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Integration of temporal and spatial patterning generates neural diversity

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Summary

In the *Drosophila* optic lobes, 800 retinotopically organized columns in the medulla act as functional units for processing visual information. The medulla contains over 80 types of neurons, which belong to two classes: uni-columnar neurons have a stoichiometry of one per column, while multi-columnar neurons contact multiple columns. We show that combinatorial inputs from temporal and spatial axes generate this neuronal diversity: All neuroblasts switch fates over time to produce different neurons. The neuroepithelium that generates neuroblasts is also sub-divided into six compartments by the expression of specific factors. Uni-columnar neurons are produced in all spatial compartments independently of spatial input; they innervate the neuropil where they are generated. Multi-columnar neurons are generated in smaller numbers in restricted compartments and later move to their final position. The integration of spatial inputs by a fixed temporal neuroblast cascade thus acts as a powerful mechanism for generating neural diversity, regulating stoichiometry and the formation of retinotopy.

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Author contributions:
C.D., T.E., X.L. and M.C. planned the project and analyzed the data; X.L., T.E. and C.B. performed the antibody screen; T.E. conducted experiments with the regional and temporal genes; X.L. focused on the Tsh/Pm1/Pm2 and 27b-Gal4 experiments. M.C. analyzed the fate of neurons in the different mutant conditions and conducted experiments on the targeting of medulla neurons. R.B. performed the 27b-Gal4 in the *Rx* mutant experiment, J.N. generated the *Vsx1*-Gal4 line and contributed to the *Rx* mutant analysis. U.A. and C.K. helped with the experiments. Z.C. performed the *Optix*>G-Trace and *Vsx1*-Gal4 single neuron flip-out experiments. R.B. generated Tm2-lexA flies. L.S., N.N. and K.P.W. generated the modENCODE antibodies that were used in the screen. The manuscript was written by T.E., M.C. and C.D. and all authors commented on it.

Introduction

The *Drosophila* optic lobes, composed of the lamina, medulla and the lobula complex, are the visual processing centers[1,2]. The lamina and medulla receive input from photoreceptors in the compound eye, process information and relay it to the lobula complex and central brain. The medulla, composed of ~40,000 cells, is the largest compartment in the optic lobe and is responsible for processing both motion and color information[3,4]. It receives direct synaptic input from the two color-detecting photoreceptors, R7 and R8[5]. It also receives input from five types of lamina neurons that are contacted directly or indirectly by the outer photoreceptors involved in motion detection[6–8]. Associated with each of the ~800 sets of R7/R8 and lamina neuron projections are 800 medulla columns, defined as fixed cassettes of cells that process information from one point in space[2,9]. Columns represent the functional units in the medulla and propagate the retinotopic map established in the compound eye. Each column is contributed to by more than 80 neuronal types, which can be categorized into two broad classes[1,2]: Uni-columnar neurons have arborizations principally limited to one medulla column and there are thus 800 uni-columnar cells of each type. Multi-columnar neurons possess wider arborizations, spreading over multiple columns. They compare information covering larger receptor fields. Although they are fewer in number, their arborizations cover the entire visual field.

The medulla develops from a crescent-shaped neuroepithelium, the outer proliferation center (OPC)[10,11]. During the third larval instar, the OPC neuroepithelium is converted into lamina on its lateral side and into medulla neuroblasts on the medial side (Fig.1a; Supp. Fig. 1a)[12,13]. A wave of neurogenesis moves through the neuroepithelial cells, transforming them into neuroblasts; the youngest neuroblasts are closest to the neuroepithelium while the oldest are adjacent to the central brain[13–17]. Neuroblasts divide asymmetrically multiple times to regenerate themselves and produce a ganglion mother cell (GMC) that divides once more to generate medulla neurons (Supp. Fig.1a)[11,18]. Recent studies have shown that six transcription factors are expressed sequentially in neuroblasts as they age (Fig.1k)[19,20]: neuroblasts first express Homothorax (Hth), then Klumpfuss (Klu), Eyeless (Ey), Sloppy-paired 1 (Slp1), Dichaete (D) and Tailless (Tll). This temporal series is reminiscent of the Hb➔Kr➔Pdm➔Cas➔Grh series observed in *Drosophila* ventral nerve cord neuroblasts that generates neuronal diversity in the embryo[21–27]. Indeed, distinct neurons are generated by medulla neuroblasts in each temporal window[19,20]. Further neuronal diversification occurs through Notch-based asymmetric division of GMCs[19]. In total, over 20 neuronal types can theoretically be generated using combinations of temporal factors and Notch patterning mechanisms[19]. However, little is known about how the OPC specifies the additional ~60 neuronal cell types that comprise the medulla.

Results

The OPC neuroepithelium is patterned along the D/V axis

To understand the logic underlying medulla development, we looked for the expression of transcription factors using 215 antibodies and identified 35 genes that are expressed in subsets of medulla progenitors and neurons[19]: The OPC neuroepithelial crescent can be sub-divided along the dorsal-ventral (D/V) axis by the mutually exclusive expression of three

homeodomain-containing transcription factors (Fig.1b–d; Supp. Fig.1b,c): Vsx1 is expressed in the central OPC (cOPC), Rx in the dorsal and ventral posterior arms of the crescent (pOPC), and Optix in the two intervening 'main arms' (mOPC)[28]. These three proteins are regionally expressed as early as the embryonic optic anlage and together mark the entire OPC neuroepithelium with sharp, non-overlapping boundaries (Supp. Fig.1d)[28]. Indeed, these three regions grow as classic compartments; lineage trace experiments show that cells permanently marked in the early larva in one OPC region do not intermingle at later stages with cells from adjacent compartments at later stages (Fig.1h–j). Of note, Vsx1 is expressed in cOPC progenitor cells and is maintained in a subset of their neuronal progeny whereas Optix and Rx expression remains restricted to the mOPC or pOPC neuroepithelium (Fig. 1c,d; Supp. Fig.1b,c). The OPC can be further subdivided into dorsal (D) and ventral (V) halves: a lineage trace with *hedgehog*-Gal4 (*hh*-Gal4) marks only the ventral half of the OPC, bisecting the cOPC compartment (Fig.1f,g). As *hedgehog* is not expressed in the larval OPC, this D/V boundary is set up in the embryo[29]. Thus, six compartments (ventral cOPC, mOPC and pOPC and their dorsal counterparts) exist in the OPC (Fig.1b). The pOPC compartment can be further subdivided by the expression of the *wingless* and *dpp* signaling genes (Fig.1e)[30]. Cells in the *wingless* domain behave in a very distinct manner from the rest of the OPC, and have been described elsewhere[31,32].

The Hth➔Klu➔Ey➔Slp1➔D➔Tll temporal progression is not affected by the compartmentalization of the OPC epithelium; we observe the same neuroblast progression in the cOPC, mOPC and *dpp*-positive half of the pOPC, ventrally or dorsally (Fig.1k,l). Thus, in the developing medulla, neuroblasts expressing the same temporal factors are generated by developmentally distinct epithelial compartments (Fig.1m).

