TEMPORAL PATTERNING AND GENERATION OF NEURAL DIVERSITY IN *DROSOPHILA* TYPE II NEUROBLAST LINEAGES

by

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DISSERTATION ABSTRACT

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The central nervous system (CNS) has an astonishing diversity of neurons and glia. The diversity of cell types in the CNS has greatly increased throughout evolution and underlies our unique cognitive abilities. The diverse neurons and glia in the CNS are made from a relatively small pool of neural stem cells and progenitors. Understanding the developmental mechanisms that generate diverse cell types from neural progenitors will provide insight into the complexity of the mammalian CNS and guide stem cell based therapies for brain repair. Temporal patterning, during which individual neural progenitors change over time to make different neurons and a glia, is essential for the generation of neural diversity. However, the regulation of temporal patterning is poorly understood.

Human outer subventricular zone (OSVZ) neural stem cells and *Drosophila* type II neural stem cells (called neuroblasts) both generate transit-amplifying intermediate neural progenitors (INPs). INPs undergo additional rounds of cell division to increase the number of neurons and glia generated in neural stem cell lineages. However, it is unknown whether INPs simply expand the numbers of a particular cell type or make diverse neural progeny. In this dissertation, I show that type II neuroblast lineages give

rise to extraordinary neural diversity in the *Drosophila* adult brain and contribute diverse neurons to a major brain structure, the central complex. I find that INPs undergo temporal patterning to expand neural diversity in type II lineages. I show that INPs sequentially generate distinct neural subtypes; that INPs sequentially express Dichaete, Grainyhead, and Eyeless transcription factors; and that these transcription factors are required for the production of distinct neural subtypes. Moreover, I find that parental type II neuroblasts also sequentially express transcription factors and generate different neuronal/glial progeny over time, providing a second temporal identity axis. I conclude that neuroblast and INP temporal patterning axes act combinatorially to specify diverse neural cell types within adult central complex; OSVZ neural stem cells may use similar mechanisms to increase neural diversity in the human brain.

This dissertation includes previously published co-authored material.

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Bu tezi anne ve babama adıyorum.
Her şey için teşekkürler.

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CHAPTER I

INTRODUCTION

The central nervous system (CNS) contains an enormous diversity of cell types. Neurons widely differ in terms of their morphology, gene expression and electro-physiological properties; neuronal diversity is central to the formation of complex neural circuits that regulate physiology and behavior. The CNS is also populated with diverse glial cells. The principal macroglial cells, astrocytes and oligodendrocytes, have long been considered homogenous, yet recent studies have begun to identify molecular and functional differences among glia [1]. In addition, neuronal and glial diversity has greatly increased through evolution and underlies the unique cognitive abilities of humans. The generation of cell type diversity in the CNS is an astonishing developmental feat as diverse neurons and glia are generated from a relatively small and morphologically homogenous pool of neural stem cells and progenitors. Understanding the developmental mechanisms that give rise to neural diversity will provide great insight into complexity of the mammalian brain, including brain evolution and disease progression. This knowledge will also guide the development of stem cell based therapies for CNS repair in the future.

There are several mechanisms that generate cell type diversity in the CNS. While neuronal activity dependent [2] or stochastic mechanisms [3] are important for cell fate specification, the developmental patterning of neural precursors is essential for the generation of diverse CNS cell types. The patterning of neural precursors is regulated by both spatial and temporal cues. Under spatial patterning, distinct neural progenitors make distinct neurons and glia. For example, in the developing mammalian forebrain, neural stem cells in the cortex give rise to excitatory pyramidal neurons while those in the medial ganglionic eminence give rise to inhibitory interneurons [4]. Spatial patterning mechanisms have been extensively studied in different parts of the CNS. In the developing spinal cord, a sonic-hedgehog (Shh) signaling gradient provides positional cues to neural progenitors along the dorsoventral axis and establishes discrete transcription factor expression domains among the progenitors; within each domain downstream transcription factors specify distinct neurons and glia [5]. Similarly,

signaling through fibroblast growth factors (FGFs) and bone morphogenic proteins (BMPs) regulates the regionalization of the developing cortex into functionally distinct areas [2].

Although spatial patterning distinguishes neural progenitors, it is not sufficient to explain the cell type diversity in the CNS as neural precursors are greatly outnumbered by the distinct cell types they make during development. Temporal patterning allows neural precursors to generate a vast array of neuronal and glial progeny. Under temporal patterning, individual neural stem cells and progenitors change over time to make different neurons and glia in a sequential manner. Several examples of temporal patterning have been described in vertebrate systems. In the retina, multipotent retinal progenitor cells (RPCs) make different neuronal and glial cell types in a stereotyped order [6]. RPCs generate retinal ganglion cells, horizontal cells, and cone receptors first, then switch to making amacrine cells, and make rod photoreceptors, bipolar cells and Muller glial cells last [7, 8]. In the spinal cord, neural progenitors switch to making glia from neurons within each spatial domain: progenitors in the pMN domain generate motorneurons and oligodendrocytes in successive waves while progenitors in the p0-p3 domains generate interneurons and astrocytes in a similar fashion [9]. Another well-known example is the temporal patterning of neural progenitors in the mammalian cortex. The mature cortex is arranged into six laminar layers that typically segregate neurons with common morphological, molecular and electrophysiological features. Lineage tracing and birthdating studies have shown neural progenitors sequentially generate the deep and upper layer neurons in the cortex [10]. In addition, transplantation experiments have shown that the fates of cortical neurons are largely specified at the time of their birth [10, 11]. Similar to neural precursors in the spinal cord, cortical progenitors switch to glia production after generating neurons [9].

Despite the important role of temporal patterning in the generation of neural diversity, the mechanisms that regulate temporal patterning are poorly understood. Temporal patterning is regulated by both cell-intrinsic and extrinsic signals, yet in vitro culture experiments across multiple systems have shown a greater role for progenitor cell-intrinsic cues in the sequential generation of cell types [12-14]. In the retina, the Krüppel family zinc-finger transcription factor Ikaros is expressed in RPCs during early

developmental stages and specifies the early-born cell types including retinal ganglion cells and horizontal cells [15]. However, the factors that specify late-born cell types from RPCs are unknown. In the spinal cord, Delta-Notch signaling and the HMG box transcription factor Sox9 have been implicated in the neuron-glia switch [16]. In the cortex, the regulation of the neuron-glia switch has been extensively studied. During early neurogenic stages, the basic helix-loop (bHLH) transcription factor Neurogenin1 suppresses the production of astrocytes [12, 17] and the high mobility group A (HMGA) proteins promote neurogenesis [18]. During late gliogenic stages, JAK-STAT, Notch and BMP signals, along with polycomb group proteins, promote astrocyte production [19, 20]. In contrast, few factors have been implicated in the generation of neuronal diversity in the cortex. The winged helix transcriptional repressor Foxg1 is expressed in late neural progenitors and suppresses the generation of early-born Cajal-Retzius neurons during late development [21, 22]. The molecular mechanisms that regulate the sequential generation of the vast array of cortical neurons from neural progenitors are largely unknown.

The *Drosophila* CNS offers an attractive model system to study temporal patterning mechanisms. *Drosophila* neural progenitors are called neuroblasts (NBs) and they proliferate largely during the embryonic and larval developmental stages (~6 days in total) to give rise to a highly stereotyped nervous system. NBs can be readily identified by several molecular markers, including the bHLH transcription factor Deadpan (Dpn) [23] and the zinc-finger transcription factor Worniu (Wor) [24], and undergo several well-characterized asymmetric cell divisions to self-renew (i.e. maintain the NB pool) and generate differentiating progeny [25, 26]. Most NBs undergo a simple “type I” cell lineage to self-renew and generate a series of smaller ganglion mother cells (GMCs) that divide only once to make two neurons and glia [25, 26].

The temporal patterning of type I NBs in the embryonic ventral nerve cord (VNC) has been extensively studied. There are 30 NBs at stereotyped positions in each half-segment of the VNC, and each gives rise to a unique and invariant lineage of neurons and glia [27-29]. NBs undergo temporal patterning to generate distinct progeny in an invariant birth order. For example, NB7-1 sequentially generates five GMCs that make five distinct motor neurons, called U1-U5 (and their siblings) respectively [28, 30, 31]. The temporal patterning of embryonic NBs is regulated by the sequentially expressed

transcription factors Hunchback (Hb), Krüppel (Kr), Pdm1/2, and Castor (Cas) [30, 32-35]. Nearly all NBs sequentially express these transcription factors and generate one GMC on average during each expression window [30]. The early expressed zinc-finger transcription factors Hb and Kr specify the early-born progeny in NB lineages (e.g. U1-U2 and U3 motor neurons in the NB7-1 lineage respectively) [30], while the late expressed POU domain transcription factors Pdm1/2 and the zinc-finger transcription factor Cas specify the late-born progeny (e.g. U4 and U5 motor neurons in NB7-1 respectively) [33-35]. Importantly, the Hb > Kr > Pdm > Cas transcription factor series regulates the temporal identity of NB progeny, but does not specify their exact cell identity. For example, while Hb specifies the first-born U1-U2 motor neurons in the NB7-1 lineage, it specifies the first-born glial cell in the spatially distinct NB6-4 lineage [30]. Thus, cell identity is specified by the combination of spatial and temporal patterning inputs. In addition, the temporal identity transitions are regulated by feedforward activation and feedback repression between the sequentially expressed transcription factors [30, 35] as well as NB "timer" genes such as the COUP-TF subfamily nuclear receptor Seven-up (Svp) [36]. Lastly, the temporal identity transitions are largely cell-intrinsic as cultured NBs progress through the Hb > Kr > Pdm > Cas series and generate molecularly distinct progeny over time [33].

