Temporal patterning in intermediate progenitors increases neural diversity

Omer Ali Bayraktar[1,2] and **Chris Q. Doe**[1,2,3,*]

[1]Howard Hughes Medical Institute, University of Oregon, Eugene, OR 97403

[2]Institute of Molecular Biology, University of Oregon, Eugene, OR 97403

[3]Institute of Neuroscience, University of Oregon, Eugene, OR 97403

Abstract

Human outer subventricular zone (OSVZ) neural progenitors and Drosophila type II neuroblasts both generate intermediate neural progenitors (INPs) that populate the adult cerebral cortex or central complex, respectively. It is unknown whether INPs simply expand or also diversify neural cell types. Here we show that Drosophila INPs sequentially generate distinct neural subtypes; that INPs sequentially express Dichaete>Grainyhead>Eyeless transcription factors; and that these transcription factors are required for the production of distinct neural subtypes. Moreover, parental type II neuroblasts also sequentially express transcription factors and generate different neuronal/ glial progeny over time, providing a second temporal identity axis. We conclude that neuroblast and INP temporal patterning axes act combinatorially to generate increased neural diversity within adult central complex; OSVZ progenitors may use similar mechanisms to increase neural diversity in the human brain.

Proper brain development requires the production of a vast array of neurons and glia from a relatively small pool of stem/progenitor cells. Spatial patterning mechanisms generate progenitor diversity along the anterior-posterior and dorso-ventral axes, but the temporal patterning cues used by individual progenitors to make different neural cell types over time remain poorly characterized[1–2]. Drosophila neural progenitors (called neuroblasts; NBs) are a model system to study temporal patterning. Most embryonic and larval NBs undergo a "type I" cell lineage to bud off a series of smaller ganglion mother cells (GMCs) that each make a pair of neurons or glia[3–8] (Fig. 1a), and transcription factors that specify temporal identity have been characterized in both embryonic NBs [3–9] and larval NBs[10,11].

We and others have recently discovered six "type II" NBs in the dorsomedial larval brain lobe (DM1–DM6) and two with more lateral positions[12–14] (Fig. 1a). Type II NBs undergo self-renewing asymmetric cell divisions to generate a series of smaller intermediate neural

progenitors (INPs); then each INP also undergoes self-renewing divisions to generate a series of ~6 GMCs, which typically each produce two neurons or glia[12–14] (Fig. 1a). Thus, both NBs and INPs generate a series of progeny over time. For clarity we say type II NBs transition from early > late over time, and INPs transition from young > old over time (Fig. 1a). Type II NBs give rise to large clones of neurons and glia that populate the adult brain central complex (CCX)[15–17]. Thus, type II NBs share features with human OSVZ progenitors: both progenitors generate INPs, and both are used to increase the number of neurons in a particular brain region[18,19]. Although there are at least 60 morphologically distinct neurons in the fly adult CCX[20], we know virtually nothing about how parental NBs or INPs generate neural diversity.

INPs sequentially express three transcription factors

We asked whether single INPs sequentially express a series of transcription factors, which would be indicative of temporal patterning. We used the previously characterized *R9D11-gal4* line driving *UAS-GFP* to mark all INPs and their progeny from the DM1–DM6 NB lineages (Fig. 1b)[15]. INPs can be identified as small Deadpan (Dpn)+GFP+ cells that are adjacent to the Dpn+GFP-type II NB (Fig. 1b'); they are distinct from Dpn-GMCs and neurons. Importantly, INP age can be determined by its distance from the parental type II NB: newly-born young INPs are close to the parental NB, whereas older INPs are displaced further from the parental NB[13,15,21] (Fig. 1b'). The ability to identify progressively older INPs allowed us to screen for transcription factors that were only present in young, middle, or old INPs.

We screened a collection of 60 antibodies to neural transcription factors (Sup Table 1), and found three that were sequentially expressed in INPs. In late larvae at 96h and 120h after larval hatching (ALH), young INPs near the parental NB contained the Sox-family transcription factor Dichaete (D)[22,23]; D was not detected in old INPs further from the parental NB (DM3 shown in Fig. 1c–d; similar expression was observed in other DM lineages; Sup Fig. 1). In contrast, the Pax6 transcription factor Eyeless (Ey)[24] was detected in old INPs but not young D+ INPs; there were very few double-negative or double-positive INPs (Fig. 1c,d). Similarly, the *R12E09-gal4* line containing a 2.7 kb *D* enhancer fragment[25] was expressed in young INPs, whereas the *OK107-gal4* enhancer trap at the *ey* locus[26] was expressed in old INPs (detailed expression patterns are shown in Sup Fig 2; henceforth called *R12E09^D* and *OK107^Ey*). The D-to-Ey series was detected in all type II lineages examined and at all larval stages (DM1–DM6 at 24–120h ALH; Fig. 1e and Sup Tables 2–3; Sup Fig 1). Thus, all INPs – from different type II NBs and from early or late NBs – sequentially express D and Ey (Fig 1h–i).

In addition, we found that "middle-aged" INPs contained the CP2 family DNA-binding factor Grainyhead (Grh)[27]. Grh was assigned to middle-aged INPs because its expression overlapped both D and Ey at their expression border (Fig. 1e). Thus, INPs transition through four molecular states (Fig. 1h,g); it is likely that several GMCs are born during each of these windows, but for simplicity only one GMCs per window is shown in our summaries. The D>Grh>Ey series was observed in INPs born from multiple type II NBs (DM2–DM6; DM1 does not have detectable Grh) and in INPs born at all larval stages (Fig. 1g and Sup Tables

4–5; Sup Fig 1). In addition to its expression in INPs, Grh is also detected in type II NBs and transiently in immature INPs (Fig. 1e). We conclude that most INPs progress through a stereotyped D>Grh>Ey transcription factor series (Fig. 1h–i).

