Molecular Characterization of the Sea Urchin Larva Photosensory System: Insights Into the Evolution of a Go-Opsin Positive Neuronal Population

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Article

Molecular Characterization of the Sea Urchin Larva Photosensory System: Insights into the Evolution of a Go-Opsin Positive Neuronal Population

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Abstract: The ability to perceive and respond to light stimuli is fundamental not only for spatial vision, but also to many other light mediated interactions with the environment. In animals, light perception is performed by specific cells known as photoreceptors and, at molecular level, by a group of GPCRs known as Opsins. Sea urchin larvae possess a group of photoreceptor cells (PRCs), deploying a Go-Opsin (Opsin3.2), which have been shown to share transcription factors and morphology with PRCs of the ciliary type, contributing to raising new questions on how this sea urchin larva PRC is specified and whether it shares a common ancestor with ciliary PRCs or it evolved independently through convergent evolution. To answer these questions, we combined immunohistochemistry and fluorescent *in situ* hybridization to investigate how the Opsin3.2 PRCs develop in the sea urchin *Strogylocentrotus purpuratus* larva. Subsequently, we applied single cell transcriptomics to investigate the molecular signature of the *Sp-Opsin3.2* cells, and show that they deploy an ancient regulatory program responsible for photoreceptors specification. Finally, we also discuss the possible functions of the Opsin3.2 cells based on their molecular fingerprint, and we suggest that they are involved in a variety of signaling pathways, including those entailing the thyrotropin-releasing hormone.

Keywords: opsin; photoreceptors; evolution; sea urchin

1. Introduction

The ability to perceive and respond to environmental conditions, such as light and temperature variations, is fundamental to all organisms. Moreover, many of these conditions undergo cyclic variations and many organisms, including animals, developed mechanisms to anticipate and synchronize their activities to the external periodicities: e.g., circadian and circalunar rhythms [1,2]. One of the main sources of information to entrain such rhythms is light [3]. Nonetheless, light is used to drive a variety of other behaviors and physiological processes. Well-known are the roles of light in spatial vision, which requires specialized structures such as eyes or eyespots and a specific organization of the nervous system [4], and the one in non-directional UV-avoidance behaviors that have been widely described in marine larvae [5].

Light perception is executed by specialized photoreceptor cells (PRCs). In order to increase the photosensitive surface available, PRCs possess different types of membrane extensions [6,7] and can be classified accordingly. Bilaterians mainly possess two types of PRCs: ciliary photoreceptors (c-PRCs) and rhabdomeric photoreceptors (r-PRCs). These two types of photoreceptors are diversified both at morphological and molecular levels, with the latter being characterized by different transcription factor (TF) repertoires guiding their specification and distinct phototransduction cascades [7].

The molecules that allow photoreceptors to translate light stimuli into intracellular signals are proteins called opsins. Animal opsins are membrane proteins, members of the G-Protein Coupled Receptor (GPCR) family, which are able to detect light stimuli due to a Schiff base linkage to a

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chromophore [8,9]. The chromophore is usually 11-*cis*-retinal and the linkage happens at a specific lysine residue situated in the VII helix (K296 in the bovine rhodopsin, the first opsin to be fully characterized at the level of primary amino acid sequence and 3D structure and often used as reference for comparisons with other opsins [8,10]. The presence of this specific residue, therefore, can be used as a fingerprint indication to identify a GPCR as bona fide opsin.

Phylogenetic analyses have revealed a widespread distribution of opsins in metazoans and have classified them into a variety of main groups, depending on which phyla were taken into account for the analysis [9,11–14]. Recent studies have classified opsins into the following groups: ciliary opsins (or c-opsins, containing vertebrate visual pigments), rhabdomeric opsins (or r-opsins, containing for example the melanopsins), neuropsins, peropsins, RGR opsins, Go-opsins and cnidopsin [13,15–19].

Apart from the opsin classification, the molecular events following the opsin activation have been studied in great detail. When the chromophore absorbs a photon, it undergoes a conformational change becoming all-*trans*-retinal and activating the protein. The activated opsin subsequently binds a G-protein (guanine nucleotide-binding protein) activating a specific phototransduction cascade, depending on which G-protein type is involved. For instance, vertebrate visual opsins recruit Gt proteins with subsequent decrease in the intracellular cGMP levels, closure of ionic channels and final cell membrane hyperpolarization, which eventually inhibits the glutamate neurotransmitter release. In contrast, melanopsins and many invertebrate opsins, have been shown to activate a Gq-mediated transduction cascade. This involves a signal cascade initiated by the enzyme Phospholipase C (PLC) and eventually induce membrane depolarization (reviewed in [14,20]). Moreover, a phototransduction cascade mediated by Go-opsins was discovered in scallop hyperpolarizing photoreceptors [21]. In contrast, Go-Opsin1 has been reported to mediate depolarization in *Platynereis* [22].

Ciliary and rhabdomeric photoreceptors were taught to be typically used for vision in deuterostome and protostome eyes, respectively. However, the finding of ciliary PRCs in protostomes [23–25], rhabdomeric photoreceptors in deuterostomes [26–28], and the molecular similarities shared by PRC types among different clades suggest that Urbilateria possessed both photoreceptor types [6,29,30]. Nonetheless, how these two different types of photoreceptors evolved still remains an open question. The sea urchin *Strongylocentrotus purpuratus* represents an interesting case to address this questions since it belongs to deuterostomes but utilizes both rhabdomeric and ciliary opsins [31,32] and its genome encodes for nine opsins: Opsin1, Opsin2, Opsin3.1, Opsin3.2, Opsin4, Opsin5, Opsin6, Opsin7 and Opsin8 [11]. Despite the description of the wide repertoire of opsins encoded in the sea urchin genome, very little is known on their expression patterns and functions. Recently, the sea urchin extraocular photoreceptor cells in *Paracentrotus lividus* at mature rudiment (when the adult rudiment formed in the left side of an 8-armed larva is fully mature and ready to go through metamorphosis) and juvenile stages was investigated. In brief, while the mature rudiment expresses only Opsin2 positive and Opsin4 positive r-PRCs, juveniles express all the aforementioned Opsins (Opsin1, Opsin2, Opsin3.1, Opsin3.2, Opsin4, and Opsin5) except Opsin6 and 7 [33]. Moreover, the study of Opsin1 and Opsin4 PRCs in the juveniles of *P. lividus* revealed an expression pattern similar to their orthologous in *S. purpuratus* juveniles [27,34], suggesting conservation of their functions in the two species. Less is known about Opsin expression in sea urchins at larval stages. The Go-Opsin, known as Opsin3.2 in *S. purpuratus* and *P. lividus*, was found expressed in two cells located at the sides of the apical organ in *S. purpuratus* [35] and *Hemicentrotus pulcherrimus* [36]. The Sp-Opsin3.2 expressing PRCs, which have been previously described as non-directional [35], also express evolutionary conserved developmental transcription factors similar to what is found in c-PRCs of other animals, typically deploying a c-opsin. However, it remains to be clarified if the regulatory programs of ciliary and Go-Opsin photoreceptor cells derive from the same ancestor photoreceptor cell program and, in this case, how they diversified during evolution.