The embryonic type I NBs are an excellent model system to study temporal patterning mechanisms, yet embryonic born neurons account for less than 10% of the neurons in the *Drosophila* adult CNS [37, 38]. NB divisions during the larval stages generate the majority of cells in the adult CNS including the ~20,000 central brain neurons that form complex functional neural circuits [39]. There are approximately 100 NBs in each larval central brain lobe and each type I NB undergoes 40-60 self-renewing divisions during larval life to generate ~100 neurons [38, 40, 41]. Each larval central brain type I NB gives rise to a unique and invariant lineage of neurons; the cell bodies of the neurons in type I lineages are spatially clustered and they project their axons within one stereotyped tract to distinct brain areas forming "clonal units" in the adult [39-41]. Despite the comprehensive characterization of the neuronal lineages generated by larval central brain NBs [40-42], temporal patterning mechanisms have only been studied in a few select type I lineages. In the mushroom body lineages, larval NBs sequentially give

rise to four neuronal subtypes in broad contiguous windows [43, 44]. A temporal gradient of the BTB-zinc finger protein Chinmo in mushroom body NB progeny specifies early versus late-born neuronal identity [44, 45]. In addition, ecdysone hormone-induced microRNA let-7 specifies late-born neurons in mushroom body lineages [46]. Amongst the antennal lobe lineages, the anterodorsal projection neuron (adPN) NB sequentially generates 40 neuronal subtypes [47]. The embryonic temporal identity factor Krüppel specifies a single neuron subtype in the embryonic portion of the adPN lineage while Chinmo is involved in the specification of several larval-born projection neuron subtypes [48]. Previous studies have shown that two genes from the embryonic temporal series, Castor and Seven-up, are also sequentially expressed in most central brain NBs during early larval life [49, 50]. The Cas > Svp transition regulates the switch from Chinmo+ neuron production to Broad+ (another BTB-zinc finger protein) neuron production in larval type I lineages [49]. However the identities of early-born Chinmo+ and late-born Broad+ neurons have not been distinguished by morphology or other molecular markers. The early Cas > Svp series also regulates a Hedgehog (Hh) signaling gradient in larval NBs [50] and this temporal cascade schedules the timely termination of NB proliferation at the larval life [49, 50]. Nevertheless, the role of Hh signaling in temporal patterning has not been studied. In summary, the temporal patterning mechanisms used by larval NBs to generate neuronal diversity in the adult brain are largely unknown. Finally, it is important to consider that previous studies have not found a significant role for embryonic temporal identity factors (Hb, Kr, Pdm, Cas) in larval temporal patterning, indicating that novel factors regulate the generation of neural diversity from larval NBs.

The type I NBs in the larval brain give rise to larger and more complex sets of neural progeny than those in the embryonic VNC, yet both progenitors undergo simple cell lineages. Recently, NBs with more complex cell “type II” cell lineages were discovered in the larval central brain [51-53]. Firstly, type II NBs can be identified as large Deadpan+ cells that, unlike type I NBs, lack the expression of the proneural transcription factor Asense (Ase) [53]. There are only eight type II NB lineages per brain lobe: six lineages are found in the dorsomedial brain in a stereotyped anterioposterior order, named the DM1-6, and two are found in more lateral positions, named the DL1-2 [54]. Type II NBs undergo asymmetric cell divisions throughout larval life to self-renew

and generate a series of smaller transit amplifying daughter cells called intermediate neural progenitors (INPs). Each INP then undergoes several asymmetric cell divisions to self-renew and make ~6 GMCs that divide only once to make two differentiated progeny [51-53]. Due to the extended proliferation of INPs, type II NBs give rise to far larger population of neurons and glia (~450 cells per lineage) than type I NBs (~100 cells) during larval life [51-54].

Type II NBs produce the largest clones of neurons in the *Drosophila* CNS. However, little is known about the neural diversity generated in type II lineages. Initial studies have suggested that type II NBs could give rise to a diverse collection of neurons and glia. Firstly, genetic lineage-tracing experiments showed that neurons derived from individual DM type II NBs contribute to many regions in the developing pupal brain [54]. This contrasts type I NBs, which give rise to clonal units of neurons that contribute to a few brain regions [40, 41], suggesting that type II lineages might harbor extraordinary neuronal diversity. Interestingly, most DM type II lineages contribute neurons to the developing central complex in the pupal brain [54]. The central complex (CCX) is an evolutionarily conserved major insect brain structure [55]. Structural and functional studies in *Drosophila* and other insects have shown that the CCX is a higher order control center for locomotion [56], and is also involved in various behaviors including visual orientation [57], place memory [58], attention [59], and courtship [60]. For example, genetic mutants with structural aberrations in the CCX are capable of basic locomotion but have defects in optimizing walking speed and leg coordination during turns [61]. The CCX is often called the most complex part of the *Drosophila* brain as it has at least 60 morphologically distinct sets of neurons that make highly ordered modular connections within the CCX and between the CCX and other brain regions [62-64]. Secondly, type II NBs contribute many glial cells to the *Drosophila* brain. Specifically, lineage-tracing experiments showed that INPs give rise to both neurons and glia in the larval brain and type II NB lineages are a major source of neuropil glia [54, 65]. Neuropil glia enwrap major structures in the adult brain including the CCX and closely associate with axonal and dendritic processes of neurons [66], and have diverse functions including removal of degenerating axons [67] and neurotransmitter uptake [68]. These observations suggest that type II NBs could make an extraordinarily diverse set of neurons and glia.

Previous studies have examined the neuronal and glial progeny of type II NBs only in the developing larval and pupal brain. The full repertoire of neural diversity that type II lineages generate in the adult brain is unknown. Do type II NBs give rise to extraordinary neural diversity in the *Drosophila* adult brain? What is the extent of type II lineage derived neurons' contributions to the adult CCX? Importantly, virtually nothing is known about how type II NBs or INPs generate neural diversity. Do type II NBs undergo temporal patterning to make diverse neurons and glia? Do single INPs undergo temporal patterning to generate diverse neural subtypes, or do they merely expand the numbers of a particular cell type? If INPs generate neural diversity, what are the mechanisms used? These are the central questions I address in this dissertation.

Another issue pertaining to neural diversity is the extraordinary size of type II lineages. What are self-renewal/differentiation mechanisms that expand the proliferation in type II lineages? One candidate factor that could distinguish type I and II NB proliferation patterns is the homeodomain transcription factor Prospero (Pros), a master regulator of neural differentiation. *prospero* is transcribed and translated in type I NBs, but Prospero protein is actively exported from the NB nucleus [69]. Prospero is asymmetrically segregated into GMCs during NB divisions [70, 71]. In GMCs, Prospero is localized to the nucleus and initiates a gene expression program that terminates proliferation and promotes neural differentiation [72, 73]. Upon loss of Prospero, GMCs fail to differentiate and form NB-like tumorigenic cells [72-76]. Similarly, the Prox1 vertebrate homolog represses neural progenitor proliferation [77] and is a candidate tumor suppressor in several cancers [78-81]. In contrast, type II NBs lack detectable Prospero protein [51-53]. Prospero is also absent from new-born immature INPs, yet INPs express Prospero upon committing to the limited transit-amplifying progenitor fate [51-53]. Recent studies have also shown that type II lineages are susceptible to forming tumors: upon loss of the translational repressor Brain-tumor (Brat) or the Notch repressor Numb, new-born INPs fail to differentiate and become type II NB-like cells [53, 76]; these tumors can be suppressed by ectopic Prospero [53, 76]. In addition, loss of Brat or Numb does not generate tumors from type I NBs that inherit Prospero to their progeny [52]. Thus, Prospero is an excellent candidate factor that could distinguish type I/II NB proliferation patterns or identity.