OPC compartments produce distinct neural cell types

To test whether the intersection of the D/V and temporal neuroblast axes leads to the production of distinct neural cell types, we focused on the progeny of Hth neuroblasts, which maintain Hth expression[19]. In late third instars, Hth neurons are found in a crescent that mirrors the OPC (Fig.2a). The Notch^{ON} (N^{ON}) progeny of Hth^{+} GMCs express Bsh and Ap[19,33], and they are distributed throughout the entire medulla crescent (Fig.2a,d). In contrast, the Notch^{OFF} (N^{OFF}) progeny, which are Bsh^{-}Lim3^{+}Hth^{+} neurons, express different combinations of transcription factors, and can be sub-divided into three domains along the D/V axis: (i) in the cOPC, N^{OFF}Hth^{+} neurons express Vsx1, Seven-Up (Svp) and Lim3 (Fig.2a–e; Supp. Fig.2a); (ii) in the pOPC, these neurons also express Svp and Lim3, but not Vsx1 (Fig.2a; Supp. Fig.2b); (iii) in the ventral pOPC exclusively, these neurons additionally express Teashirt (Tsh; Fig.2g,j; Supp. Fig.2d). We do not observe N^{OFF}Hth^{+} cells in the mOPC (Supp. Fig.3a). Rather, Cleaved-Caspase-3^{+} cells are intermingled with Bsh^{+} neurons (Fig.2k). When we prevent cell death, Bsh^{+}Hth^{+} cells become intermingled with neurons that express the N^{OFF} marker Lim3, confirming that the N^{OFF}Hth progeny undergo apoptosis in the mOPC (Supp. Fig.3b–d).

We could therefore distinguish three regional populations of Hth neurons (plus one that is eliminated by apoptosis) and a fourth population that is generated throughout the OPC. We set out to determine the neuronal identity of each of these populations:

1. Bsh is a specific marker of Mi1 uni-columnar interneurons that are generated in all regions of the OPC (Fig.2a,l–n)[19,33].

2. To determine the identity of $Hth^{+}N^{OFF}$ cOPC-derived neurons, we generated Hth^{+} single cell flip-out clones (using *hth*-Gal4) in the adult medulla. The only Hth^{+} neurons that are also $Vsx1^{+}Svp^{+}$ are Pm3 local multi-columnar neurons (Supp. Fig.2e; Fig.2f,l)[33]. Thus, Pm3 neurons are the $N^{OFF}Vsx1^{+}Svp^{+}$ neurons generated in the cOPC by Hth neuroblasts.

3. For $Hth^{+}N^{OFF}$ pOPC-derived neurons, we utilized *27b*-Gal4, which drives expression in larval pOPC Hth^{+} neurons and is maintained to adulthood (Supp. Fig.2c,d,f). Flip-out clones with *27b*-Gal4 mark Pm1 and Pm2 neurons (Fig. 2h,i,n; Supp. Fig.2f), as well as Hth^{-} Tm1 uni-columnar neurons that come from a different temporal window. Both Pm1 and Pm2 neurons (but not Tm1) express Hth and Svp (Supp. Fig.2f and Fig.2h,i). Pm1 neurons also express Tsh (Fig.2i), which only labels larval ventral pOPC neurons.

Thus, in addition to uni-columnar Mi1 neurons generated throughout the OPC, Hth neuroblasts generate three region-specific neuronal types: multi-columnar Pm3 neurons in the cOPC; multi-columnar Pm1 neurons in the ventral pOPC; and multi-columnar Pm2 neurons in the dorsal pOPC (Fig.2l–n).

Spatial inputs are only required for the specification of multi-columnar neurons

To determine the contribution of the temporal and spatial factors to the generation of the different neuronal fates, we mutated them and determined whether neuronal identity was lost. To test the temporal axis, we mutated *hth*. As previously reported[19,33], Bsh expression is lost in *hth* mutant clones. Loss of *hth* in MARCM clones also leads to the loss of the Pm3 marker Svp (Fig.3a) without affecting expression of Vsx1, indicating that Vsx1 is not sufficient to activate Svp and can only do so in the context of an Hth^{+} neuroblast. Hth is also required for the specification of Pm1 and Pm2 in the pOPC as Svp and Tsh expression is lost in *hth* mutant larval clones (Fig.3b and Supp. Fig.4a). Ectopic expression of Hth in older neuroblasts is not able to expand Pm1, 2 or 3 fates (based on the expression of Svp) into later born neurons (Fig.3c,d; Supp. Fig.4b), though it is able to expand Bsh expression (as previously reported[19]). Thus, temporal input is necessary for the specification of all Hth^{+} neuronal fates but only sufficient for the generation of Mi1 neurons.

We next determined if regional inputs are necessary and/or sufficient to specify neuronal fates in the progeny of Hth^{+} neuroblasts. In *Vsx1*-RNAi clones, Svp expression is lost in the cOPC but Bsh is unaffected (Fig.3e). Additionally, $Hth^{+}Lim3^{+}$ cells are absent, suggesting that N^{OFF} cells undergo apoptosis in these clones (Supp. Fig.3e). Conversely, ectopic expression of Vsx1 leads to the expression of Svp in mOPC Hth^{+} neurons (Fig.3f) but does not affect Bsh expression. Therefore, Vsx1 is both necessary and sufficient for the specification of Pm3 fates in the larva. However, unlike the temporal factor Hth, Vsx1 does not affect the generation of Mi1 neurons, which suggests that this uni-columnar cell type is immune to spatial input.

In *Rx* whole mutant larvae and in mutant clones, $Svp^+Lim3^+Hth^+$ larval neurons (*i.e.* Pm1 and Pm2 neurons) in the pOPC are lost (Fig.3g; Supp. Fig.4c,d). Additionally, the Pm1 marker Tsh is lost in ventral pOPC Hth^+ cells (Supp. Fig.4e,f). Consistent with the *Vsx1* mutant data, larval Bsh expression is not affected by the loss of *Rx* (Fig.3g) but no $N^{OFF}Hth^+$ neurons are observed (Supp. Fig.3f). The Svp^+Hth^+ Pm1/2 neurons are also lost in the adult medulla rim region where the Pm1/2 cell bodies are located (Supp. Fig.4g,h). Furthermore, *27b*-Gal4, which drives GFP expression in Pm1, Pm2 and Tm1 neurons, is no longer expressed in *Rx* mutants in the medulla rim (Fig.3l,m). However, uni-columnar Tm1 neurons remain in normal numbers.

Ectopic expression of Rx leads to the activation of Svp in mOPC Hth^+ neurons, but does not affect the expression of Bsh (Fig.3h,k). It also leads to the activation of Tsh, but only in the ventral half of mOPC Hth^+ neurons, suggesting that a ventral factor acts together with Rx to specify ventral fates (Fig.3k). Taken together, the above data show that Rx is both necessary and sufficient for the specification of Pm1/2 neurons but (like Vsx1) does not affect the generation of Mi1 neurons.

Finally, we analyzed the role of the mOPC marker Optix in neuronal specification. In *Optix* mutant clones, Svp is ectopically expressed in the mOPC, but Bsh expression is not affected (Fig.3i). Of note, these ectopic Svp^+ neurons fail to express the region-specific Pm markers Vsx1 or Tsh (in ventral clones), which suggests that they assume a generic Pm fate (Supp. Fig.4i). Conversely, ectopic expression of Optix leads to the loss of Svp expressing neurons in both the cOPC and pOPC but does not affect Bsh (Fig.3j). These N^{OFF} neurons die by apoptosis as $Lim3^+$ neurons are not intermingled with $Bsh^+Hth^+N^{ON}$ neurons (Supp. Fig. 3g). When apoptosis is prevented in mOPC-derived neurons, Svp is not derepressed in the persisting Hth^+N^{OFF} neurons, which suggests that Optix both represses Svp expression and promotes cell death of Hth^+N^{OFF} neurons (Supp. Fig.3c).