Cross-regulation between INP temporal transcription factors

We next wanted to determine if D, Grh, and Ey exhibit cross-regulation in INPs. We used *wor-gal4, ase-gal80*[29] to drive *UAS-D^RNAi* in a *dichaete* heterozygous background (subsequently called D^RNAi), which removed detectable D from INP lineages (Sup Fig 4). Compared to wild type, D^RNAi resulted in a significant loss of early-born Grh+Ey– INPs (Fig 2a–d), without altering the number of later-born Grh+Ey+ INPs (Fig. 2c, Sup Fig 4). The same result was observed in *D* mutant clones (Sup Fig 4). In contrast, misexpression of D did not lead to ectopic Grh expression (Sup Fig 4). Thus, D is necessary for the timely activation of Grh in INP lineages, although D-independent inputs also exist (Fig 2m).

To test whether Grh regulates D or Ey, we used *R9D11-gal4* to drive *UAS-Grh^RNAi* in a *grh* heterozygous background (subsequently called Grh^RNAi), which significantly reduced Grh levels in middle-aged INPs (Sup Fig 5). Grh^RNAi increased the number of D+ INPs at the expense of Ey+ INPs (Fig. 2e–f) without altering the total number of INPs (control 33.2 ± 5.1; Grh^RNAi 31.7 ± 3.3; p=0.57). As expected, Grh^RNAi did not change the numbers of D+ and Ey+ INPs in the DM1 lineage, which lacks Grh expression (Sup Fig 5), nor did misexpression of Grh lead to ectopic Ey expression (Sup Fig 5). We conclude that Grh represses D and activates Ey within INP lineages (Fig. 2m).

To determine if Ey regulates D or Grh, we used *R12E09^D-gal4 UAS-FLP actin-FRT-stop-FRT-gal4* to drive permanent expression *UAS-Ey^RNAi* within INPs (subsequently called *R12E09^D>>act-Gal4* or INP-specific Ey^RNAi; see Fig. 3a for summary). We confirmed that INP-specific Ey^RNAi removed Ey expression from INPs (Fig. 2g; Sup Fig 7), without affecting Ey in the mushroom body or optic lobes (Sup Fig 6). Ey^RNAi resulted in a striking increase in the number of old D-Grh+ INPs, without affecting the number of young D+ INPs (Fig. 2g–h; Sup Fig 7). Conversely, Ey misexpression in INPs significantly reduced the number of Grh+ INPs (Fig. 2i–j; Sup Fig 7) without altering the total number of INPs (control 31.7 ± 2.5; Ey misexpression 34.7 ± 3.4; p=0.11). We also observed an increase in D+ INPs (Fig. 2j; Sup Fig 7), consistent with a regulatory hierarchy in which Ey represses Grh which represses D. This effect was not due to ectopic Ey directly activating D because misexpression of Ey had no effect on D+ INP numbers in the DM1 lineage, which lacks Grh expression (Sup Fig 7). We conclude that Ey is necessary and sufficient to terminate the Grh expression window in INPs. We propose a "feedforward activation/feedback repression" model for D>Grh>Ey cross-regulation (Fig 2m).

We noticed that Ey^RNAi resulted in an increase in the total number of INPs. This could be due to a prolonged INP cell lineage, or due INPs switching to symmetric cell divisions that expand the INP population. To distinguish between these alternatives, we induced permanently-marked clones using the MARCM technique[30] within wild type and Ey^RNAi INPs at 24h ALH, and assayed them at the end of larval life (120h ALH) to determine if they maintained a single INP per clone. Wild type clones never contained an INP, showing

that the INP lineages have ended by this time (Fig. 2k), whereas Ey[RNAi] always contained a single INP within the clone (Fig. 2l). In addition, all Grh+ INPs exhibited normal INP markers (Dpn+Ase+ nuclear Pros−) and retained the ability to generate nuclear Pros+Elav+ neurons (Sup Fig 8). We conclude that Ey[RNAi] extends individual INP cell lineages beyond that of wild type INPs.

INPs generate different neurons and glia over time

Next, we asked next whether distinct neuronal or glial subtypes were generated during each transcription factor expression window. To determine the cell types produced by young D+ INPs or old Ey+ INPs, we used permanent lineage tracing (see Fig. 3a). Cells labeled by *R12E09[D]* but not *OK107[Ey]* are generated by young INPs, whereas cells labeled by *OK107[Ey]* are generated by old INPs (Fig 3b,e; Sup Fig 3). We screened our collection of 60 transcription factor antibodies and found two that labeled subsets of young INP progeny, and two that labeled subsets of old INP progeny. The transcription factors D and Brain-specific homeobox (Bsh)[31] labeled sparse, non-overlapping subsets of young INP progeny (Fig 3c–d), but not old INP progeny (Fig. 3f–g,j; Sup Fig 9). Thus, young INPs generate Bsh + neurons, D+ neurons, and many neurons that express neither gene. In contrast, the glial transcription factor Repo[16,32,33] and the neuronal transcription factor Twin of Eyeless (Toy)[34] labeled sparse, non-overlapping subsets of old INP progeny, but not young INP progeny (Fig. 3h–j; Sup Fig 9). Additional mechanisms must restrict each marker (D, Bsh, Repo, Toy) to a small subsets of young or old INP progeny; e.g. each population could arise from just early- or late-born INPs within a type II NB lineage (see below). We conclude that INPs sequentially express the D>Grh>Ey transcription factors, and they generate distinct neuronal and glial cell types during successive transcription factor expression windows (Fig. 3k). These data provide the first evidence in any organism that INPs undergo temporal patterning.

INP transcription factors are required to generate temporally distinct neural subtypes

We wanted to determine if D>Grh>Ey act as temporal identity factors that specify the identity of INP progeny born during their window of expression. First, we investigate the role of Ey in the specification of late-born INP progeny. INP-specific Ey[RNAi] resulted in the complete loss of the late-born Toy+ neurons and Repo+ neuropil glia, but did not alter the number of early-born D+ and Bsh+ neurons (Fig. 4a–i). Removal of Toy+ neurons (using Toy[RNAi]) does not alter the number of Repo+ glia, and conversely removal of Repo+ glia (using Gcm[RNAi]) does not alter the number of Toy+ neurons (Sup Fig 10), thus Ey is independently required for the formation of both classes of late INP progeny. Conversely, permanent misexpression of Ey in early INPs increased late-born Toy+ neurons and decreased early-born Bsh+ neurons (Fig. 4j–n), consistent with Ey specifying late INP temporal identity. Unexpectedly, ectopic Ey reduced the number of late-born Repo+ glia (Fig. 4n; Sup Fig 11). We conclude that Ey is an INP temporal identity factor that promotes the independent specification of late-born Toy+ neurons and Repo+ glia (Fig. 4o).