What is the relevance of studying neural diversity and proliferation in type II NB lineages to understanding nervous system development in mammals? In mammals, adult neural stem cells as well as stem cells in many non-neural tissues generate transit-amplifying progenitors [82, 83]. Yet, a newly discovered neural stem cell in the developing human brain bears the most striking resemblance to type II NBs. In the embryonic mammalian brain, neural stem cells called radial glia undergo a type I NB-like lineage pattern to generate neurons and glia in the cerebral cortex [4, 84, 85]. These type I NB-like radial glia reside in a proliferative brain region called the ventricular zone (VZ) [4, 84, 85]. Recent studies have discovered that radial glia-like progenitors are also present in a neighboring region, the outer subventricular zone (OSVZ) [86-89]. In mammals that have a small and non-folded (lissencephalic) cerebral cortex, such as rodents, the OSVZ progenitors have type I NB-like division patterns [89, 90]. In mammals that have a large and highly folded (gyrencephalic) cerebral cortex, such as humans, OSVZ is much thicker than in lissencephalic mammals and OSVZ progenitors undergo type II NB-like lineage patterns to generate transit-amplifying INPs [86, 89]. The extended proliferation of INPs is thought to contribute to the expansion of the neocortex size and complexity in humans, which underlies our unique cognitive abilities [91]. Thus, understanding the mechanisms that regulate neural diversity and proliferation in type II NB lineages could provide valuable insight into the development of the human brain.

Here I characterize a novel temporal patterning program that generates neural diversity in type II NB lineages. In Chapter II, I characterize two new genetic tools that allow specific manipulation of type II NB gene expression and lineage tracing. Initially, I show that the differentiation factor Prospero regulates the proliferation pattern in type II NB lineages, but does not distinguish type I and II NB identity. Then, I show that that type II NBs make significant contributions to the adult CCX and give rise to extraordinary neural diversity. This work was previously published in volume 5 of the journal Neural Development in October 2010 and was coauthored with J.Q. Boone, M.L. Drummond, and C.Q. Doe. In Chapter III, I show that INPs undergo temporal patterning to make distinct neurons and glia over time. I identify that INPs sequentially express three transcription factors and that these factors are required for the generation of

temporally distinct neural subtypes. I show that late-born INP progeny are required for distinct aspects of CCX development. Moreover, I show that parental type II NBs also undergo temporal patterning. I conclude that type II NB and INP temporal patterning axes act in a combinatorial fashion to specify increased neural diversity. This work is in press in the journal Nature and was coauthored with C.Q. Doe.

CHAPTER II

DROSOPHILA TYPE II NEUROBLAST LINEAGES KEEP PROSPERO LEVELS LOW TO GENERATE LARGE CLONES THAT CONTRIBUTE TO THE ADULT BRAIN CENTRAL COMPLEX

Drosophila neural progenitors, called neuroblasts (NBs), are an excellent model system to study progenitor self-renewal and differentiation mechanisms [1]. NBs divide asymmetrically to generate a larger self-renewing NB and a smaller differentiating progeny. Genetic analyses have identified proteins partitioned into the NB that promote self-renewal and proteins partitioned into the smaller progeny that promote differentiation [2].

Recent work has shown that there are two types of NBs in the *Drosophila* larval brain: type I and type II [3-5]. There are approximately 90 type I NBs per brain lobe that have nuclear Deadpan (Dpn), nuclear Asense (Ase), and cytoplasmic Prospero transcription factors. They divide asymmetrically to bud off small ganglion mother cells (GMCs) that undergo a terminal symmetric division to produce two neurons [1]. Type I NBs express all known apical/basal polarity markers. Apical markers are segregated into the NB, where they can promote aspects of NB identity [6]; basal markers such as Miranda, Prospero, Brain tumor (Brat), and Numb are segregated into the GMC, where they promote neuronal differentiation [7-11]. Axons formed by the neuronal progeny of central brain type I lineages fasciculate with each other and generally project within a single stereotyped tract to their targets [12]; this is different from type I NB lineages in the ventral nerve cord, which exhibit axon branching [13].

There are only eight type II NBs per brain lobe, and they can be identified as large Dpn+ cells that are Ase- Prospero- (unlike type I NBs). Type II NBs express all known apical/basal polarity markers except for Prospero, and they bud off small progeny that lack Prospero protein. These type II NB progeny have been called transit amplifying

GMCs [4], intermediate progenitors [3], or secondary NBs [5]. Here we will use the term intermediate neural progenitors (INPs) because it accurately reflects the position of these cells within the lineage (intermediate between NB and GMC) and the proliferation ability (intermediate between NB and GMC), and it is less likely to be confused with either NB or GMC cell types. Each INP divides between four and eight times to generate equal-sized siblings: another INP and a GMC that produces a pair of neurons (Figure 1A). Due to the extended proliferation of the INPs, each type II NB contributes a far larger population of neurons to the adult fly brain compared to a type I NB [3-5].

Recently, type II lineages have been shown to be susceptible to tumor formation: loss of the translational repressor Brat or the Notch repressor Numb or the transcription factor Earmuff from the whole brain results in tumor formation only within type II lineages [5, 9, 14]. Tumor formation is due to INPs reverting back to a type II NB-like identity; interestingly, the tumor phenotype can be suppressed by ectopic Prospero [5, 9, 14]. This raises the possibility that Prospero overexpression suppresses *brat* or *numb* tumors by transforming type II NBs to a type I NB identity. Consistent with this model, only type I NBs contain detectable levels of Prospero protein - type II NBs lack Prospero protein [3-5]. Alternatively, Prospero could inhibit proliferation in type II NBs without altering their cell fate. Consistent with this model, loss of *prospero* from embryonic or larval type I NB lineages leads to failure to repress cell cycle genes [15, 16] and 'tumor' formation [5, 9, 11, 14]. Similarly, the Prox1 vertebrate ortholog is expressed in newly differentiating neurons [17], inhibits neural progenitor proliferation [18], and is a candidate tumor suppressor gene [19-21].

Here we characterize two Gal4 lines that allow us to manipulate *prospero* expression within type II NBs and their INP progeny. We use these lines to test whether Prospero controls the difference between type I and type II NB identity, or whether it acts to limit progenitor proliferation without affecting NB identity. In addition, we use these lines to perform heritable lineage tracing to determine, for the first time, the adult brain neurons generated by the type II NB lineages.

RESULTS

Identification of 19H09, a Gal4 line expressed in type II neuroblasts and INPs

To identify Gal4 lines that would allow us to manipulate Prospero expression in type II lineages and INPs, we screened Gal4 lines available from public stock centers and enhancer-Gal4 lines targeted to the attP third chromosomal location (Manning *et al*., unpublished results) [22]. Here we describe the 19H09-Gal4 line, which is expressed in five to seven out of the eight type II NBs and their INP progeny. The line is also expressed in a few type I NBs (which are ventral and thus easy to exclude from our analyses) and some post-mitotic neurons that project to the mushroom body (Figure 1E,E').

We analyzed 19H09 expression at 24, 48, 72, 96 and 120 h after larval hatching (ALH) by driving expression of nuclear green fluorescent protein (GFP). We observed no brain expression from 24 to 72 h ALH (data not shown). At 96 h and 120 h ALH we observed expression in five type II NBs and numerous adjacent small cells (Figure 1B; Table 1). We identify the NBs as type II based on their lack of Ase and ability to generate small Ase+ Dpn+ progeny [3-5]. As expected, the 19H09-labeled type II progeny include the newly born 'immature' INPs that have not yet upregulated Dpn [5] (Figure 1D, asterisks). However, some of these immature Dpn- INPs were Ase+, showing that Ase is upregulated prior to Dpn during INP maturation (Figure 1B',D). More distant from the parental type II NB were the mature Dpn+ Ase+ INPs and the Prospero+ GMCs derived from each INP (Figure 1D). Thus, analysis of 19H09 expression confirms that type II NBs generate INPs, and shows for the first time that INPs mature by upregulating Ase followed by Dpn, prior to dividing to produce GMCs. As further confirmation that 19H09 drives expression in type II NBs and their progeny, we drove expression of a membrane-tethered GFP to trace axon projections (Figure 1C-E). We observe immature and mature INPs adjacent to the type II NBs (Figure 1C) as well as the projections of the earlier-born neurons in the lineages (Figure 1E,E'). We observed that some of these secondary axon tracts were split and targeted towards different parts of the brain (Figure 1E, white arrows) unlike type I axon projections in the central brain, which generally extended along a single tract (Figure 1E', red arrow) [12]. We also observed commissural projections from type II lineages (Figure 1E, yellow arrow). Since 19H09 is

