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Evolution of photosensory pineal organs in new light: the fate of neuroendocrine photoreceptors

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Pineal evolution is envisaged as a gradual transformation of pinealocytes (a gradual regression of pinealocyte sensory capacity within a particular cell line), the so-called sensory cell line of the pineal organ. In most non-mammals the pineal organ is a directly photosensory organ, while the pineal organ of mammals (epiphysis cerebri) is a non-sensory neuroendocrine organ under photoperiod control. The phylogenetic transformation of the pineal organ is reflected in the morphology and physiology of the main parenchymal cell type, the pinealocyte. In anamniotes, pinealocytes with retinal cone photoreceptor-like characteristics predominate, whereas in sauropsids so-called rudimentary photoreceptors predominate. These have well-developed secretory characteristics, and have been interpreted as intermediaries between the anamniote pineal photoreceptors and the mammalian non-sensory pinealocytes. We have re-examined the original studies on which the gradual transformation hypothesis of pineal evolution is based, and found that the evidence for this model of pineal evolution is ambiguous. In the light of recent advances in the understanding of neural development mechanisms, we propose a new hypothesis of pineal evolution, in which the old notion 'gradual regression within the sensory cell line' should be replaced with 'changes in fate restriction within the neural lineage of the pineal field'.

Keywords: pineal gland; vertebrate photoreceptors; opsin; melatonin; electron microscopy

1. INTRODUCTION

The pineal organ develops from the roof of the embryonic forebrain and in the adult brain constitutes, together with the habenular nuclei, the main part of the epithalamus (figure 1). In lampreys, cartilaginous and bony fishes, amphibians, turtles, lizards and birds, the pineal organ is a directly photosensory organ, containing cells that respond to changes in the environmental light conditions. By contrast, the pineal organ of mammals is not directly photosensory by virtue of intrinsic photosensory cells, although its activity is indirectly under the control of the ambient light–dark cycle (Vollrath 1981; Korf 2000). During the 1960s and early 1970s a series of extensive—now classical—comparative ultrastructural studies of the pineal organ in a wide variety of vertebrates led to a breakthrough in our understanding of pineal evolution, and formed the basis of the current state of knowledge that the main parenchymal cell type of all pineal organs, i.e. the pinealocyte, has evolved within the vertebrate radiation through a gradual loss of photoreceptor characters and a gradual increase of neuroendocrine characters. Thus, the non-sensory pinealocyte of mammals has evolved from a photoreceptor cell, similar to those present in the pineal organ of anamniotes (Collin 1969, 1971; Oksche 1971; Collin & Oksche 1981; figure 2).

Actually, a transformation from a directly photosensory pineal organ in 'lower vertebrates' to a secretory gland in mammals had been suggested by several early authors on the basis of light microscopic observations (see Bargmann 1943). Bargmann (1943, p. 484) went even further, and specified that '…die Sinneszellen werden unter Verlust ihrer rezeptorischen Abschnitte zu den Pinealzellen der Säuger.' (…the sensory cells become, through the loss of their receptoral parts, mammalian pineal cells.). The great impact of the early ultrastructural studies was enforced by the close correlation with comparative neurophysiological studies (reviewed by Dodt 1973; Meissl & Dodt 1981) and studies of indoleamine metabolism (e.g. Collin 1968*a*; Quay *et al.* 1968*a*; Wartenberg & Baumgarten 1969; Owman & Rüdeberg 1970; Owman *et al.* 1970; Collin & Meiniel 1973*a*,*b*; Ueck 1973, 1974). Subsequent studies of pineal organs, using ultrastructural, immunochemical and molecular biological methods, have supported this concept of pinealocyte evolution, which has obtained the status of a paradigm in neuroendocrine research. However, careful analysis of the original data in the light of recent advances in our understanding of neural development—in particular how multiple cell types of neuronal and glial lineage are generated in the central nervous system (CNS)—prompts us to suggest a modification and extension of this hypothesis of pineal evolution. This revision attempts to provide an insight into the evolution of photosensory pineal organs, and in particular how the variability of pineal organization has evolved.

2. THE PINEAL ORGAN: PART OF A PINEAL COMPLEX

In the early vertebrates, the pineal organ was probably one part of a bipartite pineal complex, which consisted of

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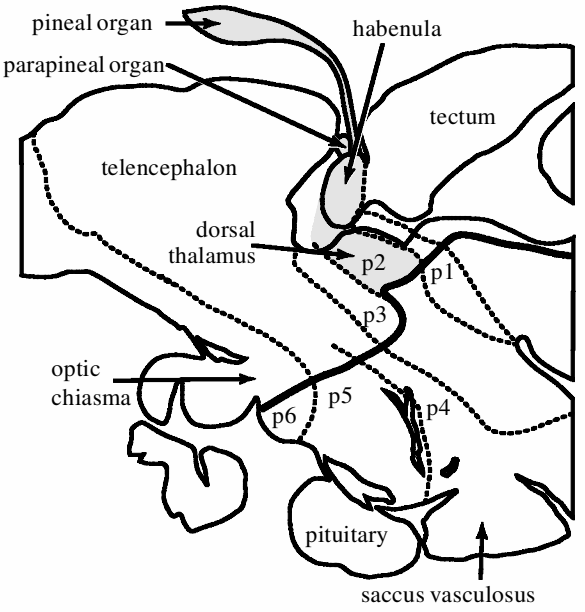

Figure 1. Overview, in a near-midsagittal section, of the forebrain of a teleost fish. The pineal complex, consisting of a pineal organ and a parapineal organ, is formed from the alar plate of the second forebrain neuromere (shaded in the figure). p1–p6, forebrain (prosencephalic) neuromeres 1–6, with approximate boundaries indicated by dotted lines. The alar/basal plate boundary is indicated by a thick black line. (cf. Ekström *et al.* (2001).)

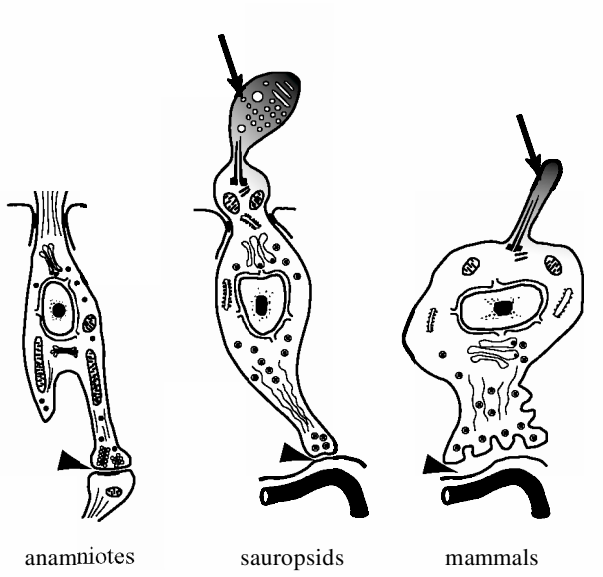

Figure 2. Schematic presentation of the evolution of the non-sensory mammalian pinealocyte from a pineal photoreceptor cell, indicated by a modification of the apical sensory cilium (arrows) and a transformation of the basal axon from a neural synaptic to a neuroendocrine connection (arrowheads). (Modified, with permission, from Korf (2000).)

a pineal organ (epiphysis cerebri) and a parapineal organ. Both structures were probably directly photosensory, as we can see in extant vertebrates with this pineal organization: lampreys, bony fishes (though not all), and many lizards. Those vertebrates with only one part of the pineal complex are all believed to have retained the pineal organ but not the parapineal. The question as to whether the pineal complex was originally bilateral, like the lateral eye retinae, has generated much heated debate over the years, and has recently been subject to a detailed review (Concha & Wilson 2001). It will not be discussed further here.

Also in extant vertebrates the pineal complex may have one intracranial portion and one extracranial portion. The pineal organ is always intracranial, as is the parapineal organ of lampreys and fishes. The extracranial portion may be endowed with lens- and/or cornea-like structures, as for the frontal organ of some anurans and the parietal eye of some lacertilians and *Sphenodon*. The frontal organ of anurans is considered to be a specialization of the distal part of the pineal organ, whereas the parietal eye is a parapineal organ. In lampreys the pineal organ lies dorsal to the parapineal organ, i.e. closer to the epidermis; the orbital cartilages of the lamprey's 'skull roof' do not cover the pineal complex (Bargmann 1943; Oksche 1965).

There is usually a clear structural subdivision within the pineal complex, in terms of predominant cell populations as well as gross morphology. When most pronounced, as in some bony fishes, the pineal organ may be subdivided into a distal pineal end-vesicle and a pineal stalk, the latter connecting the pineal organ with the diencephalic roof. The end-vesicle may consist of distinct distal, intermediate and proximal regions, recognizable primarily on account of the predominant cell types. The functional significance of this structural subdivision remains unknown, but it most probably reflects the presence of different types of photoreceptor cells and their signalling modes.

3. THE CELLULAR CONSTITUENTS OF THE PHOTOSENSORY PINEAL COMPLEX

Three basic cell types make up the bulk of the parenchyma of the photosensory pineal organ. Two are of a neuronal lineage: the photosensory pinealocytes or—as they will be referred to in the following text—pineal photoreceptors, and neurons. In the literature, the term pinealocyte is used in two senses. It is used as an inclusive term (i.e. pineal cell), encompassing all pinealocytes—sensory or not—but not neurons or glial elements. However, it is also used to specify a non-sensory 'typical mammalian' pinealocyte, with its special morphology.

Photoreceptors and neurons may come in many variants in any pineal organ, just as there may be various types of photoreceptor cells and neurons in the vertebrate retina. The third basic cell type is of a glial lineage: the so-called supportive cell, also known as the ependymal interstitial cell. Although this review focuses on the evolution of pineal photoreceptors, it is appropriate to give a brief account of the neurons and glial elements of the photosensory pineal organ.

(a) *Pineal neurons*

Neurons are typically postsynaptic to photoreceptors, and their axons form the pineal tract that primarily innervates forebrain cell groups in the pretectum and thalamus, while smaller numbers project to the habenula, hypothalamus and preoptic region. Some of these targets of the pineal tract also receive direct retinal innervation (Ekström *et al.* 1994).

There are several types of pineal neurons: at least in

those vertebrates with neural photoreceptor cells. Neuronal types have been characterized by their morphology and distribution at the light microscopic level, mainly by use of acetylcholinesterase (AChE) histochemistry and retrograde neuronal labelling. In teleost fishes and frogs, which comprise the most extensively investigated species, the pineal complex contains pseudo-unipolar, bipolar and multipolar neurons. The different types of neurons usually show a differential distribution in the pineal complex. The rostral part of the pineal may contain an aggregation of larger neurons, comprising the three basic types mentioned above, whereas smaller pseudo-unipolar neurons predominate in the rest of the pineal. In addition, bipolar neurons may be found in the pineal stalk (e.g. Ekström & Korf 1985; Ekström & Meissl 1990*a*). The differential distribution of neurons may be related to the distribution of different photoreceptor types. In some species, like the pike, neurons are present only in the most rostral part of the pineal and in the stalk portion, whereas the intermediate part is largely devoid of neurons. The latter region contains predominantly neuroendocrine photoreceptors that lack typical presynaptic specializations and do not contact neurons (Falcón 1979; Falcón & Mocqard 1979).

Most of the pineal neurons are analogous with the retinal ganglion cells, in the sense that they emit axons that innervate central brain areas (Ekström *et al.* 1994). Pineal interneurons have been identified after dye filling after intracellular recordings (Ekström & Meissl 1988) and there is a population of γ-amino-butyric acid immunoreactive (IR) neurons, which may represent pineal interneurons (Ekström *et al.* 1987, 1990). There is at present no evidence for the presence of intrinsically photosensitive second-order neurons in the pineal organ, analogous with recently demonstrated photosensitive retinal neurons (Soni *et al.* 1998; Kojima *et al.* 2000; Berson *et al.* 2002; Hattar *et al.* 2002).