The above data demonstrate that input from both the temporal and regional axes is required to specify neuronal fates (Fig.3n). The temporal factor Hth is required for both Mi1 and Pm1/2/3 specification. The spatial genes are not required for the specification of N^{ON}Mi1 neurons, consistent with the observation that Mi1 is generated in all OPC compartments. The spatial genes, however, are both necessary and sufficient for the activation (Vsx1 and Rx) or repression (Optix) of the N^{OFF} Pm1/2/3 neurons. Thus, Hth^+ neuroblasts generate two types of progeny: N^{OFF} neurons that are sensitive to spatial input (Pm1/2/3) and N^{ON} neurons that are refractory to spatial input (Mi1). Vsx1 expression in the cOPC is only maintained in Hth^+N^{OFF} neurons, suggesting that spatial information may be ‘erased’ in Mi1, thus allowing the same neural type to be produced throughout the OPC.

Cross regulation of spatial factors in the OPC

Do spatial genes regulate each other in the neuroepithelium? In *Vsx1* mutant clones, Optix (but not Rx) is derepressed in the cOPC epithelium (Fig.4a,b). Conversely, ectopic Vsx1 is sufficient to repress Optix in the mOPC and Rx in the pOPC (Fig.4c,d). Similarly, Optix, but not Vsx1, is derepressed in *Rx* mutant clones in the pOPC epithelium (Fig.4h) and ectopic Rx is sufficient to repress Optix in the mOPC (but not Vsx1 in the cOPC) (Fig.4i). In *Optix* mutant clones, neither Vsx1 nor Rx are derepressed in the mOPC epithelium (Fig.4e), but

ectopic Optix is sufficient to repress both Vsx1 in the cOPC and Rx in the pOPC (Fig.4f,g). The observation that Optix is not necessary to suppress Vsx1 or Rx in the mOPC neuroepithelium is surprising because Svp is activated in a subset of Hth^+ neurons in the mOPC in *Optix* mutant clones (Fig.3i). Nevertheless, when we abolish cell death in the mOPC, the ectopic undead N^{OFF} neurons express Lim3 but not Svp, (Supp. Fig.3b–d), which confirms that Optix represses Svp expression in mOPC neurons. Taken together, these results support a model in which Optix is sufficient to repress Vsx1 and Rx, promote the death of Hth^+N^{OFF} neurons and repress Pm1/2/3 fates (Fig.4j). Vsx1 and Rx act to promote Pm3 (Vsx1) or Pm1/2 (Rx) fates but can only do so in the absence of Optix.

Extensive cell movement from spatially produced neurons during pupation

These results suggest that multi-columnar neurons are generated at specific locations in the medulla crescent. However, since these neurons are required to process visual information from the entire retina in the adult medulla, how does the D/V position of neuronal birth in the larval crescent correlate with their final position in the adult? We performed lineage-tracing experiments with *Vsx1*-Gal4 to permanently mark neurons generated in the cOPC and with *Optix*-Gal4 for mOPC neurons, and we analyzed the position of the cell bodies of these neurons. In larvae, neurons from the cOPC or from the mOPC remain located in the same D/V position relative to where they were born (Fig. 5a,b). In adults, both populations have moved to populate the entire medulla cortex along the D/V axis (Fig. 5c,d). We next analyzed the kinetics of cell movement during development by following cOPC neurons. Neurons born in the cOPC remain tightly clustered until 20h after puparium formation (P20), after which point the cell bodies spread throughout the medulla cortex (Supp. Fig. 5a,b). By P30 the neurons are distributed over the entire D/V axis of the medulla cortex (Supp. Fig.5c). In the adult, the majority of neurons derived from the cOPC neuroepithelium are located throughout the cortex although there is an enrichment of neurons in the central region of the cortex (see below; Fig.5d; Supp. Fig.5d).

To determine whether these observed movements involve the entire neuron or just the cell body, we looked at the initial targeting of cOPC or mOPC-derived neurons in larvae before the onset of cell movement. In larvae, both populations send processes that target the entire D/V axis of the medulla neuropil (Fig 5.a′,b′). Therefore, medulla neurons first send projections to reach their target columns throughout the entire medulla. Later, remodeling of the medulla results in extensive movement of cell bodies along the D/V axis, leading to their even distribution in the cortex.

What is the underlying logic behind why some neurons move while others do not? We followed markers for the Mi1 (Bsh), Pm2 (Hth^+Svp^+), Pm1 ($Hth^+Svp^+Tsh^+$), and Pm3 ($Vsx1^+Svp^+Hth^+$) populations of neurons through pupal stages and up to the adult. Mi1s are generated evenly throughout the larval OPC and remain regularly distributed across the D/V axis at all stages (Fig.2a; Supp. Fig.6). We repeated the lineage-tracing experiment with *Vsx1*-Gal4 to follow Mi1 neurons produced by the cOPC. These neurons remain exclusively in the center of the adult medulla cortex (Fig.5e), demonstrating that Mi1 neurons do not move. In contrast, Pm3 neurons remain tightly clustered in the central region until P20, at which point they move to occupy the entire cortex (Supp. Fig.7).

However, not all multi-columnar neurons have cell bodies that move to occupy the entire medulla cortex. Unlike Mi1 and Pm3, adult Pm1 and Pm2 cell bodies are not located in the adult medulla cortex but instead in the medulla rim, at the edges of the cortex (Fig.3l). Pm1 and Pm2 markers remain clustered at the ventral (Pm1) or dorsal (Pm2) posterior edges of the medulla cortex throughout all pupal stages (Supp. Fig.6a,c). In the adult, both populations occupy the medulla rim from where they send long horizontal projections that reach the entire D/V axis of the medulla neuropil (Fig.2i). The pOPC may be a specialized region where many of the medulla rim cell types are generated. Even though most of cOPC-derived neurons move during development (Supp. Fig.5d), we have identified a cOPC-derived multi-columnar neuron (TmY_{14}) (Supp. Fig.9k) that sends processes that target the entire D/V length of the medulla neuropil but whose cell bodies remain in the central medulla cortex in the adult (Supp. Fig.8d).

Thus, the four populations of Hth neurons follow different kinetics: Mi1 neurons are born throughout the OPC and do not move, Pm3 neurons are born centrally and then move to cover the entire cortex and Pm1/Pm2 neurons are born at the ventral or dorsal posterior edges of the OPC and occupy the medulla rim in adults.

We noted that uni-columnar Mi1 neurons, whose cell bodies do not move reside in the distal cortex whereas multi-columnar Pm3 neurons, which move, reside in the proximal cortex (Fig.5e, Supp. Fig.6d). We thus tested the hypothesis that neurons whose cell bodies are located distally in the medulla cortex represent uni-columnar neurons generated homogeneously throughout the OPC that do not move. In contrast, proximal neurons, which are fewer in number and are generated in specific subregions of the medulla OPC, would be multi-columnar and might move to their final position.

We first confirmed that neurons that move have their cell bodies predominantly in the proximal medulla cortex. We analyzed the cell body position of neurons born ventrally that have moved dorsally using the *hh*-Gal4 lineage trace (Fig.5f): the cell bodies found dorsally are mostly in the proximal medulla cortex, whereas the cell bodies in the ventral region are evenly distributed throughout the distal-proximal axis of the ventral cortex. They must represent both distal uni-columnar neurons that did not move as well as proximal multi-columnar neurons that remained in the ventral region (Fig.5f).