We next tested whether D and Grh specify early and mid INP temporal identity. INP-specific D[RNAi] led to a small but significant reduction in the number of early-born Bsh+ neurons (Sup Fig 11), whereas INP-specific Grh[RNAi] severely reduced the number of early-born Bsh+ neurons (Sup Fig 11) without impairing INP proliferation (Sup Fig 5) or late INP progeny (Sup Fig 11). This is consistent with the Bsh+ neurons deriving from the D+ Grh+ expression window. Interestingly, misexpression of D or Grh did not increase Bsh+ neuron numbers (Sup Fig 11); perhaps D/Grh co-misexpression is required to generate Bsh+ neurons. We conclude that both D and Grh are required, but not sufficient, for the production of Bsh+ early INP progeny.

Late-born INP progeny are required for adult central complex morphology and behavior

The function of early- or late-born INP progeny in adult brain development is unknown. Here we determine the role of late-born INP neurons and glia in the development and function of the adult central complex (CCX), an evolutionarily-conserved insect brain structure containing many type II NB progeny[15–17]. The CCX consists of four interconnected compartments at the protocerebrum midline: the ellipsoid body (EB), the fan-shaped body (FB), the bilaterally paired noduli (NO), and the protocerebral bridge (PB); each of these compartments is formed by a highly diverse set of neurons[20,35]. First, we used permanent lineage tracing (*OK107[Ey]>>act-gal4 UAS-cd8:GFP*) to map the contribution of late-born Ey+ INP progeny to the adult CCX. We detected cell bodies in the dorsoposterior region of the CCX (data not shown), and their axonal projections extensively innervated the entire EB, FB, and PB, with much weaker labeling of the NO (Fig. 5a–d). We conclude that old INPs contribute neurons primarily to the EB, FB, and PB regions of the CCX. Second, we used INP-specific Ey[RNAi] to delete the late-born Toy+ neurons and Repo+ glia (see Fig. 4). Loss of late-born INP progeny generated major neuroanatomical defects throughout the adult CCX: the EB and NO were no longer discernible, the FB was enlarged, and the PB was fragmented (Fig. 5f–l; quantified in o; summarized in p). Subsets of this phenotype were observed following removal of Toy+ neurons or Repo+ glia (Fig. 5m–o; Sup Fig 12), showing that they contribute to distinct aspects of the CCX. Previous studies have described similar or weaker morphological CCX defects in *ey* hypomorphs[36], *toy* mutants[34], and after broad glia ablation during larval stages[37]. In addition, we found that Ey[RNAi] adults have relatively normal locomotion, but have a significant deficit in negative geotaxis (Fig. 5q; Supp Movie 1). We conclude that Ey is a temporal identity factor that specifies late-born neuron and glial identity, and that these late-born neural cell types are essential for assembly of the adult central complex.

INP temporal patterning and NB temporal patterning act combinatorially to increase neural diversity

We have found that Bsh+ neurons and Repo+ glia are sparse within the total population of young or old INP progeny, respectively, indicating that additional mechanisms must help restrict the formation of these neural subtypes. One mechanism could be temporal patterning within type II NB lineages.

To determine whether type II NBs change their transcriptional profiles over time, we assayed known temporal transcription factors[3,5,10,11,38] for expression in type II NBs at five timepoints in their lineage (24h, 48h, 72h, 96h, and 120h ALH). We observed no type II NB expression for Hunchback, Kruppel, Pdm1/2, and BrC; and Grh was expressed in all type II NBs at all timepoints. However, we identified three transcription factors with temporal expression in type II NBs. D and Castor (Cas) were specifically detected in early type II NBs: 3–4 NBs at 24h ALH, 0–1 NB at 48h ALH, and none later (Fig 6a,b). Although we never detected D simultaneously in all type II NBs at 24h, permanent lineage tracing with *R12E09D* labels all type II NBs (Sup Fig 3), indicating that all transiently express D. The third transcription factor, Seven-up (Svp), showed a pulse of expression in a subset of type II NBs at 48h ALH, but was typically absent from younger or older type II NBs (Fig 6a,b). D, Cas, and Svp are all expressed in the anterior-most type II NBs (probably corresponding to DM1–DM3), and thus at least these type II NBs must sequentially express D/Cas>Svp. We conclude that type II NBs can change gene expression over time.

Next, we wanted to determine whether type II NBs produce different INPs over time. We generated permanently-labeled clones within the type II NB lineages at progressively later timepoints (see methods; Fig. 6c–d). If type II NBs change over time to make different INPs, early and late NB clones should contain different neural subtypes. We assayed clones for Repo+ glia and Bsh+ neurons, choosing these markers because Repo+ neuropil glia have been hypothesized to be born early in type II NB lineages[17] and Bsh+ neurons were positioned far from the Repo+ glia consistent with a different birth-order. Bsh+ neuron numbers began to decline only in clones induced at the latest timepoint (Fig. 6e,g,i), showing that they are generated late in the type II NB lineage (Fig. 6j, grey). In contrast, Repo+ glia were detected in clones induced early but not late (Fig. 6f,h,i), proving that they are specifically generated by early type II NBs (Fig. 6j, blue). This allows us to assign Repo+ glia to an "early NB, old INP" portion of the lineage, and Bsh+ neurons to a "late NB, young INP" portion of the lineage (Fig. 6j). We conclude that type II NBs undergo temporal patterning, and propose that NB temporal patterning acts combinatorially with INP temporal patterning to generate increase neural diversity in the adult brain (Fig. 6k).

Discussion

We have shown that INPs sequentially express three transcription factors (D>Grh>Ey), and that different neural subtypes are generated from successive transcription factor windows. It is likely that multiple GMCs are born from each of the four known INP gene expression windows; GMCs born from a particular gene expression window may have the same identity, or may be further distinguished by "subtemporal genes" as in embryonic type I NB lineages [9]. We also show that each temporal factor is required for the production of a distinct temporal neural subtype. Loss of D or Grh leads to the loss of Bsh+ neurons; loss of Ey leads to loss of Toy+ neurons and Repo+ glia, although the fate of the missing cells is unknown. An unexpected finding was that Ey limits the lifespan of INPs. Mechanisms that prevent INP de-differentiation have been characterized – loss of the translational repressor Brat or the transcription factor Earmuff causes INPs to de-differentiate into tumorigenic type II NBs[14,21] – but factors that terminate normal INP proliferation have never before been identified.