Lampreys have the same set of pineal neuronal types as teleosts and frogs (Pombal *et al.* 1999; Yáñez *et al.* 1999), whereas sauropsids have a reduced pineal neuronal circuitry (Kappers 1967; Meissl & Ueck 1980; Sato & Wake 1983). Indeed, even the specialized 'eye-like' parietal eye of lizards contains only a small number of neurons (Engbretson & Anderson 1990). Among birds, distinct AChE-positive pineal nerve cells have been demonstrated in several species. The number of AChE-positive neurons in the pineal organ varies greatly between species. There appears to be a correlation between a well-developed neuronal network and the presence of pinealocytes with presynaptic specializations, although this has not been systematically investigated (Ueck 1970; Ueck & Kobayashi 1972; Korf *et al.* 1982; Sato & Wake 1983).

(b) *Pineal glial elements*

The clearly dominating glial-like cell type is the supportive cell, which displays some cytological characteristics of glial cells (Engbretson & Linser 1991). Current knowledge of supportive cell characteristics is poor, and in this review we shall not speculate on the functional role of supportive cells.

4. PINEAL PHOTORECEPTORS HAVE TWO SIGNALLING MODES

Pineal photoreceptors use two signalling modes to transmit information about the light conditions: a neural mode and a neuroendocrine mode (figure 3). Both modes may be used by one photoreceptor, which may then be designated a dual-mode photoreceptor. There are two basic types of pineal photoreceptors, neural/neuroendocrine (dual-mode) and neuroendocrine photoreceptors. In the following we will use the inclusive term 'neural photoreceptor' to indicate all photoreceptors that are presynaptic to pineal neurons, i.e. also dual-mode photoreceptors. Pure neural pineal photoreceptors have not been conclusively identified so far, but the term 'neural photoreceptor' stresses a clear distinction from neuroendocrine photoreceptors that are not presynaptic to pineal neurons.

The neural mode is served by synaptic contacts with second-order neurons, as in the retina. Although experimental evidence is very scarce, it is believed that pineal and retinal photoreceptors use the same basic phototransduction pathways (Meissl 1997; Falcón 1999), and that pineal photoreceptors primarily use glutamate as neurotransmitter (Meissl & George 1984; Vígh *et al.* 1995; 1997). Pineal photoreceptors using the neural signalling mode typically show morphological features similar to those of retinal photoreceptors: a 'sensory pole' with a mitochondria-rich ellipsoid and a well-developed photoreceptor outer segment, as well as a basal axon with synaptic specializations at the terminals (figure 3; Eakin & Westfall 1960; Collin 1969). The typical presynaptic specialization is the synaptic ribbon or variants thereof (Collin & Meiniel 1968). It is important to note that all 'typical photoreceptor characteristics' are not always present in the same photoreceptor cell. Indeed, this was one of the crucial observations that led to the formulation of the theory of gradual regression of sensory characters in pineal evolution.

The neuroendocrine mode uses the indoleamine melatonin as the main messenger. Melatonin is apparently neither stored nor actively secreted, but diffuses directly out of the photoreceptor after synthesis. Photoreceptors using the neuroendocrine mode are also 'polarized'. They are, however, typically endowed with less well-developed outer segments and they lack typical synaptic specializations (figure 3). In addition, they may contain special organelles that are considered to be indoleamine-storing, although not melatonin-storing. Such organelles are the 'dense bodies' in lampreys (Meiniel 1980) and the dense-core vesicles in sauropsids (Collin & Meiniel 1973*a*,*b*). There is a variation in the numbers of dense-core vesicles in pineal photoreceptors among sauropsids, a variation that may be correlated with the relative amounts of indoleamines stored in the cytoplasm and their release (Collin 1971). Indoleaminergic melatonin precursors are apparently stored in the photoreceptor cytoplasm as well, both in species with or without specialized storage organelles (Collin 1971).

The basal axonal protrusion of the typical neuroendocrine photoreceptor does not form synaptic connections with intrapineal neurons. It may, however, extend to establish contact with the basal lamina of the pineal epithelium. Dense-core vesicles are generally accumulated in the distal parts of the axon, i.e. the region closest to the basal lamina, which is adjacent to the perivascular space. In addition, synaptic ribbon-like structures may be aligned along the zone of contact with the basal lamina (Collin & Meiniel 1971). The close contact with the basal lamina is

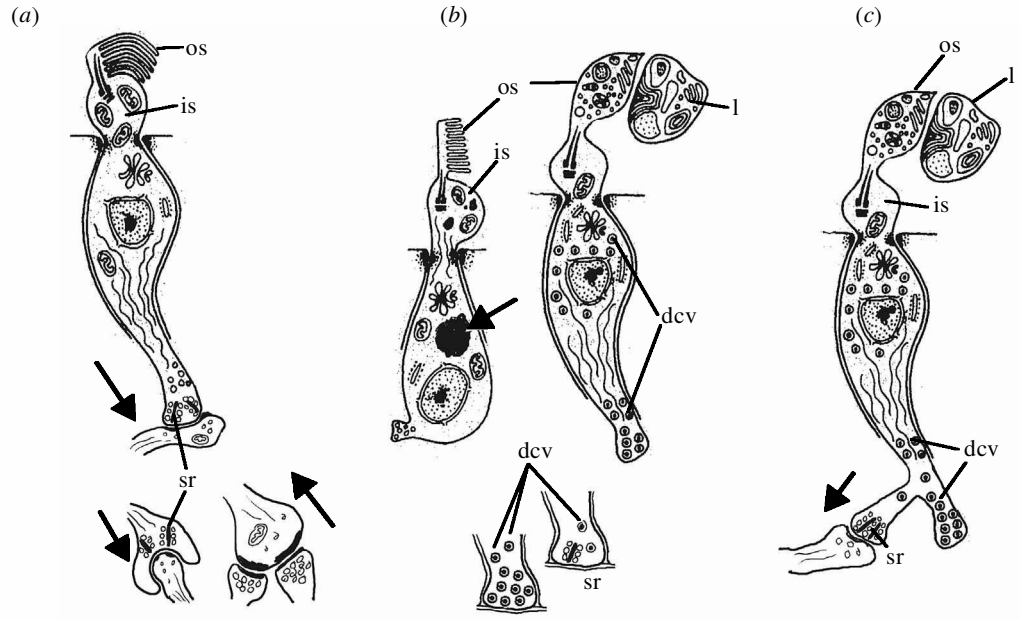

Figure 3. Pineal photoreceptors use two signalling modes: neural and neuroendocrine. The signalling mode may be reflected in the cell morphology. (*a*) Neural connections: synaptic formations. Neural mode photoreceptors have regular lamellar outer segments and a basal axon with synaptic specializations. Pineal neurons may form feedback synapses on pineal photoreceptors. (*b*) Secretory activity. Neuroendocrine mode photoreceptors have small or transformed outer segments, and organelles for indoleamine storage or synthesis: dense bodies (large arrow) or dense-core vesicles (dcv). (*c*) Synaptic formations and secretory activity. The two signalling modes may be used in parallel by pineal photoreceptors. In a lizard, typical neuroendocrine photoreceptors may also form synaptic specializations. Arrows in (*a*) and (*c*) show signalling direction. Abbreviations: is, inner segment; l, lamellar structures possibly derived from outer segments; os, outer segment; sr, synaptic ribbon.

believed to make transfer to the extravascular space more efficient. Photoreceptors of the neuroendocrine type are usually referred to as 'rudimentary photoreceptor cells' (Collin & Oksche 1981).

It is important to stress that a single photoreceptor may use both modes, and indoleamine synthesis and cellular storage may occur in the absence of specialized organelles (Collin 1971). This is obvious, for example in bony fishes, where neuroendocrine photoreceptors are morphologically similar to neural photoreceptors and neither type contains storage organelles (Falcón 1979). Actually, in fishes, neural photoreceptors appear to be the predominant type, and they do participate in indoleamine synthesis (Falcón & Collin 1985). In lampreys (Collin & Meiniel 1968), and pipid and discoglossid frogs (Ueck 1968), dense-core vesicles occur in the basal pedicles of otherwise typical 'neural' pineal photoreceptors. In the pineal organ of the lizard *Takydromus*, individual photoreceptor cells may have two types of basal processes, i.e. both 'neural' with synaptic ribbons and 'neuroendocrine' with dense-core vesicles (Ohshima *et al.* 1999).

5. PINEAL EVOLUTION: BRIEF OVERVIEW OF THE PARADIGM

The pioneering comparative ultrastructural and neuroanatomical studies of Oksche and Collin and their colleagues, together with data from other important early electron microscopical studies, led to today's paradigm that the main parenchymal cell type, the pinealocyte, has undergone a remarkable evolution. All pinealocytes, even mammalian non-photosensory pinealocytes, have gradually evolved from photoreceptor cells. Furthermore, during amniote evolution, the neuroendocrine signalling mode has become dominant, as typical photoreceptors and pineal neurons have become less abundant. This view of pineal evolution is usually shown in a diagrammatic form (figure 2) which shows that pineal photoreceptor cells have gradually lost their sensory and neural characters and switched to an indirect control of their neuroendocrine function (Collin 1971; Collin & Oksche 1981).

The current paradigm is based on two lines of evidence. First, the relative occurrence of pinealocytes with more or less pronounced photoreceptor morphology varies in different vertebrate classes. Specifically, three homologous cell types of the 'receptor line' were distinguished in the pineal organ: photoreceptor cells, rudimentary photoreceptor cells and (non-photosensory) pinealocytes. Evolution had thus taken place within a receptor cell line: photoreceptor cells changed progressively into rudimentary photoreceptor cells, and the latter transformed into pinealocytes (Collin 1971; Collin & Oksche 1981). Their photoreceptor cells would correspond to our neural-mode photoreceptors, while the rudimentary photoreceptors correspond to our neuroendocrine photoreceptors.

The second line of evidence came from developmental studies that indicated a regression of photosensory capacity during ontogenetic development of the pineal organ in amniotes. The ontogenetic data were interpreted as evidence for a gradual evolutionary transformation, where pineal photoreceptor cells had evolved to a mammalian non-sensory pinealocytes through intermediary stages similar to those of sauropsidian pineal rudimentary photoreceptors (Collin 1977; Meiniel 1981; Oksche 1983).

As will be discussed in detail below, there is actually little direct support for such a gradual modification. First and foremost, birds and lizards are not the ancestors of mammals, and it is more than likely that pineal photoreceptors have evolved, within the sauropsid radiation, independently of pinealocyte evolution within the mammalian radiation. Second, ultrastructural observations of selected developmental stages in a few species do not form a solid basis for extrapolation of changes in specific cellular characteristics during evolution.

6. PINEAL EVOLUTION: A MODIFICATION OF THE VIEW

Perhaps the most important aspect of the diagrammatic representation of pineal photoreceptor evolution (figure 2) is that it is a representation of the main types of pinealocytes in different vertebrate taxa. Those represent the actual observations; the receptor cell line theory is built upon these observations. Figure 2 shows that in anamniotes 'typical' photoreceptors are most abundant, in sauropsids (reptiles and birds) rudimentary photoreceptors are most abundant, while mammals have non-photosensory pinealocytes. If we return to those original observations, supplement them with data achieved in later studies, and plot the basic types of photoreceptor cells on a cladogram showing the phylogenetic relationship among vertebrates (figure 4), one may observe the following.