We next analyzed the pattern of movement of Tm2 uni-columnar neurons from the ventral and dorsal halves of the OPC using *hh*-based lineage-trace. The cell bodies of Tm2 neurons are located throughout the D/V axis in the medulla cortex but are colabeled with the *hh* lineage marker only in the ventral half (Supp. Fig.8c). Thus, like Mi1, Tm2 uni-columnar neurons do not move. Furthermore, uni-columnar Tm1 neurons, labeled by *27b*-Gal4 are born throughout the D/V axis of the OPC crescent with distal cell bodies (Supp. Fig.8a,b), suggesting that they also remain where they were born.

Conversely, we asked whether neurons that are specified in only one region, such as the Vsx^+ neurons of the cOPC, are multi-columnar in morphology. By sparsely labeling cOPC derived neurons using the *Vsx1*-Gal4 driver, we characterized thirteen distinct cell-types that retain Vsx1 expression in the adult medulla (Supp. Fig.9). Strikingly, all are multi-columnar

in morphology, further supporting the model that it is the multi-columnar neurons that move during pupal development.

Finally, we generated MARCM clones in the OPC neuroepithelium and visualized them using cell-type specific Gal4 drivers in the adult medulla. We observed two classes of adult clone distribution: Clones in which neurons are tightly clustered, and clones in which neurons are dispersed. Consistent with our model, the clustered clones are those labeled with uni-columnar neuronal drivers (Tm3, Fig.5g; Mi1, Supp. Fig.8e), whereas the dispersed clones are those labeled with a multi-columnar driver (Dm12, Fig.5h).

Taken together, the above data demonstrate that neurons that do not move are uni-columnar (with cell bodies in the distal cortex), whereas most multi-columnar neurons (with cell bodies in the proximal cortex) move (Fig.6)

Discussion

We show here that combinatorial inputs from the temporal and spatial axes act together to promote neural diversity in the medulla. We showed previously that a temporal series of transcription factors expressed in medulla neuroblasts allows for a diversification of the cell types generated by the neuroblasts as they age[19,20]. We now show that input from the D/V axis leads to further diversification of the neurons made by neuroblasts at a given temporal stage, producing the same uni-columnar neuronal type globally as well as generating locally a smaller number of many cell types that either move to occupy the entire medulla, or innervate the entire medulla by sending horizontal projections. This situation is reminiscent of the mode of neurogenesis in the *Drosophila* ventral nerve cord in which each neuroblast also expresses a (different) temporal series of transcription factors that specifies multiple neuronal types in the lineage[21–23]. Spatial cues from segment polarity, D/V and Hox genes then intersect to impart unique identities to each of the lineages[34, 35,36,37,38]. However, neuroblasts from the different segments give rise to distinct lineages to accommodate the specific function of each segment. In contrast, in the medulla, the entire OPC contributes to framing the repeating units that form the retinotopic map. It is therefore likely that each neuroblast produces a common set of neurons that connect to each pair of incoming R7 and R8 cells, or L1–L5 lamina neurons. This serves to produce 800 medulla columns with a 1:1 stoichiometry of medulla neurons to photoreceptors. The medulla neurons that are produced by neuroblasts throughout the D/V axis of the OPC appear to be uni-columnar (Fig.6a). The production of the same neuronal type along the entire OPC could be achieved by selectively ‘erasing’ spatial information in uni-columnar neurons, as it happens in Mi1 neurons.

Regional differences in the OPC confer further spatial identities to neuroblasts with the same temporal identity, and lead to specific differences in the lineages produced in the compartments along the D/V axis of the medulla. These differences produce smaller numbers of multi-columnar neurons whose stoichiometry is much lower than 1:1 (Fig.6b). These neurons often move during development in order to be uniformly distributed in the medulla cortex. This combination of regional and global neuronal specification in the medulla presents a powerful mechanism to produce the proper diversity and stoichiometry of neuronal types and generate the retinotopic map.

Methods

Antibodies and Immunostaining

To identify transcription factors that are involved in optic lobe development we screened 215 antibodies for expression in the optic lobe at the 3rd instar and/or adult stages. Sources for the antibodies consisted of: (a) the polyclonal antibody collection against *Drosophila* segmentation proteins[41], (b) the modENCODE project[42], (c) the Developmental Studies Hybridoma Bank, and (d) generous gifts from the *Drosophila* community. The following antibodies were used for this study: guinea-pig anti-Vsx1 (1:500) (Howard Lipshitz), rabbit anti-Rx (1:500) (Uwe Walldorf), rabbit anti-Optix (1:200) (Francesca Pignoni), mouse anti-Svp (1:100) (Yasushi Hiromi), rabbit anti-Hth (1:500) (Richard Mann), guinea-pig anti-Bsh (1:500) (Makoto Sato), rat anti-Slp1 (1:200) (Ken Cadigan), rabbit anti-Teashirt (1:300) (Stephen Cohen) and guinea-pig anti-Lim3 (1:500) (Jim Skeath). Commercial antibodies used were: rabbit anti-GFP (1:300) (Invitrogen), sheep anti-GFP (1:500) (AbD Serotec), rabbit anti-cleaved Caspase-3 (1:100) (Cell Signaling Technology) and goat anti-βgal (1:2500) (Biogenesis). The following antibodies were obtained from the Developmental Studies Hybridoma Bank: mouse anti-Dachshund (1:20), rat anti-DE-Cadherin (1:20), rat anti-DN-Cadherin (1:20), mouse anti-Eyeless (1:20) and mouse anti-Eya (1:20). Secondary antibodies were obtained from Jackson Immunolabs or Invitrogen and used at 1:400.

Immunostaining was performed as previously described[43]. Images were acquired using a Leica SP5 confocal and processed with Adobe Photoshop.

Fly stocks and genetics

For lineage analysis, either “yw; act-FRT-STOP-FRT-lacZ; UASFLP” or “UAS-Flip; ; Act-FRT-y+-FRT-LexA::VP16, LexAop-myr::GFP” (FLEXAMP)[30] was crossed to MzVUM-Gal4 (to report for Vsx1)[44], Optix-Gal4 (Justin Kumar) or hedgehog-Gal4. “UAS-Red-Stinger, UASFLP, ubi-FRT-STOP-FRT-NuGFP”(G-TRACE)[45] was used with Optix-Gal4. To generate ‘flip-out’ clones, hsFlp; UAS-FRT-STOP-FRT-CD8::GFP flies were crossed to either 27b-Gal4 or MzVum-Gal4; the progeny were heat shocked at 37 °C for 30 minutes at late pupal stages and dissected as adults. R83H09-Gal4[46] was used to specifically label TmY14 neurons in adult.

To generate *hth* mutant MARCM clones, *y,w,hsFLP,UASCD8GFP; ; tub*-Gal4, *FRT82B tub-gal80/TM6B* flies were crossed with *FRT82B* hth^{P2}*/TM6B* flies (Richard Mann). Additional mutant alleles used were: *FRT42D*, $Optix^{1}$ (Rui Chen) and *FRT42D*, Rx^{ex8}. *Vsx1* knock-down was achieved using UAS-*Vsx1* RNAi from the VDRC (KK collection, ID:107684). To generate GOF clones, hsFLP; Actin-FRT-STOP-FRT-Gal4, UAS-GFP flies were crossed to the following UAS lines: UAS-Vsx1 (Howard Lipshitz), UAS-Hth:GFP (Bloomington), UAS-Rx(1) (Uwe Walldorf) and UAS-Optix (Justin Kumar). Larvae were heat-shocked at 37°C for 5 or 20 minutes at the L1–L2 stage.