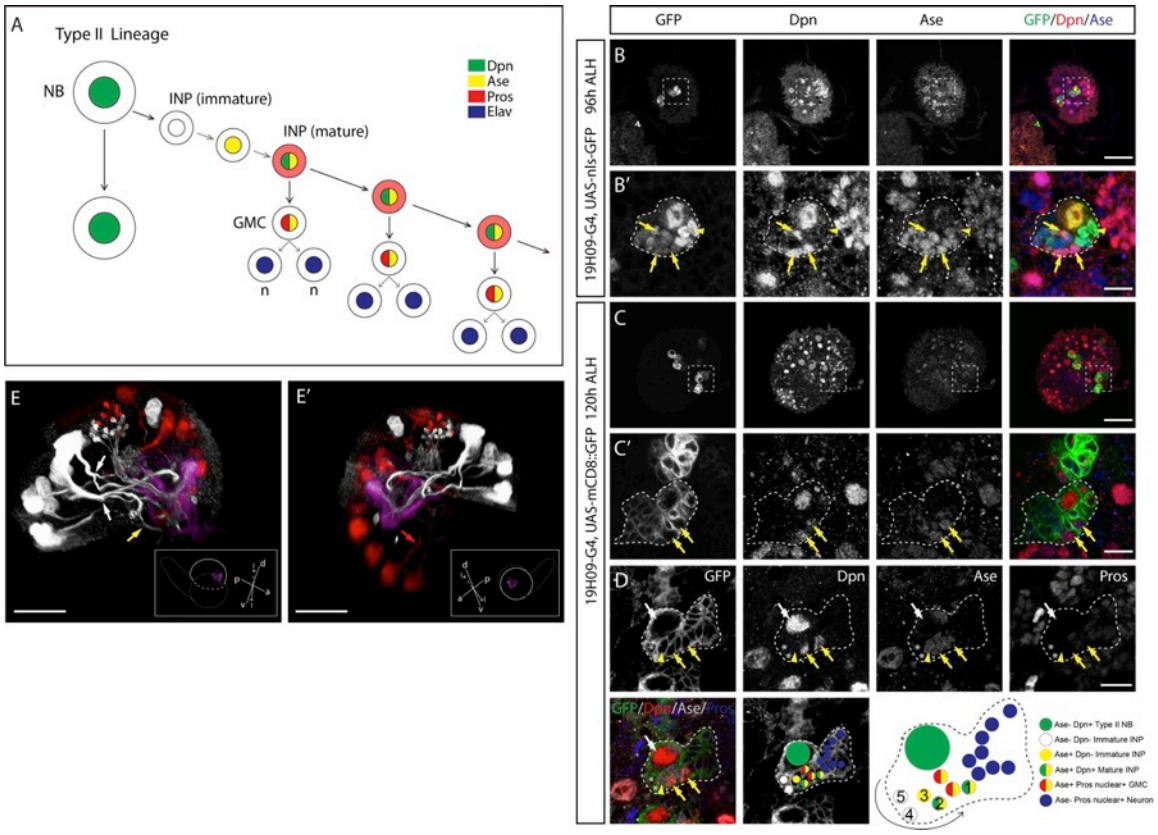


Figure 1. 19H09-Gal4 labels a subset of type II neuroblasts and their progeny.
(A) Type II NB lineage summary, modified from [4]. **(B-D)** Confocal images of third instar larval brains expressing nls::GFP (B) or mCD8::GFP (C) under 19H09-Gal4 stained for indicated markers (white box). Low magnification images of single brain lobes are presented in (B,C) and high magnification images of boxed areas are presented in (B',C'), respectively. (D) A high magnification image of a type II NB and associated progeny from a different brain. The white dotted outlines represent the green fluorescent protein (GFP)-labeled areas. Type II NB are large Dpn+ Ase- cells (white arrows). Ase- Dpn- immature INPs next to type II NBs are indicated with asterisks, Ase+ Dpn- immature INPs close to type II NBs are indicated with arrowheads and mature INPs are indicated with yellow arrows. The type II lineage shown in (D) is diagrammed below indicating the different kinds of cells in the lineage. The birth order of the cells was inferred from their relative position to the parental NB: newly born cells (5) are in direct contact with the type II NB while earlier-born cells (1) and their progeny are displaced and found further away. **(E,E')** Three-dimensional reconstruction of medial (E) and lateral (E') views of a 120 h ALH (after larval hatching) brain lobe expressing mCD8::GFP under control of 19H09-Gal4. Type II lineages and their axonal projections are in white, the mushroom body, visualized by FasII, in magenta, and type I lineages and their projections in red. Additionally, a subset of neurons that project to the mushroom body are visualized by the driver. The optic lobes have been removed and the brain cropped for a clearer view. Brains (gray outline) are in the orientations shown in the insets, with imaged lobes indicated with a white dashed line and their mushroom bodies shown. The rest of the brain apart from the imaged lobes is indicated in white outline. Split axon tracts of type II lineages are indicated with white arrows, the yellow arrow points at a commissural projection from a type II lineage, and the red arrow points at a type I projection. Orientation: d, dorsal; v, ventral; p, posterior; l, lateral; m, medial. Scale bars: (B,C) 50 μM; (B',C',D) 10 μM; (E,E') 40 μM.

not expressed before 72 h ALH, only a subset of secondary axon projections of type II lineages were labeled. Our observations confirm and extend the findings from clonal analysis of type II lineages [23]. We conclude that 19H09 can be used to drive gene expression in type II NBs and their INP, GMC, and neuronal progeny beginning at late larval stages.

Table 1. Analysis of 19H09 expression in wild-type and *prospero* misexpression brains.

Genotype and stage	Type II NB[a]	GFP+ type II NB[b]	GFP+ type I NB[c]	GFP+ INP[d]	GFP+ type II progeny[e]	Sample size[f]
19H09-G4, UAS-nls-GFP @ 25°C						
96 h ALH	8.0	4.6 ± 0.5	1.9 ± 1.3	18.9 ± 5.9	58.9 ± 17.4	7
120 h ALH	8.0	5.4 ± 0.5	6.6 ± 1.9	62.9 ± 6.4	138.1 ± 14.1	8
19H09-G4, UAS-mCD8::GFP @ 25°C						
96 h ALH	8.0	5.5 ± 0.7	1.9 ± 1.4	31.7 ± 5.9	84 ± 16.3	10
120 h ALH[g]	8.0	6.5 ± 0.8	8.3 ± 1.5	81.9 ± 9.0	232.5 ± 14.2	8
19H09-G4, UAS-mCD8::GFP @ 30°C						
120 h ALH[g]	8.0	5.8 ± 0.4	7.0 ± 1.8	75.5 ±3.7	242.7 ± 34.5	6
19H09-G4, UAS-mCD8::GFP, UAS-pros @ 30°C						
120 h ALH	7.3 ± 0.5	4.6 ± 0.7	7.3 ± 1.5	9.8 ± 2.6	108.8 ± 13.5	9

[a]Large Dpn+ Ase-. [b]Large GFP+ Dpn+ Ase-. [c]Large GFP+ Dpn+ Ase+. [d]Small GFP+ Dpn+. [e]Small GFP+ cells in type II lineages. [f]Brain lobes. [g]At 120 h ALH, 19H09-driven mCD8::GFP weakly labels one or two extra type II lineages.

Identification of 9D11, a Gal4 line expressed in INPs and their progeny

The 9D11-Gal4 line was generated by fusing *cis*-regulatory DNA from the *earmuff* gene to Gal4 [22]; it shows expression in the dorsomedial and centromedial larval brain region with axon projections similar to those shown for type II NB progeny (compare Image 3 in Pfeiffer et al. 2008 with Image 2 in Boone and Doe 2008 and Images 2 to 7 in Izergina et al. 2009). We found that 9D11-Gal4 is specifically expressed in an increasing number of INPs from 24 h to 96 h ALH (Figure 2A-C; Table 2) but not in the type II NB (Figure 2D'-F', white arrows). In the type II lineages, mature INPs (Figure 2F', yellow arrow) but not the Ase- and Ase+ immature INPs (Figure 2E',F', asterisks and arrowhead, respectively) were labeled, showing that 9D11 expression correlates with INP maturation.

The absence of 9D11 expression from immature INPs is consistent with previous observations [14]. No other cells in the central brain expressed 9D11, but expression was found in the optic lobe (data not shown). We confirmed that 9D11 is expressed in INPs by crossing it to UAS-mCD8::GFP and observing axon projections that match the previously identified axon projection pattern of type II progeny (Figure 2G,G') [4, 23]. 9D11 is strongly expressed in INPs within the six medial type II lineages (Figure 2G,G'); these are likely to be the DM1-DM6 NBs) [12, 23]. 9D11 is also expressed weakly in the remaining two lateral lineages beginning at 96 h ALH (Figure 2E,G,G').

As in the case with 19H09, the type II axon projections labeled with 9D11 were split into several branches and targeted towards different parts of the brain (Figure 2G,G'). These projections included commissural (Figure 2G, arrows) and descending ipsilateral (Figure 2G', arrowheads) bundles; the former were observed from all six medial type II lineages. Type II axonal fibers entered the larval commissure at different sites but a significant portion of labeled projections were targeted to the dorsoposterior commissure (DPC; Figure 2G, yellow arrow), which is a part of the larval precursor to the central complex of the pupal brain [12]. Upon labeling with 9D11 it was difficult to trace trajectories due to dense staining, yet we were still able to individually identify 9D11+ type II lineages by the positional information of cell body clusters (that is, stereotypical anterior-to-posterior arrangement of the medial lineages) and by matching the visible projections to previous data (Figure 2G,G', lineages labeled) [12, 23]. We conclude that the medial type II lineages make complex secondary axon projections and project a subset of their axons to the interhemispheric commissure.