The D>Grh>Ey INP temporal identity factors are all used in other contexts during Drosophila development. Many embryonic NBs sequentially express D>Grh[3]. Ey is expressed in mushroom body NBs[39], and is required for development of the adult brain mushroom body[40]. Interestingly, mammalian orthologs of D and Ey (Sox2 and Pax6, respectively) are expressed in neural progenitors[41], including OSVZ progenitors[19], but have not been tested for a role in temporal patterning.

We have shown that there are two axes of temporal patterning within type II NB lineages: both NBs and INPs change over time to make different neurons and glia, thereby expanding neural diversity. It will be important to investigate whether INPs generated by OSVZ neural stem cells undergo similar temporal patterning (perhaps using Sox2 and Pax6), and whether combinatorial temporal patterning contributes to the neuronal complexity of the human neocortex.

Methods Summary

Larvae were staged to 120h ALH based on age and morphology unless otherwise indicated; adult females were 3–5 days old. Immunohistochemistry was performed essentially as described[15] and imaged using Zeiss700/710 microscopes. INPs and GMC/neuronal progeny were distinguished by Dpn staining. $R12E09^D>>act\text{-}gal4$ and ubiquitously-expressed temperature-sensitive Gal80 were used for inducible lineage tracing. Standard methods were used to assess geotaxis behavior[42]. Data represent mean ± s.d. Two-tailed Student's t-tests were used. $*P<0.05$, $**P<0.01$, $***P<0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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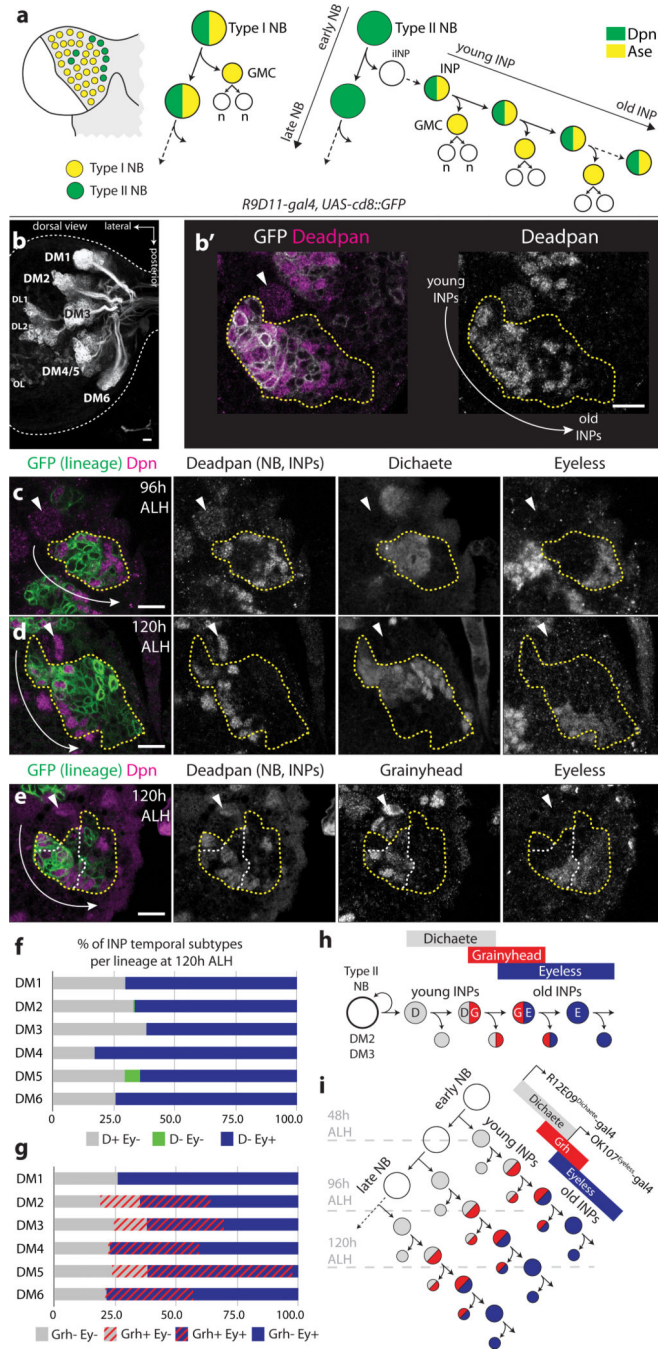
Fig 1. INPs sequentially express candidate temporal identity factors

a, Position of type II NBs (left). Cell lineage of type I and II NBs (right). iINP, immature INP. n, neurons.

**b**, Type II NB lineages in one brain lobe, z-projection, *R9D11-gal4 UAS-cd8::GFP*.

b', High magnification view of the DM3 lineage showing the parental NB (Dpn+GFP−, arrowhead), the smaller INPs (Dpn+GFP+), and GMCs/neurons (Dpn− GFP−). Yellow line surrounds GFP+ cells.

c–e, Dichaete marks young INPs and Eyeless marks old INPs; DM3 lineage shown. *R9D11-gal4 UAS-cd8::GFP* marks INPs and their progeny (yellow line). (**e**) Quantification. *n*=6 brains, lineages in a single lobe counted, percentages per each lineage were averaged.

f–g, Grainyhead marks middle-aged INPs, which include the oldest Dichaete+ INPs and the youngest Eyeless+ INPs; DM3 lineage shown. *R9D11-gal4 UAS-cd8::GFP* marks INPs and their progeny (yellow line) and Grainyhead+ cells (white line). In addition, Grh+ GFP− immature INPs are observed between the parental NB and the GFP+ INP pool. (**g**) Quantification as in **e**.