Wild-type MARCM clones were generated by crossing *y,w,hsFLP,UASCD8GFP; ; tub*-Gal4, F*RT82B tub-gal80/TM6B* flies with *FRT82B/TM6B* flies. MARCM clones were induced in L1–L2 larvae via heat shock at 37°C for 1 hour and dissected 72–96 hours later as wandering 3rd instar larvae. Cell-type specific MARCM clones were generated using a 5

minute heat shock at 37°C. To visualize these clones *tub*-Gal4 was replaced with either Tm3-Gal4 (R13E12[46]), Mi1-Gal4 (*bsh*-Gal4) or Dm12-Gal4 (R47G08[46]). To rescue cell death in mOPC-derived neurons *Optix*-Gal4 was crossed to "*UAS-Flip; LexAop-P35; Act-FRT-y+-FRT-LexA::VP16, LexAop-myr::GFP*" (FLEXAMP-P35). $Dronc^{I24}$ mutants were used to abolish cell death in all neurons.

Rx^{ex8} mutants[47] were balanced over Cyo,pAct-GFP to allow for the identification of mutants at the larval stage. Rx^{ex8} mutants are pharate adult lethal[47]. For each genotype at least 3 individual flies were analyzed.

Generation of the Tm2-lexA driver

A 1.6kb fragment from the third intron of the *otd* gene, which was previously identified as an eye enhancer[48], was amplified from an otd-Gal4 construct (donated by Cook T.) and inserted into a LexA-GAD construct (Pacman-hspbp-LexAGAD-SV40). Transgenic flies were obtained using the ΦC31 transposase system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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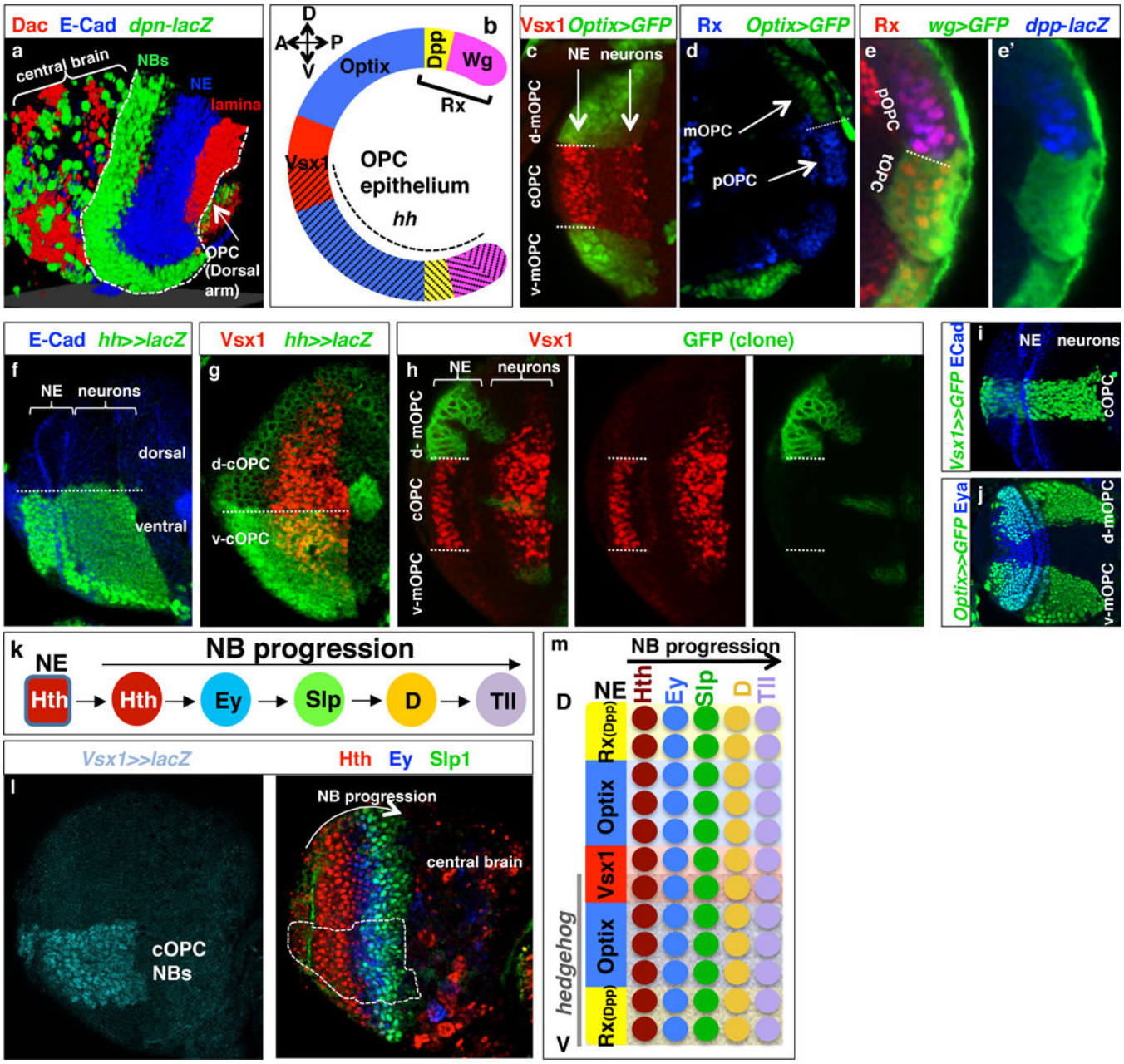

Figure 1. The OPC NE is patterned along its dorsal/ventral axis

a. Lateral view of a three-dimensional reconstruction of a third instar larval brain. The dotted line outlines one arm of the OPC. Dac labels the lamina (red), E-Cad marks the neuroepithelium (blue) and neuroblasts are marked by *deadpan-lacZ* (green).

b. Cartoon schematic of the compartmentalization of the OPC neuroepithelium.

c. Vsx1 (red) labels the central region (cOPC) and *Optix*>GFP (green) marks the adjacent ventral and dorsal main regions (v-mOPC and d-mOPC).

d. Rx (blue) marks the pOPC and *Optix*>GFP (green) marks the mOPC.

e and e′. Rx (red) labels the pOPC. The pOPC is subdivided into *wg*-positive (*wg*>GFP in green) and *dpp*-positive (*dpp-lacZ* in blue) domains (e′).

f and g. E-cad labels the NE (blue) and *hh*-lineage trace is detected with βgal (green). *hh* is active in only the ventral half of the OPC, including the cOPC marked by Vsx1 (red).
h. A MARCM GFP clone (green) located in the d-mOPC extends to, but cannot invade, the cOPC compartment, which is marked with Vsx1 (red).
I and j. A lineage trace experiment with *Vsx1*-Gal4 (i) and *Optix*-Gal4 (j) (GFP in green) marks only the cOPC (i) or the mOPC (j) and its progeny in the lamina and medulla. The OPC (in blue) is labeled with DE-Cadherin (i) or with Eya (j).
k. Cartoon schematic depicting the sequence of transcription factors expressed during the temporal progression of medulla neuroblasts.
l and l′. The progression of temporal neuroblast transcription factors is the same throughout the OPC. The cOPC is marked with the *Vsx1* lineage trace (cyan in l and an outline in l′).
m. Cartoon schematic of the NB progression. The sequence is the same throughout the crescent.

