Spatio-temporal relays control layer identity of direction-selective neuron subtypes in Drosophila

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Visual motion detection in sighted animals is essential to guide behavioral actions ensuring their survival. In Drosophila, motion direction is first detected by T4/T5 neurons. Their axons innervate one of the four lobula plate layers. How T4/T5 neurons with layer-specific representation of motion-direction preferences are specified during development is unknown. We show that diffusible Wingless (Wg) between adjacent neuroepithelia induces its own expression to form secondary signaling centers. These activate Decapentaplegic (Dpp) signaling in adjacent lateral tertiary neuroepithelial domains dedicated to producing layer 3/4-specific T4/T5 neurons. T4/T5 neurons derived from the core domain devoid of Dpp signaling adopt the default layer 1/2 fate. Dpp signaling induces the expression of the T-box transcription factor Optomotor-blind (Omb), serving as a relay to postmitotic neurons. Omb-mediated repression of Dachshund transforms layer 1/2- into layer 3/4-specific neurons. Hence, spatio-temporal relay mechanisms, bridging the distances between neuroepithelial domains and their postmitotic progeny, implement T4/T5 neuron-subtype identity.
Visual signals received by the retina are generally not stationary because objects in the environment and/or the bodies of animals move. To detect motion, visual circuits perform complex spatio-temporal comparisons that convert luminance changes collected by photoreceptors into signals containing information about direction or speed. Despite the seemingly divergent anatomy of vertebrate and insect visual systems, they display remarkable parallels in the computations underlying motion vision and the neuronal elements performing them. In most sighted animals, this involves neurons that respond to motion signals in specific directions. Direction-selectivity emerges from differences in the connectivity of their dendrites. Motion-direction preferences by their axons are represented by layer-specific innervation. Thus, anatomical characteristics such as layer-specificity seem to be intricately linked with motion-directionality. However, how these are implemented during circuit development is poorly understood.

The Drosophila visual system has emerged as a powerful model for elucidating the neural circuits and computations underlying motion detection. Photoreceptors (R-cells) in the retina extend axons into the optic lobe consisting of the lamina, medulla, lobula plate, and lobula (Fig. 1a). Neuronal projections in these ganglia are organized into retinotopically arranged columnar units. The medulla, lobula plate, and lobula are additionally subdivided into synaptic layers. They are innervated by more than 100 neuronal subtypes that extract different visual features in parallel pathways. T4 and T5 lobula plate neurons are the first direction-selective circuit elements. Each optic lobe hemisphere contains ~5300 T4/T5 neurons. T4 dendrites arborize within medulla layer 10, and T5 dendrites in lobula layer L1. Their axons project to one of the four lobula plate layers, thereby defining four different neuron subtypes each (Fig. 1a). Axons separate according to their motion-direction preferences. Thus, front-to-back, back-to-front, upward, and downward cardinal motion directions are represented in lobula plate layers 1–4. T4 neurons are part of the ON motion detection pathway reporting brightness increments, while T5 neurons are part of the OFF pathway reporting brightness decrements. Distinct neuron sets in the lamina and medulla relay ON and OFF information to T4 and T5 neurons. Direction-selectivity emerges within T4/T5 dendrites and involves the non-linear integration of input from these upstream neurons for enhancement in the preferred direction and suppression in the null-direction. Dendritic arbor of the four T4 neuron subtypes have characteristic orientations, that correlate with the direction preferences of lobula plate layers innervated by their axons. Thus, direction-selectivity involves the establishment of neuron subtypes, each with distinct spatial connectivities. Here, we address when and how T4 and T5 neuron subtypes with different layer identities are specified during development.

Optic lobe neurons originate from two horseshoe-shaped neuroepithelia, called the outer and inner proliferation centers (OPC and IPC; Fig. 1b). These are derived from the embryonic optic lobe placode and expand by symmetric cell divisions during early larval development. At the late 2nd instar larval stage, neuroepithelial (NE) cells from the medial OPC edge begin to transform into medulla neuronal stem cells, called neuroblasts (Nbs). These undergo asymmetric divisions during development. In contrast to controls (n = 13/13), either one (n = 18/30) or two (n = 12/30) of the four lobula plate layers were missing. Moreover, T4/T5 neuron numbers were reduced by approximately 25% in three-layered and 50% in two-layered samples, respectively (Fig. 1h). To determine which layers were affected, optic lobes were immunolabeled with the cell surface molecule Connectin, a specific marker of lobula plate layers 3/4. In contrast to controls (n = 26/26), Connectin was expressed either in one (n = 16/30) or none (n = 14/30) of the lobula plate layers in wg(KO;NRT-wg) flies (Fig. 1i–k). Thus, T4/T5 neurons innervating layers 3/4 were preferentially affected in flies solely expressing membrane-tethered Wg.