First, many types of pineal photoreceptors, and cells that have the characteristics of non-sensory pinealocytes, may occur together in one taxon. This multiplicity of cell types was observed in several original ultrastructural studies (cf. Collin 1971). Among the most intriguing findings were that in the clearly photosensory pineal complex of lampreys not only typical neural photoreceptors occurred, but also neuroendocrine photoreceptors and even pinealocyte-like cells without any differentiated sensory pole (Meiniel 1980). There are also 'pinealocytes' in teleosts (Ekström & Meissl 1990*b*) and snakes (Vivien 1964; Petit 1971). Similarly, photoreceptors with regular outer segments occur in the pineal organs of all poikilotherms (except snakes, as far as we know).

Second, because the sauropsid line (reptiles and birds) is distinct from that of mammals, data from today's reptiles give only little information about the evolution of either avian pineal photoreceptors or mammalian pinealocytes. Most data are from turtles and lizards, while crocodiles, the closest extant relatives of birds, have no pineal organ (Studnicka 1905).

Still, these data do show that a regression of the sensory structures and loss of neural signalling have occurred in the pineal complex within the radiation of reptiles, birds and mammals. This has traditionally been interpreted as a gradual transformation where mammalian pinealocytes have passed through intermediary stages resembling those of lizards, turtles and birds (see above; Collin 1971, 1977; Collin & Oksche 1981). However, the most parsimonious interpretation of the evolution of pineal photoreceptors and non-sensory pinealocytes suggests: (i) that the common ancestors of sauropsids and mammals (node X in figure 4) still possessed neural photoreceptors, possibly alongside typical neuroendocrine photoreceptors; and (ii) that pineal photoreceptors and pinealocytes have evolved independently in sauropsids and mammals.

7. PINEAL EVOLUTION: A NEW APPROACH

Our interpretation retains the basic assumption that the ancestral pineal organ was a photosensory organ and that, in this sense, all pinealocytes—photosensory or not—are modifications of ancestral pineal cells of neuronal lineage. However, our interpretation is based on what we know about the development of multiple cell types in specific regions of the CNS, and it incorporates all cells of the neuronal lineage, not only the pineal photoreceptors. It also takes into account what we know about the evolution and phylogeny of amniotes.

In the CNS, multipotent neural stem cells generate pluripotent neural progenitor cells that give rise to multiple cell types. As the pineal organ is part of the CNS, this general mechanism should apply to the formation of the pineal complex as well (figure 5), and it would accommodate the multiple types of photoreceptors, neurons and glial elements observed in all pineal organs. Pineal cell types may be distinguished using various criteria, and it is appropriate to scrutinize some of these criteria to validate our assumption of the presence of numerous distinct pineal cell types, as opposed to the continuous modification of a few distinct cell lines. In the following, we shall use the term cell 'class' to denote photoreceptor cells, neurons and glial elements, where each class may encompass several types, for example photoreceptor types and neuronal types.

8. ARE THERE REALLY MANY TYPES OF PINEAL PHOTORECEPTORS?

(a) *Criteria for distinguishing true 'types'*

The receptor cell line theory relies on observations of morphological differentiation of photoreceptors, detection of indoleamines, and direct physiological studies of light sensitivity. But what criteria are useful to distinguish different photoreceptor 'types'?

The degree of development of 'typical' photoreceptor characteristics, such as well-developed outer and inner segments or the presence of a basal axon with terminal formations containing presynaptic specializations, is obviously of prime importance. The presence or absence of specialized organelles, like those considered to serve as indoleamine storage sites (see below), are obviously also of significance.

Active indoleamine synthesis may be a general property of pineal photoreceptor cells. Thus, distinguishing criteria lie rather at the level of cellular specializations for storage and release, than at a dichotomy presence/absence of indoleamine synthesis. Direct identification of melatonin by immunocytochemistry has been questioned on methodological grounds (Ekström & Meissl 1997). The presence of indoleamines has usually been demonstrated by biochemical and autoradiographic methods (Meiniel *et al.* 1972; Collin *et al.* 1982; Falcon & Collin 1985; Meiniel 1987), formaldehyde-induced fluorescence (Collin 1968*a*; Ueck 1973; Juillard *et al.* 1977; Meiniel 1978; Falcón *et al.* 1980), or immunocytochemistry for detection of serotonin (Falcón *et al.* 1984; Araki *et al.* 1992; Ohshima *et al.* 1999;

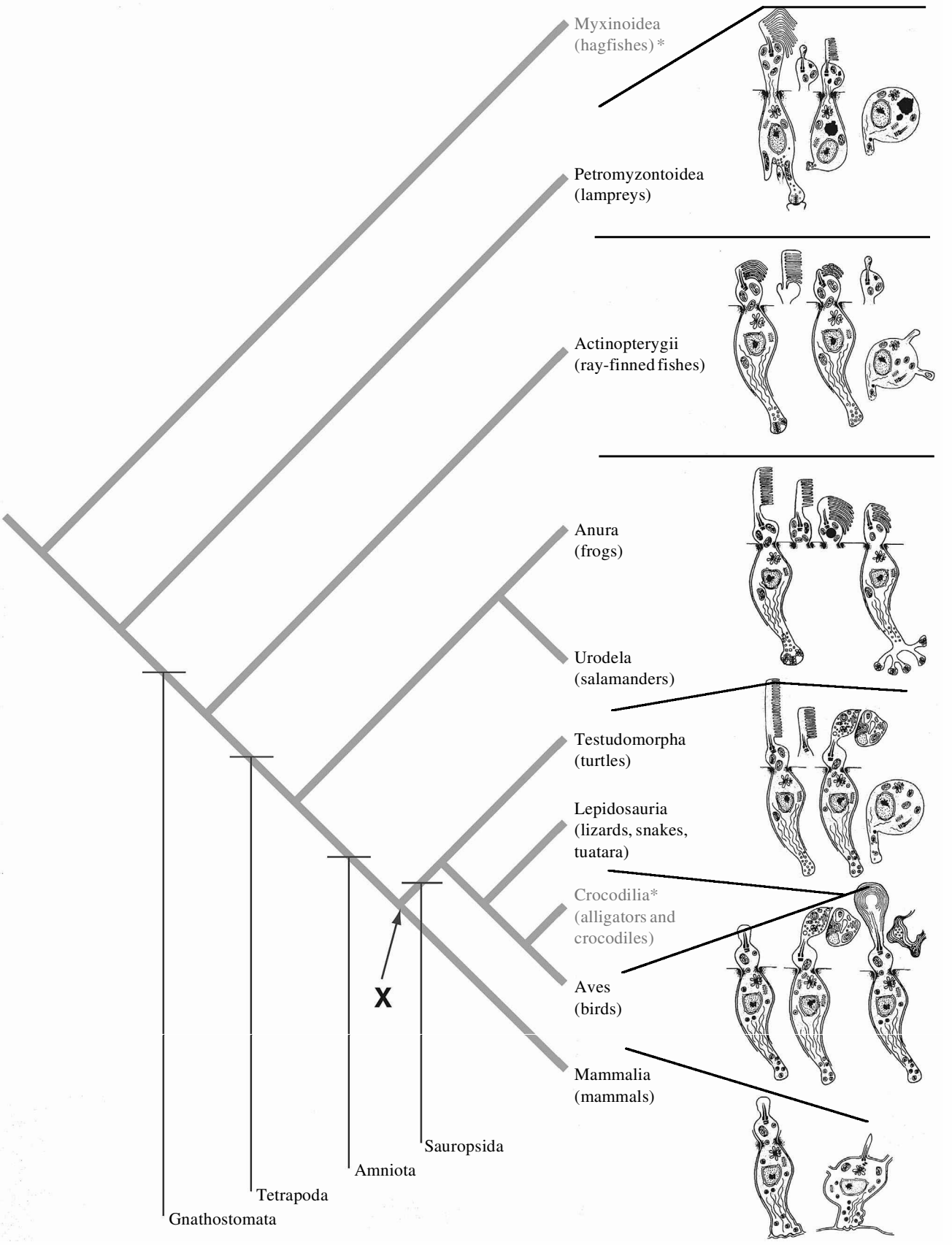

Figure 4. Cladogram showing the phylogenetic relationships, and the different types of pinealocytes (redrawn and adapted from Oksche (1983); cf. figures 6 and 7) in the vertebrate groups discussed in this paper. Node X indicates the divergence of sauropsids and mammals. An asterisk denotes that a pineal complex is not present in hagfishes and crocodiles.

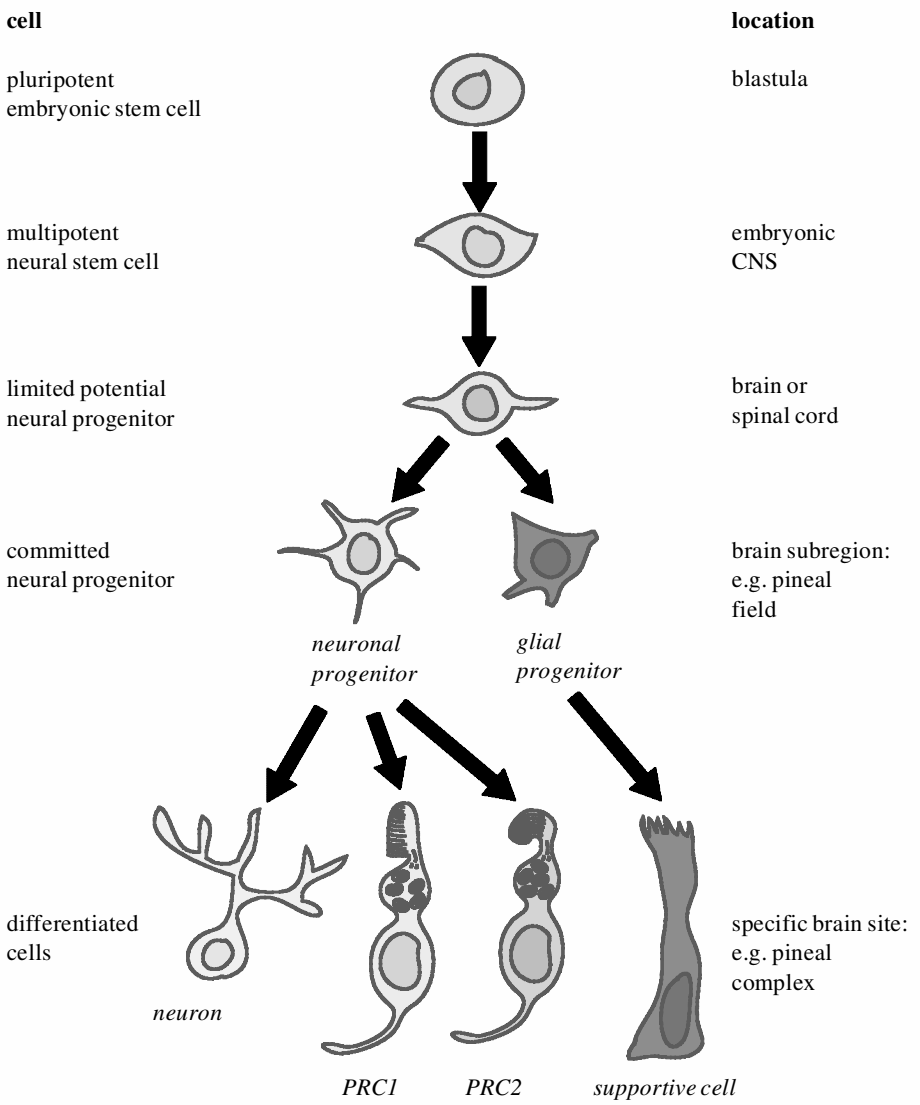

Figure 5. Proposed cell lineage in the developing photosensory pineal complex. The neuronal lineage comprises neurons and photoreceptor cells (PRCs), while the glial lineage comprises supportive cells (and a small number of oligodendrocytes; not shown). (Redrawn and adapted, with permission, from Gage (2000).)

