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# Functional heterogeneity in the pineal projection neurons of zebrafish

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## Highlights :

- A subpopulation of pineal projection neurons expresses the melanopsin gene *opn4xa*
- This new pineal cell type shares a developmental program with other projection neurons
- The pineal gland contains both *opn4xa*<sup>-</sup> LIGHT OFF and *opn4xa*<sup>+</sup> LIGHT ON projection neurons

## Summary

The zebrafish pineal organ is a photoreceptive structure containing two main neuronal populations (photoreceptors and projections neurons). Here we describe a subpopulation of projection neurons that expresses the melanopsin gene, *opn4xa*. This new pineal cell type, that displays characteristics of both projection neurons and photoreceptors, share a similar dependency for BMP and Notch signalling pathways

with classical non-photosensitive projection neurons (PN). Functionally, however, whereas classical, *opn4xa*-negative PNs display an achromatic LIGHT OFF response, the novel cell type we describe exhibit a LIGHT ON character that is elicited by green and blue light. Taken together, our data suggest a previously unanticipated heterogeneity in the projection neuron population in the zebrafish pineal organ raising the question of the importance of these differences in pineal function.

## **Introduction**

The zebrafish pineal gland is a neuroendocrine structure of the dorsal diencephalon that plays a key role in mediating the effects of the circadian clock on sleep/wake rhythms. An important part of this activity is controlled by pineal photoreceptors (PhRs) that secrete the sleep-promoting hormone melatonin (Ben-Moshe Livne et al., 2016; Gandhi et al., 2015). The PhR population is heterogeneous and contains three described subtypes: cells expressing *red cone opsin* (15 cells at 48-54 hpf), those expressing *exorhodopsin* (20 cells at 48-54 hpf; Cau et al., 2019; Clanton et al., 2013) and cells coexpressing *parietopsin* (a green-sensitive opsin) and the UV-sensitive opsin, *parapinopsin1* (6 cells at 48-54 hpf; Cau et al., 2019; Koyanagi et al., 2015; Wada et al., 2018). In addition to PhRs, the pineal gland contains projection neurons (PNs, 20 cells at 48 hpf) which project to the ventral diencephalon (Wilson and Easter, 1991) and a population of AgRP2+ cells that do not express neuronal markers nor exhibit a neuronal morphology, and share molecular characteristics with retinal-pigment epithelium cells (Shainer et al., 2017, 2019).

While a significant amount is known concerning the subpopulations of pineal PhRs and their function (Ben-Moshe Livne et al., 2016; Cau et al., 2019; Clanton et al.,

2013; Gandhi et al., 2015; Koyanagi et al., 2015; Wada et al., 2018), much remains to be discovered concerning a possible corresponding heterogeneity in the PN population. PN functions have not been investigated. Electrophysiological experiments in lampreys and goldfishes suggest that PNs receive and integrate inputs from the PhRs (Meissl et al., 1986; Uchida et al., 1992). Most projection neurons are thought to function in a LIGHT OFF fashion. Indeed, electrophysiological experiments performed in the rainbow trout suggest that teleost pineal projection neurons fire constantly in the dark and exhibit a LIGHT OFF response. This response is elicited at all visible wavelengths, although maximum firing occurs at green wavelengths. While it is not clear whether individual projection neurons (PNs) respond to all wavelengths, nevertheless, this response was referred to as 'achromatic' as it is elicited by a wide range of wavelengths (Dodt, 1963; Morita, 1966). In contrast, in the same species, few projection neurons show a chromatic response. This response is excitatory at medium and long wavelengths and inhibitory in the violet-UV range (Morita, 1966). PN achromatic LIGHT OFF responses were also recorded in other species such as frog, pike and turtle (Falcón and Meissl, 1981; Meissl and Ueck, 1980; Morita and Dodt, 1965) while chromatic LIGHT ON responses were described in lizards, frogs and pikes (Dodt and Heerd, 1962; Dodt and Meissl, 1982; Meissl and Donley, 1980). These results suggest heterogeneity in the population of pineal projection neurons in a number of species. However, whether a similar heterogeneity exists in zebrafish is unclear, as are the molecular mechanisms that permit PN responses to changes in brightness and wavelength.

In the main photosensitive structure of the vertebrate, the retina, there is a clear heterogeneity in the population of projection neurons. Indeed, based on morphological, molecular, electrophysiological and/or functional criteria around 30 categories of

Retinal Ganglion Cells, or RGCs, have been described so far in the mouse (see Sanes and Masland, 2015 for a review). Strikingly, among these subpopulations, a subset of the RGCS has been shown to be directly photosensitive, owing to the expression of melanopsin, a blue-green photosensitive pigment. These intrinsically photosensitive RGCs (ipRGCs), integrate light information from rods and cones, as well as from their innate photosensitivity, to regulate photo-entrainment of the circadian system (Lucas et al., 2012). Therefore, ipRGCs form a class of atypical photoreceptors that can both directly sense light and project to distinct brain areas to influence circadian rhythms, suggesting they function both as photoreceptors and projection neurons.

In this paper, we describe that the expression of the zebrafish melanopsin gene *opn4xa* is restricted to a subset of pineal PNs and analyse their development. The development of PhRs and PNs require specific signalling pathways: the Bone Morphogenetic Protein (BMP) pathway is necessary and sufficient to trigger PhR fate as well as to activate the Notch pathway in these cells, which inhibits a PN fate (Cau and Blader, 2009; Cau et al., 2008; Quillien et al., 2011; Sapède and Cau, 2013). Analysis of embryos deficient for the BMP or the Notch signalling pathways suggest that Notch inhibits the production of *opn4xa+* cells, as it does with the rest of the PN population, and that the BMP pathway is dispensable for *opn4xa+* cell development. These results suggest that *opn4xa+* cells and classical PNs share a common basic developmental program. Finally, monitoring of the induction of the immediate early gene *c-fos* in different illumination regimes highlights two distinct activities in the PN population. A subpopulation of *opn4xa-* cells show a LIGHT OFF response when the embryos are adapted in white, blue, red or green light. In contrast, *opn4xa+* PNs exhibit

a LIGHT ON response following a pulse of white, blue or green light. We thus propose a parallel between *opn4xa*<sup>+</sup> PNs and the ipRGCs of the retina.