Results

Layer 3/4 innervating T4/T5 neurons depend on Wg secretion. Wnt family members are evolutionary conserved signaling proteins that orchestrate tissue patterning and growth during development. Recent findings showed that flies expressing membrane-tethered instead of normally secreted Wingless (Wg) were viable and had normal bodies and well-patterned, albeit slightly smaller wings. This indicated that long-range spreading of Wg is not essential for the development of many tissues. As the general fitness of these flies was reduced, Wg release could possibly be required in tissues other than imaginal discs. In the visual system, Wg is expressed and required in the GPC areas for neuron specification. To explore whether Wg plays a role in the IPC and the spreading of this signaling molecule is essential, we examined the brains of adult homozygous flies, engineered to solely express Wg fused to the type-2 transmembrane protein Neurotactin (wg(KO;NRT-wg); Fig. 1c). We uncovered a specific and highly penetrant phenotype in the lobula plate (Fig. 1d–g): unlike in controls (n = 13/13), either one (n = 18/30) or two (n = 12/30) of the four lobula plate layers were missing. Moreover, T4/T5 neuron numbers were reduced by approximately 25% in three-layered and 50% in two-layered samples, respectively (Fig. 1h). To determine which layers were affected, optic lobes were immunolabeled with the cell surface molecule Connectin, a specific marker of lobula plate layers 3/4. In contrast to controls (n = 26/26), Connectin was expressed either in one (n = 16/30) or none (n = 14/30) of the lobula plate layers in wg(KO;NRT-wg) flies (Fig. 1i–k). Thus, T4/T5 neurons innervating layers 3/4 were preferentially affected in flies solely expressing membrane-tethered Wg.
To elucidate the underlying causes, we examined the expression of wild-type Wg in the 3rd instar larval optic lobe. Consistent with previous reports, Wg protein was detected in the dorsal and ventral GPC areas adjacent to the OPC (Fig. 1l, n, o). Apposing the ventral p-IPC shank, Wg was expressed in surface (s-)IPC NE cells, which generate two lobula neuron clusters. Additionally, Wg was expressed in a small Nb clone adjacent to the dorsal p-IPC shank (Fig. 1m–o). Relying on the perdurance of reporter gene expression by two wg-Gal4 drivers to mark the progeny of these domains.
Wg secretion from the GPC areas induces wg in secondary domains. We next asked how the prevention of Wg release might cause the lobula plate-specific defects. In the GPC areas, Wg blocks the expression of the transcriptional regulator Homothorax (Hth), a temporal series factor in the OPC22. Hth expression was correctly suppressed in wg[KO;NRT-wg] flies, suggesting that NRT-Wg functions as wild-type Wg in this context (Supplementary Fig. 2a, b). However in the IPC (Fig. 2a, b; Supplementary Fig. 2c–f), NRT-Wg expression was absent in the s-IPC adjacent to the ventral p-IPC and the Nb clone adjacent to the dorsal p-IPC in approximately half of the samples (n = 22/41). In the remaining samples, residual NRT-Wg expression was found either in the s-IPC (n = 9/41), the Nb clone (n = 8/41), or both (n = 2/41). The s-IPC and its progeny developed normally, because Hth was expressed and two lobula neuron clusters were formed (Supplementary Fig. 2g–j). Wg protein and reporter gene expression were detected in the GPC areas before the s-IPC in late 2nd instar larvae (Fig. 2c, d). Furthermore, wg expression in the s-IPC of wg[KO;NRT-wg] mid 3rd instar larvae was absent when Wg normally is detected (Fig. 2e, f). Thus, GPC-derived Wg could induce Wg expression in the s-IPC and Nb clone.

Failure to induce NRT-Wg expression could conceivably not be due to lack of Wg release but instead to sub-optimal signaling activity of the NRT-Wg protein. We therefore asked whether boosting NRT-Wg expression could overcome observed defects. This was tested by combining a UAS-NRT-wg transgene, known to be highly active with the knock-in null allele wg[KO;Gal4], serving also as a driver that restricts activation to endogenous expression domains.30 UAS-FLP and wg[KO;FRT wg+ FRT NRT-wg]60 ensured that the endogenous locus only produced NRT-Wg following the induction of recombination events. A tub-Gal80B transgene controlled the timing of expression (for full genotypes, see Supplementary Table 1). Thus, at a restrictive temperature, Wg expression is normal and no NRT-Wg is produced. After a shift to a permissive temperature, UAS-NRT-wg is activated and the Wg allele switches to expressing NRT-Wg. We found that following a temperature shift at the mid 3rd instar larval stage, NRT-Wg was expressed in the GPC areas and the s-IPC (Fig. 2g; n = 16/16). However, following an early temperature shift at the 1st instar larval stage, no expression could be detected in the s-IPC in the majority of samples (76%; n = 26/34) (Fig. 2h). We suggest that this partial phenotype is due to imperfect allele switching, i.e., residual wild-type Wg is produced by the GPC areas, and that Wg release from the GPC areas is required between the 1st and mid 3rd instar larval stage for inducing Wg expression in the s-IPC. Thus, impaired Wg release and not reduced signaling activity in wg[KO;NRT-wg] flies accounts for the loss of NRT-Wg expression in secondary domains.

Next, we performed converse allele switching experiments using wg[KO;FRT NRT-wg FRT wg+] to express wild-type Wg in a wg[KO;NRT-wg] background.60 We used R46E01-Gal4 (Supplementary Fig. 2k, l) and UAS-FLP to induce recombination specifically in the GPC areas. Expression of wild-type wg in the GPC areas rescued the s-IPC-specific loss of NRT-Wg (Fig. 2i, j; n = 16/23), demonstrating that Wg release specifically from the GPC areas is essential.

Consistent with Wg signaling-dependent induction of wg, the target genes frizzled 3 (fz3) and notum were expressed in the s-IPC (Fig. 2k, l). Moreover, Wg expression in the s-IPC was abolished following the simultaneous knockdown of the Wg receptors frizzled 3 (fz3) and frizzled 2 (fz2) (Fig. 2m, n) in the IPC by combining the fas3-Gal4 driver23 (cf. Supplementary Fig. 7a) with UAS-RNA interference (RNAi) transgenes. Hence, Wg released from the GPC areas is required to induce Wg in the s-IPC and Nb clone (Fig. 2o).