Prospero misexpression suppresses proliferation in type II NBs but does not induce type I NB identity

After characterizing the type II and INP Gal4 lines 19H09 and 9D11, we next used these lines to test whether misexpression of Prospero could induce a type II to type I NB transformation. Type I NBs contain cytoplasmic Prospero at interphase, form Prospero basal cortical crescents during mitosis, and generate only nuclear Prospero+

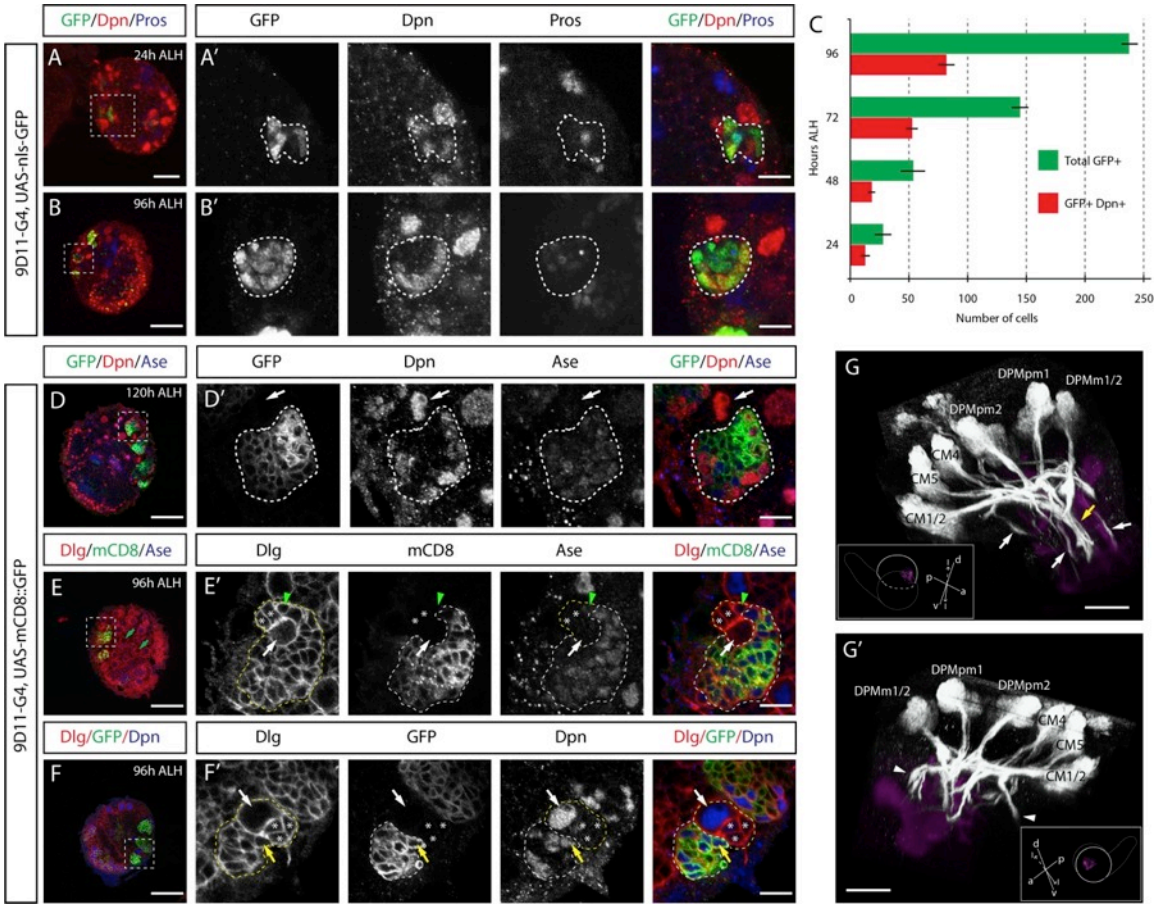


Figure 2. 9D11 specifically labels INPs and their progeny within the central brain. **(A,B,D-F)** Confocal images of first (A) and third (B,D,E,F) instar larval brains expressing nls-GFP (A,B) or mCD8::GFP (D-F) under 9D11-Gal4 stained for indicated markers (white boxes). Low magnification images of single brain lobes are presented in (A-F), and high magnification images of boxed areas are presented in (A'-F'), respectively. White outlines represent the GFP-labeled areas; yellow outlines represent NB lineages visualized by Discs-large (Dlg) staining. Type II NBs are indicated with white arrows; Ase- Dpn- immature INPs next to type II NBs are indicated with asterisks; Ase+ Dpn- immature INPs close to type II NBs are indicated with arrowheads. Mature INPs are small Dpn+ cells in white outlined areas or indicated with yellow arrows. (E) Green arrows point at the two lateral type II NBs. **(C)** Histogram showing increasing number of INPs and total cells labeled by nls-GFP driven by 9D11-gal4 between 24 and 96 h ALH. Error bars indicate standard deviation. **(G,G')** Three-dimensional reconstruction of medial (G) and lateral (G') views of a 120 h ALH brain lobe expressing mCD8::GFP under control of 9D11-Gal4. Type II lineages and their axonal projections are shown in white, and the mushroom body, visualized by FasII, is shown in magenta. The optic lobe is removed and the brain cropped for a clearer view. See text for lineage labels. Brains (gray outline) are in the orientations shown in the insets, with imaged lobes indicated with a white dashed line and their mushroom bodies shown, same as in Fig. 1. White arrows point at commissural projections and arrowheads point at descending ipsilateral projections from type II lineages. The yellow arrow points at the dorsoposterior commissure. Type II lineages were labeled according to [12]. Orientation: d, dorsal; v, ventral; p, posterior; l, lateral; m, medial. Scale bars: (A) 20 μM; (B-F) 50 μM; (A'-F') 10 μM; (G,G') 40 μM. CM, centromedial lineages. DPMm, medial dorsoposterior lineages, medial subgroup. DPMpm, medial dorsoposterior lineages, posteromedial subgroup.

Table 2. Analysis of 9D11 expression in wild-type and *prospero* misexpression brains.

Genotype and stage	GFP+ INP[a]	GFP+ type II progeny[b]	Sample size[c]
9D11-G4, UAS-nls-GFP @ 25°C			
24 h ALH	12.4 ± 3.5	27 ± 5.8	8
48 h ALH	18.1 ± 2.4	52.6 ± 9.7	8
72 h ALH	51.7 ± 4.4	142.2 ± 6.6	13
96 h ALH	80.1 ± 6.2	232.7 ± 6.2	10
9D11-G4, UAS-mCD8::GFP @ 25°C			
24 h ALH	11.8 ± 5.8	25.6 ± 5.8	5
48 h ALH	16.11 ± 5.5	53.44 ± 18.3	8
72 h ALH	52.1 ± 10.3	527.5 ± 24.4	9
96 h ALH	83.5 ± 2.5	548.9 ± 14.9	7
9D11-G4, UAS-mCD8::GFP @ 30°C			
96 h ALH	86.6 ± 6.3	619.3 ± 20.1	7
120 h ALH	710.8 ± 14.1	97.6 ± 2.3	5
9D11-G4, UAS-mCD8::GFP, UAS-Pros @ 30°C			
96 h ALH	14.2 ± 2.8	197.6 ± 20.6	10
120 h ALH	18.6 ± 2.8	214.8 ± 18.3	8

[a]Small GFP+ Dpn+. [b]Small GFP+. [c]Brain lobes.

GMCs that undergo a terminal cell division. In contrast, type II NBs lack detectable Prospero protein at all stages of the cell cycle and generate nuclear Prospero- INPs that can divide multiple times [3-5]. We used the 19H09-Gal4 line to drive low levels of Prospero in type II NBs, and observed cytoplasmic Prospero at interphase and basal cortical Prospero at mitosis (100%, n = 11 mitotic NBs; Figure 3), similar to type I NBs [3-5]. However, the NBs remained Ase- and generated bifurcating axon projections characteristic of type II NBs in the central brain (Figure 3B,I). Expression of higher levels of Prospero did not give a type II to type I transformation, but rather led to the loss of type II NBs via death or differentiation (Table 1), as previously reported for misexpression of Prospero in type I NBs [24]. We propose that these NBs are missing due to differentiation because we observe large cells with both nuclear Prospero and Dpn as well as large cells with just nuclear Prospero (Figure 3F-H), consistent with Prospero inducing downregulation of Dpn as the first step in differentiation. We conclude that

misexpression of Prospero in type II NBs does not transform them to a type I NB identity.