## **Results**

### **A subset of pineal projection neurons expresses the melanopsin gene *opn4xa***

To describe photoreceptor heterogeneity in the pineal, we previously analysed the expression of classical opsin photo-pigments (Cau et al., 2019). We also considered the expression of the five zebrafish melanopsin genes and found a small population of *opn4xa*<sup>+</sup> cells at the left and right borders of the pineal organ (Fig. 1A) (Matos-Cruz et al., 2011). Combining *in situ* hybridisation for *opn4xa* with immunostaining against GFP in the transgenic lines *Tg(aanat2:GFP)<sup>y8</sup>* and *Tg(elavl3:EGFP)<sup>knu3</sup>*, which label PhR and PN respectively (Fig 1B, (Cau et al., 2008)), revealed that *opn4xa* expression is not detected in classical PhR (Fig. 1C,D,E) but in PNs (Fig. 1F,G,H). *opn4xa* expression can be first detected at 22-23 hours post-fertilisation (hpf) in 1-2 *elavl3:EGFP*-positive cells. At 23 hpf an average of 4 *opn4xa*<sup>+</sup> cells are observed and this number remains stable to at least 10 days post-fertilisation (dpf; Fig. 1L). To further characterise *opn4xa*<sup>+</sup> cells, we looked for different markers displaying similarly restricted expression within the pineal organ. We found that the gene encoding the Wnt transcriptional effector *Tcf7* (Veien et al., 2005) is expressed in a small subset of PNs (Fig S1 A-C). We took advantage of a *tcf7* enhancer trap line *Et(T2KHG)<sup>nns8</sup>* (Nagayoshi et al., 2008) to characterise *tcf7* expression in the pineal further. We first confirmed that expression of GFP from the *Et(T2KHG)<sup>nns8</sup>* line co-localises with endogenous *tcf7* expression in PNs (Fig. S1 A-C). We next assessed co-labelling of the *Et(T2KHG)<sup>nns8</sup>* enhancer trap line with *opn4xa* mRNA at stages ranging from 24 hpf to 4 dpf. Up to 30 hpf, the majority of GFP<sup>+</sup> cells were also *opn4xa*-positive (Fig. 1 I,J,K Fig. S1 G). In contrast, from 2 dpf onwards, a population of cells showing

a lower level of GFP appeared in the pineal of *Et(T2KHG)<sup>nns8</sup>* that do not express *opn4xa* (Fig. S1 D-G). Altogether, we describe a subpopulation of PNs expressing *opn4xa* that can be labelled specifically with the *Et(T2KHG)<sup>nns8</sup>* enhancer trap line up to 30 hpf.

### **Specification of the *opn4xa+* pineal fate does not require Wnt signalling**

The fact that the *tcf7* enhancer trap line *Et(T2KHG)<sup>nns8</sup>* is expressed in *opn4xa+* cells prompted us to investigate whether Wnt signalling was involved in the specification of the *opn4xa+* fate. First, we tested the expression of *opn4xa* in *Et(T2KHG)<sup>nns8</sup>* homozygous embryos as the enhancer trap insertion abolishes expression of the endogenous *tcf7* gene (Nagayoshi et al., 2008). Pineal *opn4xa+* cells were observed in normal numbers in all GFP+ embryos coming from *Et(T2KHG)<sup>nns8</sup>* +/- incrosses suggesting that *tcf7* is not required for their specification (Fig 2 A,B) as one third of GFP+ embryos are expected to be homozygous for the insertion. To look at a possible redundancy with other Wnt effectors, we analysed the expression of *tcf7l2* and *lef1* in the pineal and compared it with the *Et(T2KHG)<sup>nns8</sup>* enhancer trap. Only the former is expressed in the pineal but did not show co-expression with the *tcf7* enhancer trap (Figure S2). Finally, we modulated Wnt activity using conditional overexpression of Dkk1 or Wnt8 in heat-shocked *Tg(hsp70l:dkk1b-GFP)<sup>w32</sup>* and *Tg(hsp70:wnt8-GFP)<sup>w34</sup>* embryos, respectively (Stoick-Cooper et al., 2007). Heat-shocked embryos carrying the *Tg(hsp70l:dkk1b-GFP)<sup>w32</sup>* transgene show normal expression of *opn4xa* (Fig 2B) suggesting that Wnt activity is dispensable for the specification of *opn4xa+* cells. Similarly, heat-shocked embryos transgenic for the *Tg(hsp70:wnt8-GFP)<sup>w34</sup>* transgene show normal numbers of *opn4xa +* cells suggesting that Wnt activity is not

sufficient to drive the *opn4xa+* fate. We conclude that Wnt signalling does not play a role during specification of the *opn4xa+* fate.

### **Specification of *opn4xa+* cells does not require BMP signalling and is inhibited by Notch signalling**

Classical PhRs are specified by a combination of Notch and BMP pathway activity with BMP activity being necessary and sufficient to activate the PhR fate and Notch being required to inhibit the inappropriate PN program (Cau et al., 2008; Quillien et al., 2011). To understand whether *opn4xa+* cells are closer to PNs or PhRs developmentally speaking, we analysed the effects of reducing BMP or Notch activities on the specification of these cells.

We used *Tg(hsp70l:dnXla.Bmpr1a-GFP)<sup>w30</sup> ; Tg(hsp70l:nog3)<sup>fr14</sup>* double transgenic embryos heat-shocked at 14hpf to reduce BMP signalling, a condition that we have previously shown reduces the number of classical PhRs (Quillien et al., 2011). Under these conditions, the number of *opn4xa+* cells was similar compared to wildtype embryos (Fig 2A, C); pineal *opn4xa+* cells in BMP loss-of-function embryos exhibit an aberrant medial location which is likely due to the absence of classical photoreceptors normally found in the centre of the pineal gland (Fig2A). The effects of impairing Notch activity was analysed in the *mindbomb* (*mib*) mutant (*mib<sup>ta52b</sup>* (Itoh et al., 2003)). We found that *mib* embryos exhibit twice the number of *opn4xa+* cells at 48 hpf compared to controls (Fig 2A,D). Altogether these results suggest that pineal *opn4xa+* cells behave as classical pineal PNs with respect to their requirement for BMP and Notch activity.

**In contrast to classical PNs, *opn4xa* + projection neurons respond to light in a LIGHT ON fashion**

Electrophysiological experiments performed in the rainbow trout, pike, frog and turtle have shown that projection neurons are continuously activated in the dark suggesting that they function in a LIGHT OFF mode (Dodt, 1963; Falcón and Meissl, 1981; Meissl and Ueck, 1980; Morita, 1966; Morita and Dodt, 1965). To assess whether *opn4xa*+ PNs are also LIGHT OFF, we analysed the expression of *c-fos* after a 30 minute dark-pulse in 48 hpf light-adapted embryos (Fig 3A); *c-fos* is an immediate early gene that is extensively used as a read out of neuronal activation (reviewed in Guzowski et al., 2005). While light-adapted embryos fixed at the same circadian time show no *c-fos* expression in the pineal (Fig 3Bi), a pulse of dark induced expression of *c-fos* expression in  $8.36 \pm 2.13$  cells per embryo, which are located laterally in the pineal (from n=14 embryos) (Fig 3Bii). The same experiment performed in *Et(T2KHG)<sup>nns8</sup>* or *Tg(elavl3:EGFP)<sup>knu3</sup>* embryos showed that these LIGHT OFF cells are *tcf7*:GFP- but *elavl3*:EGFP+ and thus correspond to classical, *opn4xa*- projection neurons (Fig 3C,D). To address if *opn4xa*+ PNs are LIGHT ON, next we examined the effect of a light pulse in dark-adapted embryos (Fig 4A). We found that a 30 minute pulse of white light delivered to 48 hpf dark-adapted embryos induced *c-fos* expression in  $3 \pm 1.41$  cells (from n=17 embryos) located laterally within the pineal organ (Fig 4Bii) in cells that strongly express the *Et(T2KHG)<sup>nns8</sup>* transgene (Fig 4C); *c-fos* transcript was not detected in the pineal of non-light stimulated embryos fixed at the same circadian time (Fig 4Bi). Similar results were obtained in 27 hpf and 3 dpf dark-adapted embryos (Fig S3). In mammals, the pineal gland is connected to the retina via a multi-synaptic pathway (Sapède and Cau, 2013). To rule out that the pineal LIGHT ON response we describe is caused by photosensitivity in the retina, we assessed the presence of this

response in *lakritz (lak)* mutant which lack neurotransmission from the retina owing to a complete absence of RGCs (Kay et al., 2001). The pineal LIGHT ON response is similar in *lak+/+* and *lak-/-* embryos at 48 hpf suggesting that the retina is not involved in this activity (Fig 4D,E).