Pombal *et al.* 1999; Yáñez *et al.* 1999) or the terminal enzyme in the melatonin biosynthetic pathway, hydroxyindole-*O*-methyltransferase (Voisin *et al.* 1988; Falcón *et al.* 1994). Taken together, the data indicate that active indoleamine synthesis can be indirectly shown by serotonin immunoreactivity in the pineal organ.

Direct physiological measurements have unequivocally shown that pineal photoreceptors use a variety of photopigments with different spectral sensitivity, or with other differences in their light response properties. The large majority of these studies were extracellular recordings from postsynaptic pineal neurons, and they thus give only an indirect assessment of the presence of different types of photoreceptors. Still, there is strong evidence of the presence of more than one photopigment in the pineal complex of several species (for reviews see Dodt 1973; Meissl & Dodt 1981). Single-cell electrophysiological recordings or single-cell microspectrophotometry have only rarely been performed.

Instead of direct physiological measurements of light responses, the expression of specific opsins or other phototransduction-related proteins may serve as a tentative identification characteristic, possibly implicating the spectral sensitivity of the photoreceptor cell. The identification of opsins in the pineal organ was long dependent on the use of antibodies against retinal opsins from mammals or birds. In the early studies, specificity problems in terms of epitope recognition strongly hampered conclusive analysis. Interpretation of pineal photoreceptor labelling is not straightforward as N-terminal as well as C-terminal epitopes may be shared by rod and cone opsins, and by different classes of cone opsins (Röhlich & Szél 1993).

Pineal photoreceptors have been detected using specific antibodies against retinal rod opsins/rhodopsins, red/green cone opsins and blue cone opsins (table 1), as well as with antibodies against pinopsin (P-opsin). It is notable that immunoreactivity for the 'pineal-specific' pinopsin is less pronounced in anamniotes than in reptiles and birds (Vígh *et al.* 1998). Rod opsin antibodies label pineal photoreceptors in all vertebrate classes examined except lacertilians. It is intriguing that photoreceptors in the lacertilian parietal eye have so far not been shown to be IR with any opsin antibodies, although Kawamura & Yokoyama (1997) have identified the mRNAs of three visual opsins

Table 1. Opsin antibodies used to detect photoreceptor cells and pinealocytes in the pineal complex.
(A, anuran amphibians; B, birds; C, lampreys (cyclostomes); F, teleost fishes; L, lizards; T, turtles; U, urodele amphibians. For details, see text.)

antibodies	specificity	positive in	references[a]
rod opsins/rhodopsins			
2235/nos17–45	rod opsin	CFATB	Papermaster (1982)
CERN-JS839	rod opsin	FB	Jansen *et al.* (1987)
CERN-858	rod opsin	F	Schalken (1987), Janssen (1991)
CERN-886	rod opsin	F	Schalken (1987), Janssen (1991)
CERN-922	rod opsin	C	Meléndez-Ferro *et al.* (2002)
1H9, 2F3	rhodopsin	B	Ishikawa *et al.* (1987)
AO	rhodopsin N-terminal (rods; blue and green cones in birds)	CFUAL	Röhlich & Szél (1993)
Rh-AS	toad rod/opsin (rods and green cones)	A	Okano *et al.* (2000)
cone opsins			
R2, R3	iodopsin	CB	Shichida *et al.* (1989)
CERN-874	chicken cone opsins	CF	Foster *et al.* (1993)
COS-1	red/green cone opsins C-terminal (red/green cones; rods in fishes)	CFUAL	Röhlich & Szél (1993)
OS-2	blue cone opsin C-terminal (green and blue cones; rods)	CFAL	Röhlich & Szél (1993)
pinopsins			
P7 and P9	P-opsin C-terminal	B	Okano *et al.* (1997)
3033	P-opsin C-terminal (QRTGKASPGTPGPH)	FALB	Fejér *et al.* (1997)

[a] Original description of the antiserum, or more extensive specificity tests.

and pinopsin in both the pineal organ and parietal eye in the American chameleon (green anole) *Anolis carolinensis*.

It should be pointed out that most immunocytochemical studies have been performed at the light microscopic level. In many cases, labelling patterns have not been analysed at the cellular level beyond identification of photoreceptor-like cells. However, differential distribution of different opsin-immunoreactivities within the pineal complex has been observed in several species. Such results indicate the presence of multiple populations of photoreceptor cells, expressing different opsins.

Similarly, the use of molecular biological methods have contributed to the identification of several 'new' types of opsins and other putative photopigments in the pineal complex, like parapinopsin (Blackshaw & Snyder 1997*a*), vertebrate ancient opsins (Soni *et al.* 1998; Moutsaki *et al.* 2000), exo-rhodopsin (Mano *et al.* 1999), extra-retinal rod-like opsin (Philp *et al.* 2000*a*) and cryptochromes (Kobayashi *et al.* 2000). Unfortunately, the *in situ* hybridization labelling patterns have not so far been correlated with morphologically identified pineal photoreceptor types. It should also be noted that for most of the opsins it has not been shown that the expressed opsin combines with a chromophore to form a functional photopigment.

(b) *Photoreceptor cells in the pineal complex of anamniotes*

The lamprey pineal complex consists of a pineal organ and a parapineal organ (figure 6). The pineal organ consists of a principal distal portion (end-vesicle), and a proximal atrium that is joined with the brain via a pineal stalk. The dorsal and ventral walls of the distal portion may be markedly different. The ventral wall is thicker and is called the retina, while the dorsal wall—the pellucida—is thinner and may show a lens-like appearance, although it does contain photoreceptors (Studnicka 1905).

At a conservative estimate, there are at least three types of photoreceptor cell in the pineal complex of lampreys (figure 6). In the pineal organ of *Lampetra planeri*, Vígh-Teichmann *et al.* (1989) distinguished three main types of photoreceptors on the basis of morphology and presence or absence of opsin immunoreactivity at the ultrastructural level. In the same species, Meiniel (1980) had previously described three structural variants of pineal cells with serotonin-containing dense bodies. The most common is a typical pineal photoreceptor with a short but fully differentiated cone-like outer segment, while a smaller number have reduced outer segments. The third type has spherical or oval cell bodies, and lacks inner and outer segments. This type is very similar to ophidian and mammalian pinealocytes. Indoleamine metabolism has been verified experimentally in the photoreceptor cells (Meiniel 1987), but not in the pinealocyte-like cell type. Observations of the distribution of different types of serotonin-IR cells in the pineal complex of lampreys (Pombal *et al.* 1999) largely corroborate the observations of Meiniel (1980, 1987).

In the pineal organ of *L. japonica*, almost all of the scattered serotonin (5-hydroxytryptamine; 5-HT)-IR photoreceptors in the dorsal pellucida and the ventral retina possessed rod-opsin-IR outer segments. By contrast, the large majority of photoreceptors with rod-opsin-IR outer segments in the end-vesicle were 5-HT-immunonegative. Iodopsin-IR outer segments were found mainly in the end-vesicle and most distal part of the atrium, with the largest density in the ventral retina. Some of these cells were also 5-HT-IR, indicating that there may be indoleamine-

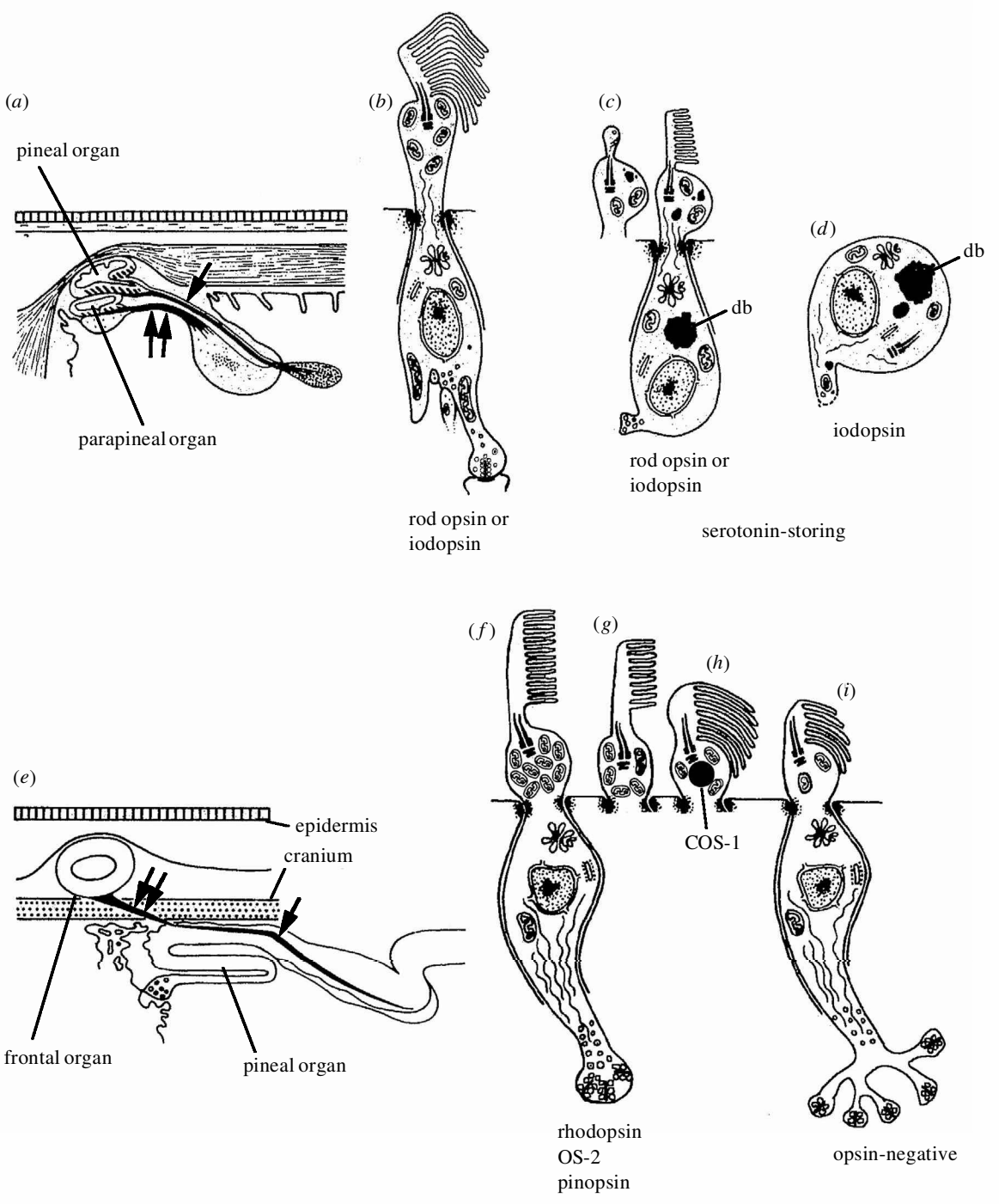

Figure 6. The pineal complex of anamniotes and its different photoreceptor/pinealocyte types. (*a–d*) Lamprey pineal complex, (*e–i*) frog pineal complex. (*a*) The pineal complex of lampreys comprises a pineal organ and a parapineal organ. Afferent fibres in the pineal tract (single arrow) and the parapineal tract (double arrow) convey sensory information to the brain. (*b*) Typical neural photoreceptors are IR for rod opsin or iodopsin. (*c*) Photoreceptors with serotonin-storing dense bodies (arrow) have small lamellar segments or bulbous sensory cilia, and are IR for rod opsin or iodopsin. (*d*) Pinealocyte-like cells, with no apparent sensory specialization, contain a dense body (arrow) and are iodopsin-IR. (*e*) The pineal complex of frogs comprises a pineal organ and a frontal organ. Afferent fibres in the pineal tract (arrow) and the pineal nerve (double arrow) convey sensory information to the brain. (*f*) Large 'rod-like' photoreceptors are strongly IR for rhodopsin, and less strongly IR for OS-2 and pinopsin. (*g*) Small 'rod-like' photoreceptors are strongly IR for OS-2, and less strongly IR for rhodopsin and pinopsin. (*h*) Large 'cone-like' photoreceptors are strongly IR for COS-1, and have an oil droplet (O) in the inner segment. (*i*) Small 'cone-like' photoreceptors are immunonegative for all opsin antibodies tested, and have a different configuration of the basal axon terminals. (Redrawn and adapted, with permission, from Oksche (1983).)

producing photoreceptors with different spectral sensitivities. Also in this species, large numbers of spherical or oval (pinealocyte-like) 5-HT-IR cells were observed in the atrium. Iodopsin-IR, but not rod-opsin-IR structures were found in the atrium (Tamotsu *et al.* 1990, 1994).