Sea urchin larvae also express the Opsin2, in a few cells localized in the oral and post oral arms, likely belonging to a mesenchymal cell population [37]. Sea urchin Opsin2 was originally identified as belonging to the echinoderm specific Echinopsin A type [11], but most recently it has been re-

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clustered in a new group called Bathyopsins [17]. Nonetheless, nothing else is known about the sea urchin larva Opsin2 photoreceptors in terms of regulatory state and cell identity.

The single cell omics approaches have enabled the identification of cell types and helped in reconstructing their evolution at an unprecedented level. Single cell RNA sequencing (scRNA-seq) has been successfully applied to echinoderm embryonic and larval stages to investigate animal evolution from a cell type perspective [38–40]. In addition, scRNA-seq of *P. lividus* mature rudiments has revealed the presence of distinct PRC populations utilising an evolutionary conserved photoreceptor regulatory program [33].

Here we used fluorescent *in situ* hybridization (FISH) and immunohistochemistry (IHC) to identify and trace, during *S. purpuratus* development, a Go-Opsin (Sp-Opsin3.2) positive neuronal cell type. Using single cell transcriptomics, we were able to reconstruct the molecular identity of these photoreceptor cells, contributing to disentangling their evolution and predicting their function. Moreover, we compared the profiles of the Opsin3.2 positive cells with the single cell transcriptomes of the other PRCs present in the *S. purpuratus* larva, the Opsin2 positive cells [37]. Through this analysis, we showed that the Opsin3.2 PRCs employ an evolutionary conserved photoreceptor genetic program and are able to produce multiple neurotransmitters, suggestive of multifunctionality; we also partially reconstructed the putative phototransduction cascade activated within these cells, as predicted by single cell transcriptomics. Furthermore, comparison between the two photoreceptors showed many similarities but also significant differences at the level of TFs expressed and activated transduction cascade. For example, while the Opsin3.2 PRCs are neurons associated to the apical plate of the larva, thus possibly from ectodermal origin, the Opsin2 PRCs have a mesodermally derived muscle and immune cell identity. Last but not least, we investigated the presence of the circadian rhythm machinery in the Opsin3.2 and Opsin2 PRCs finding no significant expression of genes that were suggested to be part of the sea urchin master clock [41].

2. Materials and Methods

2.1. Animal Husbandry

Adult *S. purpuratus* were obtained from Patrick Leahy (Kerckhoff Marine Laboratory, California Institute of Technology, Pasadena, CA, 648 USA) and maintained in circulating seawater at Stazione Zoologica Anton Dohrn in Naples at 15°C. Gametes were obtained by vigorous shaking the adult sea urchins. The sperm was collected dry using a Pasteur pipette and stored at 4°C until usage. To collect eggs, females were inverted over a beaker filled with diluted 9:1 (9 parts Mediterranean Sea seawater and 1 part distilled water) FMSW. About 20 ml of eggs were fertilized, adding a few drops of sperm diluted 1:10000. Embryos were transferred in FMSW and reared at 15 °C under a 12h light/12h dark cycle. Larval cultures were maintained by exchanging half of the FMSW with fresh FMSW 2 times per week. After 3 days post fertilization (dpf) pluteus stage, the larvae were fed 3 times per week with the unicellular micro-algae *Dunaliella sp* at an approximate concentration of 1000 cells/mL.

2.2. Fluorescent In Situ Hybridization (FISH) and Immunohistochemistry (IHC)

Whole mount RNA Fluorescent *in situ* hybridization and combined FISH-IHC were performed as described in [42,43]. Summarizing, specimens at different developmental stages were collected and fixed in Fixative I (4% PFA in 0.1M MOPS and 0.5M NaCl) for at least one night at 4°C. subsequently, samples were washed 3 times with MOPS buffer (0.1M MOPS, 0.5M NaCl, 0.01% Tween 20) for 15 minutes at RT, dehydrated in 70% ethanol and finally stored at -20°C until usage. Antisense probes were transcribed from linearized DNA and labelled during transcription using Digoxigenin (Roche) or Fluorescein (Roche) labelled ribonucleotides following the manufacturer's instructions. Trh probe, instead, was DNP labelled as described in details in [42,43]. Primer sequences used for cDNA isolation and probes synthesis are included in Supplementary Table S1. Fluorescent signal was developed via using fluorophore conjugated tyramide technology (Perkin Elmer, Cat. #NEL752001KT). For combined FISH-HIC, after tyramide amplification step, samples were incubated in blocking (containing 1 mg/ml Bovine Serum Albumin and 4% Sheep Serum in PBS) for 1h at RT,

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then transferred in primary antibody diluted 1:400 in blocking O/N at 4°C. Samples were washed 4-6 times with PBS 1x, then stained with appropriate Alexa Fluor secondary antibodies (488 rabbit, 555 rat) diluted 1:1000 in blocking, and finally washed 4-6 times with PBS 1x. DAPI (10 mg/ml stock) was added to the samples at a final dilution of 1:10000 to stain nuclei. Specimens were imaged using a Zeiss LSM 700 confocal microscope and pictures analyzed using ImageJ. To stain the Opsin3.2 positive cells we used an anti Sp-Opsin3.2 gifted by Dr. Robert D Burke. To stain the TRHergic cells we used a custom antibody produced for us by GenScript adapting the method used by [44]. Briefly, the Sp-TRH mature amidated peptide (QYPGa) was coupled to an immunogen (Keyhole limpet haemocyanin, KLH) via an N-terminal cysteine. Subsequently the KLH-CQYPGa was used to immunize rabbits. Sera were affinity purified against the antigen by the company which obtained nine antibody fractions. All the fractions were tested by Elisa with different sea urchin neuropeptides to find the most specific ones which were used to perform immunohistochemistry [45].

2.3. Larvae Dissociation

Dissociation of the five dpf *Strongylocentrotus purpuratus* plutei into single cells was performed as described in [40]. In brief, Larvae were collected, seawater was removed, and larvae were resuspended in Ca2+ Mg2+-free artificial sea water and then passed to dissociation buffer containing 1 M glycine and 0.02 M EDTA in Ca2+ Mg2+-free artificial sea water. Larvae were incubated for 10 min on ice and mixed gently via pipette aspiration every 2 min. From that point and onwards the progress of dissociation was monitored. Cell viability was assessed via using Propidium Iodide and Fluorescein diacetate and only specimens with cell viability ≥ 90 % were further processed. Single cells were counted using a haemocytometer and diluted according to the manufacturer's protocol (10x Genomics). Throughout this procedure samples were kept at 4 °C.