Taken together, our results indicate heterogeneity in the light-response within the PN population with *opn4xa+* PNs functioning in a LIGHT ON fashion and classical PNs in a LIGHT OFF mode.

### **LIGHT ON and LIGHT OFF responses are elicited by different range of wavelengths**

Apart from *Opn4xa*, which is sensitive to blue-green light (Davies et al., 2011), the classical PhR in the pineal express opsin photo-pigments (*exorhodopsin*, *red cone opsin* and *parietopsin*) that are sensitive to green and red light (Cau et al., 2019). We next examined whether LIGHT ON in blue, red or green wavelengths were involved in the induction of *c-fos* expression. We observed that 30 minute light pulses in the blue and green range induce *c-fos* expression while similar pulses of red light could not induce a LIGHT ON response (Fig 5 A-C, G). Given that the absorbance spectrum of the *Opn4xa* photo-pigment overlaps with the emission of the green and blue LEDs used in this study, we propose that this LIGHT ON response reflects the direct photosensitivity of *opn4xa+* photo-pigment. In marked contrast with the results obtained for the LIGHT ON response, LIGHT OFF was elicited when the embryos were adapted in either red, blue or green light (Figure 5 D-F, H) suggesting that this response is elicited by a wide-range of wavelengths and can, therefore, be referred to as achromatic.

Our results suggest that classical, *opn4xa*<sup>-</sup> projection neurons function in a LIGHT OFF mode and most likely transduce the light input received by classical PhRs. In contrast, *opn4xa*<sup>+</sup> projection neurons are activated by light and most likely directly photosensitive.

## Discussion

In this paper, we describe a new population of cells in the zebrafish pineal gland. These cells express the green-blue photosensitive pigment Opn4xa and share developmental characteristics with classical, non-photosensitive projection neurons. Intriguingly, while classical PNs exhibit a LIGHT OFF response, a LIGHT ON response is observed in *opn4xa*<sup>+</sup> PN upon illumination. These results suggest a previously unanticipated cellular diversity in the projection neuron population within the pineal gland. In the following section, we discuss the specification and potential function of the *opn4xa*<sup>+</sup> PNs.

Projection neurons and photoreceptors of the pineal gland differ in their requirement for Notch and BMP signalling pathways: photoreceptors require BMP activation (while projection neurons do not) and BMP is in turn needed to activate the Notch pathway in these cells, which inhibits the projection neurons fate (Cau et al., 2008; Quillien et al., 2011). This led us to postulate that decoupling of the activation of BMP and Notch activity could represent an attractive mechanism to further diversify cell fates. Indeed, activation of the BMP signalling pathway without activation of Notch should lead to the generation of cells with a mixed photoreceptor/projection neuron identity (Cau and Blader, 2009). In contrast with this hypothesis, *opn4xa*<sup>+</sup> cells, that exhibits both characteristics of photoreceptors and projection neurons, do not require BMP activity suggesting that they are more closely related to projection neurons than

to classical photoreceptors. Similarly, Notch activity inhibits the production of both *opn4xa*<sup>-</sup> and *opn4xa*<sup>+</sup> projection neurons to the same extent. Therefore, neither BMP nor Notch activities contribute to specifying *opn4xa*<sup>+</sup> versus *opn4xa*<sup>-</sup> PN fates.

Since *opn4xa*<sup>+</sup> and classical projection neurons show similar behaviour upon BMP or Notch loss of function, what are the signals that could specify these distinct identities? The Wnt effector *tcf7* is specifically expressed in *opn4xa*<sup>+</sup> PN and so is the *Et(T2KHG)<sup>nns8</sup>* enhancer trap line at early stages. Nevertheless, *tcf7* mutants show a normal number of *opn4xa*<sup>+</sup> pineal cells. Moreover, neither gain nor loss of Wnt activity affects the number of *opn4xa*<sup>+</sup> cells, which suggests that Wnt activity is neither necessary nor sufficient for specification of *opn4xa* expression. It remains to be addressed whether *tcf7* (and/or Wnt signalling) is required to specify other aspects of the phenotype of these atypical PNs. Despite the absence of an observable function for *tcf7*, the *Et(T2KHG)<sup>nns8</sup>* enhancer trap line provides a useful marker for *opn4xa*<sup>+</sup> cells at early stages which will help us in analysing the projection pattern of these cells.

Our analysis confirms previous results obtained in another teleost, the rainbow trout, as well as in numerous other species showing that an important part of the projection neuron population function in a LIGHT OFF manner (Dodt, 1963; Falcón and Meissl, 1981; Meissl and Ueck, 1980; Morita, 1966; Morita and Dodt, 1965). Within the projection neuron population only *opn4xa*<sup>+</sup> PNs function in a LIGHT ON fashion. Given that this activity is only seen using blue and green light and not red light, it likely requires the function of *Opn4xa*, a photo-pigment with an absorbance peak at 470 nm (Davies et al., 2011). This result contrasts with the observation that a LIGHT ON (excitatory response) is observed at medium to long wavelengths in the rainbow trout (Morita, 1966). A surprising degree of variation is indeed observed in the wavelengths triggering ON and OFF chromatic responses in different species. For instance, green

light triggers the excitatory response in lizards and frogs while red light elicits this response in the pike. Similarly, the chromatic inhibitory response is stronger in the ultraviolet range in fish and frogs but mainly due to blue light in lizards (see Dodt and Meissl, (1982) for a review). It would be interesting to understand whether the LIGHT ON chromatic response observed in lizard, frog and pike require a direct photosensitivity from specific projection neurons. Alternatively, in these species, pineal photoreceptors could elicit a dual excitatory/inhibitory action on their targets similar to that described for retinal photoreceptors, which trigger a different response in bipolar cells depending on the type of glutamate receptor they express (Euler et al., 1996; Masu et al., 1995; de la Villa et al., 1995).

Are there other LIGHT ON projection neurons in the zebrafish pineal gland? Adaptation with white light allowed us to identify an average 8 LIGHT OFF PNs for around 3 LIGHT ON PNs. Since at 48 hpf the pineal gland contains 20 PNs, either some of the PN responses have not been identified or the LIGHT OFF cells do not systematically activate *c-fos* following a 30 min dark pulse, in the present testing conditions. It thus remains possible that there are other unidentified projection neurons that respond in a LIGHT ON fashion for instance in response to UV light.