**wg is required to induce dpp in adjacent p-IPC subdomains.** How can wg in these secondary domains control T4/T5 neurogenesis in the p-IPC/d-IPC? The Drosophila Bmp family member Decapentaplegic (Dpp) is a known target of wg in the OPC and is expressed adjacent to the IPC areas in dorsal and ventral OPC subdomains26 (Fig. 3a). Similarly in the IPC, Wg expression in the s-IPC and Nb clone was detected adjacent to dpp-positive ventral and dorsal p-IPC subdomains (Fig. 3b–d). dpp reporter gene expression persisted in the two progenitor streams arising from these subdomains23 (Fig. 3c, d). Consistent with stepwise inductive events, dpp in the p-IPC did not precede wg expression in the s-IPC in late 2nd instar larvae (Fig. 3e). Furthermore, fz3 and notum (Fig. 2k, l) were expressed similarly to dpp in p-IPC subdomains and progenitor streams. In wg[KO;NRT-wg] flies, dpp-lacZ in the p-IPC was either absent (Fig. 3g, h; n = 16/32) or showed only residual labeling in one progenitor stream (Supplementary Fig. 3a; n = 16/32), in line with the penetrance and expressivity of phenotypes observed in adults. By contrast, OPC expression was unaffected, suggesting that releasable Wg is not required in this region (Fig. 3g, h). Constitutively active Wg signaling induced by IPC-specific expression of Armadillo resulted in ectopic dpp-lacZ labeling (Fig. 3i; Supplementary Fig. 3b), confirming that dpp is a Wg target in the p-IPC. IPC-specific wg knockdown abolished dpp-lacZ labeling, corroborating that wg is required in the s-IPC and Nb clone, and not the GPC areas for induction (Fig. 3j). Since Dpp signaling mediates EMT of migratory progenitors in the Dpp-expression domains, cell...
streams from these areas were affected in wg[KO;NRT-wg]/ flies and following IPC-specific knockdown of wg and the Dpp type I receptor thickveins (tkv) (Fig. 3h, j, k). Although the overall d-IPC morphology was altered (Supplementary Fig. 3c–f), progenitors and Nbs generated from the remaining p-IPC showed wild-type marker expression (Supplementary Fig. 3g–n). In adults, similar to wg[KO;NRT-wg]/ flies, lobula plate layers 3/4 were absent following IPC-specific knockdown of fz, fz2, or tkv (Fig. 3l–n).

Hence, wg from the s-IPC and Nb clone regulates dpp expression in adjacent p-IPC subdomains and EMT of progenitors that mature into Nbs producing T4/T5 neurons for layers 3/4 (Fig. 3o).

**Layer 3/4 T4/T5 neurons arise from Dpp-positive subdomains.**

To provide evidence that T4/T5 neurons innervating layers 3/4 specifically originated from the Dpp-positive subdomains in the
p-IPC, we next conducted lineage-tracing experiments. Because dpp-Gal4 is expressed in the entire optic lobe, we searched for a driver with activity restricted to the IPC Dpp-expression domains. Expression of R45H05-Gal4 (Fig. 4a) is controlled by an enhancer fragment of dorsocross 1, which encodes a T-box transcription factor and known Dpp target gene34. Reporter gene expression was detected in dorsal and ventral p-IPC subdomains, cell streams, Nbs, and postmitotic progeny in larval optic lobes and persisted throughout pupal development into adulthood, where it specifically labeled T4/T5 neurons innervating the layers 3/4 but not 1/2 (Fig. 4b; Supplementary Fig. 4a, b). As the expression was weak, we combined the FLPout approach with tub-Gal80 to permanently label R45H05-Gal4 expressing progeny, and again solely detected T4/T5 neurons projecting into layers 3/4 (Fig. 4c). Finally, we used the Flybow approach to label lineages in different colors. All clones contained T4/T5 neurons innervating either layers 1/2 or 3/4 but not both (Fig. 4d; n = 43 clones in 26 optic lobes), consistent with distinct origins of these neuron subtypes.

In lineage-tracing experiments using R45H05-Gal4, clones included C2, the T2-variant T2a, and/or T3 neurons, as judged by their characteristic cell body positions and terminals in the lamina and lobula, respectively (Fig. 4e). While C3, T2, and T3 neurons were not affected, C2 neurons were fully or partially absent in wg[KO;NRT-wg] flies with two or three remaining lobula plate layers, respectively (Fig. 4f, g; Supplementary Fig. 4c–i). Due to the lack of specific markers, we could not conduct tests for T2a neurons. Thus, T4/T5 neurons innervating layers 3/4, C2, and possibly T2a neurons are specifically derived from the dorsal and ventral Dpp-expression domains, while the p-IPC core generates T4/T5 for layers 1/2, as well as C3, T2, and T3 neurons (Fig. 4h).

**Fig. 2** The GPC areas release Wg to induce wg in the s-IPC. **a, b**(950,797),(1000,820)(836,798),(886,822) Schematic in **a** highlights the region of interest shown in subsequent panels. Unlike in controls (**a**), Wg immunolabeling (green) was absent in the s-IPC (dashed line, double arrowhead) in many wg[KO;NRT-wg] flies (**b**). The GPC areas (arrowhead) were not affected. **c, d** In 2nd instar larvae (2L), Wg protein (green) and wg(KO;Gal4) UAS-cdbGFP (d, green) were detected in GPC areas (arrowhead), but not in the adjacent IPC (dashed line). **e, f** S-IPC-specific wg(KO;Gal4) UAS-cdbGFP expression (green, double arrowheads) in mid 3rd instar larvae (**e**) was absent in wg[KO;NRT-wg] flies (**f**). Arrowheads indicate expression in GPC area. **g, h** In controls, wg(KO;Gal4) UAS-FLP-mediated wg[KO;FRT wg] FRT NRT-wg allele switching and simultaneous UAS-NRT-wg overexpression were induced at the mid 3rd instar larval stage (g, wg+ background). Allele switching at the 1st instar larval stage (h, NRT-wg background) did not rescue s-IPC-specific Wg loss (green). **i, j** Unlike in controls (**i**), R46E01-Gal4 UAS-FLP-mediated GPC areas-specific wg[KO;FRT NRT-wg FRT wg+] allele switching (j) rescued s-IPC-specific NRT-Wg loss (green). Filled and open triangles in transgenic schematics represent FRT and loxP sites, respectively (g–j). **k** The Wg target gene reporter lines fzs-GD0357-GFP (k, green) and notum-W48-JacZ (l, green) show expression in the GPC areas, the s-IPC, and in migratory progenitors (arrow) originating from the adjacent p-IPC. **m, n** Unlike in controls (**m**), fas3N2281-Gal4-mediated IPC-specific fz and fzs knockdown (**n**) caused loss of Wg (green) in the s-IPC. **o** Summary of wg function in the GPC areas. For genotypes and sample numbers, see Supplementary Table 1. Scale bars, 50 μm.