h,i, Summary of Dichaete, Grainyhead, and Eyeless sequential expression in INPs. Gal4 lines expressed in INPs are indicated. Scale bars, 10 μm.
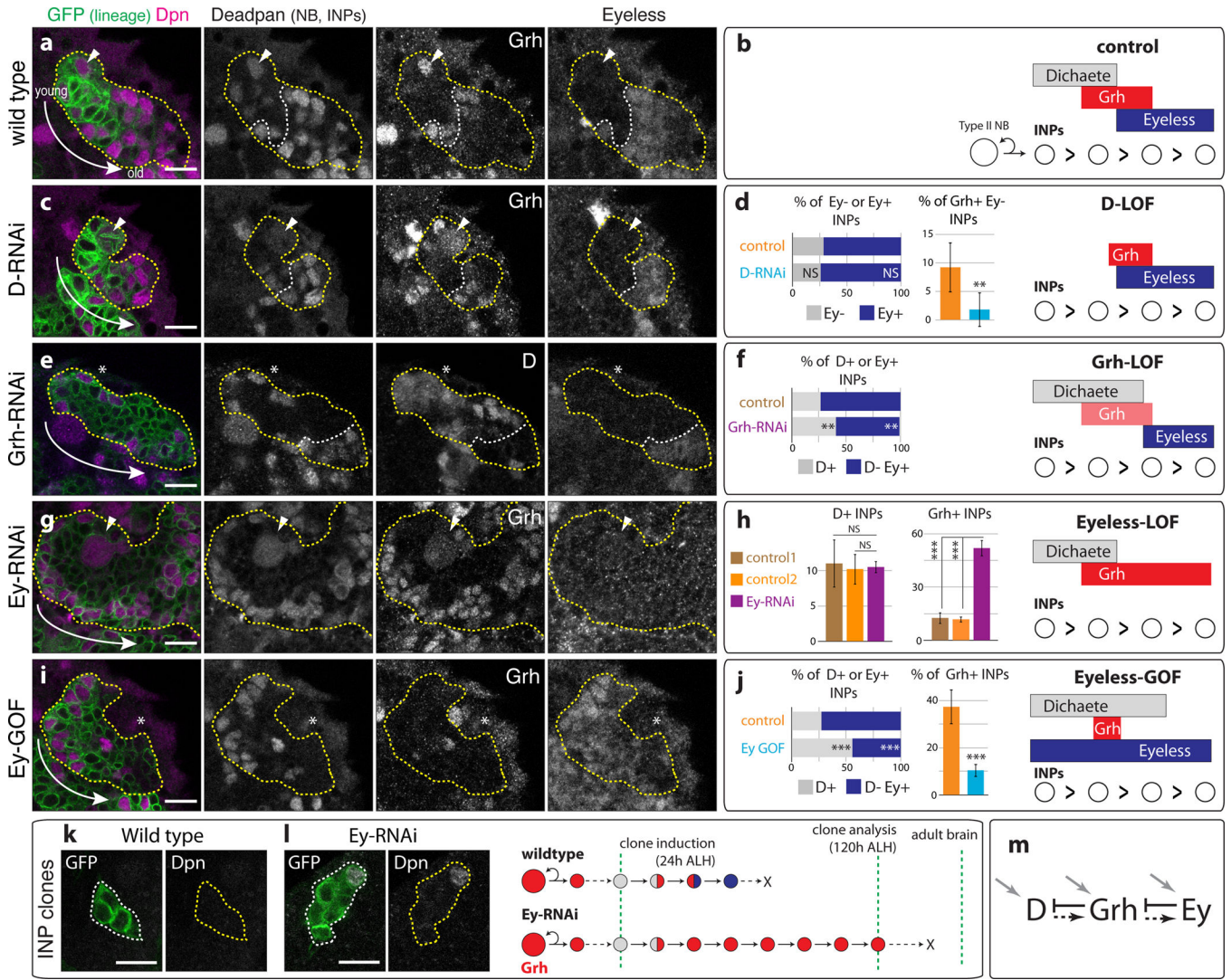
Fig 2. Cross-regulation between INP temporal transcription factors

INP temporal transcription factor expression in DM2 lineage at 120h ALH. INPs were marked with GFP (yellow outline) driven by: *wor-gal4 ase-gal80* (**a,c**), *R9D11-gal4* (**e,i**), or *R12E09^D^-gal4* (**g**). See supplemental methods for full genotypes. Ey border, white line. The parental type II NBs, arrowhead, or asterisk when out of focal plane.

a–b, Wild type expression of Grh and Ey in INPs.

c–d, D^RNAi^ delays Grh expression in INPs, such that no Grh+Ey− INPs are observed. For Ey see Sup Fig. 4a,b. (**d**) Quantification of Ey+ and Grh+Ey− INP numbers (*n*=6).

e–f, Grh^RNAi^ extends D expression and delays Ey expression in INPs. (**f**) Quantification (*n* 5).

**g–h**, Ey^RNAi^ extends Grh expression in INPs. (**h**) Quantification (*n* 4).

i–j, Ey misexpression reduces Grh expression in INPs. (**j**) Quantification (*n* 5).

k–l, Ey^RNAi^ extends the INP cell lineage. (**k**) Wild type MARCM clones induced early in single INPs never contain an INP at the end of larval life; (**l**) Ey^RNAi^ MARCM clones maintain a single INP at the end of larval life (*n* 10 clones).

m, Summary. Black arrows, positive regulation; black T-bars, negative regulation; gray arrows, external positive regulation.