While a complete description of the neurotransmitter/neuromodulator content of all zebrafish pineal neurons is lacking, glycinergic cells with a photoreceptor morphology have been observed in the pineal gland (Marquart et al., 2015; Moly et al., 2014)). These observations suggest that at least a proportion of pineal photoreceptors exert an inhibitory action on projection neurons, therefore providing a possible mechanism for the LIGHT OFF response we describe. Alternatively, photoreceptors could constantly produce an excitatory neurotransmitter in the dark, which would be blocked by light leading to an interruption of projection neuron activity through a

“disfacilitation mechanism” (Uchida et al., 1992). A more detailed analysis of the neurotransmitter/neuromodulator content of all pineal neurons, and *opn4xa*<sup>+</sup> PNs in particular, would be useful. Finally, the fact that the LIGHT OFF response is observed independently of the wavelength at which the embryos are adapted suggests that LIGHT OFF projection neurons receive inputs from several types of pineal photoreceptors.

Although direct photosensitivity of *opn4xa*<sup>+</sup> PNs remains to be confirmed, they share many characteristics with retinal ipRGCS raising intriguing questions regarding the functions of these pineal cells. Indeed, in mammals ipRGCs exhibit crucial roles during the control of circadian, wake/sleep rhythms as well as in mediating the direct effects of illumination on physiology and behaviour (see Lazzerini Ospri et al., (2017); Lucas et al.,(2012) for reviews)). In particular, ipRGCs transmit light information, via their own intrinsic photosensitivity as well as inputs from rod and cone PhRs, to the mammalian ‘master clock’ the suprachiasmatic nucleus (Hannibal and Fahrenkrug, 2004; Hattar et al., 2002, 2006). It is unclear whether a suprachiasmatic nucleus is present in the larval zebrafish and whether *opn4xa*<sup>+</sup> retinal or pineal cells project onto such a structure. On the other hand, the zebrafish pineal gland has long been thought to play a ‘master clock’ role owing to its direct photosensitivity, its capacity to generate intrinsic rhythms of melatonin synthesis/secretion and the fact that disrupting the function of the molecular clock in pineal PhRs affect circadian rhythms of locomotor activity (Ben-Moshe Livne et al., 2016; Bolliet et al., 1997; Cahill, 1996, 1997; Falcón et al., 1989). Could *opn4xa*<sup>+</sup> PNs play a role alongside classical pineal PhRs in the function of the circadian system? Analysis of an *opn4xa* mutant allele combined with retina-specific and/or pineal specific transgenic tools would help us understand whether *opn4xa*<sup>+</sup> PNs are important players in this field.

## **Experimental Procedures**

### Zebrafish lines and developmental conditions

All animals were handled in the CBI fish facility, which is certified by the French Ministry of Agriculture (approval number A3155510). The project was approved by the French Ministry of Teaching and Research (agreement number APAFIS#3653-2016011512005922).

Embryos were reared at 28 degrees in a 14 hours Light/ 10 hours Dark cycle and staged according to standard protocols. All the transgenic and mutant lines have been described previously: *Tg(aanat2:GFP)<sup>y8</sup>* (Gothilf et al., 2002), *Tg(elavl3:EGFP)<sup>knu3</sup>* (Park et al., 2000), *Et(T2KHG)<sup>nns8</sup>* (Nagayoshi et al., 2008), *Tg(hsp70l:dkk1b-GFP)<sup>w32</sup>* and *Tg(hsp70:wnt8-GFP)<sup>w34</sup>* (Stoick-Cooper et al., 2007), *Tg(hsp70l:dnXla.Rbpj-MYC)<sup>vu21</sup>* and *Tg(hsp70l:nog3)<sup>fr14</sup>* (Chocron et al., 2007), *mib<sup>ta52b</sup>* (Itoh et al., 2003), *lakritz* (Kay et al., 2001). Conditions of heat-shock were: *Tg(hsp70l:dnXla.Rbpj-MYC)<sup>vu21</sup>* and *Tg(hsp70l:nog3)<sup>fr14</sup>* : 30 minutes at 39.5°C; *Tg(hsp70l:dkk1b-GFP)<sup>w32</sup>* and *Tg(hsp70:wnt8-GFP)<sup>w34</sup>* : 1 hour at 38°C. The protocol for genotyping *lakritz* embryos is available upon request.

### Application of different light regimes

To assess LIGHT ON responses, two different protocols were used. In the first protocol, the light was turned on at 9 am and the embryos fixed at 9.30 am. In the second one, the embryos were dark adapted for 2 hours (from 9.30 to 11.30) before a 30 mn light pulse and a fixation at 12 am. We did not notice a difference in the responses between these two protocols. On the other hand, for the assessment of the

LIGHT OFF responses, the embryos were light-adapted from 9 am to 12 am after which light was turned off and the embryos were fixed 30 mns after. The comparison of adapted and pulsed embryos fixed at the same circadian time allowed us to conclude that the observed *c-fos* expression was caused by the pulse rather than by circadian rhythms.

Illumination with white light was performed using a regular neon lamp (80 lux). Illumination with blue, green and red light was performed in a MWP unit (Zantiks) equipped with standard blue, green and red LEDs. The characteristics of the light emitted from these LEDs is:

Blue light: 92 lux,  $\lambda_{\max}$ = 449nm, half-band width: 25,4nm

Green light: 109 lux,  $\lambda_{\max}$ = 512 nm, half-band width: 33,07 nm

Red light: 34 lux,  $\lambda_{\max}$ = 627 nm, half-band width: 21,67 nm

Scripts controlling the different light regimes are available upon request.

### Immunohistochemistry and in situ hybridisation

Immunohistochemistry and in situ hybridisation were performed as previously described and fluorescent in situ hybridisation were revealed using either Fast Red (Sigma) or the TSA Plus System (TSA-Fluorescein, Perkin Elmer; Cau et al., 2019). Immunohistochemistry against Tcf7l2 was performed as described in Hüsken et al., (2014) using an anti-Human TCF3,4 antibody (1:400, Biomol, clone 0.T.148).

Probes used in this study were: *opn4xa* (Matos-Cruz et al., 2011), *c-fos* (Ellis et al., 2012), *lef1* and *tcf7* (Veien et al., 2005).

### Cell counting and statistical Analysis

Fluorescent cells were counted using the ImageJ software on confocal data sets with 2  $\mu\text{m}$  interval optical planes. Statistical tests and number of embryos used are stated in each figure and/or figure legend.

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## **Declaration of competing interest**

The authors declare that no competing interests exist.