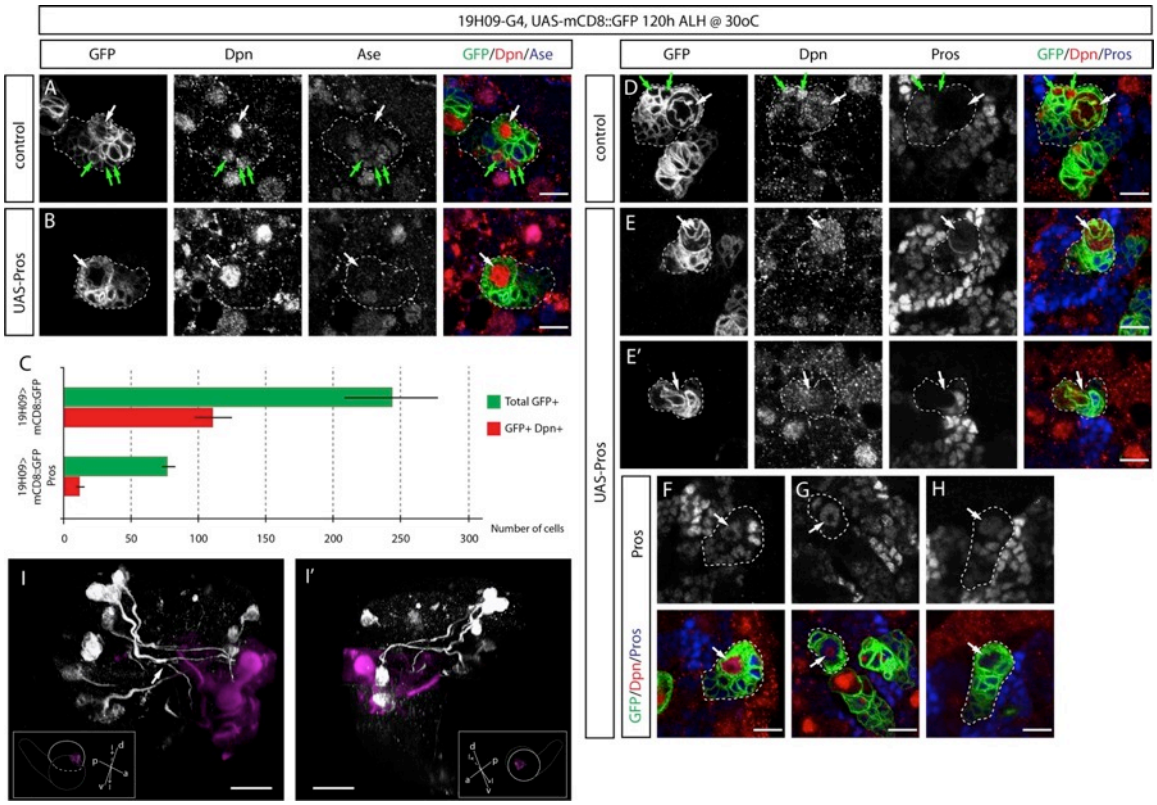


Figure 3. Prospero misexpression in type II NBs reduces lineage size but does not induce type I NB identity.

(A,B,D-H) High magnification confocal images of type II NBs and associated progeny from third instar larval brains expressing mCD8::GFP (A,D) or mCD8::GFP and Pros (B,E-H) under control of 19H09-Gal4. White outlines represent the GFP labeled areas. Type II NBs are indicated with white arrows and mature INPs are indicated with green arrows. **(C)** Histogram showing number of INPs and total cells labeled by mCD8::GFP driven by 19H09-gal4 in control and *pros* overexpression brains at 120 h ALH. Error bars indicate standard deviation. **(I,I')** Three-dimensional reconstruction of medial (I) and lateral (I') views of a 120 h ALH brain lobe expressing mCD8::GFP and Pros under control of 19H09-Gal4. Type II lineages and their axonal projections are showm in white, and the mushroom body, visualized by FasII, is shown in magenta. The optic lobe is removed and the brain cropped for a clearer view. Brains are in the orientations shown in the insets, with imaged lobes indicated with a white dashed line and their mushroom bodies shown. The white arrow points at a split axon tract from a type II lineage. Orientation: d, dorsal; v, ventral; p, posterior; l, lateral; m, medial. Scale bars: (A-E) 10 μM; (F,F') 40 μM.

Prospero misexpression suppresses INP proliferation

Misexpression of Prospero in type II NBs and their progeny using the 19H09 driver resulted in many Prospero+ small progeny around the NB (Figure 3E), and a large reduction in the number of neurons generated by each type II NB (Figure 3B,C; Table 1). The reduction of clone size could be due to reduced proliferation of the parental NB or the INPs. To distinguish between an effect on the NB versus INPs, we used the INP-specific Gal4 line 9D11 to misexpress Prospero. We found no difference in NB numbers, but we observed a striking reduction in the number of INPs and total cells at both 96 h and 120 h ALH (Figure 4A-E; Table 2; 96 h ALH). We conclude that misexpression of Prospero does not affect type II lineage identity, but rather it suppresses INP proliferation, and that type II lineages are much larger than type I lineages, in part due to the absence of Prospero from the new-born INP progeny.

9D11 is expressed in a small subset of neurons in the adult brain that project to the fan-shaped body of the central complex

We and others have shown that although there are only 8 type II NBs among the approximately 100 central brain NBs, the type II NBs generate a disproportionately high percentage of the total neurons in the late larval brain [3-5]. We were curious to know if the shared developmental history of the type II neurons directs them to form a specific structure in the adult brain, or whether these neurons are dispersed throughout the adult brain. Recent work has shown that clones generated within type II NBs preferentially contribute to the central complex of the pupal brain [23], supporting a 'common function' model. The *Drosophila* central complex is a major neuropil in the adult brain that has been implicated in several behaviors, including locomotion, flight, and visual pattern memory [25-27], and consists of four interconnected substructures located on the midline of the protocerebrum: the protocerebral bridge (PB), the fan-shaped body (FB), the paired noduli (NO) and the ellipsoid body (EB). These neuropils are closely associated with the accessory areas, lateral accessory lobes (LAL; also known as ventral bodies) and bulbs (BUs; also known as lateral triangles) [28-30]. In addition, central complex neurons can be classified as either large-field or small-field. Large-field neurons link a single central complex substructure to regions outside the central complex; most project to one of the