2.4. Single-Cell RNA Sequencing and Data Analysis

Single cell RNA sequencing was performed using the 10x Genomics single-cell capturing system. Specimens from two independent biological replicates were loaded on the 10x Genomics Chromium Controller. Single cell cDNA libraries were prepared using the Chromium Single Cell 3' Reagent Kit (Chemistry v3). Libraries were sequenced by GeneCore (EMBL, Heidelberg, Germany) for 75 bp paired-end reads (Illumina NextSeq 500). ScRNA-seq output reads were aligned and analyzed using Cell Ranger Software Suite 3.0.2 (10x Genomics) The genomic index was made in Cell Ranger using the *S. purpuratus* genome version 3.1 [46,47]. Cell Ranger output matrices for two biological replicates were used for further analysis in the Seurat v4 R package [48]. The analysis was performed according to the Seurat scRNA-seq R package documentation [48,49]. Genes that are transcribed in less than three cells and cells that have less than a minimum of 250 transcribed genes were excluded from the analysis. Datasets were normalized and variable genes were found using the vst method with a maximum of 2000 variable features. Data integration was performed via identification of anchors between the two different objects. Nearest Neighbor (SNN) graph was computed with 36 dimensions (resolution 1.0) to identify the clusters. Uniform Manifold Approximate and Projection (UMAP) was used to perform clustering dimensionality reduction. Cluster markers were found using the genes that are detected in at least 0.01 fraction of min.pct cells in the two clusters. The 3 dpf scRNA-seq [40,50] and 5 dpf datasets were merged using the harmony package (v0.1.0._and 30 dimensions) [51] Transcripts of all genes per cell type were identified by converting a Seurat DotPlot with all these transcripts as features into a table (ggplot2 3.2.0 R package). Subsetting analysis was performed by selecting all the cells present in neurons 4 and immune cells that express Opsin3.2 and Opsin2 respectively, through the Subset function incorporated into the Seurat R package.

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3. Results

3.1. Expression Pattern of Opsin3.2 (Go-Opsin) during Larval Development

To investigate the expression pattern of the *Sp-Opsin3.2* gene during *S. purpuratus* larva development, we combined IHC and FISH performed on larvae collected at different developmental stages, from the early 2-armed pluteus (3 dpf) to the 8-armed pluteus (5 wpf). At the 3 dpf pluteus stage, *Sp-Opsin3.2* FISH revealed the presence of one or two positive cells located in the area above the mouth, distally to the larval apical organ (Figure 1, A-B'). The apical organ is a sensory organ present in many marine larvae involved in settlement and metamorphosis [52–54]. In sea urchin larvae, it is located between the oral arms, just above the mouth, and mainly consists of a group of serotonergic neurons [31,52,55]. In the 4 dpf larvae, the number of cells positive for *Sp-Opsin3.2* transcripts increases to two or three (Figure 1, C-D'), while at 5 dpf they reach a total number of 3 to 4 cells (Figure 1, E-F). At 6-arm pluteus stage (4 wpf), we could detect the presence of Opsin3.2 ganglia (Figure 1, G-G'), situated in the same region in respect to the apical organ right above the mouth and below the joint with the oral arms. These ganglia remain up to the 8-arm competent to metamorphosis pluteus larva (5 weeks post fertilization) (Figure 1, G-H'').

Previous studies have demonstrated the presence of neurons that produce the Thyrotropin releasing hormone neuropeptide (TRH) in cells located in similar regions as the Opsin3.2 positive neurons [40,56]. In order to understand whether the TRH positive cells (TRHergic neurons) are in close proximity to the Opsin3.2 PRCs and if TRH could be produced by the Opsin3.2 PRCs, we performed double FISH and double IHC for Sp-Opsin3.2 and Sp-Trh. Interestingly, the co-localization of both proteins and *Sp-Trh* transcripts in the same neurons of the 3 dpf pluteus larva (Figure 1, B and B') indicate that Opsin3.2 PRCs are able to produce the neuropeptide TRH. Similarly, at 4 and 5 dpf pluteus stages, all the Sp-Opsin3.2 positive cells are also TRHergic, as shown by *Sp-Opsin3.2*/*Sp-Trh* double FISH combined with Sp-TRH immunostaining (Figure 1, F) and TRH/Opsin3.2 double immunostaining combined with *Sp-Trh* FISH (Figure 1, B-D')

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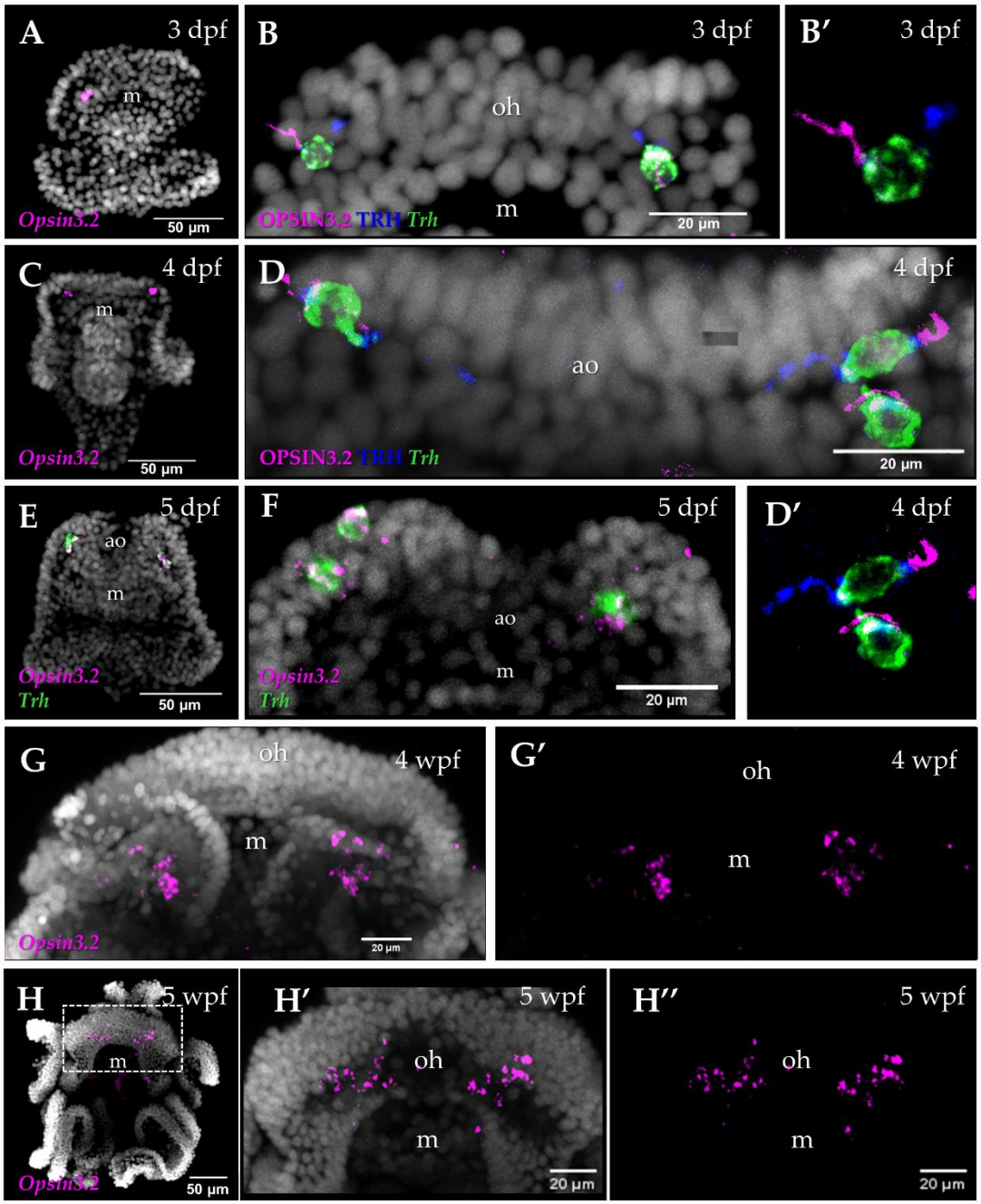