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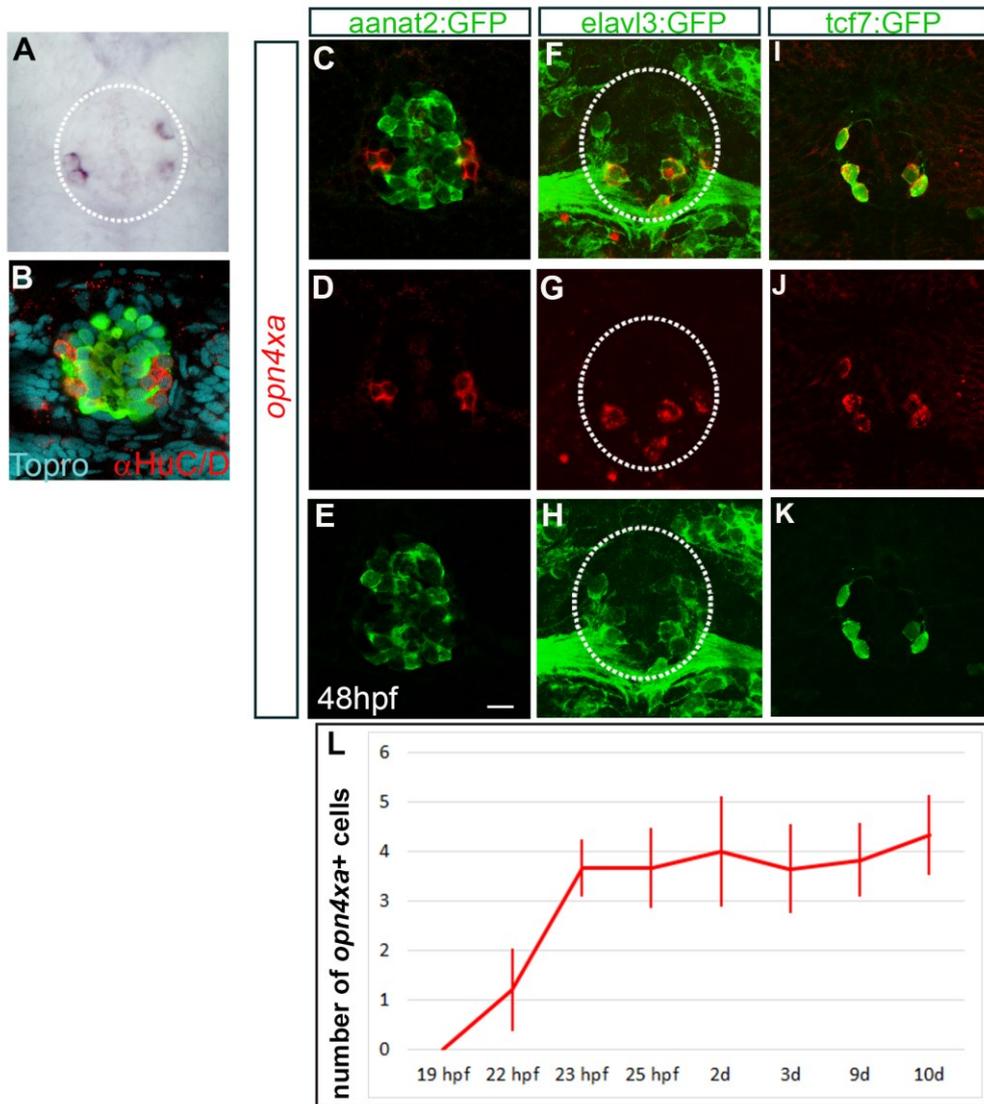
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## **Figure and Figure Legends**



**Figure 1: *opn4xa* is expressed in a restricted population of projection neurons within the pineal gland.**

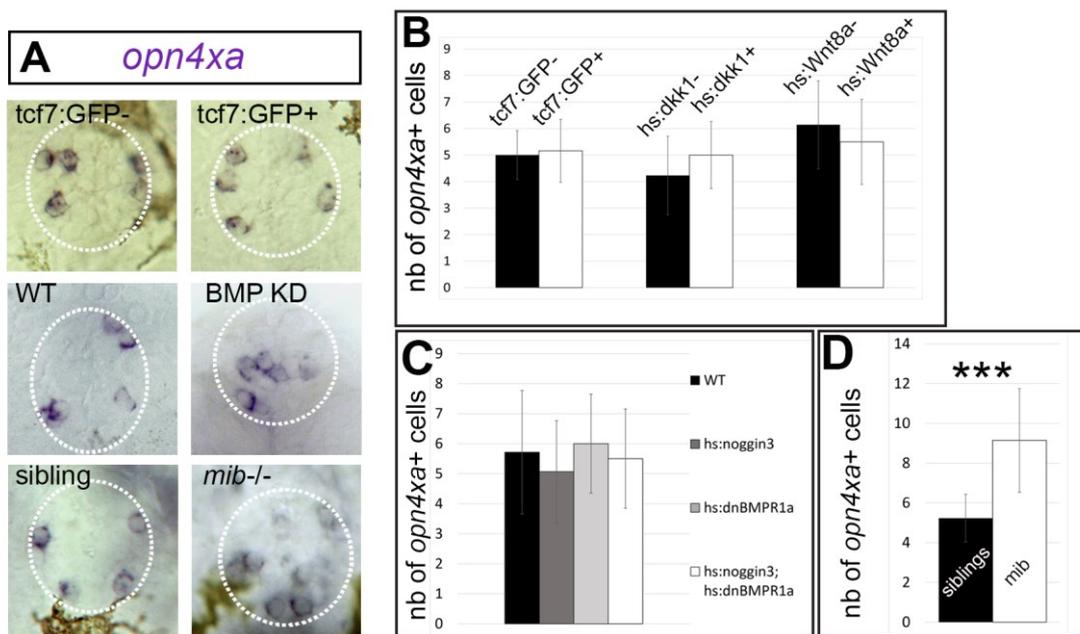
(A) *in situ* hybridisation at 48hpf showing pineal *opn4xa*+ cells. The pineal is highlighted with a white dotted circle.

(B) Confocal projection showing the relative distribution of PNs (red, labelled with an anti-HuC/D antibody) and PhRs (green, labelled with an anti-GFP in a Tg(*aanat2:GFP*)<sup>y8</sup> background) at 48 hpf. Topro (cyan) labels cell nuclei.

(C-K) Co-expression of *opn4xa* mRNA (in red) on the one hand with the Tg(*aanat2:GFP*)<sup>y8</sup> ('*aanat2:GFP*', C,D,E), the Tg(*elavl3:EGFP*)<sup>knu3</sup> ('*elavl3:EGFP*', F,G,H) and the *Et(T2KHG)*<sup>nns8</sup> ('*tcf7:GFP*', I,J,K transgenes (in green).

(A-K) Anterior is upwards. Scale bars: 10 µm.

(L) Time course of *opn4xa* expression in the developing pineal organ. Average numbers of *opn4xa*+ cells are quantified at different time points indicated in hours post-fertilisation (hpf). Values are mean ±SD, n=4, 5, 3, 6, 9, 26, 6 and 6, respectively.