**Fig. 3** DAC controls layer 3/4 T4/T5 neuron identity. **a** A schematic model of the IPC cell lineages. **b** In lineage-tracing experiments using R45H05-Gal4, clones included C2, the T2-variant T2a, and/or T3 neurons, as judged by their characteristic cell body positions and terminals in the lamina and lobula, respectively (Fig. 4e). While C3, T2, and T3 neurons were not affected, C2 neurons were fully or partially absent in wg[KO;NRT-wg] flies with two or three remaining lobula plate layers, respectively (Fig. 4f, g; Supplementary Fig. 4c–i). Due to the lack of specific markers, we could not conduct tests for T2a neurons. Thus, T4/T5 neurons innervating layers 3/4, C2, and possibly T2a neurons are specifically derived from the dorsal and ventral Dpp-expression domains, while the p-IPC core generates T4/T5 for layers 1/2, as well as C3, T2, and T3 neurons (Fig. 4h).

**Notch controls the choice between T4 and T5 neuron identity.** In the OPC, Notch signaling in asymmetric GMC divisions contributes to generating neuronal diversity35, involving differential apoptosis in region-specific lineages56. We therefore assessed whether this pathway could mediate the distinction of layer 1/2 and 3/4 innervating T4/T5 neurons downstream of Dpp. We did not detect apoptotic cells in the 3rd instar larval lobula plate (Supplementary Fig. 6a). However, expression of an activated, ligand-independent form of Notch (N(tnt)) in d-IPC Nbs in the second competence window and their progeny using the late R9B10-Gal4 driver affected T4 neuron formation, because neurites were missing in the medulla of 3rd instar larvae (Fig. 6a, b). Adults exhibited a milder phenotype, in which mostly T4 neurons connecting to the anterior proximal medulla were affected (Supplementary Fig. 6b–h). Conversely, IPC-specific knockdown of the transcriptional regulator Suppressor of Hairless (Sos(H1)) using R17B05-Gal4 caused the absence of T5 neurons in adults, whereas T4 neurons were present (Fig. 6c, d; Supplementary Fig. 6f). In these flies, lobula plate layers 3 and 4 could not be discriminated (Supplementary Fig. 6i). The lobula plate and lobula neuropils were severely disorganized, likely because of an early requirement of Notch in p-IPC NE cells during the 3rd instar larval stage27 (Supplementary Fig. 6j, k). Hence, Notch controls the choice between T4 and T5 neuron fate (Fig. 6e), whereas the distinction between layer 1/2 Dac-positive and layer 3/4 Dac-negative T4/T5 neurons is mediated by a Notch-independent mechanism.

**Dpp-dependent specification of layer 3/4 T4/T5 neurons by omb.** While dpp reporter gene activity extended from the p-IPC...
Fig. 3 s-IPC-derived Wg is required for Dpp-dependent EMT in the p-IPC. a, b In 3rd instar larvae (3L), dpp-lacZ (blue) was detected in subdomains adjacent to wg(KO;Gal4) UAS-cd8GFP (green) regions in the dorsal and ventral OPC (a, arrowheads) and p-IPC (b). Double arrowhead indicates GFP-positive Nb clone adjacent to the dorsal p-IPC. c, d dpp-lacZ (blue) was maintained in progenitor streams (arrows) from the ventral and dorsal p-IPC subdomains. e In 2nd instar larvae (2L), dpp-lacZ (blue) was present in the OPC (arrowhead) adjacent to wg(KO;Gal4) UAS-cd8GFP-positive GPC areas (green), but was absent in the IPC (dashed line). f Schematic illustrating Wg and Dpp expression domains. Arrow indicates progenitor stream originating from the ventral p-IPC subdomain. g–k Unlike in controls (g, arrow), dpp-lacZ (green) was absent from the IPC in wg(KO;NRT-wg) flies (h, asterisk). The OPC was not affected. dpp-lacZ was ectopically induced in the IPC (arrowheads) by h1J3-Gal4-mediated expression of UAS-armS10 (i). dpp-lacZ was absent following fas3NP1233-Gal4-mediated IPC-specific wg knockdown. In the Dpp-expression domain, this caused EMT defects and loss of progenitor streams (j, asterisks). Similar defects were caused by IPC-specific tkv knockdown. dpp-lacZ remains expressed in the p-IPC (k, double arrowheads). l–n Compared to controls (l), fas3NP1233-Gal4-mediated IPC-specific knockdown of fz and fz2 (m) and tkv (n) caused the loss of lobula plate (Lop) layers 3/4 labeled with Connectin in adults. o Summary of wg and dpp function in the GPC areas, the s-IPC/Nb clone, and p-IPC. For genotypes and sample numbers, see Supplementary Table 1. Scale bars, 50 μm.
to the d-IPC, phospho-Mad (pMad) labeling was restricted to p-IPC NE cells (Fig. 7a). Consistently, knockdown experiments using Gal4 lines with progressively restricted activities (Supplementary Fig. 7a–c) revealed a requirement of tkv for layer 3/4 neuron formation in p-IPC NE cells (cf. Fig. 3n), but not in d-IPC Nbs and T4/T5 neurons (Supplementary Fig. 7d–f). Therefore, an additional mechanism must relay Dpp signaling activity in p-IPC NE cells to distant postmitotic T4/T5 neurons. The T-box transcription factor Optomotor blind (Omb) is a Dpp target in the p-IPC. In 3rd instar larval brains, Omb expression is maintained in progenitors, Nbs, and T4/T5 neurons derived from the Dpp-positive p-IPC subdomains (Fig. 7b, c). Expression persisted in adult T4/T5 neuron subsets (Supplementary Fig. 7g). In wg[KO;NRT-wg] flies, omb expression was severely reduced in