Scale bars, 10 μm. All data represent mean ± s.d. NS, not significant. **$P<0.01$, ***$P<0.001$.
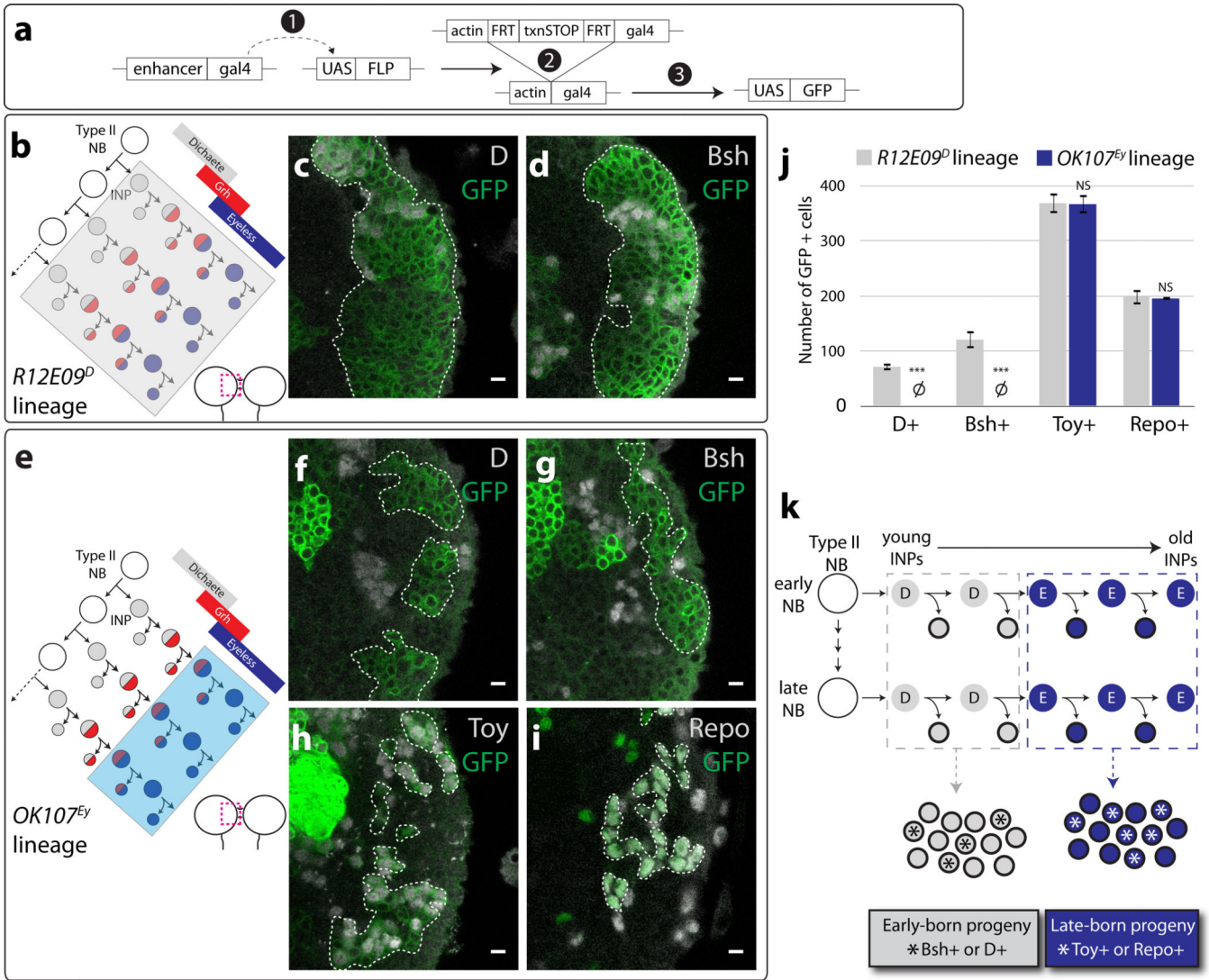
**Fig 3. INPs sequentially generate distinct temporal identities**

a, Genetics of permanent lineage tracing.

b–d, Permanent lineage tracing of all INP progeny using *R12E09^D*-gal4. Summary of GFP expression (**b**); expression of D and Bsh in the GFP+ INP progeny (**c,d**); dashed line surrounds GFP+ cells.

e–i, Permanent lineage tracing of old INP progeny using the late INP *OK107^Ey*-gal4 line. Summary of GFP expression (**e**); D+ and Bsh+ neurons are excluded from late INP progeny (**f,g**) whereas Toy+ neurons and Repo+ glia are among the late-born INP progeny (**h,i**); dashed line surrounds GFP+ cells.

j–k, Quantification (**j**) and summary (**k**). GFP+ INP progeny in DM1–6 lineages were counted, *n* 3 brain lobes for each marker. Region of dorsomedial brain imaged at 120h ALH (boxed in cartoon).