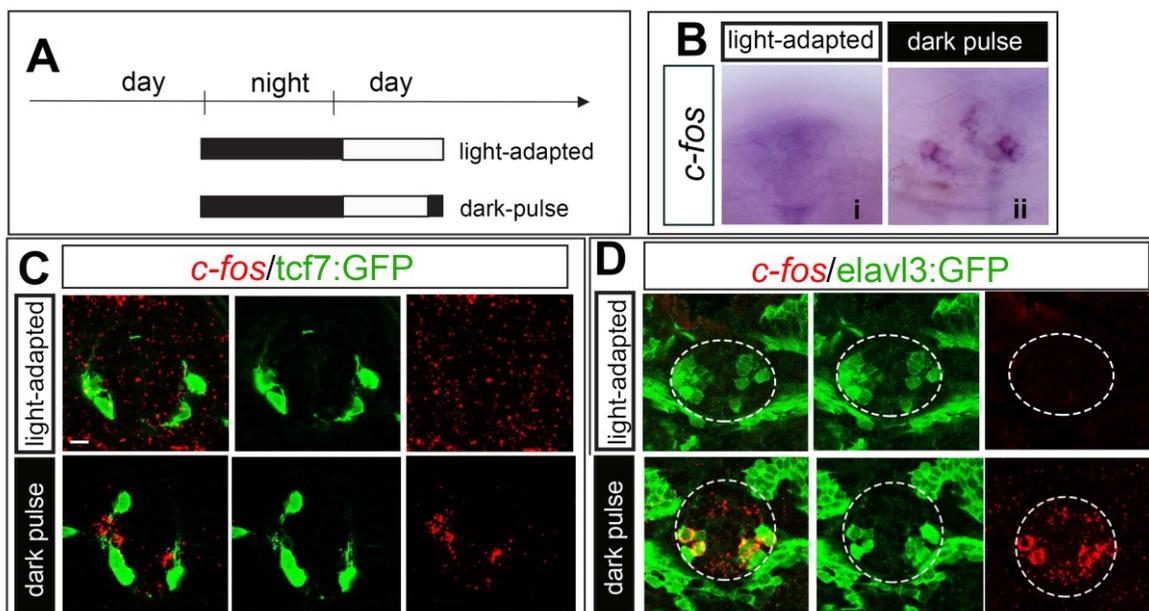
**Figure 2: Effect of manipulating Notch, BMP and Wnt pathways on the *opn4xa*+ fate**

(A) Representative pictures of *in situ* hybridisations for *opn4xa* in the pineal organ upon BMP, Notch and Wnt modulation (as indicated) and their corresponding controls. (B-D) Average numbers of pineal *opn4xa*+ positive cells upon manipulation of Wnt (B), BMP (C) or Notch signalling activity (D). Values indicated on the graphs are mean ±SD (error bars). Two tailed Mann Whitney non parametric tests, were used for B and D. \*P<0.05; \*\*\*P<0.001.

(B) Effect of gain or loss of Wnt activity on the number of pineal *opn4xa*+ cells: Embryos from *Et(T2KHG)<sup>nns8</sup> +/-* incrosses were assessed for GFP fluorescence; countings were performed at 30hpf. The size of the GFP- and GFP+ populations ('*tcf7:GFP-*' and '*tcf7:GFP+*') were respectively n=8 and n=31. Embryos carrying the *Tg(hsp70l:dkk1b-GFP)<sup>w32</sup>* transgene after an heat shock at 18 hpf, were compared with negative siblings at 48 hpf (n=13 for the '*hs:dkk1-*' and n=11 for the '*hs:dkk1+*'). Embryos carrying the *Tg(hsp70l:wnt8-GFP)<sup>w34</sup>* transgene (n=14) after an heat shock at 14 hpf, were compared to siblings (n=14) at 48 hpf.

(C) Effect of a reduction in Notch activity: Control embryos (n=30) were compared to homozygous *mindbomb* (*mib*<sup>-/-</sup>; n=7) mutants at 48 hpf

(D) Effect of a reduced BMP signalling: Embryos transgenic for *Tg(hsp70l:dnXla.Bmpr1a-GFP)<sup>w30</sup>* (*hs:dnBMPR1a*, n=12) or *Tg(hsp70l:nog3)<sup>fr14</sup>* (*hs:noggin3*, n=30) or both (n=18) were compared to siblings (n=7). Kruskal-Wallis test with Dunn's multiple comparisons test, P=0.44; not significant.



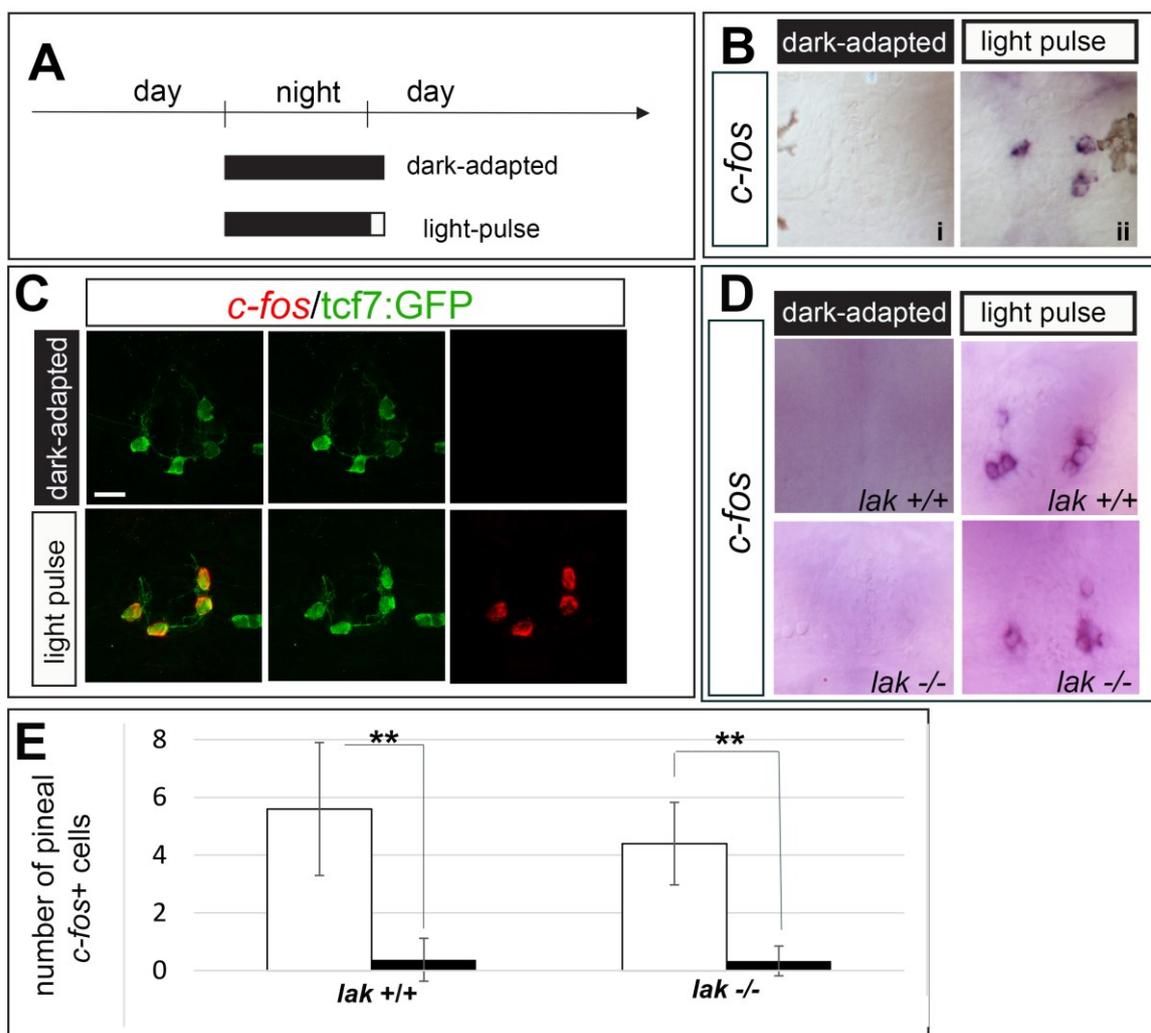
**Figure 3: Identification of a population of *opn4xa*- PNs functioning in a LIGHT OFF mode:**