Thus, there is consistent evidence for the presence of

different types of photoreceptor cells that are differentially distributed in the pineal complex of lampreys. Taken together, there is evidence for three major morphological types of pineal photoreceptors with different degrees of outer segment elaboration. These may be IR for rhodopsin, iodopsin, or use other as yet undetermined opsin(s), and it is likely that many serotonin-expressing photoreceptors probably use opsin(s) other than rhodopsin and iodopsin (Samejima *et al.* 1989; Tamotsu *et al.* 1990, 1994; Yáñez *et al.* 1999).

Teleost fishes have not been examined for multiple types of pineal photoreceptors in such detail as lampreys and frogs, but available data indicate the presence of regional variations with regard to different types of photoreceptors and neurons (Falcón 1979; Falcón & Mocquard 1979; Ekström & Meissl 1997).

The pineal complex of frogs consists of an extracranial frontal organ and an intracranial pineal organ (figure 6). At least four different types of photoreceptor cells may be distinguished by their ultrastructural morphology and opsin expression in the pineal organ of frogs. The majority of photoreceptor cells in the pineal organ are of two types, named large and small 'rod-like' photoreceptors. Both have a large outer segment and a large ellipsoid filled with numerous mitochondria, and one large bulbous axon terminal containing numerous synaptic ribbons. The 'large rod-like' photoreceptors are strongly rhodopsin-IR and moderately OS-2-IR (table 1), whereas the 'small rod-like' cells are strongly OS-2-IR and moderately rhodopsin-IR (Vígh-Teichmann & Vígh 1990). Both types are weakly pinopsin-IR (Vígh *et al.* 1998).

The two other types were designed as 'cone-like'. They have smaller and tapering outer segments, ellipsoids containing smaller numbers of mitochondria, and are rhodopsin-immunonegative. The 'large cone-like' photoreceptors contain an oil droplet in the ellipsoid, and have a large bulbous axon terminal similar to those of the rod-like photoreceptors. Their outer segments are IR for COS-1 (table 1), but immunonegative for pinopsin. The 'small cone-like' photoreceptors have smaller outer segments, lack oil droplets and possess branching basket-like axon terminals. They are immunonegative for all tested opsin antibodies (Vígh-Teichmann & Vígh 1990).

'Rod-like' and 'cone-like' photoreceptors have also been identified in the frontal organ. In the frontal organ 'cone-like' rhodopsin-immunonegative photoreceptors predominate (Vígh & Vígh-Teichmann 1986).

While most pineal photoreceptors form local synaptic connections with intrapineal neurons, there is yet another type of pineal photoreceptor that emits long axons along the pineal stalk to the brain. In lampreys they appear to be fairly randomly distributed in the pineal organ, possibly with a predominance in the distal atrium (Samejima *et al.* 1989; Pombal *et al.* 1999). One central target for the long photoreceptor axons appears to be the optic tectum (Pombal *et al.* 1999); if there are others it is presently not known. Centrally projecting photoreceptors have also been demonstrated in the pineal organ of teleosts (Ekström 1987), and in the pineal organ of frogs (Ekström & Meissl 1990*a*).

The evidence for multiple types of photoreceptors in the pineal organ of anamniotes is supported by electrophysiological recordings from pineal photoreceptors and neurons demonstrating the presence of more than one photopigment in several species (Meissl & Dodt 1981; Ekström & Meissl 1997).

(c) *Photoreceptor cells in the pineal complex of sauropsids*

Sauropsids comprise reptiles and birds. Among reptiles, lizards have been studied in the greatest detail with regard to photoreceptor subtypes, but there are corroborative observations from turtles and snakes. Our current knowledge of pineal photoreceptor types in birds largely derives from studies of domestic chicken and quail.

The pineal complex of many, but not all (Gundy & Wurst 1976), lacertilians consists of an intracranial pineal organ and an extracranial parietal eye, the latter being considered a parapineal organ homologue. The lizard pineal complex contains multiple types of photoreceptor cells, both typical neural and typical neuroendocrine photoreceptors (figure 7*a–f*).

Ultrastructural studies have consistently shown that the pineal complex of lizards contains two basic types of photoreceptor cells, the 'typical' neural photoreceptor cell and the 'secretory rudimentary photoreceptor', i.e. neuroendocrine photoreceptors (Collin 1969; see above). The neural and neuroendocrine photoreceptors are differentially distributed in the pineal complex. The pineal organ contains mainly neuroendocrine photoreceptors, while smaller numbers of neural photoreceptors may be found (Collin 1968*b*; Oksche & Kirschstein 1968) in restricted locations (Ohshima *et al.* 1999). The parietal eye contains almost exclusively neural photoreceptors (Eakin & Westfall 1960; Oksche & Kirschstein 1968; Jenison & Nolte 1979). However, the parietal eye does synthesize melatonin in at least some lacertilians (Firth & Kennaway 1987; Tosini & Menaker 1998), and 5-HT-IR photoreceptors and photoreceptors containing numerous dense-core vesicles have been observed in the parietal eye of one lizard species (Ohshima *et al.* 1999).

Differential opsin expression indicates that there are different types of photoreceptors in the pineal complex. In three *Lacerta* species, and *Phelsuma laticauda*, photoreceptors with small outer segments were IR with the cone-specific anti-opsins OS-2 and COS-1 (table 1). Photoreceptors with large pear-shaped outer segments were pinopsin-IR. By contrast, the photoreceptors of the parietal eye were immunonegative with these antisera, as well as with rhodopsin antisera (Vígh *et al.* 1998).

It is notable that all developing photoreceptors in the pineal organ share morphological characteristics until late embryonic development, when the two types of photoreceptors begin to differentiate (Meiniel 1976*a*). Indoleamine metabolism is associated exclusively with the neuroendocrine photoreceptor type, both during development and in the adult pineal (Collin & Meiniel 1973*a*; Meiniel 1976*b*; Ohshima *et al.* 1999).

In adult turtles and snakes, the pineal complex consists solely of a pineal organ. Only a few species have been investigated with regard to the presence of different cell types. In turtles neuroendocrine photoreceptors are clearly the dominant type, but a small number of neural photoreceptors were located in the posterior wall of the pineal organ in *Pseudemys* (Vivien-Roels 1970). Adult snake pinealocytes share structural characteristics with mam-

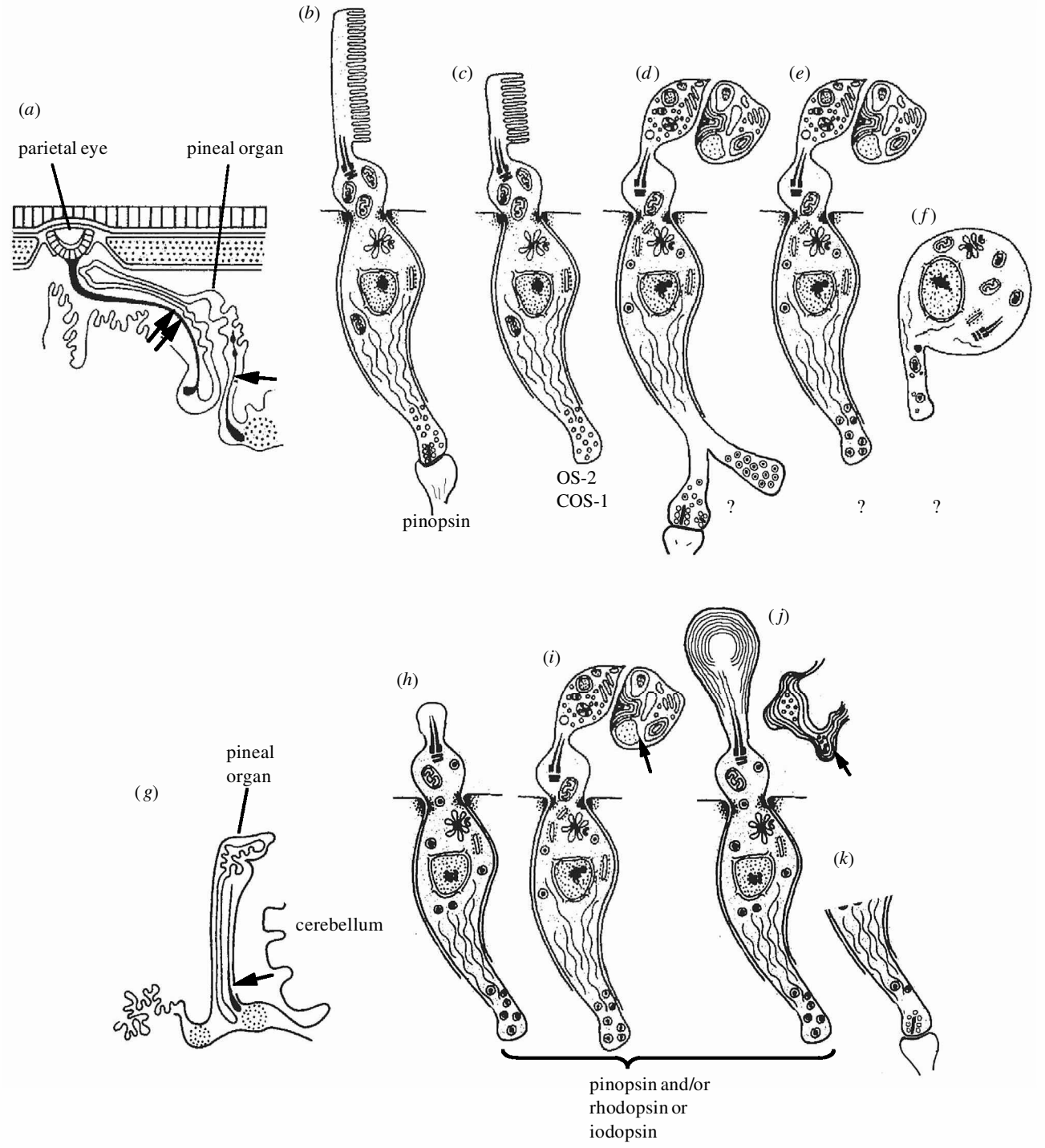

Figure 7. The pineal complex of sauropsidians (excluding crocodiles) and its different types of photoreceptor/pinealocytes. (*a–f*) Reptiles, (*g–k*) birds. (*a*) The pineal complex of lizards comprises a pineal organ and a parietal eye (parietal organ). Afferent fibres in the pineal tract (arrow) and the parietal nerve (double arrow) convey sensory information to the brain. (*b*,*c*) Photoreceptors in the lizard pineal organ with different outer segment and axonal morphologies are IR for pinopsin or OS-2 and COS-1. Parietal eye photoreceptors are immunonegative with all opsin antibodies tested. (*d*,*e*) The dominating type of photoreceptor in the pineal organ of lizards and turtles, characterized by modified outer segments and dense-core vesicles indicative of indoleamines, has not been identified with respect to opsin immunoreactivity. (*f*) Pinealocyte-like cells, constituting the main cell type of the neuronal lineage in the ophidian pineal organ, have not been identified with respect to opsin immunoreactivity. (*g*) The pineal complex of birds comprises only a pineal organ. A pineal tract (arrow) is present in some species, but it has not been experimentally established whether it conveys afferent sensory signals to the brain. (*h*) Photoreceptor with sensory cilium, that may be modified as a bulbous cilium. (*i*) The sensory cilium may be modified into an irregular outer segment-like structure, or (*j*) into a pear-shaped outer segment with concentric lamellae. Aggregations of lamellar and tubular whorls (arrows) often occur adjacent to sensory cilia. (*k*) In species with a relatively well-developed pineal neuronal circuitry, photoreceptors with basal synaptic specializations may be found. (Redrawn and adapted, with permission, from Oksche (1983).)

malian non-sensory pinealocytes (Vivien 1964; Petit 1971), and direct photosensitivity has so far not been demonstrated in the pineal organ of snakes. Still, there is some evidence for multiple types of pinealocytes: cells with a morphology similar to neuroendocrine photoreceptors were demonstrated in the pineal organ of *Bungarus caeruleus* (Naz *et al.* 1999).