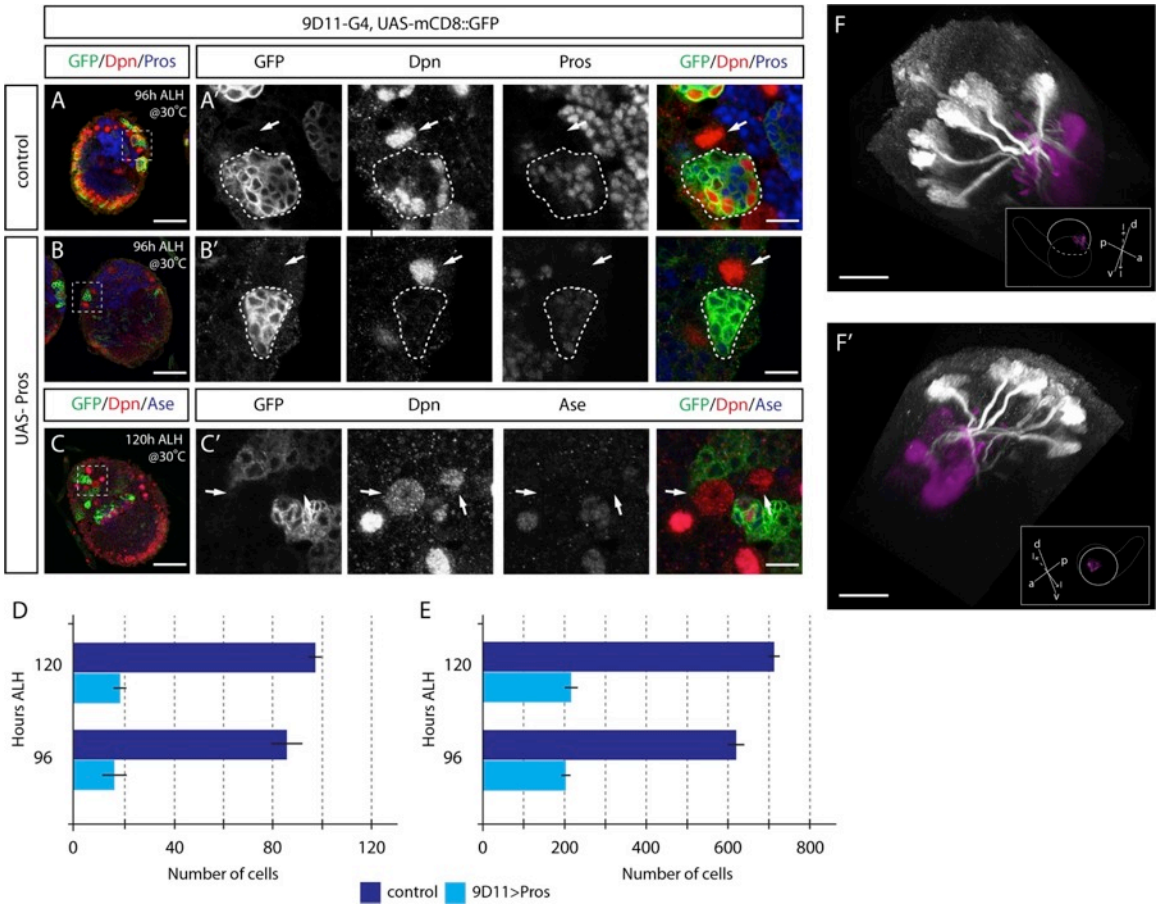
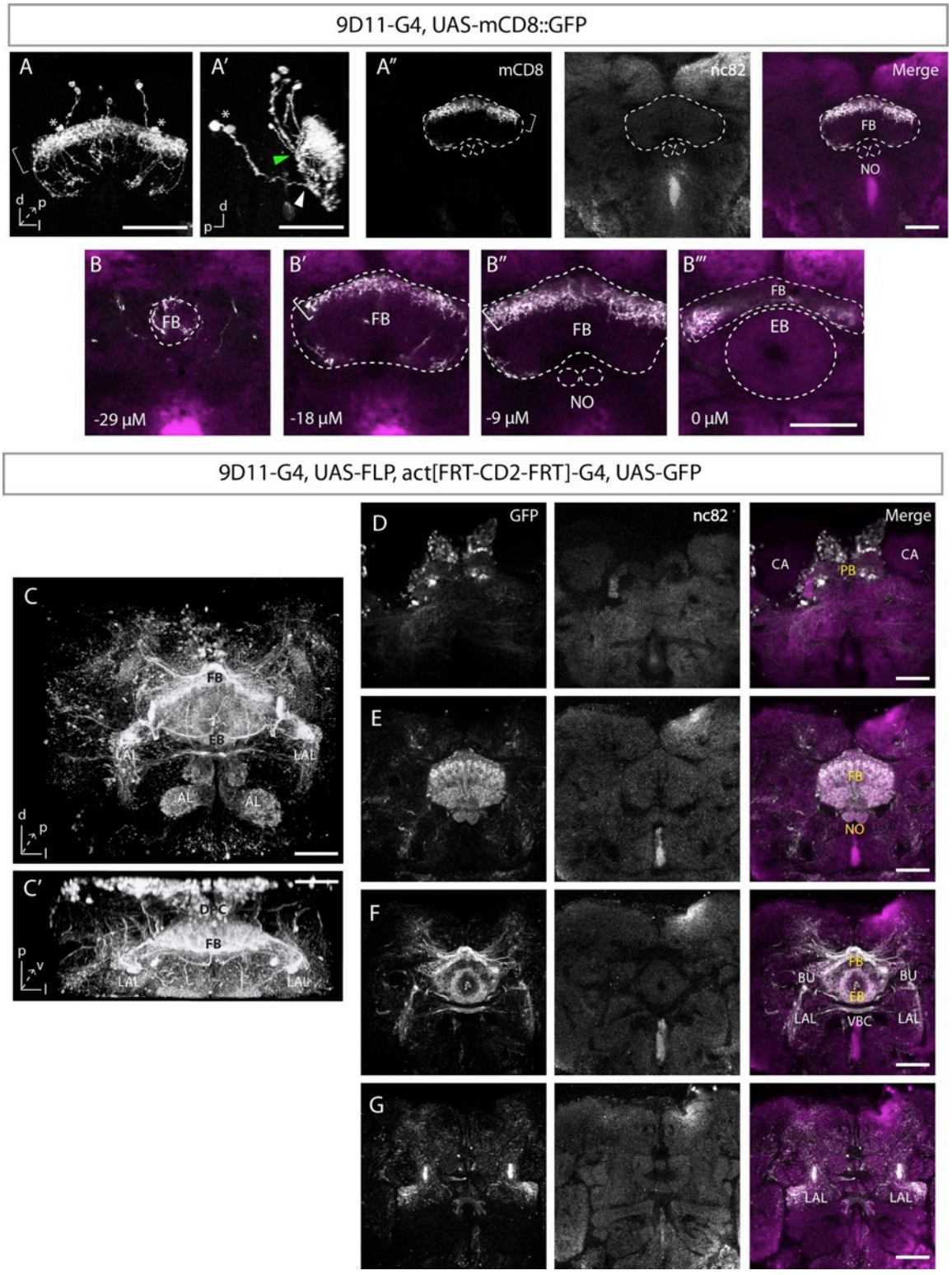


Figure 4. Prospero misexpression suppresses INP proliferation.
(A-C) Confocal images of third instar larval brains expressing mCD8::GFP (A) or mCD8::GFP and Pros (B,C) under 9D11-Gal4 stained for indicated markers (white boxes). Low magnification images of single brain lobes are presented in (A-C), and higher magnification images of boxed areas are presented in (A'-C'), respectively. White outlines represent the GFP-labeled areas. Type II NB are indicated with white arrows; mature INPs are small Dpn+ cells in white outlined areas. **(D,E)** Histograms showing number of INPs (D) and total cells (E) labeled by mCD8::GFP driven by 9D11-gal4 in control and *prospero* overexpression brains at 96 and 120 h ALH. **(F,F')** Three-dimensional reconstruction of medial (F) and lateral (F') views of a 120 h ALH brain lobe expressing mCD8::GFP and UAS-Prospero under control of 9D11-Gal4. Type II lineages and their axonal projections are shown in white, and the mushroom body, visualized by FasII, is shown in magenta. The optic lobe is removed and the brain cropped for a clearer view. Brains are in the orientations shown in the insets, with imaged lobes indicated with a white dashed line and their mushroom bodies shown. Orientation: d, dorsal; v, ventral; p, posterior; l, lateral; m, medial. Scale bars: (A-C) 50 µM; (A'-C') 10 µM; (F,F') 40 µM.

accessory areas. Small-field neurons are primarily intrinsic to the central complex, where they innervate a single substructure or link two to three substructures in a columnar fashion [29, 30].

To trace the projections of type II NB progeny, we used the INP-specific 9D11-Gal4 line to assay for adult brain expression directly, as well as to induce expression of a heritable genetic marker in INPs during larval stages and assay cell body position and axon projections in the adult brain. First, we observed that 9D11 was expressed in a small subset of neurons in the adult brain that projected to the FB region of the central complex (Figure 5A,B). The cell bodies were located in the dorsal posterior complex medial to the mushroom body calyces and their projections entered the FB at different sites (Figure 5A,A'). They formed a dense layer of arborizations at the top sections of the dorsal FB, and in a columnar fashion along the vertical staves throughout the rest of the FB (Figure 5A,B). The projections were confined to the FB and did not enter the NO or EB (Figure 5B-B'''). The position and projections of these neurons match the P3 or P4 small-field pontine neurons that are intrinsic to the FB [29, 30]. Our observations are consistent with a previous study on the adult brain expression pattern of 9D11 [22]. We conclude that 9D11 is expressed in pontine neurons, small-field neurons of the adult FB [29, 30]. These results are consistent with those showing type II lineages projecting to the central complex at pupal stages [23], but we can not definitely say that these neurons are derived from type II lineages solely based on adult 9D11 expression. Thus, we next turned to inducing permanent expression of GFP in the 9D11+ INP progenitors during larval stages, and assaying their position and projection in the adult brain.

Figure 5 (next page). Lineage tracing with 9D11 labels the adult central complex and associated regions. (A,B) 9D11 expression in the adult brain stained for mCD8 (white) and synaptic marker nc82 (magenta). (A,A') Frontal (A) and sagittal (A') views of the three-dimensional reconstruction of mCD8::GFP confocal z-stacks, close up on the FB. Three cell pairs are located dorsal to the FB while two cell pairs are more ventral at the level of the dorsal FB (asterisks). The projections from the dorsal cell pairs enter the dorsal FB at medial sites (green arrowhead) while the projections of ventral pairs enter the dorsal FB at more anterolateral sites (white arrowhead). (A'') Single frontal confocal section of the same brain at the level of the FB. (B-B''') Serial higher magnification frontal confocal sections of the central complex from posterior to anterior. Cell bodies are posterior to (B). White brackets indicate the dense dorsal layer of innervations at the FB. White outlines represent the neuropils visualized by nc82 staining (labeled). (G). CA, calx. VBC, ventral body commissure. See Supplementary Figure 1 for more representative stacks, and Supplementary Figure 2 for high magnification images of the central complex. Orientation: d, dorsal; v, ventral; p, posterior; l, lateral. Scale bars: 40 μM.