Fig. 4 The Dpp-expression domain generates C2 and T4/T5 neurons for layers 3/4. a R4S05-Gal4 UAS-cd8GFP (green) colocalizes with the dpp-lacZ (blue) expression domain in the IPC (arrow) but not the OPC (arrowhead) in 3rd instar larvae (3L). b R4S05-Gal4 UAS-cd8GFP (green) was weakly maintained in T4/T5 neurons innervating Connectin-positive lobula plate (Lop) layers 3/4 (red, arrow) in adults. c Permanent GFP-labeling (green) of the R4S05-Gal4 expression domain using act>FRT106-FRT-stopFRT>Gal4 was specific to T4/T5 neurons innervating lobula plate layers 3/4. d R9810-Gal4 in conjunction with Flybow transgenes labeled T4/T5 neuron clones either innervating lobula plate (Lop) layers 1/2 (mCitrine, yellow) or 3/4 (mCherry, red). e Permanent GFP labeling (green) with R4S05-Gal4 included C2, as well as T2a and/or T3 neurons with characteristic axon terminals in the lamina (La, arrow) and lobula (Lo, arrowhead). f, g Unlike in controls (f), C2 neuron-specific R17C06-Gal4 UAS-cd8GFP expression (green) with axon terminals (arrow) in the lamina (La) was absent in wg[KO;NRT-wg] flies that had two remaining lobula plate layers (g). h Schematics illustrating the neuron subtypes derived from the Dpp-expression domain and the core p-IPC in 3rd instar larvae and adults. For genotypes and sample numbers, see Supplementary Table 1. Scale bars, 50 μm.
the p-IPC and progeny, but not in the OPC (Fig. 7d, e). Consistently, Omb was also decreased following IPC-specific tkv knockdown (Fig. 7f, g).

To determine the function of omb, we conducted knockdown experiments using fas3-Gal4 and two validated UAS-RNAi transgenes (Supplementary Fig. 7h–k). Experimental animals were raised at 18 °C and shifted to 29 °C at the early 3rd instar larval stage, because omb is expressed in the embryonic optic lobe placode and null mutations cause severe disorganization or complete loss of adult optic lobes37. Under these conditions, omb knockdown did neither affect dpp expression nor the EMT of progenitors from the p-IPC (Fig. 7h, i). However in adults, lobula plate layers 3/4 were absent (Fig. 7j, k). In contrast to tkv, omb knockdown using Gal4 lines with progressively restricted activities revealed that this transcription factor is required in Nb in the second competence window and postmitotic T4/T5

\[ R8B10 > cd8GFP \]

\[ dace7a23 > cd8GFP \]

\[ R9B10 > cd8GFP \]

\[ dac1 > MARCM \]

\[ GPA > MARCM \]

\[ fas3 > cd8GFP \]

\[ dac1, ato1 > MARCM \]

\[ Control \]

\[ dac1, ato1 > MARCM \]

\[ Control \]
neurons for layer 3/4 neuron specification (Fig. 7l, m). Thus, \textit{omb} could relay subdomain-specific Dpp signaling effects in p-IPC NE cells across intermediate cellular states, i.e., migratory progenitors, Nbs, and T4/T5 progeny (Fig. 7n).

\textbf{omb mediates layer 3/4 T4/T5 neuron specification by Dac repression.} Finally, we assessed whether Omb could be responsible for downregulating Dac. In 3rd instar larvae, Omb was coexpressed with high levels of Dac in younger and low levels in older T4/T5 neurons (Fig. 8a). From 24 h after puparium formation (APF) (Fig. 8b) to adulthood (Supplementary Fig. 8a), Omb and Dac showed mutually exclusive expression in T4/T5 neurons. While Dac was maintained in \textasciitilde50% of adult T4/T5 neurons in controls (Fig. 8c, h), 95% of T4/T5 neurons expressed Dac and adopted layer 1/2 identity following \textit{omb} knockdown (Figs. 7m, 8d, b). Conversely, when \textit{omb} was overexpressed, none of the T4/T5 neurons expressed Dac and all acquired layer 3/4 identity (Fig. 8e, h, j). Under both conditions, many T4/T5 neurons underwent apoptosis in 3rd instar larvae (Supplementary Fig. 8b–d). Consistently in adults, their numbers were reduced by 33% and 48%, respectively (Fig. 8h), possibly because excessive T4/T5 neurons either for layers 1/2 or 3/4 compete for limited trophic support. To demonstrate that \textit{omb} is sufficient, we took advantage of \textit{wg\textsuperscript{KO;NRT-wg}} flies, in which all T4/T5 neurons adopted layer 1/2 identity and Dac was downregulated in 97% of these (Fig. 8f, h; Supplementary Fig. 8e, f). In this background, \textit{omb} overexpression only mildly affected neuron numbers (19% reduction) and Dac was downregulated (Fig. 8g, h). Importantly, concomitant upregulation of Connectin suggested that these differentiated into layer 3/4 innervating T4/T5 neurons (Fig. 8i, k). Thus, \textit{omb} is required and sufficient for specifying T4/T5 neurons innervating layers 3/4 by downregulating Dac (Fig. 8l).

\section*{Discussion}

The spread of Wg is dispensable for patterning of many tissues\textsuperscript{30}. However, our study uncovered a distinct requirement for diffusible Wg in the nervous system, where it orchestrates the formation of T4/T5 neurons innervating lobula plate layers 3/4. Their generation depends on inductive mechanisms (Fig. 8m) that are relayed in space and time. The spatial relay consists of a multistep-signaling cascade across several NE domains: Wg from the GPC areas induces \textit{dpp} expression in the s-IPC and Nb lineage adjacent to ventral and dorsal p-IPC subdomains; this secondary Wg source activates \textit{dpp} expression. \textit{Dpp} signaling mediates EMT of migratory progenitors from these subdomains. The p-IPC core produces Dac-positive layer 1/2 specific T4/T5 neurons. \textit{Dpp} signaling in p-IPC NE subdomains triggers a temporal relay across intermediate cellular states by inducing \textit{omb}. Omb in turn suppresses Dac, conferring layer 3/4 identity to postmitotic T4/T5 neurons.