Also in adult birds, the pineal complex has only one component, the pineal organ. The pineal parenchyma may be saccular, organized in a system of tubules and follicles, or have a compact non-follicular appearance. In some species, one organization is maintained throughout the pineal organ, whereas in others combinations are found (Quay 1965).

There are different morphological types of avian pineal photoreceptors (figure 7). They are usually referred to as pinealocytes in the literature, but they are directly photosensory (Binkley *et al.* 1978; Deguchi 1981; Okano *et al.* 1994; Max *et al.* 1995). We will adhere to common practice and use the designation pinealocyte when referring to birds. By far the most abundant type of pinealocyte appears to be of more or less modified neuroendocrine photoreceptor type. It has an inner segment-like structure with a cilium that may expand into more or less irregular bulbous structures. It also has a non-synaptic basal neurite and an abundance of indoleamine-storing dense-core vesicles: primarily in the basal process. This type has been found in the pineal organs of most avian species (Oksche & Vaupel-von Harnack 1966; Quay *et al.* 1968*b*; Collin 1969; Oksche & Kirschstein 1969; Oksche *et al.* 1969; Collin *et al.* 1982). In addition, pinealocytes with irregular lamellar structures resembling photoreceptor outer segments, and/or basal neurites containing synaptic ribbons adjacent to neuronal dendrites have been observed in some species (Quay *et al.* 1968*b*; Bischoff 1969; Ueck 1970).

Another morphological distinction is made between follicular and parafollicular cells. Follicular cells line the follicular lumina, while parafollicular cells lie basal to the follicular cells (Ohshima & Matsuo 1988). Follicular cells are clearly of the neuroendocrine photoreceptor type, whereas parafollicular cells are often irregular and multipolar, without apparent photoreceptor morphology (Goto *et al.* 1989). For this reason, and because of their lack of direct contact with the pineal lumen, they have been referred to as pinealocytes in contradistinction to the other (photoreceptor) cell types (Oksche & Vaupel-von Harnack 1966; Ohshima & Matsuo 1988). However, it has recently been shown that parafollicular cells may possess pinopsin-IR bulbous cilia or concentric lamellar structures (Hirunagi *et al.* 1997).

The large majority of avian pinealocytes express pinopsin. In fact, photoreceptors with all known types of outer segment-like configurations are pinopsin-IR (Hirunagi *et al.* 1997). In addition, follicular cells may also express rhodopsin- and iodopsin-like opsins (Araki *et al.* 1992). These opsins have not yet been demonstrated in parafollicular cells. It is at present not clear whether rhodopsin- and iodopsin-like opsins are expressed by the small subset (*ca.* 10%) of the follicular photoreceptors that do not express pinopsin, or if either may co-localize with pinopsin. In any case, rhodopsin- and iodopsin-IR photoreceptors occur in much smaller numbers than pinopsin-IR ones (Yamao *et al.* 1999).

Although follicular and parafollicular cells have clearly distinct morphologies, they share some cytochemical and cytological characteristics. Both follicular and parafollicular cells are involved in indoleamine synthesis (Ohshima & Matsuo 1991), and both types express immunoreactivity for visinin, a protein specific for retinal cones in non-mammals (Goto *et al.* 1989). Pinopsin-IR follicular and parafollicular cells also express the α-subunit of pineal transducin, which is identical to retinal rod-specific α-subunit of transducin (Kasahara *et al.* 2000; Matsushita *et al.* 2000).

9. EVIDENCE FOR SENSORY REGRESSION DURING ONTOGENETIC DEVELOPMENT

The evidence for a gradual regression of photosensory cells during ontogenetic development in amniotes is mainly based on observations of a few species of lizards, birds and mammals. The studies have concentrated on the development of pineal photoreceptors and pinealocytes. Among the studies of non-mammals, only the investigations of the lizard *Lacerta vivipara* cover a continuum of characterized developmental stages *and* fully mature animals.

In a series of carefully conducted studies of pineal development in the lizard *La. vivipara*, Meiniel and co-workers followed the differentiation of pineal photoreceptor types. Between developmental stages 35 and 37, differentiation of the basal (axonal) protrusions reflect development of two photoreceptor types: 'typical' neural mode photoreceptors with synaptic specializations and 'rudimentary secretory' neuroendocrine mode photoreceptors with dense-core vesicles. Both types are endowed with a distinct inner segment, which carries a sensory cilium. Through stages 38 and 40 (immediately before birth), sensory cilia with lamellar structures that increase in size with age are observed on both types. The lamellae are still small and each cilium carries a small number of lamellae: the maximum number observed at stage 40 was 32 lamellae on one cilium. Three days after birth the typical photoreceptors retain lamellar outer segments, while the photoreceptors with numerous dense-core vesicles and without synaptic specializations have highly irregular non-lamellar outer segments (Meiniel 1976*a*). Only the latter photoreceptor type accumulated exogenous ^{3}H-5-hydroxytryptophan, indicating that they are capable of indoleamine synthesis (Meiniel 1976*b*). These data indicate a regression of the outer segment in the indoleamine-synthesizing photoreceptors during ontogeny.

What do these observations imply? First, that neural and neuroendocrine pineal photoreceptors differentiate early and acquire recognizable phenotypes before the sensory cilium differentiates outer segment-like morphology. Second, that while the sensory cilium of the neural photoreceptor pursues the 'canonical' path of outer segment development (cf. Eakin & Westfall 1961), the neuroendocrine photoreceptor fails to form regular lamellae. Some apparently start to form lamellae but those cells disappear soon after birth, which has been interpreted as a regression.

The failure by neuroendocrine photoreceptors to form

a structurally well-organized outer segment may be owing to the lack of an appropriate signal for assembly of outer segment lamellae, or even an active inhibition. Even if there were a 'default developmental pathway' that would lead to the elaboration of sensory cilia outer segments, this pathway is obviously not sustained by the (intra- or extra-) cellular environment. Instead, development follows another pathway that leads to the neuroendocrine photoreceptor morphology. It is noteworthy that the cells have attained neural or neuroendocrine phenotypes already before 'regression' of the outer segment commences in the neuroendocrine photoreceptors.

The evidence for a sensory regression, i.e. a loss of outer segment-like structures, during avian ontogenetic development is weak and inconsistent. In domestic ducks, Collin (1966) observed that in 26-day-old embryos there appears to be only one type of pinealocyte, endowed with inner segment-like structures and bulbous cilia, whereas pinealocytes with this morphology were rarely observed in adult ducks. However, Oksche & Vaupel-von Harnack (1966) and Oksche (1968) noted that irregular lamellar complexes, associated with ciliary protrusions from the apical pole of the pinealocytes, were more often observed in adult ducks than in embryos. In chickens, the distinct pineal follicles are progressively lost with age, as the pineal parenchyma becomes more solid. This has been interpreted as evidence that the avian pineal is transformed from a pineal organ to a 'pineal gland' during ontogeny (Sato 2001), a notion which implies loss of photoreceptor characteristics. However, according to Omura (1977) photoreceptor-like structures gradually become more abundant (or easier to detect) with increasing age in the pineal organ of the brown leghorn chicken. In the Japanese quail, there is an initial decrease in numbers of paraboloids and regular stacks of lamellae after hatching, accompanied by an increase in the numbers of synaptic ribbons, but during later development more developed lamellar whorls appear in the follicular lumen (Ohshima & Hiramatsu 1993). Bischoff (1969) observed pinealocytes with lamellar whorls extending from their cilia in adult chickens and quails. It should be kept in mind that these data stem from studies of domestic or laboratory-reared species, and differences in observations may be due to the use of different strains.

Mammalian pineal organs are not directly photosensory, as far as we know. Still, pinealocytes with a morphology more or less reminiscent of sauropsid photoreceptors (especially avian pinealocytes) have been observed in a few mammalian species during development (Clabough 1973; Zimmerman & Tso 1975; Pévet 1980; Vígh & Vígh-Teichmann 1993), and in adults (Pévet & Collin 1976; Pévet *et al.* 1977). However, there is no evidence that individual pinealocytes undergo regression of their apical pole.

10. IF NOT ONTOGENETIC SENSORY REGRESSION, THEN WHAT?

If ontogeny should reflect phylogeny in terms of pinealocyte development, it would be reasonable to assume that all members of a given clade should show a similar developmental sequence. Photoreceptors with outer segment-like structures would be observed at early stages, and disappear at later stages, regardless of the mechanism behind this change! As reviewed above, there is little support for such a generality.

In our view, an alternative interpretation of the data should be considered: an interpretation that is more in line with the probable mode of generation of a neural lineage in pineal organs. The parenchymal cells of the pineal organ are derived from multipotent neural stem cells that gradually become more and more restricted in their developmental potential (figure 5). Neural progenitors give rise to a sequence of different types of photoreceptors and neurons. As development proceeds, the possible fate of the offspring of the neural progenitors is gradually restricted, leading to an increasing bias towards the generation of specific cell type(s). The cellular environment influences fate restriction, and as development proceeds, the cellular environment changes too.

Early pineal cells (pinealoblasts, in analogy with retinoblasts in the developing retina) are multipotent. Cells from developing pineal organs of embryonic quails may differentiate *in vitro* into pinealocytes and neurons (Araki *et al.* 1992, 1993), but also into pigment cells, lens cells and muscle cells (Watanabe *et al.* 1985, 1988, 1992), depending on time in culture and culture conditions. In particular, culture in the presence of retinal diffusible factors enhances the differentiation of iodopsin-IR, but not rhodopsin-IR pinealocytes (Araki 1997), indicating the importance of the cellular environment.

Similarly, cells from neonatal rat pineal organs differentiate *in vitro* into neurons, pinealocytes, photoreceptor-like cells and muscle cells, depending on the culture conditions. When cultured in the absence of norepinephrine, cells from neonatal rat pineal organs are induced to express rhodopsin immunoreactivity (Araki 1992, 2001), and respond to light by a decrease in norepinephrine-induced melatonin synthesis (Tosini *et al.* 2000). Cultured in the presence of norepinephrine, rhodopsin expression is suppressed and the pineal cells do not become photosensory. These results have been interpreted as evidence that the developing sympathetic noradrenergic input suppresses development of photosensitivity (Tosini *et al.* 2000), and may be compared with observations made in developing chickens and quails. In these species, a well-developed system of intrapineal AChE-positive neurons appears in the embryonic pineal organ, but gradually disappears during post-hatching development (Sato & Wake 1984). In the adult avian pineal the distribution of AChE-positive fibres is largely complementary to that of adrenergic sympathetic fibres (Sato & Wake 1983), and it appears that regression of the AChE-positive neuronal network mirrors the invasion of adrenergic fibres during development (Sato & Wake 1984). These results strongly indicate that the cellular environment in the developing pineal organ influences fate restriction at the level of cytodifferentiation.

