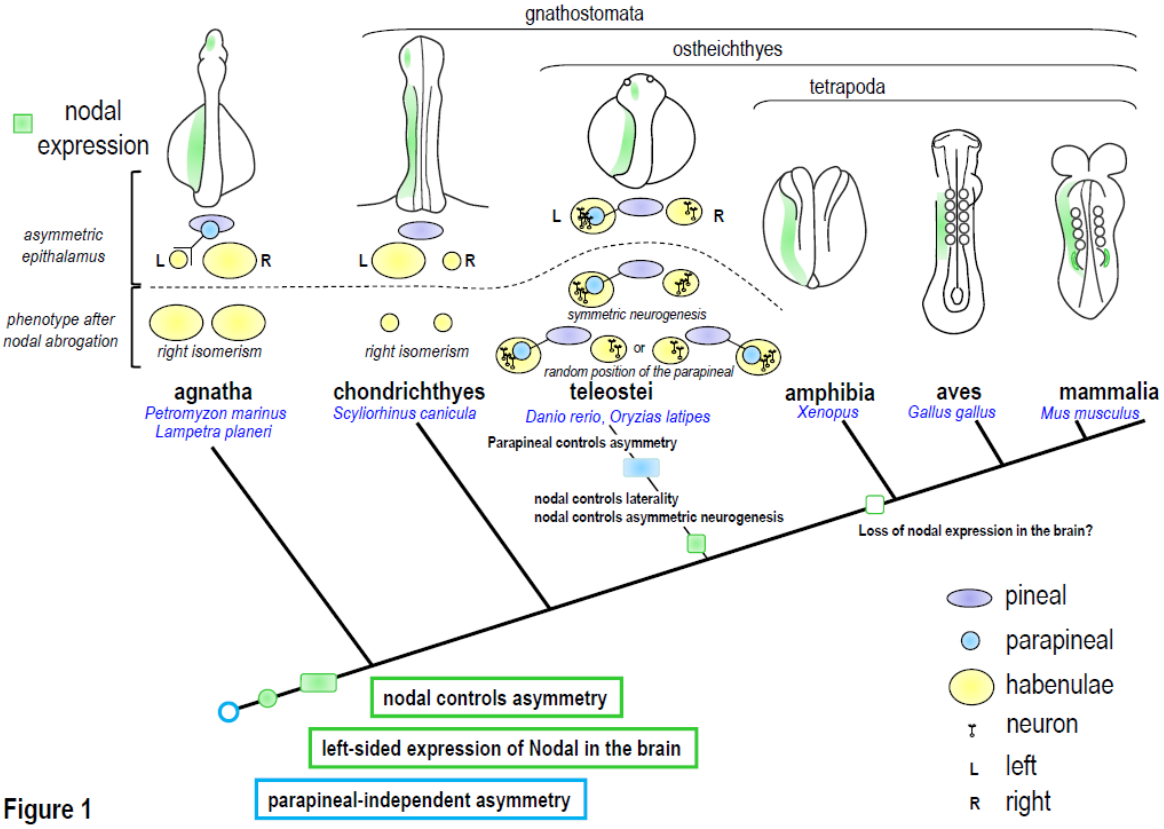

Figure 1

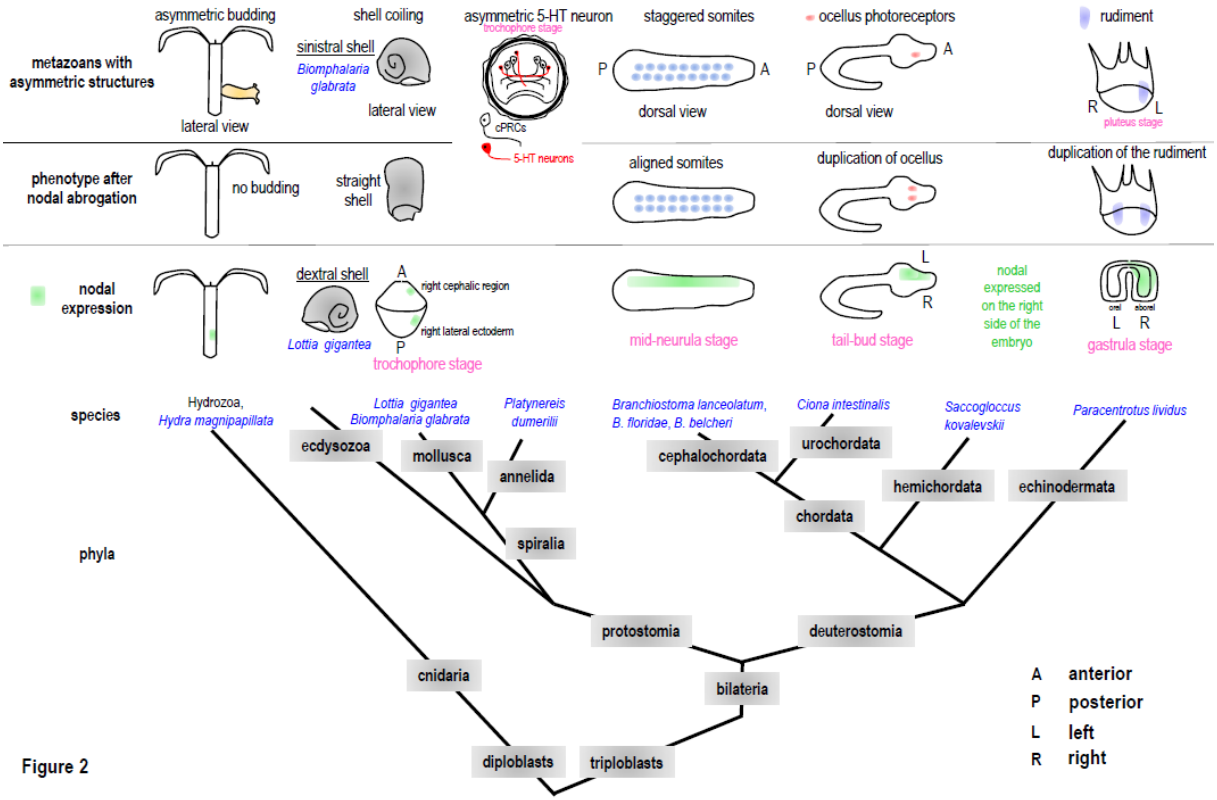

Figure 2

Table 1: Molecular guide to look for photosenstive structures outside vertebrates that could account for epithalamus origin in vertebrates. References can be found in the text together with species used in the study. Expression patterns generally refer to those described in embryonic structures.

	mollusca (snails)	**annelida**	**tunicata**	**cephalochordata**
Description of the asymmetric photosensitive structure in the anterior part of the body				
	No asymmetric photoreceptors but an asymmetric "cephalic" expression of Nodal is reported	Paired ciliary photoreceptors (c-PRCs) associated to asymmetric serotoninergic cell	2 sensory organs located on the right side at the tail bud stage: ocellus and otolith	2 unpaired photoreceptive modules composed of c-PRCs: frontal eye and lamellar body
Circadian behaviour associated to the structure		Diel vertical migration (DVM) regulated by melatonin level at larval stage		Lamellar body suggested to control DVM at larval stage
Genes involved in melatonin synthesis / circadian rhythm				
aanat			no *aanat* genes in urochordate genomes	*anaat* genes present in the amphioxus genome but no expression pattern available
hiomt		expressed in c-PRCs		
baml/clock		expressed in c-PRCs		
Genes involved in photoreceptor specification / eye formation				
***crx/otx* family**			*Ci-otx* expressed in anterior neural structure	
Pax6			expressed in the anterior brain vesicle	expressed in the cerebral vesicle which form the frontal eye and the lamellar body
Rx/rax		expressed in c-PRCs	*Ci-rx* essential for ocellus formation	*rx* expressed in the anterior part of the cerebral vesicle
Transcription factors				
Tbx2		*Tbx2/3* expressed in the asymmetric serotoninergic cell		
Genes involved in photoreception / phototransduction				
opsin		*c-opsin-1* expressed in c-PRCs	*Ci-opsin1* expressed in the ocellus	*Opsin* expressed in the frontal eye
arrestin			*Ci-arrestin* expressed in the ocellus	