![Fig. 5](image5) \textbf{Fig. 5} \textit{dac} and \textit{ato} are required for T4/T5 neuron formation. \textbf{a} Schematic illustrating the expression of Ase (turquoise), Ato (red), and Dac (blue) in d-IPC Nbs during the first (1) and second (2) competence windows and their progeny. \textbf{b, c} Dac (red) was expressed in all \textit{R9B10-Gal4 UAS-cd8GFP} (green) labeled T4/T5 neurons in 3rd instar larvae (b). It was downregulated (arrowheads) in approximately 50% of adult T4/T5 neurons (c). \textbf{d} \textit{dac\textsuperscript{mutant}} labeled T4/T5 neurons (green) faithfully reported Dac (red, double arrowhead) expression in adults (d), specifically labeling T4/T5 neurons innervating lobula plate (Lop) layers 2 and 3 (e). \textbf{f} Schematic illustrating Dac expression (blue) in adult T4/T5 neurons. \textbf{g–l} Unlike in controls (g, i), \textit{dac\textsuperscript{mutant}} mutant T4/T5 neurons adopted T2/T3 neuron morphologies with \textit{R9B10-Gal4 UAS-cd8GFP} (green), displaying neurite extensions into medulla (Me) layer M9 (arrows) and more distal layers (arrowhead) (h), and \textit{UAS-brp-RFP} labeled synaptic terminals (red, arrows) in lobula (Lo) layers 2 and 3 (j). \textbf{k} Unlike in controls (k), \textit{fas3\textsuperscript{mutant}} labeled T4/T5 neurons (green) mediated IPC-specific simultaneous knockdown of dac and ato generated neurons that failed to form a four-layered lobula plate (Lop) neuropil and dendrites in medulla (Me) layer 10 and lobula (Lo) layer 1 (l). \textbf{m, n} Unlike in controls (m), Fas3-positive (red) T4/T5 neurons were absent following IPC-specific simultaneous knockdown of \textit{dact} and ato mediated by \textit{fas3\textsuperscript{mutant}} (green) (n). For genotypes and sample numbers, see Supplementary Table 1. Scale bars, 50 μm.

![Fig. 6](image6) \textbf{Fig. 6} Notch controls the choice between T4 and T5 neuron fate. \textbf{a, b} Unlike in controls (a), \textit{R9B10-Gal4 UAS-cd8GFP} labeled T4 neurites were absent in 3rd instar larvae following \textit{UAS-N\textsuperscript{intra}} overexpression in d-IPC Nbs and their progeny during the second competence window (b). T5 neurites in the lobula (Lo) were unaffected. \textbf{c, d} Unlike in controls (c), following \textit{R17B05-Gal4 UAS-cd8GFP}–mediated IPC-specific \textit{Su(H)} knockdown, T5 neurites were absent in the adult lobula (d). T4 neurites were present. \textbf{e} Schematic illustrating the Notch-dependent specification of T5 neurons. For genotypes and sample numbers, see Supplementary Table 1. Scale bars, 50 μm.
When Wg is membrane-tethered, the first step of this cascade is disrupted. This defect is not caused by decreased signaling activity of NRT-Wg protein in \textit{wg} \textit{KO;NRT-wg} flies. First, wild-type Wg signaling activity inside the GPC areas and the adjacent OPC was not affected. Second, in allele switching experiments, ectopic expression of a highly active UAS-NRT-wg transgene in the GPC areas was unable to rescue. By contrast, restoring wild-type \textit{wg} function in the GPC areas was able to rescue, supporting the notion that Wg release and spread from the GPC areas are required to induce its own expression in the s-IPC and the Nb clone.

Although Wg release is essential, the range of action is likely limited. Wg expression in the s-IPC commences in early 3rd instar larvae, when it is still in close proximity with the GPC. Half of the \textit{wg} \textit{KO;NRT-wg} flies showed residual \textit{dpp} expression in one progenitor stream at the 3rd instar larval stage and a 25% reduction of T4/T5 neurons, correlating with three lobula plate layers in adults. The other half lacked \textit{dpp-lacZ} expression and
showed a 50% reduction of T4/T5 neurons correlating with two remaining layers. While this partial phenotypic penetrance is not fully understood, NRT-Wg likely partially substituted for Wg because of the initial close proximity of the GPC areas and the s-IPC and Nb clone. Occasional residual NRT-Wg expression in the s-IPC argues against an all-or-nothing inductive event and suggests a model, whereby cell-intrinsic signaling thresholds have to be reached. Theoretically, the dpp expression defect in the p-IPC of wg[KO;NRT-wg] flies could reflect the dependence on long-range Wg from the GPC areas. However, as we have shown, IPC-specific wg knockdown leads to dpp loss in the p-IPC. Propagation of sequential Wnt signaling could explain long-range...
Drosophila eye\textsuperscript{30}, suggesting that the regulatory mechanism observed in the optic lobe might be employed in several contexts. The different outcomes of early and late allele wg to NRT-wg allele switching indicate that Wg secretion is required for the induction but not long-term maintenance of wg expression in the s-IPC. The GPC areas become rapidly separated from the s-IPC and Nb clone by compact rows of newly generated neurons. As part of a relay system, diffusible Wg may therefore be required to bridge distances over a few cell diameters during the initial phase of neurogenesis. The s-IPC in wg\texttextsuperscript{fl(ko);NRT-wg} flies expressed Hth and generated two neuron clusters as in wild-type. Thus, the sole function of wg in the s-IPC is to relay the GPC-derived Wg signal to induce dpp expression in the p-IPC. Since Wg release is not required in the GPC areas to induce dpp in the adjacent OPC, this secondary wg function in the s-IPC is most likely juxtacrine. Compared to approximately 80 medulla neuron subtypes derived from the OPC\textsuperscript{36}, the specification of 13 distinct subtypes originating from the p-IPC appears simple. However, the distinct mechanisms employed are surprisingly complex. We previously showed that cross-regulatory interactions between D and tll regulate a Nb competence switch from generating early-born C2, C3, T2, T2a, and T3 neurons to eight distinct layer-specific T4/T5 subtypes. Ato and Dac are expressed in the second Nb competence window and depend on tll\textsuperscript{23}. Our functional studies showed that dac mutant T4/T5 neurons adopted early-born T2/T3 neuron-like morphologies. Similarly, ato mutant T4/T5 neurons displayed neurite connectivity defects\textsuperscript{41}. Notably, simultaneous knockdown of dac and ato resulted in the absence of T4/T5 neurons, demonstrating that both are required together for the ability of d-IPC Nbs to produce new neuron subtypes in the second competence window.