Taken together, these observations suggest that cells of the developing pineal organ are generally competent to form photosensory cells, also in mammals. Developing mammalian pinealocytes apparently pass through a temporal window, in which they may express certain photoreceptor characteristics. The mRNAs for several principal components of the phototransduction cascade are expressed in the pineal organ of the neonatal rat

(Blackshaw & Snyder 1997*b*), but the expression levels decline with age. The competence to differentiate photosensory cells is also obvious during the temporal window when lacertilian neuroendocrine photoreceptors first start developing outer segment lamellae, but then subsequent development restricts this capacity in some cells: the photoneuroendocrine photoreceptors.

The existence of developmental temporal windows when particular photoreceptor characters are expressed (or may be induced) is not a strong argument for pineal cytodifferentiation through regression in sauropsids. In our opinion it does not imply that avian and mammalian pinealocytes have *evolved* through regression. Even if, as a general rule, they would develop ontogenetically through regression this could hardly be interpreted as reflecting evolutionary steps through rudimentary outer segments like those of sauropsidian photoreceptors. As figure 4 shows, the lacertilian and avian radiations have diverged far from the stem reptiles. Thus, the ontogenetic regression of lacertilian neuroendocrine photoreceptors is not a valid argument for evolutionary regression of mammalian pinealocytes (Collin 1977).

The concept of a gradual regression of pinealocytes during phylogeny assumes that there is an original restriction within the neural lineage in the pineal organ: a restriction dictating that there can only be a specific number of cell lines: one 'receptor cell line' (Collin 1971; Collin & Oksche 1981) or two 'sensory cell lines' (Meiniel 1981). This restriction would have been established when the first vertebrates emerged, and the first photosensory pineal cell type(s) differentiated. As a consequence, all evolution would have to take place within these cell lines and all 'intermediate cell types' observed in any pineal organ would either be at different developmental stages or represent different levels of evolution/regression. In our opinion, it is more probable that evolution has occurred in the regulation of fate restriction of the cells in the part of the embryonic CNS that will give rise to the pineal complex. In other words, it is the regulatory linkage mechanisms of fate restriction that have evolved in the pineal. Thus, one may question the notion of specific cell lines, and rather see the different types of pineal photoreceptors and pinealocytes as the result of differential combinations of regulatory mechanisms in different vertebrates. All photoreceptors, pinealocytes and neurons are of a neuronal lineage, but have evolved differentially according to evolutionary adaptations of the different vertebrate taxa.

11. NEURAL LINEAGE IN THE PINEAL COMPLEX AND IN THE RETINA

Summarizing so far, there are several distinct cell types and classes of the neuronal lineage in the pineal organ. All belong to the basic classes of photoreceptors and neurons (cf. figure 5). Comparing the major vertebrate groups, it is evident that even though many types of photoreceptors (and neurons) are present in all groups, each group has a bias towards predominant cell types. This variety and bias probably reflect that the embryonic pineal anlage has a general competence to develop a photosensory structure, but that the details of the regulation of this development have changed during evolution and are not identical throughout the vertebrate radiation.

It is not unreasonable to assume that the basic photosensory competence of the pineal anlage is similarly specified and regulated as that of the embryonic eye field in the neural plate. The eye field gives rise to the neural retina, the retinal pigment epithelium, the optic nerve and chiasma, and the chiasmatic region (including the suprachiasmatic nucleus). So, how are photoreceptors, neurons and glia generated in the vertebrate retina?

In the retina, progressive fate restriction of the progenitor cells determines final cellular phenotype. Cell division occurs at the retinal periphery, in the ciliary marginal zone. Most peripherally, the stem cells are located. Stem cells divide to form retinoblasts, which divide and give rise to progenitor cells. Subsequent divisions of the progenitor cells generate the different neuronal and glial cells in a specific sequence of formation. Although several details concerning the control of this sequence remain unknown, the sequence appears to be conserved among vertebrates in its basic pattern. Ganglion cells are generated first, followed by horizontal cells and cone photoreceptors, then amacrine cells, rod photoreceptors, bipolar cells and finally Müller glia. The generation/differentiation periods for the different cell types overlap so that more than one cell type can be generated at any time. Intriguingly, in zebrafish and goldfish rod opsin mRNA is detectable before cone opsin mRNAs, and there is an overlapping sequence of appearance of the different cone opsins (for reviews see Cook & Chalupa 2000; Marquardt & Gruss 2002).

This developmental sequence is under the control of sequential activation and inactivation of transcription factors, and the influence of the external milieu: for example, growth factors that may regulate transcription factor expression (Harris 1997; Harris & Perron 1998; Marquardt & Gruss 2002). Changes in this regulatory network during evolution would lead to changes in the development of specific adult cell morphologies and functions, in the retina as well as in the pineal organ.

12. PHOTORECEPTOR DIFFERENTIATION DURING PINEAL DEVELOPMENT

The photoreceptor cells, neurons and glial elements of the photosensory pineal organ are generated from neural stem cells and progenitors, as elsewhere in the CNS. Is there any evidence for the presence of committed progenitor cells that each generate a limited number of photoreceptor, neuronal or glial cell classes? Or, is there any evidence for the generation of different cell classes through progressive fate restriction of the progenitor cells?

A fate map for the neural plate has been made for only a small number of vertebrates (for review see Rubenstein *et al.* 1998). However, in all species the eye field has an anteromedian position, whereas the 'pineal field' is located somewhat more caudally at the lateral margins of the neural plate (figure 8). During closure of the neural tube, the eye field will end up in the most anteroventral part of the neural tube, whereas the pineal fields will unite and form a pineal anlage in the dorsal diencephalon.

The specification of the pineal field and its subsequent development have been studied in some detail in the zebrafish embryo (Masai *et al.* 1997). The gene *floating head* (*flh*) specifies the pineal field. The expression of *flh* is

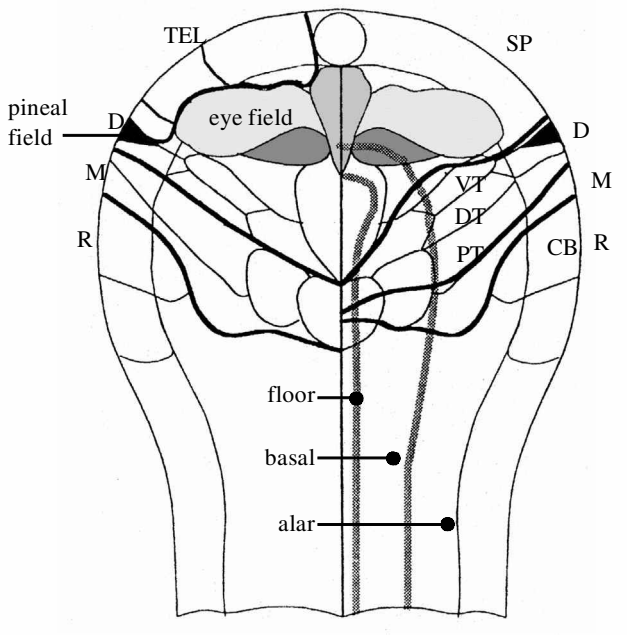

Figure 8. Locations of the eye field and pineal field in stage 15 *Xenopus* embryos. Dark grey areas, chiasma; mid-grey areas, supra chiasmatic nucleus; light grey areas, eye; black areas, pineal. Abbreviations: CB, cerebellum; D, diencephalon; DT, dorsal thalamus; M, mesencephalon; PT, pretectum; R, rhombencephalon; SP, secondary prosencephalon; TEL, telencephalon; VT, ventral thalamus. (Redrawn and adapted, with permission, from Rubenstein *et al.* (1998).)

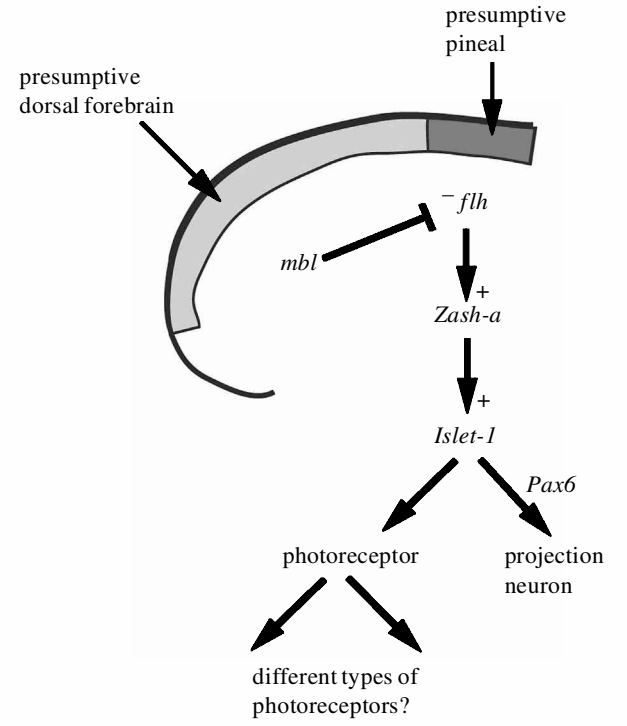

Figure 9. Pineal development in zebrafish depends on the expression of *floating head* (*flh*), which is restricted to the pineal field by the repressive action of *masterblind* (*mbl*). *Zash-1*, zebrafish achaete-scute homologue. (Redrawn and adapted, with permission, from Masai *et al.* (1997).)

restricted by *masterblind* (*mbl*), a critical gene in forebrain development (figure 9). In *mbl* null mutants the telencephalon and the optic vesicles are absent or drastically reduced in size, Flh expression expands over the entire dorsal forebrain, and a very large pineal organ is formed (Heisenberg *et al.* 1996; Masai *et al.* 1997). The limitation of *flh* expression to the pineal field appears to be mediated by Mbl inhibition of Wnt (vertebrate homologue of *Drosophila* Wingless) signalling pathways (Heisenberg *et al.* 2001).

Flh is required for neurogenesis to proceed in the pineal region, and it induces the expression of *Zash-1a*, a zebrafish homologue of the *Drosophila* proneural gene *achaete-scute*. Zash-1a is only transiently expressed during the time it induces the expression of *islet-1* (*isl-1*), which specifies the neuronal lineage in the pineal. Differentiation of the neuronal lineage then commences: projection neurons express Pax6 and Isl-1, whereas photoreceptors express Isl-1. Thus, representatives of the two main cell classes of the neuronal lineage, neurons and photoreceptors, first appear at approximately the same developmental stage (prim20) (Masai *et al.* 1997). Also in other teleost embryos the first photoreceptors differentiate at the same time as the first pineal neurons (Ekström *et al.* 1983; Östholm *et al.* 1988; Forsell *et al.* 2001, 2002).

The studies of zebrafish indicate that pineal neurons and photoreceptor cells are of a common lineage, but it has not yet been determined whether there is one type of neuronal progenitor (cf. figure 5) that gives rise to all types of photoreceptor cells and neurons, or if distinct sets of neuronal and photoreceptor progenitor cells are generated. The first pineal neurons and photoreceptors appear together within a narrow time-span, but when do different types of photoreceptors differentiate?

When more than one type of opsin is present in the developing pineal organ of teleost fishes, they appear sequentially. In herring, photoreceptors IR for rod opsin (antibody CERN-886; see table 1 for antibody specifications) and short wavelength (SW)-cone/rod opsin (OS-2) appear first, followed by cone opsin (CERN-874) (Forsell 2000). There may also be a progressive bias, with increasing age, towards the formation of photoreceptors expressing specific opsins. In the halibut embryo, transcripts of halibut pineal organ 1 (HPO1) (green-like opsin) and HPO4 (ultraviolet (UV)-like opsin), as well as immunoreactivity for SW cone/rod opsins become detectable at the same developmental stage. However, during subsequent development putative UV-opsin expressing cells become selectively expressed in the pineal stalk region, while green-opsin ones are ubiquitously distributed (Forsell *et al.* 2002). This suggests that putative UV-opsin expressing photoreceptors are generated only during early development, while green-opsin photoreceptors are generated over a longer period and form the bulk of the distal end-vesicle.