Figure 1. Molecular characterization of the Opsin3.2 cells at different developmental stages. (**A**) FISH of *Sp-Opsin3.2* stains 1 cell at 3 dpf. (**B**) FISH for *Sp-Trh* was combined with double TRH/Opsin3.2 immunolocalization at 3 dpf, highlighting the bipolar structure of these sensory/neurosecretory neurons with the Opsin3.2 localized on one a ciliated-like structure located on the external side of the larva. The TRH peptide, instead, is concentrated in the opposite side of the cell and it appears to be transported along the projections of the cells (directed toward the larva apical organ). (**B′**) Details of the cell showed in B on the left. (**C**) FISH of *Sp-Opsin3.2* at 4 dpf. (**D-D′**) FISH for *Sp-Trh* was combined with double TRH/Opsin3.2 immunolocalization at 4 dpf. All the 3 cells have a bipolar organization. (**E- F**) Double FISH of *Sp-Opsin3.2* and *Sp-Trh* at 5 dpf stains 3-4 cells. FISH of *Sp-Opsin3.2* at 4 wpf (**G-G′**) and 5 wpf (**H-H″**) detect two cluster of cells located at the base of the oral arms. All images are stacks of merged confocal Z sections. Nuclei are shown in white.

3.2. Characterization of the Go-Opsin3.2 and the Opsin2 Positive Cell Types at a Single Cell Resolution

ScRNA-seq is a powerful technique allowing to identify cell clusters or populations and finely disentangle their regulatory state (i.e. TFs expressed) and predict possible functions and interactions

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among cell types (based on which genes, signaling molecules or receptors are expressed). Such an approach was successfully applied to identify and characterize sea urchin larval cell populations [40,50,57] at early stages. Compared to the 3 dpf pluteus, more Opsin3.2 positive cells are present in the 5 dpf pluteus (See Figure 1). This observation, prompted us to perform scRNA-seq on *S. purpuratus* 5 dpf pluteus larvae and to combine it with the already available 3dpf pluteus dataset to investigate PRC diversity and the Go-Opsin3.2 molecular fingerprint. Our analysis included single cell transcriptomes from 32,116 cells and resulted in the identification of 21 clusters corresponding to distinct cell types or closely related cell type families (Figure 2, B and C). The quality of the clustering analysis was assessed using gene markers previously shown to label distinct cell type families at the 3 dpf *S. purpuratus* pluteus stage: *Sp-Fbsl_2* and S*p-Btub2/3* (ciliary band); *Sp-Hbn* and *Sp-Frizz5/8* (apical plate); *Sp-Spec2a* and *SPU_006199* (aboral ectoderm); *Sp-SynB* and *Sp-Chrna9_4* (neurons); *Sp-Mhc* and *Sp-Mlckb* (esophageal muscles), S*p-Nan2* and *Sp-Vasa* (coelomic pouches);, *Sp-MacpfA2* and *Sp-Pks1* (immune cells); *Sp-185/333B3d* and *Sp-Fcoll/II/IIIf* (blastocoelar cells); *Sp-Msp130* and *Sp-Sm37* (skeletogenic cells); *Sp-Hox11/13b*, *Sp-Cdx* and *Sp-Rfxc1l* (posterior gut*), Sp-Nkx6.1* and *Sp-Pdx* (pyloric sphincter); *Sp-Hnf4*, *Sp-ManrC1a* (stomach); *Sp-Ptf1a* and *Sp-Cpa2L* (exocrine pancreas-like); *Sp-Ahrl* and *Sp-Trop1* (cardiac sphincter); *Sp-Alpi* and *Sp-Brn1/2/4* (esophagus/oral ectoderm). Plotting of the average expression of the aforementioned gene markers resulted in a meaningful clustering reconstructing most of the well known larval cell types (Figure 2, B and C).

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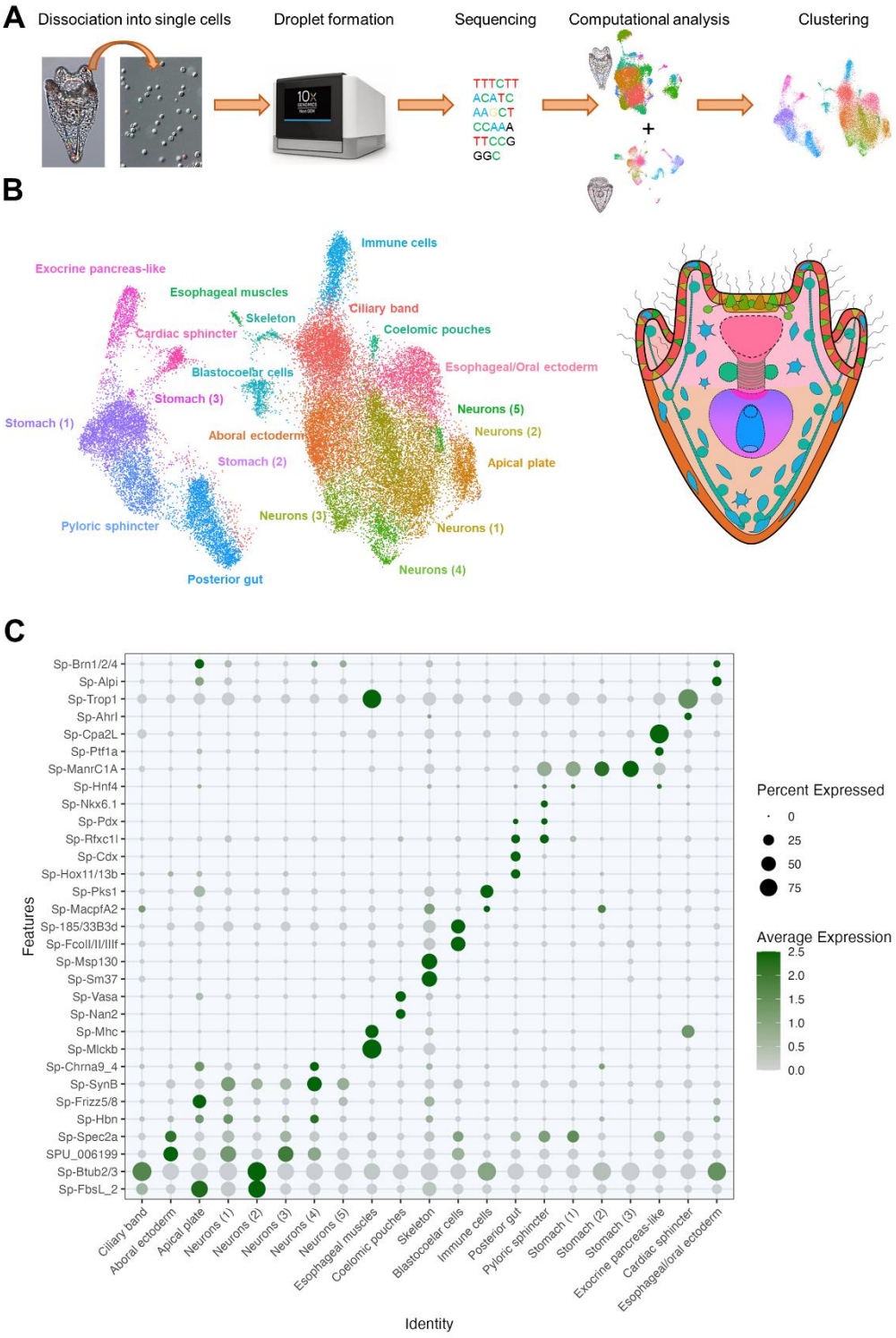