Dac is initially expressed in all T4/T5 neurons but only maintained in layer 1/2 innervating subtypes. This suggests that an essential step for the specification of layer 3/4 innervating neurons is the downregulation of Dac and the suppression of the T4/T5 default neuron fate, i.e., layer 1/2 identity. Although the mode of this inhibitory mechanism depends on the outcome of the Nb-specific switching mechanism in the d-IPC, it is already primed in p-IPC NE cells. Thus, layer-specificity and therefore motion-directionality are determined early in the NE precursors of T4/T5 neurons. Molecularily, it involves the Omb-mediated relay of Dpp-signaling-dependent NE cell patterning information across intermediate cell states to postmitotic T4/T5 neurons resulting in the repression of Dac. In contrast to the OPC\textsuperscript{36}, we found no link between NE patterning in the p-IPC and Notch-dependent differential apoptosis of region-specific T4/T5 subtypes. Instead, Notch controls the choice between T4 and T5 identity, likely during the second competence window, indicating that the distinction between layer 1/2 and 3/4 fates precedes T4 and T5 neuron specification.

The mechanisms controlling the maintenance of omb expression, and Omb-mediated downregulation of Dac are unclear. Hypotheses regarding the latter have to be reconciled with the fact that dac, together with ato, is required for the formation of all T4/T5 neurons and hence is expressed in all d-IPC Nbs during the second competence window. Omb and Dac are initially co-expressed in Nbs and young T4/T5 neurons, suggesting that Omb does not directly repress dac transcription. Yet, expression of the dac\textsuperscript{Omb} enhancer trap Gal4 line showed that dac is only transcribed in layer 1/2 neurons in adults. A possible scenario is that Omb could break Dac autoregulation by triggering degradation of Dac. Since T-box genes can act as transcriptional activators and repressors\textsuperscript{42} and their effects are influenced by various cofactors\textsuperscript{13}, future studies will need to explore the molecular details underlying Omb-mediated repression of Dac. It will also be important to determine whether layer 3/4 specification is mediated solely by Dac downregulation, or whether omb has additional instructive roles.

Consistent with the observation that C2 and C3 neurons have distinct developmental origins\textsuperscript{44}, we found that Nbs derived from the Dpp-expression domain produce C2 and possibly T2a neurons during the first Nb competence window, while the core p-IPC generates C3, T2, and T3 neurons. dac mutant T4/T5 neurons adopt T2/T3-like morphologies suggesting that this is the default neuron fate in this neuron group. While Omb is maintained in C&T neurons derived from the Dpp-expression domain, Dac is not expressed, suggesting that Omb interacts with other molecular determinants in these neurons. While we did not explore how layer 1 and 2 neurons or layer 3 and 4 neurons become distinct from each other because of the lack of specific markers, our data suggest a possible contribution of Ato/Dac and Notch signaling, as these are active within the d-IPC. Findings in a concurrent study of Pinto-Teixeira et al.\textsuperscript{45} align with our data concerning the role of Dpp and Notch signaling. Furthermore, a second study of Mora et al.\textsuperscript{46} reported an additional role for Ato in controlling the transient amplification of d-IPC Nbs by symmetric cell division to ensure that the correct number of T4/T5 neurons is produced. It will be fascinating to identify the transcriptional targets of Notch, Ato/Dac, and Omb that mediate ganglion- and layer-specific targeting of T4/T5 dendrites and axons, respectively. Finally, future behavioral studies of layer 3/4-deficient flies will address what effect direction selectivity is affected or compensatory mechanisms are in place.

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Fig. 8 Omb converts layer 1/2 into layer 3/4 T4/T5 neurons by Dac downregulation. a, b In the 3rd instar larvae (a). Omb (red) and Dac (blue) were initially co-expressed (arrows) in new-born R8910-Gal4 UAS-cdbGFP (green) labeled T4/T5 neurons close to the d-IPC. In some more mature T4/T5 neurons, that were positive for Omb, Dac expression was low (arrowheads) and generated two neuron clusters as in wild-type. While in others, Dac expression was high (double arrowheads) and Omb expression was absent. At 24 h after puparium formation (APF) (b), Dac (double arrowheads) and Omb (arrowheads) show mutually exclusive expression.