In zebrafish, it has not yet been determined whether different types of photoreceptor cells express the two opsins identified in the zebrafish pineal, and if they appear sequentially. Red cone opsin is first expressed at 48 h post-fertilization (Robinson *et al.* 1995), whereas exo-rhodopsin (Mano *et al.* 1999) has so far been studied only in adults.

There are few data on the differentiation of photorecep-

tor and neuronal types in lampreys, amphibians or reptiles. However, in birds a sequential appearance of pinealocyte types suggests that there is a progressive fate restriction during pineal development. In the developing pineal organ of the chicken, follicular pinealocytes and supportive cells differentiate before parafollicular cells (Oshima & Matsuo 1988). Also in chickens, pinopsin-IR pinealocytes appear 5 days before rhodopsin- and iodopsin-IR ones (Yamao *et al.* 1999). In the embryonic quail, there is a time-lag of 2 days between the appearance of rhodopsin-IR and iodopsin-IR pinealocytes (Araki *et al.* 1992). Moreover, *in vitro* studies have shown that the pineal organ of the embryonic quail contains multipotent progenitor cells that may generate different cell types under different culture conditions (Araki *et al.* 1992; Araki 2001). Norepinephrine significantly suppresses differentiation of rhodopsin-IR photoreceptors, and the efficiency of this suppression decreases with age (Araki *et al.* 1992). Growth factors differentially promote the differentiation of pineal neurons or rhodopsin-IR photoreceptors, and it appears the expression of pinopsin, rhodopsin and iodopsin is differentially regulated (Araki 2001).

The sequential appearance of different opsins in the developing pineal organ could indicate that there is a progressive fate restriction with respect to photoreceptor cell types, but it does not discriminate whether different photoreceptor types are sequentially generated by common progenitors, or different committed progenitors are activated at different developmental stages. The observations need to be further refined by birth-dating the photoreceptors expressing different opsins, or by direct tracing of the cell lineages.

13. WHAT CONFERS PHOTOSENSITIVITY TO THE DEVELOPING PINEAL COMPLEX AND RETINA?

In the early neural plate, a number of key controller genes are expressed in the anterior median eye field: *six3*/*6*, *pax6* and *rx* genes. These genes appear to act together in a network of eye-determination genes, which interacts with rostral-patterning genes like *emx* and *otx*. The *six3*/*6*, *pax6* and *rx* genes regulate the expression and function of other genes required for proper retinal development, and in some species some of them (and/or other members of the same gene families) are expressed also in the pineal field or early pineal anlage (*six3* (Ghanbari *et al.* 2001), *pax6* (Masai *et al.* 1997; Kawakami *et al.* 1997) and *Xrx1* (Casarosa *et al.* 1997)).

It is striking that there are marked differences between species with regard to the 'hierarchy' or regulatory linkage of these eye-determination and rostral-patterning genes. For example, in zebrafish and *Xenopus rx* may act downstream of *pax6* and *six3*/*6*, whereas in mice *rx* may act upstream. In *Xenopus*, the expression domain of *Xrx1* is complementary to that of *otx2*, whereas in zebrafish the *rx* expression domain is initially contained within the *otx2* domain, and downregulation of *otx2* takes place later within the *rx* expression domain (for discussion see Chuang & Raymond 2001).

There are also differences with regard to the expression of members of these gene families in the retina versus the pineal organ. In *Xenopus*, *Xrx1* is expressed both in the developing pineal organ and retina, whereas in zebrafish *rx1*/*2* gene expression is confined to the developing eye (Casarosa *et al.* 1997; Chuang *et al.* 1999). In chickens, *pax6* is strongly expressed in the developing retina but only weakly in the pineal organ, whereas *pax7* is strongly expressed in the pineal but not at all in the retina (Kawakami *et al.* 1997).

The genes that regulate the expression of genes necessary for photoreceptor differentiation in the developing zebrafish pineal organ include *flh*, *otx5* and *crx*. The orthodenticle homeobox gene *otx5* and the cone–rod homeobox gene *crx* are expressed in the pineal organ and retina of all vertebrates examined so far (e.g. Casarosa *et al.* 1997; Chen *et al.* 1997; Vignali *et al.* 2000; Liu *et al.* 2001; Sauka-Spengler *et al.* 2001; Gamse *et al.* 2002). In the zebrafish, *otx5* is selectively transcribed in the pineal organ and retina, and regulates genes that show circadian expression like *aanat2* (arylalkylamine-*N*-acetyltransferase 2: 'serotonin *N*-acetyltransferase'; the rate-limiting enzyme of melatonin biosynthesis), *reverbα* (an orphan nuclear receptor) and *irbp* (interphotoreceptor retinoid-binding protein) (Gamse *et al.* 2002).

Similar to *otx5*, zebrafish *crx* is expressed specifically in the retina and pineal complex, where it appears to be restricted to photoreceptor cells. Zebrafish Crx has a weak, but significant, transactivating effect on the bovine rhodopsin proximal promoter region (Liu *et al.* 2001). Crx transactivates photoreceptor-specific and pineal-specific genes and is essential for normal development of retinal photoreceptors in mammals (Chen *et al.* 1997; Furukawa *et al.* 1997).

It is possible that the functions of *flh*, *otx5* and *crx* are complementary with regard to different photoreceptor functions, but the interactions between *otx5* and *crx* genes, on the one hand, and *pax*, *six* and *rx* genes on the other, are unfortunately not known. Flh is expressed well in advance of Pax6, and in the absence of Flh neurons and photoreceptors differentiate only in severely reduced numbers (Masai *et al.* 1997). Otx5 downregulation does not affect *flh* expression (Gamse *et al.* 2002), consistent with the role of *flh* as an early determinator of pineal neural lineage (Masai *et al.* 1997).

It is worth noting that teleost fishes may possess pineal-specific rod-like opsins, exo-rhodopsins (Mano *et al.* 1999; Philp *et al.* 2000*a*) alongside the retinal rod (rhod) opsins. The zebrafish retinal rod opsin promoter contains conserved *cis*-elements that act as binding sites for transcription factors like Crx and Rx. However, the promoter is either not identical to that of the pineal exo-rhodopsin, or is identical but critical transcription factors are not present in the pineal organ, because the promoter drives enhanced green fluorescent protein in retinal rods only (Kennedy *et al.* 2001). Either way, the expression pattern of the transgenic rod opsin promoter supports our notion that changes in regulatory linkage—here the interactions between transcription factors and *cis*-elements—has led to the evolution of different photoreceptor types: retinal rods and pineal photoreceptors expressing exo-rhodopsin.

14. WHY IS THE MAMMALIAN PINEAL ORGAN NOT PHOTOSENSORY?

As discussed above, developing mammalian pinealocytes apparently pass through a temporal window in which they may express certain photoreceptor characteristics. Depending on the species, a variable number of adult pinealocytes retain the expression of selected phototransduction-related proteins (Foster *et al.* 2003). Crx continues to be expressed in the adult mouse pineal (Furukawa *et al.* 1997), and it has been suggested that the continued expression of Crx may be related to the presence of several phototransduction proteins in the pineal organ of adult mammals (Foster *et al.* 2003).

However, this cannot be the only explanation. If Crx transactivates pineal-specific genes like serotonin *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT), as well as photoreceptor-specific genes like rhodopsin, then why do so few mammalian pinealocytes express rod opsin? Only a fraction of the pinealocytes are opsin-IR in the adult mammalian pineal organ (Foster *et al.* 2003). Because the majority of the pinealocytes in the adult pineal express Crx (Chen *et al.* 1997), this would mean that Crx does not activate phototransduction genes in all pinealocytes throughout life, although it apparently is needed to maintain normal levels of NAT and HIOMT expression (Furukawa *et al.* 1999). Crx interacts with other transcription factors (Chau *et al.* 2000; Mitton *et al.* 2000; Zhu & Craft 2000; Wang *et al.* 2002), but obviously in different combinations for photoreceptor genes than for melatonin synthesis genes. It could also be that the efficient combinations change during the lifespan, or the 'partner(s)' necessary for photoreceptor gene expression is only expressed during a brief developmental period.

Using the 'receptor cell line theory' of Collin and Oksche to account for the expression of Crx by a majority, and opsin by a minority, of the pinealocytes, would infer either that not all mammalian pinealocytes are evolutionarily derived from photoreceptor cells (or we would have to accept a larger number of cell lines), or that different pinealocyte populations have evolved at different rates (opsin-expressing pinealocytes would be less regressed). Neither explanation appears overtly plausible. However, in the framework of our hypothesis, it could be explained by an analogy with the photoreceptor determination in the zebrafish pineal organ where there is an initial choice between neuronal or photoreceptor phenotype (Masai *et al.* 1997). A similar choice could be envisaged in the mammalian pineal organ, where 'photoreceptors' are formed only during a brief period, and those formed are not well sustained by their maturing cellular environment. The interplay between patterning genes and cytodifferentiation genes, and the cellular environment, has evolved so that crucial elements in the phototransduction or photoneuroendocrine pathways are not expressed. Although rod opsin is expressed in the developing pineal gland of neonatal hamsters, the chromophore 11-*cis* retinaldehyde is not present (Foster *et al.* 2003). Even in the presence of exogenous 11-*cis* retinaldehyde, the pineal rod opsin fails to form a functional photopigment (Foster *et al.* 1989; Kramm *et al.* 1993). So, although there may be a competence to differentiate photosensory cells during the temporal window when mammalian pinealocytes express phototransduction proteins in abundance, crucial factors are lacking and subsequent development further restricts the capacity to form functional photoreceptors in the mammalian pineal organ. However, some of the 'photoreceptor determinants', like Crx, are used in the regulatory linkage of functions other than phototransduction, and are therefore retained in (some of the) adult pinealocytes.

15. CONCLUSION

The pineal organ and the eyes develop from well separated regions of the neural plate. Development of these regions is under the control of transcriptional regulators and signalling molecules that are largely common, but not identical sets. Clearly, we have the potential for generation of photoreceptor organs in both morphogenetic fields, but under slightly different control. In this perspective, it is not surprising that we do not find clear-cut 'cone-type' and 'rod-type' photoreceptors in the pineal organ, or that we find opsins and other phototransduction-related proteins in the chiasmatic region, melatonin synthesis in the retina, and circadian clocks in these three regions (e.g. Binkley *et al.* 1978; Yoshikawa *et al.* 1994; Cahill & Besharse 1995; Cahill 1996; Miyamoto & Sancar 1998; Provencio *et al.* 1998; Whitmore *et al.* 1998; Zylka *et al.* 1998; Blackshaw & Snyder 1999; van der Horst *et al.* 1999; Philp *et al.* 2000*b*; Oishi *et al.* 2001; Tosini *et al.* 2001) in various combinations among vertebrates.

We suggest that the developing pineal field has the potential to form photosensory structures in all vertebrates, but that this potential has become restricted with evolution and is differentially restricted in different vertebrate taxa. The differential restriction is evident from the abundance of different pineal photoreceptor types found among vertebrate taxa, and from the expression of numerous components of the phototransduction pathway in mammalian pineal organs.

Finally, we suggest a revision of the theory of pinealocyte evolution according to Collin and Oksche. We do not envisage pinealocyte evolution as an evolution within one, or possibly two, particular 'sensory cell line(s)'. Instead, we suggest that evolutionary changes in the regulatory linkage of developmental processes and cellular functions in the embryonic 'pineal field' of the neural plate have shaped the different types of pineal photoreceptors we see in today's vertebrates. It is not the mature photoreceptors that evolve; it is the developmental processes that shape them.

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