(A) Experimental paradigm for the 'dark pulse versus light-adapted' comparison

Embryos were light-adapted for three hours before being submitted to a 30 min dark pulse ('dark pulse') or maintained in the light ('light-adapted')

(B) Representative pictures of *c-fos* induction in the pineal organ after application of the light regimes described in A). i) light-adapted, ii) dark-pulse

(C-E) Induction of *c-fos* in the different light regimes in embryos carrying the *Et(T2KHG)<sup>nns8</sup>* ('*tcf7:GFP*', C) or the *Tg(elavl3:EGFP)<sup>knu3</sup>* ('*elavl3:EGFP*', D) transgenes (in green). Scale bars: 10  $\mu$ m. All embryos shown are 48 hpf.



**Figure 4: *opn4xa*+ PNs function in a LIGHT ON mode:**

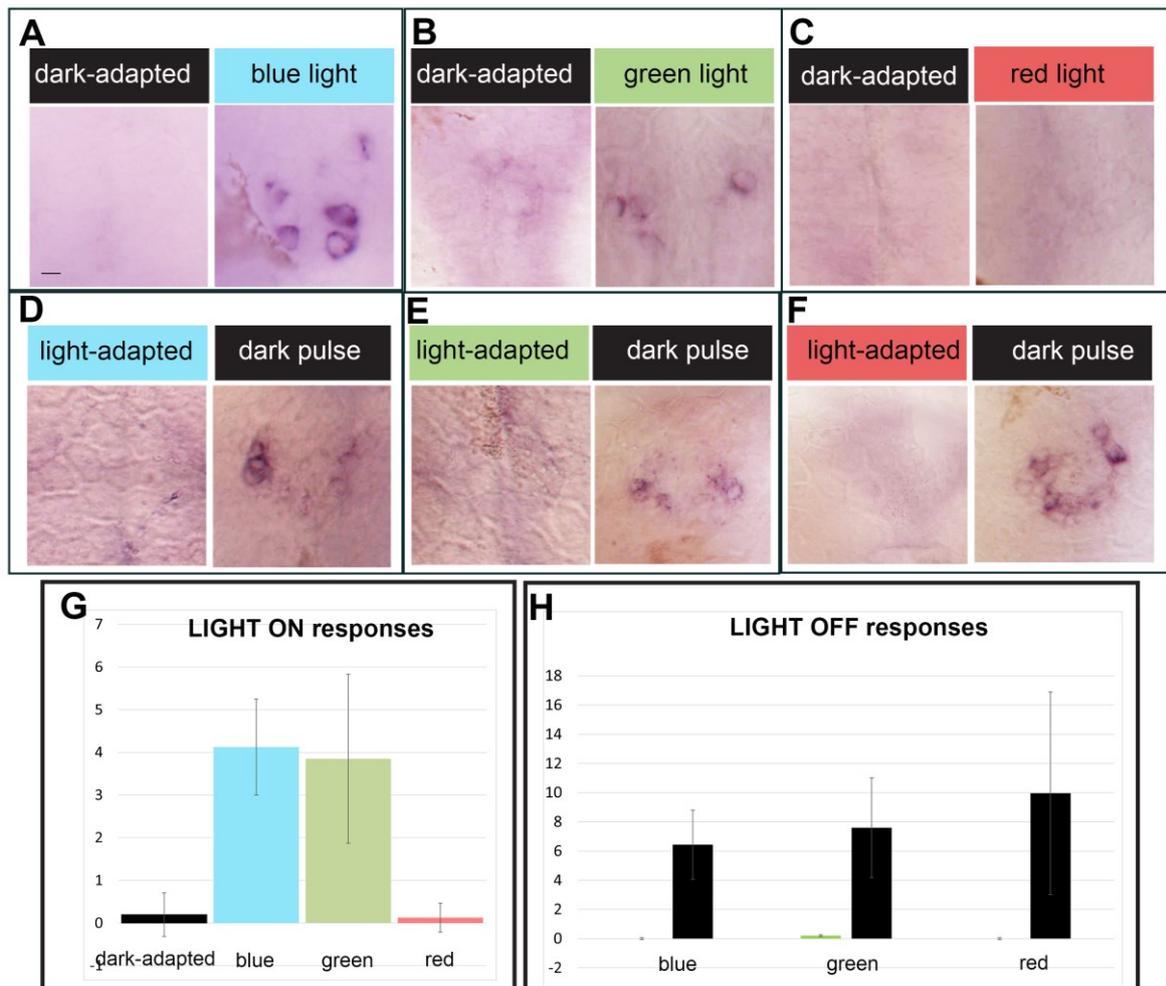
(A) Experimental paradigm for the 'light-pulse versus dark-adapted' comparison. Embryos were dark-adapted for more than 14 hours and submitted to a 30 min light pulse ('light pulse') or maintained in the dark ('dark-adapted') before fixation.

(B) Representative pictures of *c-fos* induction in the pineal organ after application of the light regimes described in A). i) dark-adapted, ii) light-pulse

(C) Induction of *c-fos* in the different light regimes in embryos carrying the *Et(T2KHG)<sup>nns8</sup>* ('*tcf7*:GFP') transgene (in green). Scale bars: 10  $\mu$ m.

(D) Induction of *c-fos* upon a 30mn light pulse in *lak* +/+ and *lak* -/- embryos.

(E) Quantification of the number of pineal *c-fos*+ cells in light-pulsed (white bars) and dark-adapted (dark bars) *lak*+/+ and *lak*-/- embryos at 48 hpf. Two tailed Mann Whitney non parametric tests, were used \* $P < 0.05$ . Values are mean  $\pm$ SD, n=5,8,10 and 6, respectively.