Figure 2. Cell type atlas of the *S. purpuratus* larvae at 3 and 5 dpf pluteus stages. (**A**) Schematic representation of the scRNA-seq pipeline. (**B**) UMAP representing the cell clustering obtained through harmony of the 3 and 5 dpf pluteus single cell datasets and their localization in a schematic representation of a 4-armed *S. purpuratus* larva. (**C**) Dotplot showing the average expression of a subset of genes used as markers to annotate the different cell clusters.

To identify which cell clusters express our Go-Opsin of interest, the average expression of *Sp-Opsin3.2* was plotted in each cluster (Figure 3, A and B). These data show that only the Neurons (4) cluster contains cells that highly express the Opsin3.2 (Figure 3, B). Notably, this is also the cluster having the most significant *Sp-Trh* expression levels (Figure 3, A). We next plotted the *Sp-Opsin2*

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average expression and found it enriched in esophageal muscles and immune cells suggesting a mesodermal origin for these cells, as already shown in another sea urchin species [37]. Moreover, our single cell data indicate that the *Opsin2* positive cells belonging to the immune cells cluster could possibly be pigment cells, as judged by the co-expression of the pigment cell marker *Sp-Pks1* in (Figure 3, A). For our analysis, we choose to focus on the Opsin2 PRCs belonging to the Immune cell population (Figure 3, B), therefore all of the following plots including Opsin2 positive cells have been produced selecting only the Pks1/Opsin2 positive cells.

Finally, to confirm the quality of our data, we plotted the average expression of transcription factors that were found through FISH to be either co-expressed with Opsin3.2 cells or in the Opsin3.2 region [18]. The list includes, but it's not limited to *Sp-Otx*, *SpTbx2/3* and *Sp-Six3* which have been suggested to be part of an ancestral photoreceptor specification code (Figure 3, C) [6]. The average expression of the selected genes was plotted in all the cell clusters and the main accumulation was found in the Apical Plate and Neuron (4) cells, while the Immune Cells had high expression only of the transcription factor *Sp-Id* (IDs are a family of helix-loop-helix proteins involved in regulating cell proliferation and differentiation) and low expression levels of *Sp-Tbx2/3*.

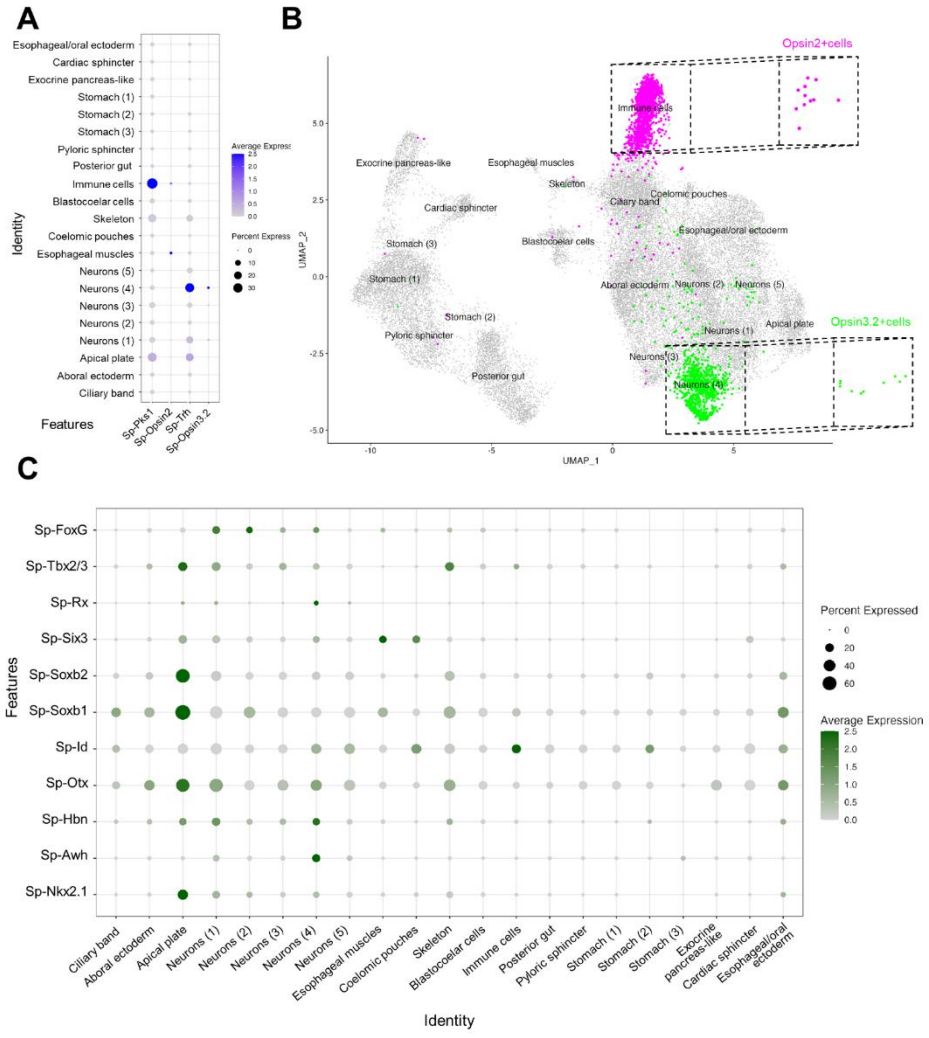

Figure 3. Identification of the Opsin3.2 and Opsin2 PRCs. (**A**) Dotplot showing the expression of *Sp-Osin3.2* and *Sp-Osin2* and of the neuropeptide *Sp-Trh* and of the Immune cells marker *Sp-Pks1*. (**B**) UMAP illustrating that Opsin3.2 PRCs have been isolated from the Neuron (4) cluster while Opsin2 have been selected from the Immune cells cluster. (**C**) Dotplot showing the expression pattern of genes selected from Valencia et al., 2021.

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3.3. Molecular Signature of Photoreceptor Cells in S. purpuratus Larvae

To gain additional information on how the Opsin3.2 cells work, we exploited the scRNA-seq data to explore their molecular signature and compared it to the molecular signature of the Opsin2 PRCs. In particular, the analysis was specifically targeted to the Opsin3.2 expressing cells and the Opsin2 positive cells belonging to the Immune cell cluster.