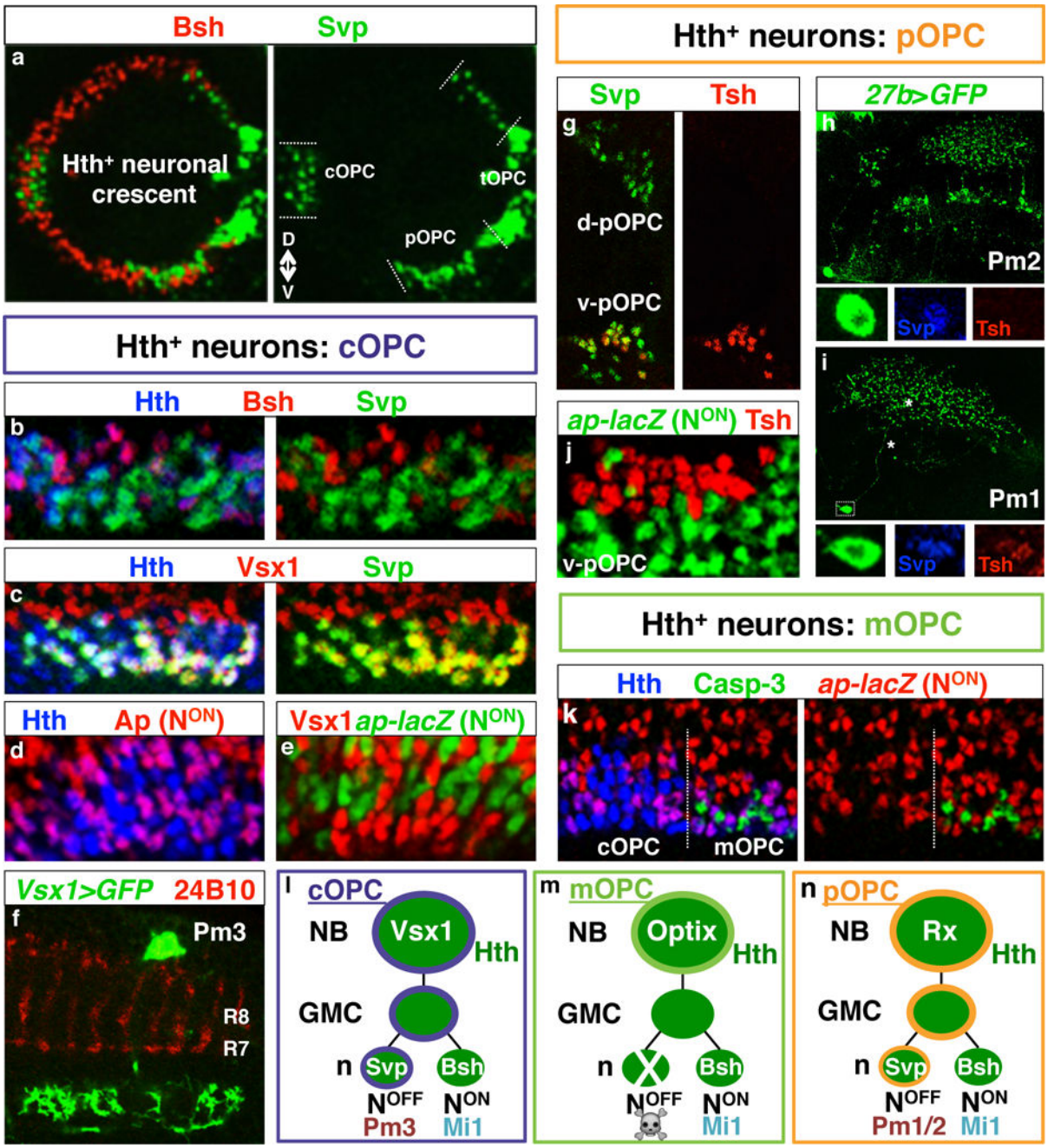

Figure 2. Distinct neuronal cell types are generated along the D/V axis of the OPC

a. Lateral view of the third instar medulla cortex with Bsh (red) and Svp (green).

b. Hth^{+} neurons (blue) in the cOPC can be divided into mutually exclusive Bsh (red) and Svp (green) populations.

c. Svp (green) and Vsx1 (red) are co-expressed in a subset of cOPC-derived Hth^{+} neurons (blue).

d. Hth^{+} neurons in the cOPC (blue) can be divided into Notch^{ON} (Ap in red) and Notch^{OFF} (Hth alone) populations.

e. Vsx1 (red) is not expressed in the Notch^{ON} (*ap-lacZ* in green) Hth^{+} neurons of the cOPC.

f. A Pm3 neuron (GFP in green) generated by flip-out with *Vsx1*-Gal4. Photoreceptors are labeled with 24B10 (red).

g. Cross-section view of the Svp-positive neurons of the pOPC (green). Only the ventral population of pOPC neurons expresses Tsh (red).

h–i. Single cell flip-out clones using *27b*-Gal4 label Pm2 (h) and Pm1 (i) neurons.

j. Tsh (red) is not expressed in the $Notch^{ON}$ (*ap-lacZ* in green) Hth^{+} neurons of the pOPC.

k. Cleaved-caspase-3 (green) marks dying cells that are intermingled with N^{ON} Hth^{+} in the mOPC.

l–n. Schematic models summarizing neurogenesis in Hth^{+} neuroblasts of different compartments along the D/V axis of the OPC.