Scale bars, 5 μm. All data represent mean ± s.d. NS, not significant. ***$P<0.001$.
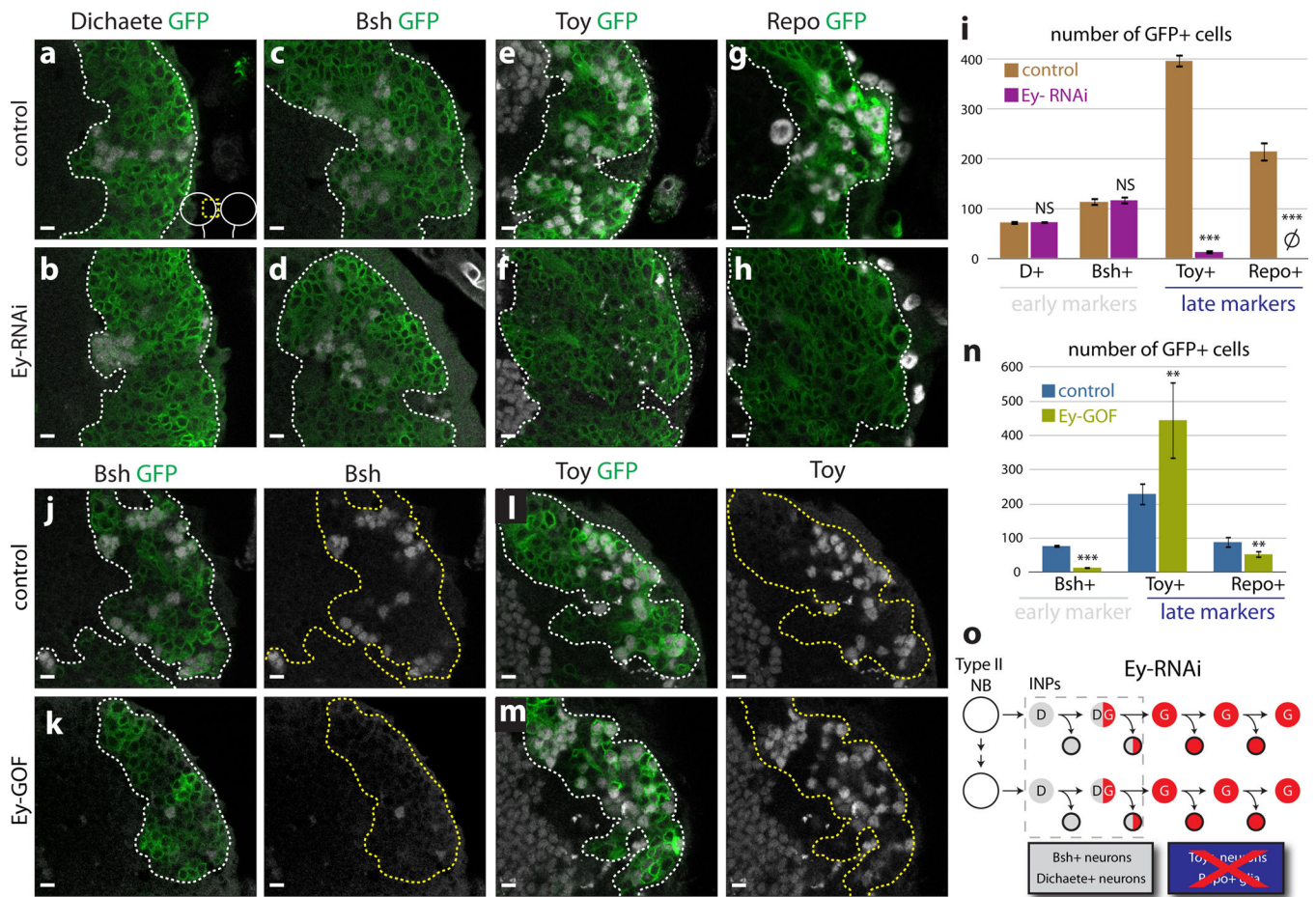
Fig 4. Eyeless is a temporal identity factor for late-born INP progeny
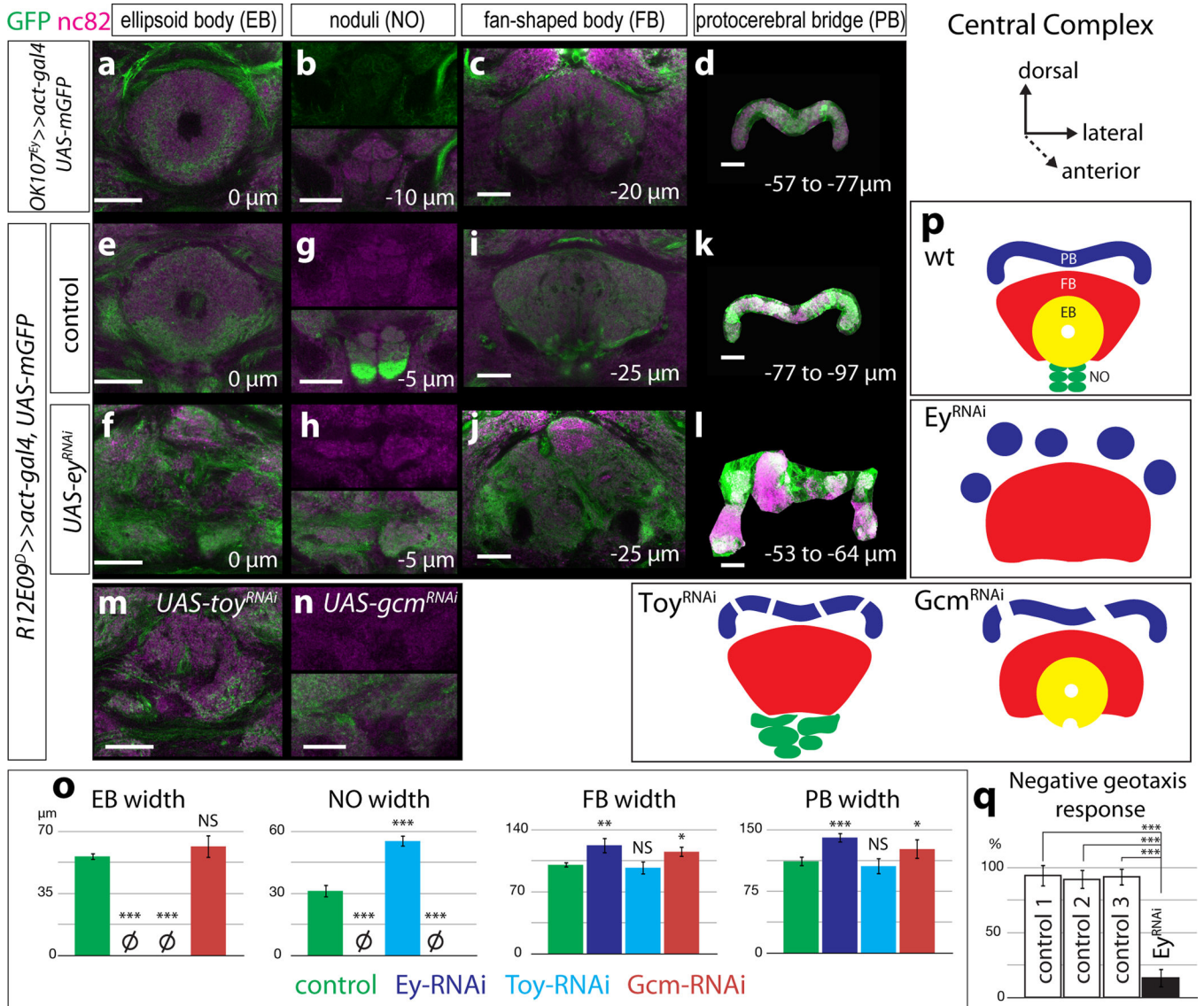
a–i, Ey^RNAi in INP lineages does not affect early-born INP progeny (**a–d**), but eliminates late-born Toy+ neurons (**e–f**) and Repo+ neuropil glia (**g–h**). (**i**) Quantification (n 4 brain lobes).