Go-Opsin photoreceptors in invertebrates have been found to be either depolarizing, *P. dumerilii* [22] or hyperpolarizing, scallop [21]. To determine which type of signaling is activated by the stimulation of the Opsin3.2 PRCs in the sea urchin larva, the expression levels of all the *S. purpuratus* genes that are known to be part of phototransduction cascades in other organisms were plotted in the Opsin3.2 cells (Figure 4, A). Such gene expression profiles were plotted also in the Opsin2 cells belonging to the Immune cells to investigate the differences of the two cascades (Figure 4, A). Unexpectedly, the G-protein type most highly expressed in the Go-Opsin PRCs are the *Sp-Gs*, *Sp-Gi*, and *Sp-GbA*, while the G proteins annotated as *Sp-Go* and *Sp-GoI* (https://www.echinobase.org/entry/) are lowly expressed, suggesting that the first types of G proteins activated by the Go-Opsin3.2 are Gs, Gi and GbA. Nonetheless, other putative components of the phototransduction cascade are only lowly expressed and it is therefore difficult to make any further prediction on the activated cascade. In any case, intriguing is the expression, although in a small percentage of cells and at low levels, of many genes annotated as phosphodiesterases (PDEs), which might be indicative of a hyperpolarizing cascade, similarly to what happens in mammalian eye photoreceptor cells (see the example in [14]. Looking at the genes from the phototransduction cascade expressed in the Opsin2 cells, the overall picture is relatively similar to the Opsin3.2 PRCs in terms of G-protein expressed and their expression levels, however the Opsin2 cells do not show any significant expression of the other putative genes involved in the phototransduction cascade.

Furthermore, we investigated the possibility that the Opsin3.2 protein might be involved in the entrainment of circadian clock (Figure 4, B). The *S. purpuratus* genome encodes for several putative orthologues of the components of the canonical animal circadian clocks: e.g. Clock (*Sp-Clock*), Timeless (*Sp-Tim*), Brain and muscle Arnt-like protein (*Sp-Bmal*), Cryptochromes (*Sp-Dcry* and *Sp-Vcry*), Hepatic leukemia factor (*Sp-Hlf*) [31]. Interestingly, of the canonical core genes, the Opsin3.2 positive PRCs express only *Sp-Clock* and at low levels (Figure 4, B). Nonetheless, they also express *Sp-Shaggy* which, in other species, is responsible for the phosphorylation of Tim, and therefore regulates its function. The Opsin3.2 PRCs also highly express *Sp-Hlf*, which was found to oscillate during the diel cycle (but not in free running condition) [41]. Finally, *Sp-Sim*, *Sp-Slimb*, *Sp-Hey*, and *Sp-Nfil3* are expressed at low levels in the Opsin3.2 PRCs. On the contrary, the Opsin2 PRCs (Figure 4, B) express only *Sp-Hlf* and *Sp-Shaggy* and at levels comparable to those found in the Opsin3.2 positive cells. In addition, they have low levels of *Sp-6_4photolyase* (photolyases are enzymes that repairs DNA damages caused by UV-light, [58]) and *Sp-Ncoa*.

Based on the evidence that the Opsin3.2 positive cells are neuron belonging to the Neuron (4) cluster, we investigated which type of neuronal genes are found in these PRCs taking advantage of the available comprehensive characterization of the larval nervous system [40] (Figure 4, C). Notably, the Opsin3.2 cells express not only genes involved in photoreceptor specification (*Sp-Otx*, *Sp-Six3*, and *Sp-Rx*) but also genes involved in neuron/anterior neuroectoderm patterning (*Sp-Nkx2.1*, *Sp-Hbn*, *Sp-Soxb1*, *Sp-SoxC* and *Sp-Brn1/2/4*). Looking at the signaling molecules, the gene having the highest expression level is the *Sp-Trh*, neuropeptide, which is evidence for the double function of the Go-Opsin PRCs as both photoreceptor and neurosecretory cells. Moreover, it has been shown, both combining IHC and FISH and by scRNA-seq, that the TRHergic cells also produce another neuropeptide, Sp-Salmfap [56,59].

To conclude, we found also that both Opsin3.2 and Opsin3 cell populations express the TFs *Sp-Otx* and *Sp-Id* which are fundamental for general photoreceptor specification; while *Sp-Rx*, expressed in ciliary photoreceptors in many animals [18], is expressed only by the Opsin3.2 positive cells (Figure 4, E).

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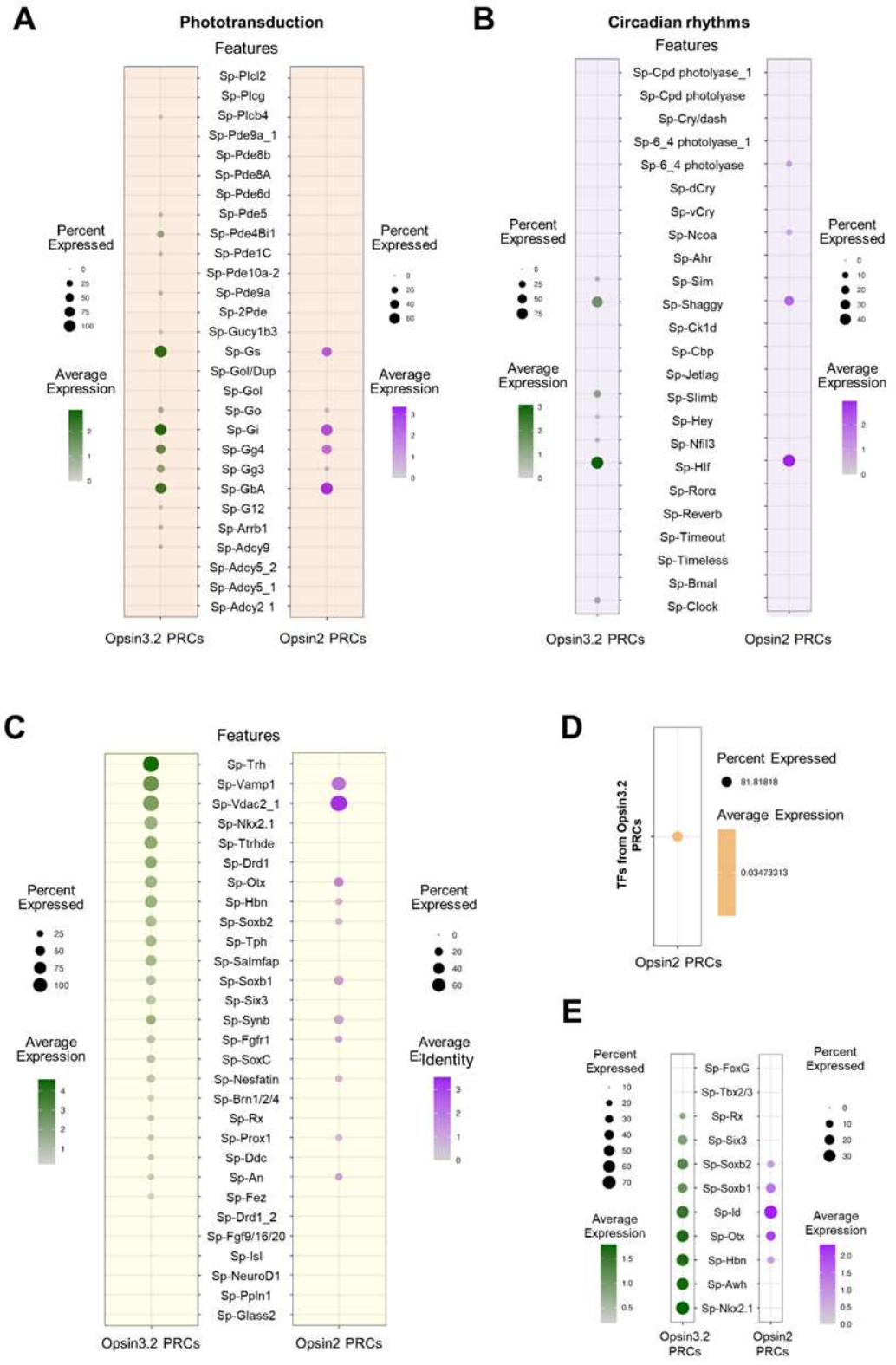