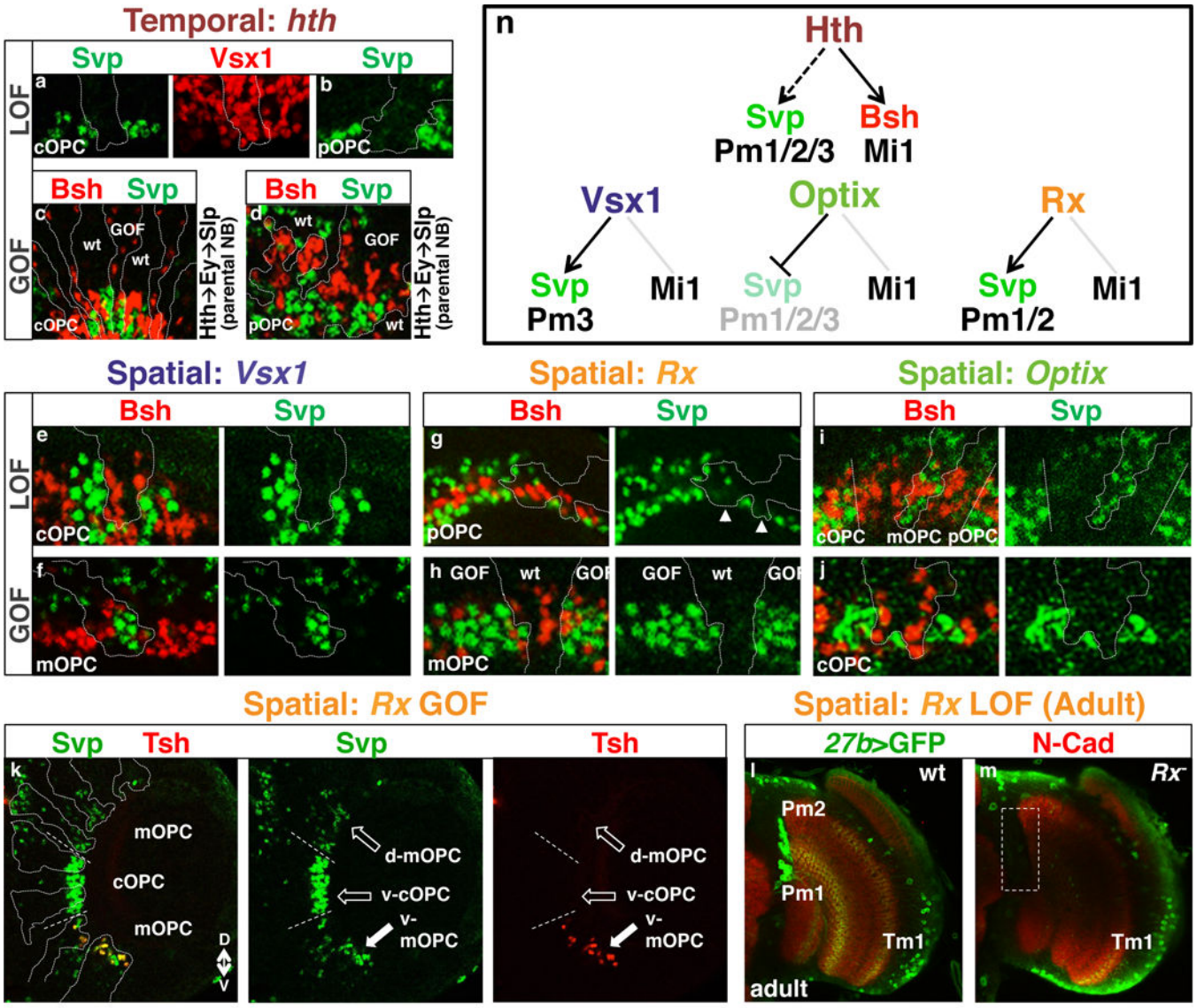

Figure 3. Temporal and spatial inputs are required for neuronal specification in the medulla

a. In *hth* mutant MARCM clone, Svp expression (green) is lost but Vsx1 (red) is unaffected in the cOPC.

b. Svp expression (green) in lost in the pOPC in *hth* mutant MARCM clone.

c and d. Bsh (red) is ectopically expressed in the progeny of older neuroblasts in *hth* gain-of-function (GOF) clones in the cOPC (c) or in the pOPC (d). Svp expression (green) is unaffected in both case (c and d).

e–j. Bsh (red) and Svp (green) expression in different LOF and GOF.

e. *Vsx1* RNAi loss-of-function (LOF) clone in the cOPC.

f. *Vsx1* GOF clone in the mOPC.

g. *Rx* LOF mutant MARCM clone in the pOPC.

h. *Rx* GOF clones in the mOPC.

i. *Optix* LOF mutant MARCM clone in the mOPC,.

j. *Optix* GOF clone in the cOPC.

k. Lateral view of the third instar medulla cortex. Svp (green) is ectopically expressed in *Rx* GOF clones. The Pm1 marker Tsh (red) is ectopically expressed in ventral mOPC clones (solid arrow) but is not in clones in the dorsal mOPC or ventral cOPC (outlined arrows).
l. Wild-type adult optic lobe with *27b*-Gal4 driving GFP expression (green) in Pm1 and Pm2 neurons in the medulla rim and Tm1 neurons in the cortex. The neuropil is labeled in red with N-Cadherin.
m. In *Rx* mutant adult optic lobe Pm1 and Pm2 neurons are absent from the medulla rim (boxed region). Tm1 neurons are unaffected.

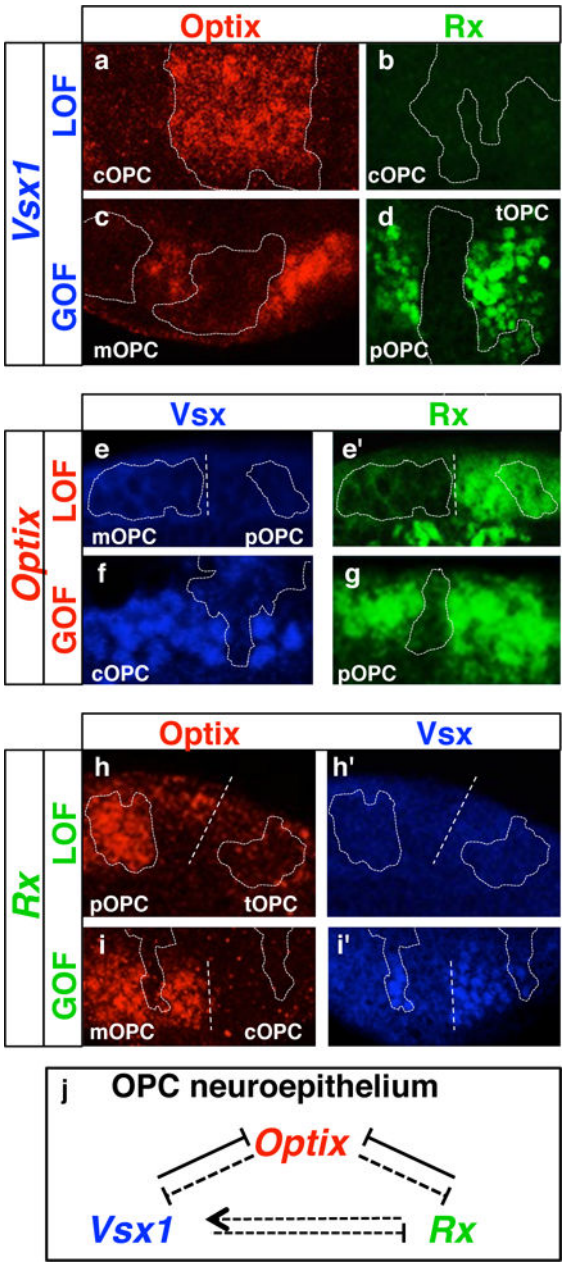

Figure 4. Spatial genes cross-regulate each other in the OPC neuroepithelium

a–i′. Expression of Optix (red), Rx (green), and Vsx1 (blue) in different clones.

a. and b. Expression of Optix and Rx in *Vsx1* RNAi LOF clones in the cOPC neuroepithelium.

c. and d. Expression of Optix and Rx in *Vsx1* GOF clones in the OPC NE.

e. and e′. Expression of Vsx and Rx in *Optix* mutant MARCM clones in the OPC NE.

f. and g. Expression of Vsx and Rx in *Optix* GOF clones in the OPC NE.

h. and h′. Expression of Optix and Vsx in *Rx* mutant MARCM clones in the OPC NE.

i. and i′. Expression of Optix and Vsx in *Rx* GOF clones in the cOPC NE.

j. The spatial genes negatively cross-regulate each other in the OPC NE. Filled line indicates that genes are necessary and sufficient while dotted line indicates sufficiency only.