**Figure 5: Different spectral sensitivities for the LIGHT ON and the LIGHT OFF responses within the pineal gland:**

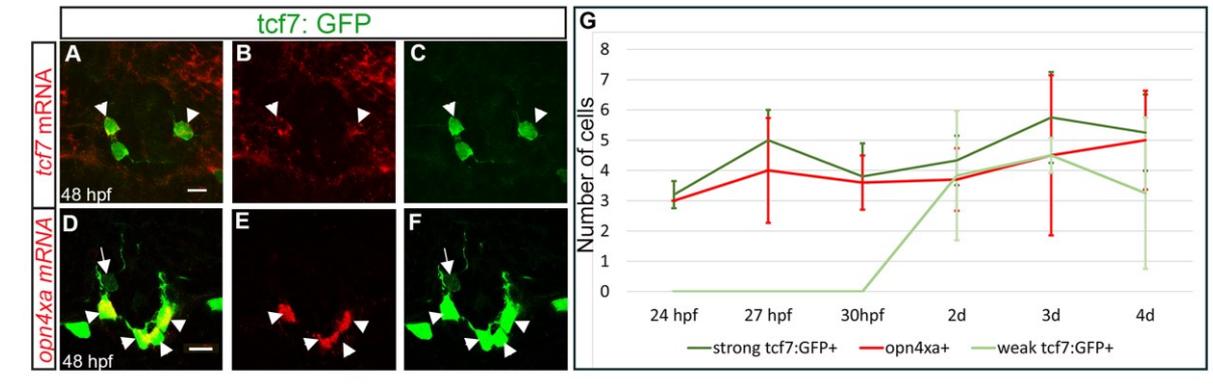
(A-F) Representative pictures of *c-fos* induction in the pineal organ after application of light pulses in the blue, green or red range compared to dark adapted embryos (A-C) or after light adaptation with blue, green or red light followed by a dark pulse ('dark pulse') or maintained in the same lighting conditions ('light-adapted') (D-F)

(G, H) Quantification of the number of cells activating *c-fos* after a light pulse (G, LIGHT ON responses) or a dark pulse (H, LIGHT OFF responses). G) Numbers of embryos : n= 41, 8, 20,16 respectively

H) Numbers of embryos n=10,16,5,25,21,22 respectively

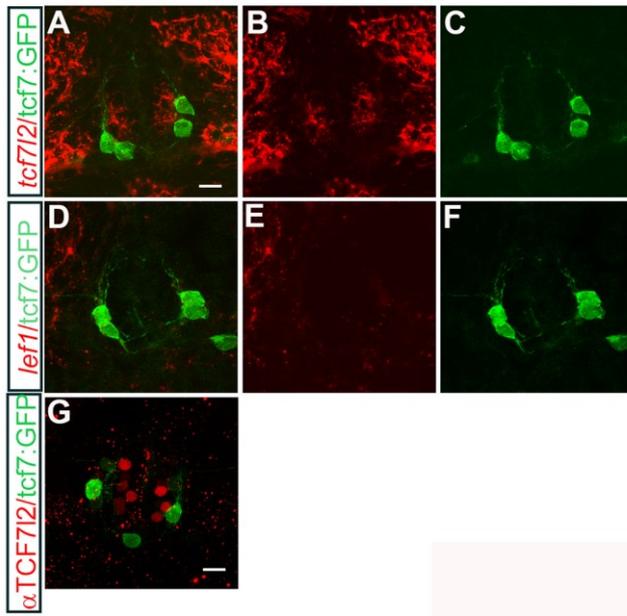
Scale bar: 10  $\mu\text{m}$ .

### Supplemental figure legends:



### **Supplemental Figure 1: Characterisation of the expression driven by the *Et(T2KHG)<sup>nns8</sup>* ('*tcf7:GFP*') enhancer trap in the pineal gland :**

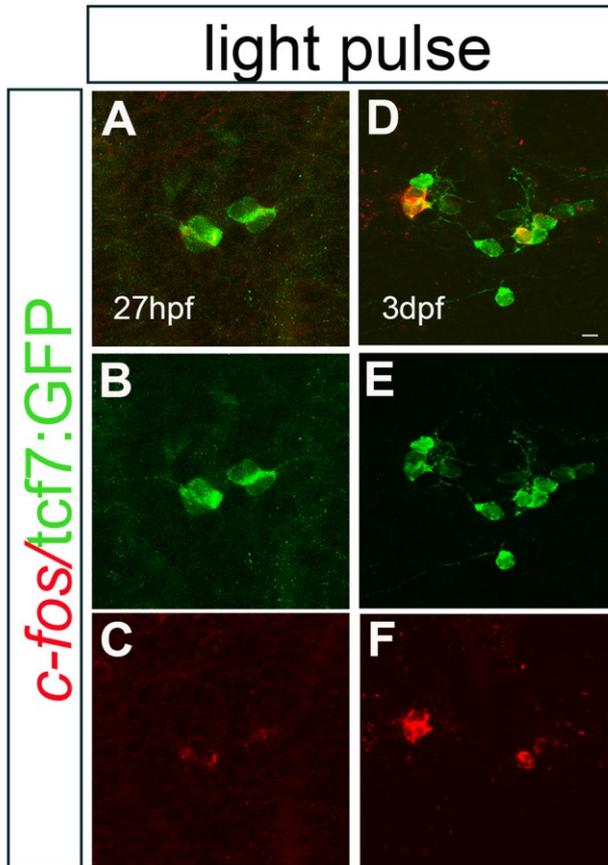
(A-F) Comparison of *tcf7* (A-C) or *opn4xa* (D-F) expression (in red, in situ hybridisation) with GFP expression from the *Et(T2KHG)<sup>nns8</sup>* ('*tcf7:GFP*') transgene (in green, immunohistochemistry). White arrowheads highlight co-expressing cells. White arrow show a weak *tcf7:GFP+* *opn4xa* – cell. Scale bars: 10  $\mu\text{m}$ . (G) Comparison of the number of cells expressing *tcf7:GFP* strongly or weakly and *opn4xa*. Means  $\pm$  S.D are shown.



**Supplemental Figure 2: Comparison of the expression of *tcf712* and *lef1* with the *Et(T2KHG)<sup>nns8</sup>* ('*tcf7:GFP*') enhancer trap in the pineal gland**

(A-F) Expression of two other TCF encoding genes: *tcf712* (A-C) or *lef1* (D-F) (in red, in situ hybridisation) with expression from the *Et(T2KHG)<sup>nns8</sup>* ('*tcf7:GFP*') transgene (in green, immunohistochemistry). (G) Double immunostaining with anti-GFP (green) and anti-TCF712 (red) in pineal neurons.

Anterior is up. All embryos are 48 hpf. Scale bars: 10  $\mu$ m.



**Supplemental Figure 3 : Induction of *c-fos* expression following a light pulse in the pineal gland at 27 hpf and 3 dpf.**

Expression of *c-fos* (in red, *in situ* hybridisation) compared with expression from the *Et(T2KHG)<sup>nns8</sup>* ('tcf7:GFP') transgene (in green, immunohistochemistry) at 27hpf (A-C) and 3dpf (D-F). Scale bars: 10  $\mu$ m.