Figure 4. Molecular signature of *S. purpuratus* PRCs at pluteus stages. (**A**) Dotplot showing the average expression of genes putatively involved in the phototransuction cascade activated by the opsins stimulation. (**B**) Dotplot showing the average expression of genes involved in the regulation circadian rhythms. (**C**) Dotplot showing the average expression of genes selected to further describe the molecular identity of the Opsin3.2 and Opsin2 PRCs. (**D**) Dotplot showing the percentage of TFs common to the two PRCs. (**E**) Dotplot showing the average expression of known TFs involved photoreceptor specification.

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4. Discussion

Main aim of this work was to characterize the Opsin3.2 photoreceptors found in *S. purpuratus* larvae. Nonetheless, during our analysis we encountered a second photoreceptor cell type known at this stage, the one expressing the Sp-Opsin2. Opsin2 PRCs have been already described not only in *S. purpuratus* [18], but also in other sea urchin species (*Hemicentrotus pulcherrimus*, S. Yaguchi et al., 2022) and showed to be a peculiar photoreceptor group, probably rising from a mesenchymal cell population. Indeed, our data also found a group of Opsin2 positive cells belonging to the Immune cell cluster. Comparison of the molecular signature of Opsin3.2 and Opsin2 PRCs showed many similarities in the TFs involved in their specification (both express *Sp-Soxb1*, *Sp-Soxb2*, *Sp-Id*, *Sp-Otx*, and *Sp-Hbn*), in the phototransduction cascade activated and in genes deployed for circadian rhythm establishment, with minor differences. Subsequently, despite the evidence that Opsin2 PRCs do not belong to a neuronal population, they still express typical neuronal markers. Major differences arose when we looked deeply into the molecular identity of the two PRCs in terms of neuropeptides, receptors and other TFs involved in neuronal specification. For example, Opsin2 do not express the *Sp-Trh* neuropeptide, and the *Sp-Drd1* dopamine receptor. Nonetheless, further comparison of the differences between the two photoreceptor cell types was outside the scope of this work and we will not further discuss the data collected about Opsin2.

4.1. Opsin3.2 Cells Characterization during S. purpuratus Larval Development

The presence of two Opsin3.2 positive cells at the sides of the apical organ in the *S. purpuratus* sea urchin larva was already reported [18,35] but no information was available about their developmental origin. Therefore, first we combined immunohistochemistry and fluorescent in situ hybridization to identify the Opsin3.2 producing cells in larvae ranging from early 4-armed to late 8-armed pluteus stages (Figure 1). Interestingly, the number of Opsin3.2 cells appeared to increase during larval development in a non-stereotypical way, going from 1-2 cells at early pluteus to two clusters of up to 6 cells bilaterally distributed at either side of the apical organ, at the late pluteus stage (4-5 wpf) (Figure 1). Moreover, combined staining of *Sp-Opsin3.2* and *Sp-Trh* provided the first evidence for a dual sensory/neurosecretory role of those cells, also displaying a bipolar morphology, with a cilium-like structure hosting the Go-Opsin molecules on one side and the axon transporting the TRH neuropeptide on the other side of the cell. Nonetheless, to prove that these cells are ciliary PRCs, the morphology of the ciliated structure requires further investigation using Electron Microscopy.

4.2. Opsin3.2 cells utilize an ancestral gene regulatory toolkit

Furthermore, we investigated the molecular fingerprint of the Sp-Opsin3.2 photoreceptors and showed that they utilize an ancestral regulatory toolkit. Namely, they express TFs required for neuron (*Sp-SoxC* and *Sp-Brn1/2/4*), and anterior neuroectoderm (*Sp-Hbn*, *Sp-Six3*) specification in sea urchins [60]. Moreover, the expression of *Sp-Otx* and *Sp-Six3* supports the ancestral module suggested by [6] to be present in the precursor of all animal photoreceptors. According to this hypothesis, the ancestral photoreceptor cells utilized a variety of Opsin types (at least 9 in the Bilaterian ancestror [17]) and gave rise to the two sister cell types known as ciliary and rhabdomeric photoreceptors. Each of them also co-opted specific TFs such as Rx in ciliary photoreceptors. Intriguingly, Sp-Go-Opsin3.2 PRCs also express the TF *Sp-Rx* and have been described as belonging to the ciliary-type [18]. The hypothesis for the common evolutionary origin of ciliary and rhabdomeric photoreceptors is supported by the evidence that *P. dumerilii* possesses rhabdomeric photoreceptors expressing not only r-opsins but also Go-Opsin1 both in the adult eye and in the larval eyespot [22]. This latter opsin type, is most commonly associated with ciliary type PRCs (e.g., in scallop and sea urchins [18,21]. This hypothesis is also supported by the existence of a r-PRCs utilizing both xenopsin and r-opsin in the larval eye of the mollusk *Leptochiton asellus* [19]. However, whether Urbilateria, the common ancestor of Bilateria, already possessed both ciliary and rhabdomeric photoreceptors, or still had a single bimodal ciliary/rhabdomeric precursor cell is not

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yet clear and only analyzing the regulatory toolkit, the morphology and the opsin repertoire of photoreceptor cells in diverse early branching bilaterians animals will help disentangling this mystery.

4.3. Opsin3.2 Cells as Sensory and Neurosecretory Unit

In addition to their photoreceptor signature, Opsin3.2 PRCs are also able to produce a variety of signaling molecules, mostly neuropeptides (TRH, An, FSALFa/Salmfap, and Nesfatin), and acetylcholine [40,56]. Therefore, they look to be both sensory and neurosecretory cells.