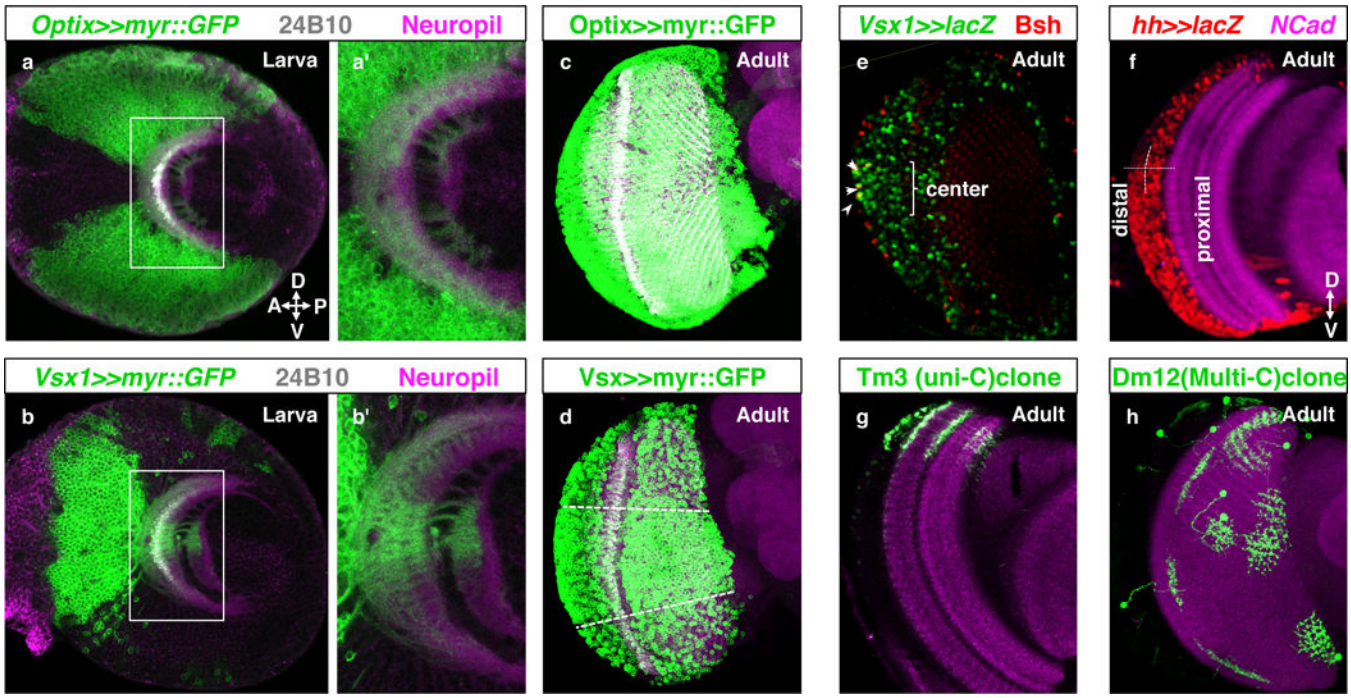

Figure 5. Neuronal movement during medulla development is restricted to multi-columnar cell-types

a–d. Lineage trace of mOPC-derived neurons using *Optix*-Gal4 (a and c) or cOPC-derived neurons using *Vsx1*-Gal4 (b and d) in larva (a and b) or adult (c and d). The dotted lines indicate the enrichment of GFP^+ neurons in the center of the medulla cortex in d.

e. *Vsx1* lineage-trace in the adult medulla. cOPC derived cells (βgal in green) are located throughout the medulla cortex but only overlap with Bsh (red) in the center. Note that Bsh nuclei are located in the distal medulla cortex.

f. *hh* lineage trace in the adult medulla. Ventrally derived cells (βgal in red) are located throughout the medulla cortex but enriched ventrally. Cells that have moved dorsally are mainly located in the the proximal half of the cortex whereas.

g. and h. MARCM clones generated in 3^{rd} instar larvae and visualized in the adult medulla (green). (g) Cells in clones labeled by a Gal4 driver for the uni-columnar cell-type Tm3 remain clustered in the adult (arrows). (h) Cells in clones marked with a Gal4 driver for the multi-columnar cell type Dm12 are dispersed in the adult (arrows).

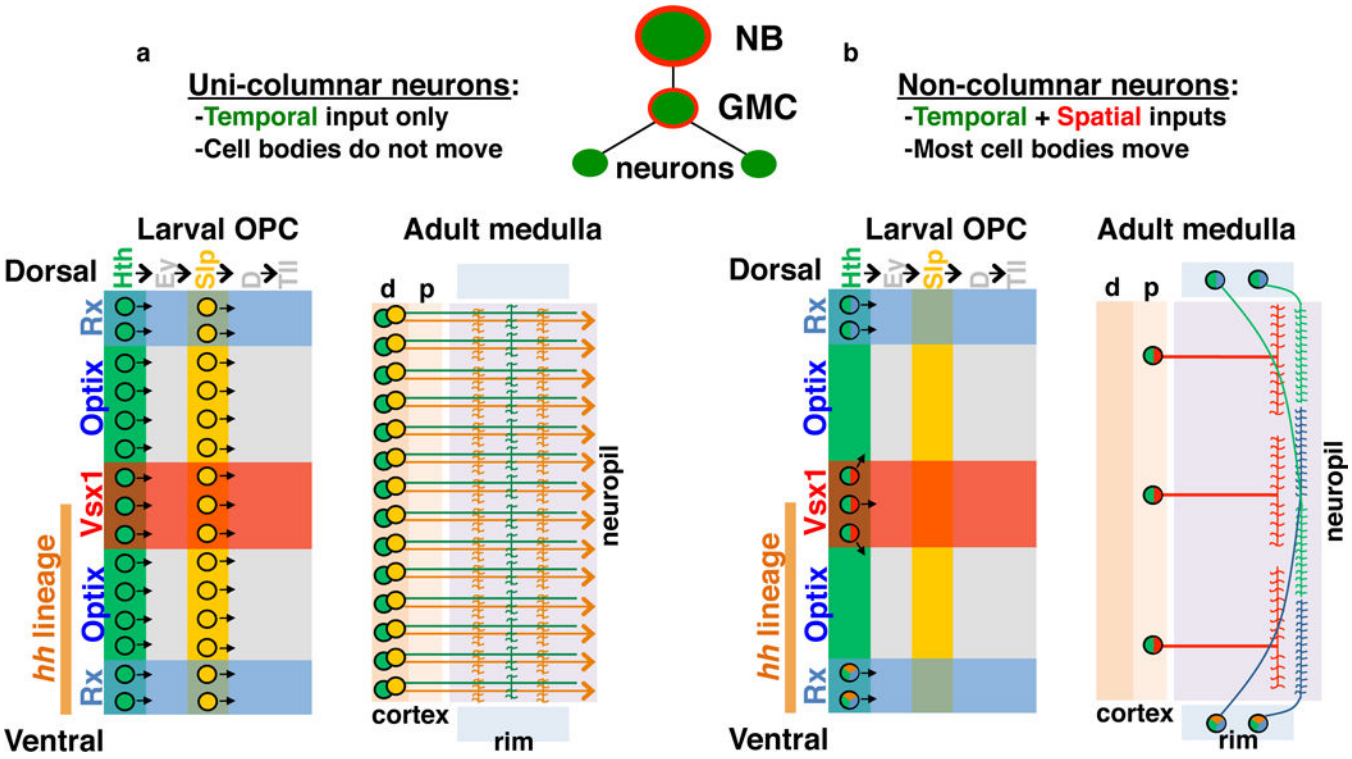

Figure 6. Model for the generation of retinotopy in the *Drosophila* medulla

a. Uni-columnar neurons are refractory to regional inputs. Uni-columnar neurons (Mi1, Tm1, Tm2…) are born throughout the dorsal-ventral axis of the larval OPC and remain in place through to adulthood. In the adult each neuron innervates only one column. Uni-columnar cell bodies are located in the distal medulla cortex. Two examples are shown.

b. Non-columnar neurons (Pm1, Pm2, Pm3…) are regionally specified and either move to take up their final position in the medulla cortex or send long tangential projections into the neuropil from the medulla rim. Three examples are shown: Red+green neurons are born in the cOPC and receive input from both Hth neuroblasts and Vsx1. Blue+green and/or brown neurons are generated in the ventral and dorsal pOPC regions and receive input from Hth neuroblasts and Rx. They populate the adult medulla rim and send large branches into the medulla neuropil. Non-columnar cell bodies are located in the proximal medulla cortex in the adult.