c-g In controls (c). Dac (red, double arrowheads) was expressed in approximately 50% of T4/T5 neurons. Arrowheads indicate Dac-negative R8910-Gal4 UAS-cdbGFP (green) labeled T4/T5 neurons. Dac was maintained in almost all T4/T5 neurons following omb knockdown (d), and downregulated following omb overexpression (e). Dac was expressed in all T4/T5 neurons in wg\texttextsuperscript{(KO);NRT-wg} flies with two lobula plate layers (f). Ectopic Omb was sufficient to downregulate Dac in these flies (g). h Quantification of all and Dac-positive T4/T5 neuron numbers following omb manipulations. The scatter plot with bars shows data points and means with 95% confidence interval error bars (n = 15 corresponding to three serial optical sections, 6-μm apart, from five samples per genotype). Unpaired, two-tailed Student’s t-test not assuming equal variance: P = 5.57 × 10^-12, P = 6.19 × 10^-15, P = 1.26 × 10^-10, P = 1.98 × 10^-17, P = 3.60 × 10^-15, P = 4.79 × 10^-15, P = 0.020, P = 0.017, P = 0.0015, P = 2.42 × 10^-13. ** P < 0.01; *** P < 0.001; **** P < 0.0001. i-k Unlike in controls (i), R8910-Gal4-mediated ectopic UAS-omb expression in T4/T5 neurons of wild type (j) or wg\texttextsuperscript{(KO);NRT-wg} (k) flies resulted in ectopic Connectin expression in the lobula plate (Lop). l Schematic illustrating Dac and Omb expression in adults. m Working model for spatial and temporal relay mechanisms regulating the formation and specification of layer-specific T4/T5 neurons. For genotypes and sample numbers, see Supplementary Table 1. Scale bars, 50 μm.
Signaling centers, also called organizers, pattern tissues in a non-autonomous fashion. The vertebrate roof plate and the cortical hem, for instance, both release Wnts and Bmps to pattern NC cells in the developing dorsal spinal cord and in the surrounding forebrain, respectively. In the Drosophila visual system, the GPC areas express Wg and Bmp signaling induces the expression of the GPC-derived invertebrate and vertebrate nervous system development. At the onset of neurogenesis, the GPC areas express Wg release from the GPC areas could coordinate the timely onset of neurogenesis in the OPC patterning and neurogenesis, this firmly establishes the GPC areas as local organizers of optic lobe development. At the onset of neurogenesis, Wg is first expressed in the GPC areas followed by the s-IPC, explaining the well-established delay in neurogenesis between the IPC and OPC. Wg release from the GPC areas could coordinate the timely onset of neurogenesis in the OPC and IPC to safeguard the alignment of matching partner neurons across several retinotopically organized neureules. The intercalation of new-born neurons between both neuroepithelia may have driven the need for a relay system using primary and secondary sources of Wg. Wg induces Dpp to both neuroepithelia may have driven the need for a relay system of matching partner neurons across several retinotopically organized neureules. The temporal relay using primary and secondary sources of Wg. Wg induces Dpp to both neuroepithelia may have driven the need for a relay system of matching partner neurons across several retinotopically organized areas. The temporal relay mediated by Omb represents an efficient strategy to pass the memory of spatial NC patterning information by Dpp signaling on to postmitotic neurons generated at a distance. It is thus intricately tuned to the distinct neurogenesis mode of the p-IPC essential for spatially matching birth-order-dependent neurogenesis between the IPC and OPC. Interestingly, the progressive refinement of NC patterning by the induction of secondary signaling centers plays a central role in vertebrate brain development. Furthermore, similar signaling cascades have been recently identified in mammalian optic tissue cultures where sequential Wnt and Bmp signaling induces the expression of the Omb-related T-box transcription factor Tbx5 to specify dorsal retinal NC cells. Hence, such cascades could encode conserved regulatory modules that are employed repeatedly during invertebrate and vertebrate nervous system development.

Methods

Genetics. Drosophila melanogaster strains were maintained on standard medium at 25°C and except for Gal80 and RNAi experiments, in which progeny were shocked for 40–60 min at 40–48 °C and 72–96 h at 29 °C. For knockdown experiments using UAS-ntr-expressing flies were crossed with (1) wg{KO;NRT-wg}/CyO; UAS-FLP FRT NRT-wg and shifted to 29 °C at the late 3rd instar larval stage. For UAS-NRT-wg gain-of-function experiments, wg{KO;FRT-wg} UAS-cd8GFP; R12G08-Gal4 flies were crossed with wg{KO;FRT-wg}/CyO; FRT40A; UAS-brp-RFP flies and kept at 18°C. While experimental animals were shifted to 29°C at the 1st instar larval stage, control animals were maintained at 18°C until the mid 3rd instar larval stage and then shifted to 29°C. wg{KO;FRT-wg} FRT NRT-wg flies are described in ref. 52. For fluorescence microscopy, fluorescence microscopy of the adult brain, larval brain, and retinal explants was performed using a microscope (Olympus BX61; excitation filters: Cy3: #112-166-003; Alexa Fluor 647: #115-606-003), further, goat antibody to chicken IgY (H+L) (Alexa Fluor 655, Molecular Probes, #A21437, 1:400) was used. Images were collected with a Leica TCS SP2 II laser scanning confocal microscope and processed using Adobe Photoshop and Fiji software programs.

Quantifications and statistics. Statistical details of all experiments are reported in the figures and figure legends. To quantify T4:T5 neuron numbers, adult optic lobes were imaged in horizontal orientations and cell numbers were collected from several independent biological sections (five biological sections in five flies; or 250 cells from two biological sections in one fly). The number of identified genotypes that were not limited in repetitively. The calculations of 95% confidence interval error bars and unpaired two-tailed Student’s t-test P values for independent samples (Excel software, 480th, Cyber-T, and GraphPad Prism 7 for type 3, not assuming equal variance).
assumption of normality in one or both tests. Quantifications are presented as scatter plots and bar graphs with means ±95% confidence interval error bars.

**Data availability** Image data sets generated and analyzed in this study are available from the corresponding author upon reasonable request. A source data file for quantifications shown in Figs. 1h and 8h is provided with this manuscript (Supplementary Data 1).

Received: 23 January 2018 Accepted: 4 May 2018
Published online: 12 June 2018

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Acknowledgements
We are grateful to C. Alexandre, A. Baena-Lopez, and J. P. Vincent (The Francis Crick Institute, London) for all Wg reagents and their invaluable advice for this study. We thank A. Brand (The Gurdon Institute, Cambridge), A. Gould (The Francis Crick Institute), Y. N. Jan (HHMI, San Francisco), R. Mann (Columbia University, New York), G. Pflugfelder (University of Mainz), F. Pignoni (SUNY Upstate Medical University), S. Sigrist (EU Berlin), J. Skeath (Washington University, St. Louis), L. Tsuda (NCGG, Obu), U. Walldorf (University of Homburg), the Bloomington Drosophila Stock Center, the Drosophila Genomics Resource Center, the Vienna Drosophila Resource Center, and the Developmental Studies Hybridoma Bank for fly strains and antibodies. We thank A. Yuen and W. Wang for help with Flybow and MARCM experiments. We thank H. Pynor for advice on the 3D drawing of the larval optic lobe. We thank J. P. Vincent and C. Alexandre, as well as A. Avola, C. de Miguel Vijandi, E. L. Powell, and R. Kaschula (The Francis Crick Institute, London) for critical reading of the manuscript. This work was supported by the Francis Crick Institute which receives its core funding from Cancer Research UK (FC001151), the UK Medical Research Council (FC001151) and the Wellcome Trust (FC001151), and by the UK Medical Research Council (U117581332).

Author contributions
H.A. and I.S. conceived and designed the study. H.A. performed the experiments and analyzed the data. H.A. and I.S. prepared the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-04592-z.

Competing interests: The authors declare no competing interests.

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