It has been proposed that the Urbilateria already possessed a simple nervous system containing cells which were both sensory and neurosecretory. In animals having a more complex nervous system, the sensory signal collected by a specified sensory cell is transferred to the central nervous system through a series of interneurons. Here, the information is integrated with the signals received by other sensory cells and the final output is then transferred to an effector neuron which might be a motor neuron or endocrine cells for example. In less complex nervous systems, such as the case of Urbilateria, on the contrary, one cell can both collect the sensory stimulus and elicit the response, thus producing a very simple circuit or minimal module capable of controlling behavioral or physiological process [61,62]. Among these minimal modules, photosensitive-neuroendocrine cells could control neurohormones secretion under particular light conditions (Tessmar-Raible et al. 2007). The Opsin3.2 cells could be the descendants of such cells, retained in the sea urchin larva. Intriguingly, a cluster of non-visual photoreceptors expressing the vertebrate ancient long opsin b (valopb) was found in the brain of adult *Danio rerio* [63], suggesting an interesting evolutionary scenario for an ancestral TRHergic/photoreceptor cell cluster, which could be tested looking for the existence of TRHergic/Opsin positive neurons in other phyla.

4.4. Opsin3.2 Cells Possible Function(s)

Considering the sensory-neurosecretory nature of the Opsin3.2 cells, it is even more interesting to understand their function. Since these are photoreceptors, the first hypothesis is that they might be involved in controlling swimming behavior in response to light. In previous works, it has been suggested that light response activated by the Go-Opsin3.2 PRCs might be directional, namely that the larva is able to distinguish from which direction light hits the photoreceptor [18]. However, in all known cases of 3D phototaxis in the water column, the cells containing shading pigments are in close juxtaposition to the photosensitive cells (as reviewed by [64]. Sea urchin pigment cells are not fixed in their position and migrate in response to infections [65,66], therefore representing an unreliable source of shading.

Nonetheless, non-directional perception of light intensity can be used by aquatic animals to adjust their vertical position in the water column [22] and to avoid high UV levels [5,29,67]. Interestingly, Yaguchi and colleagues [37] showed that *Hemicentrotus pulcherrimus* and *Temnopleurus reevesii* larvae change their swimming behavior in response to strong photoirradiation. Although the authors seem to exclude an involvement for the Go-Opsin in this response, thorough experiments including knock-down or knock-out of this opsin exposed to different wavelengths are necessary to assess the role of these PRCs in light mediated swimming behaviors. Moreover, in a previous paper, Yaguchi and collaborators [36] provide evidence for Go-Opsin PRCs controlling sphincter opening in response to light through a serotonergic signaling in *H. pulcherrimus* larva and it would be interesting to test if this is a conserved function also in *S. purpuratus* larvae.

Intriguingly, the Opsin3.2 cells in our data are also predicted to express the dopamine receptor *Sp-Drd1*. Since a dopamine signaling is involved in mediating phenotypic (developmental) plasticity in response to food availability [68], we can hypothesize that the Sp-Opsin3.2 PRCs could control this process. Another possibility is that the light stimulus perceived by the Go-Opsin3.2 is used to entrain a circadian clock. To investigate this possibility, we looked at the genes putatively involved in this process (Figure 3, D). Our scRNA-seq data do not show a prominent expression of putative core genes in the Sp-Opsin3.2 PRCs in the 3 and 5 dpf *S. purpuratus* larva, thus suggesting that the Opsin3.2 cells are involved in light perception more than circadian rhythms regulation.

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While all the evidence collected so far contributes to build hypotheses on the function of the Opsin3.2/TRHergic cells, functional experiments to knock-down or knock-out Opsin3.2, coupled with phenotypic and behavioral observations, are necessary to comprehend the function of the Opsin3.2/TRHergic cells in sea urchin larvae.

5. Conclusions

The sea urchin *S. purpuratus* larva feature a set of bilaterally symmetrical photoreceptor cells expressing the Go-Opsin3.2. This cell type deploys an ancient conserved regulatory module for photoreceptors specification. This makes them an important component to reconstruct photoreceptor/opsin system evolution, especially if combined with the analysis of photoreceptors in early branching animals. Additionally, the wide set of neuropeptides, signaling molecules and receptors expressed by these cells (including for example TRH, RX, Salmfap, An, Drd1) (Figure 5) strongly suggests their involvement in multiple processes. Nonetheless, knock-down and/or knock-out experiments are required to really understand in which process(es) the Opsin3.2 are involved.

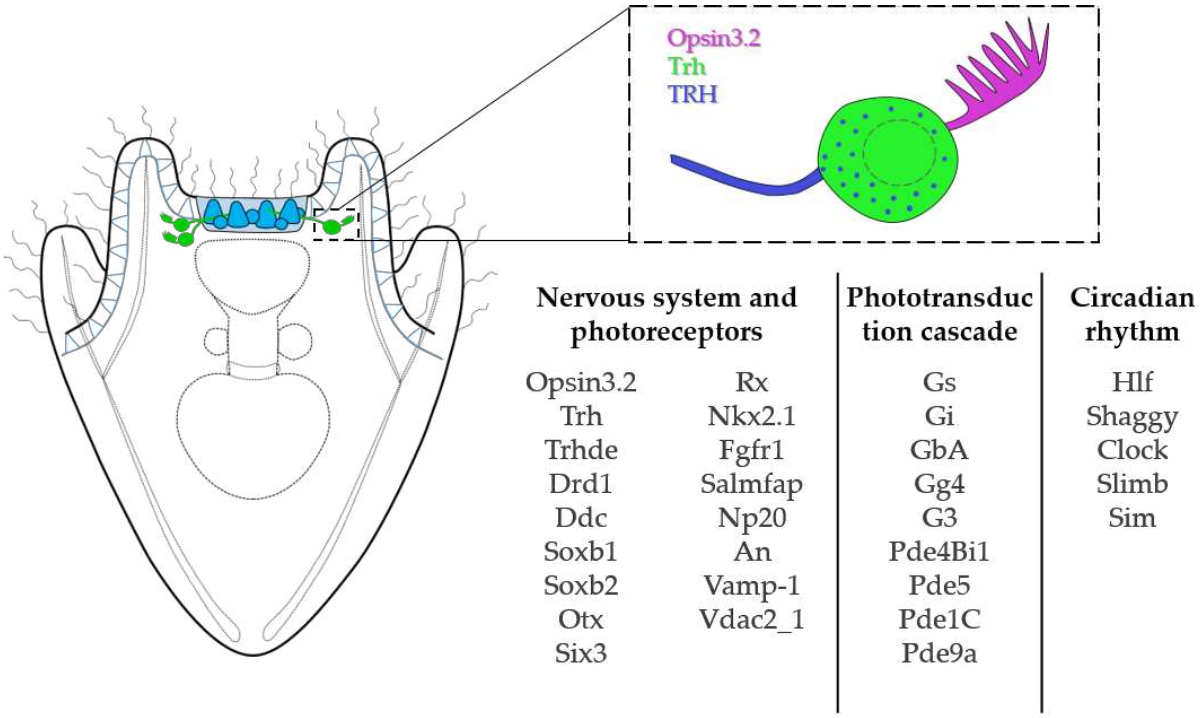

Figure 5. Opsin3.2 PRCs summary. Schematic reconstruction of the morphology, topology and molecular signature of the Opsin3.2 PRCs.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. The raw reads for the sequencing data obtained for this study are available at NCBI Gene Expression Omnibus (accession numbers TBA) Supplementary Table S1: Primers used to clone the gene of interests.

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