j–n, Ey misexpression in INP lineages leads to loss of early-born Bsh+ neurons (**j,k**), and increases the number of late-born Toy+ neurons (**l,m**). (**n**) Quantification (n 5).
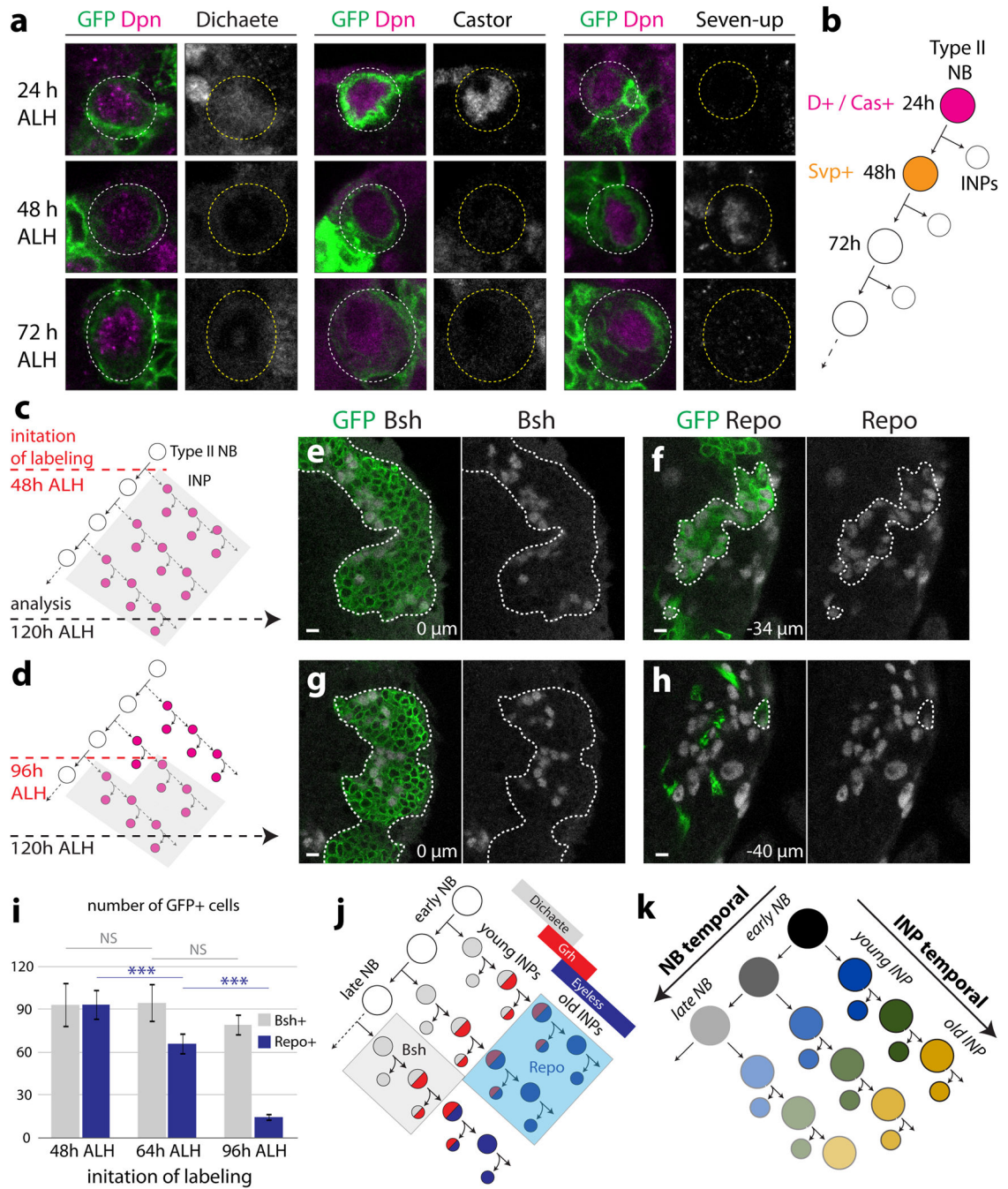
o, Summary.

Region of dorsomedial brain imaged at 120h ALH (boxed in cartoon). Scale bars, 5 μm.

All data represent mean ± s.d. NS, not significant. **$P<0.01$, ***$P<0.001$.

Fig 5. Eyeless is required for adult brain central complex morphology and behavior

a–d, Permanent lineage tracing of old INPs and their progeny (*OK107^Ey^ >>act-gal4*) extensively labels the adult central complex.

e–n, Ey^RNAi^ (**f–l**), Toy^RNAi^ (**m**), or Gcm^RNAi^ (**n**) in INPs lineages produce distinct defects in CCX morphology. Adult brains, frontal view. The z-coordinates of single confocal sections are shown relative to EB position. The PB was cropped out of the brain and displayed as a projection of indicated z-coordinates in (**d,k,l**). Scale bars, 20 μm.

o, Quantification of the width of CCX compartments (*n* 5).

p, Summary of CCX morphology upon loss of late-born INP progeny.

q, Ey^RNAi^ flies have deficits in negative geotaxis.

All data represent mean ± s.d. NS, not significant. *P<0.05, **P<0.01, ***P<0.001.

Fig 6. INP temporal patterning acts combinatorially with NB temporal patterning to increase neural diversity

a–b, Expression of D, Castor (Cas), and Seven-up (Svp) in the anterior-most type II NBs. Type II NBs are identified with *pointed-gal4 UAS-GFP* (green) and Dpn (magenta).

c–d, Schematics of INP permanent lineage tracing with *R12E09D-gal4* induced at early (**c**) or late (**d**) larval stages; all timepoints analyzed at 120h ALH. Gray shading, labeled INP and progeny.

e–f, Bsh+ neurons and Repo+ glia are both marked by permanent labeling early in type II NB lineages. Focal planes: Bsh, near NB; Repo, further from the NB (−34 μm).

g–h, Bsh+ neurons, but not Repo+ glia, are marked by permanent labeling late in type II NB lineages. Focal planes: Bsh, near NB; Repo, further from the NB (−40 μm).

Scale bars, 5 μm.

i, Quantification. *n*=5 for each timepoint. All data represent mean ± s.d. NS, not significant. ***$P < 0.001$.

j, Distinct neural progeny are born from early versus late type II NB lineages.

k, NB and INP temporal patterning act combinatorially to generate neural diversity.