9D11-G4, UAS-mCD8::GFP
A
A′
A″
mCD8
nc82
Merge
FB
NO
B
B′
B″
B‴
FB
EB
NO
-29 µM
-18 µM
-9 µM
0 µM
9D11-G4, UAS-FLP, act[FRT-CD2-FRT]-G4, UAS-GFP
C
C′
D
E
F
G
GFP
nc82
Merge
CA
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Lineage tracing of 9D11-expressing cells labels the central complex and associated regions in the adult brain

To identify the structures type II lineages contribute to adult brain, we crossed 9D11 to *UAS-FLP*, *actin[FRT-CD2-FRT]gal4*, *UAS-GFP* and induced FLP-out clones at larval stages to permanently express GFP in INPs and their neuronal progeny. We found that type II lineages primarily contribute to the central complex of the adult brain, as well as some optic lobe labeling due to 9D11 expression in this tissue.

A detailed analysis of the adult brain pattern revealed the majority of the labeled cell bodies in the dorsal posterior cortex (Figure 5), similar to the few neurons that maintain 9D11 expression in the adult brain (previous section). Additional cell bodies were seen in the anterior cortex lateral to the anterior LAL and other areas (Supplementary Figure 1).

We next describe the adult brain axon projection patterns for the type II lineages, although the high density of labeling made it difficult to link axon projections to specific cell bodies. We observed labeling of all four central complex neuropils, the two central complex accessory areas and several other regions in the central brain (Figure 5C-G; Supplementary Figure 1).

Central complex: protocerebral bridge neuropil

The PB neuropil is the most posterior of the central complex and is divided into 16 segments [29]. The PB was diffusely labeled with its lateral edges showing slightly denser staining, and the segments were not distinguishable (Figure 5D; Supplementary Figure 2A; compare Supplementary Figure 1C to 1D for denser labeling of lateral PB). Several types of small-field neurons connect the PB to other central complex neuropils but we could not distinguish them by their dispersed projections in the PB. The projections we observed in other neuropils suggest that small-field types, such as ventral fiber system (VFS) and horizontal fiber system (HFS) neurons, which connect the PB to the FB, and pontine, pb-eb-no, and eb-pb-lal neurons are labeled (see sections below) [29].

Central complex: fan-shaped body neuropil

The FB is the largest structure in the central complex and is divided into several vertical staves and horizontal stratifications [29]. Small-field neurons, which typically have their cell bodies in the DPC, contribute largely to the vertical staves while large-field neurons, which are found in both the posterior and anterior cortex, form most of the horizontal strata [29]. The FB was heavily innervated throughout, revealing its vertical and horizontal layers (Figure 5E,F; Supplementary Figures 1D-H and 2B-D). A single horizontal layer in the dorsal FB was more heavily innervated than other sections (Figure 5E; indicated with yellow dashed lines in Supplementary Figure 2B-D). We also observed dense staining in tracts dorsal to the anterior FB that are connected to arborizations in the posterior superior medial protocerebrum (psmpr) and middle superior medial protocerebrum (msmpr) regions; these projections appear to connect to the LALs as well (Figure 5F; Supplementary Figure 1E-I). We propose that these tracts are part of the anterior commisure of the FB [29].

The cell bodies and the projection pattern of several small-field types match our observations in the FB and other central complex neuropils. These include VFS and HFS neurons that project along the vertical staves (Image 5 and 6 in [29]), pontine neurons that innervate all parts of the FB (Image 9 in [29]), fb-eb neurons that innervate two horizontal layers in the FB (Image 7 in [30]) and fb-no neurons that are restricted to few staves and horizontal layers (Image 11c,d in [29]). The cell bodies and the projection pattern of some large-field F neurons (fan-shaped neurons) also match our observations in the FB. The F*m*1 and F*m*3 subtypes (fan-shaped medial neurons) have cell bodies in the DPC, and F*l* subtypes (fan-shaped lateral neurons) are primarily in the anterior cortex ventrolateral to LALs. The F*m*1 and F*m*3 neurons project anterior to the FB then posterior through the EB canal to form arbors in the second ventral layer of FB, whereas F*l* neurons project to all layers of the FB [29]. Some F*l* neurons project through the anterior commisure and innervate the msmpr (Image 22g in [29]). Another type of F*l* neuron, ExF*l*2 (an extrinsic fan-shaped neuron), has its cell body located in the DPC lateral to mushroom body calyces and forms arbors at psmpr before innervating a dorsal horizontal FB layer in a segmented fashion (Image 13 in [30]). These projections are remarkably similar to those made by type II-derived neurons, especially the tracts dorsal

to anterior FB that are connected to arbors in the psmpr and msmpr (Supplementary Figure 1) and the dense segmented dorsal layer of innervations at the FB (Supplementary Figure 2, indicated with yellow dashed lines).

Central complex: ellipsoid body neuropil

The EB neuropil is anterior to the FB and can be divided into a posterior and anterior ring. The posterior EB is innervated by small-field neurons while large-field R neurons (ring neurons), which have cell bodies ventrolateral to the LALs in the anterior cortex, fill the anterior and the median parts of the EB in concentric rings [29, 31]. However, certain R neurons are known to innervate only fragments of the EB, and ExR2, a rare extrinsic type of R neuron, is known to innervate the posterior EB only [29]. Parts of the EB were also innervated (Figure 5F; Supplementary Figure 1G,I). The posterior ring of EB was innervated in a ring-like fashion (dorsoposterior part in Supplementary Figure 2C and the middle ring in Supplementary Figure 2D); however, the more dorsoanterior parts were less innervated (Supplementary Figure 2E,F). The innervation of the anterior ring of the EB was weaker and found in a radial, evenly spaced fashion rather than a continuous ring (Supplementary Figure 2E,F). Projections through the EB canal were also observed (Supplementary Figure 2D-F, circle inside the anterior ring).

While the R neurons that project to fragments of EB could contribute to the staining of the posterior ring of EB, it is more likely generated by the small-field types such as fb-eb and pb-eb-no neurons or the rare ExR2 neuron [29], which mostly innervate the posterior ring of EB [29].

Central complex: noduli neuropil

The NO neuropil is ventral to the FB and is divided into three horizontal layers. Several small-field types innervate the NO [29, 30]. The NO was also heavily innervated (Figure 5E; Supplementary Figure 1F,G). The three horizontal layers of the NO were revealed and the top layers were heavily innervated (Supplementary Figure 2C) [30]. This pattern matches the projections of fb-no and pb-eb-no small-field neurons, which innervate only the dorsal segments of the NO [29].

Central complex: accessory areas

Few small-field neurons project to small regions of LALs, while many large-field neurons innervate the whole LAL neuropil [29]. BUs are also innervated by both small-field and large-field neurons and they are connected to the contralateral LALs [29]. In addition to the four central complex neuropils, the LAL and BU accessory areas were also labeled (Figure 5F,G; Supplementary Figure 1G-K). There were widespread arborizations in the LALs, including the ventral body commissure that connects LALs across the midline (Figure 5F). Small regions in the lateral sides of the dorsoanterior LALs, bound dorsally by the mushroom body medial lobes and ventrally by the antennal lobes (ALs), were innervated heavily (Figure 5G; Supplementary Figure 2J,K). We also observed labeling of BUs and connections between BUs and ipsilateral LALs (Figure 5 F). The extensive labeling of LALs accompanied with dense staining of small regions and the labeling in BUs is consistent with the notion that large-field types, like F*l* neurons, and small-field types, such as eb-pb-lal, HFS, and pb-eb-bu neurons, are derived from type II lineages [29]. We conclude that type II lineages contribute to all central complex neuropils and accessory areas in the adult brain.

Outside the central complex, we observed dense innervation in a region that lies dorsal to the LALs, posterior to the mushroom body medial lobes, and lateral to the anterior EB (Figure 5G; Supplementary Figure 1). The central and anterior parts of medial protocerebrum were also labeled (Supplementary Figure 1F-M). Interestingly, projections were observed in the mushroom body vertical and medial lobes (Supplementary Figure 1L,M) as well as specific glomeruli in the AL (Supplementary Figure 1J-M). The labeling we observe outside the central complex could be connections between the central complex and other brain regions or non-central complex neurons made in type II lineages.

DISCUSSION

The recent identification of the type II lineages containing transit amplifying intermediate progenitors provides an important new model for investigating progenitor self-renewal and differentiation [3-5, 14]. However, we know little about their development, cell biology, gene expression, and functional importance in the *Drosophila* central nervous

system. This is primarily due to a lack of genetic tools and markers that are specifically expressed in type II NBs and/or INPs. Here we characterize the 19H09-Gal4 line expressed in type II NBs, and the 9D11-Gal4 line expressed in INPs but not their parental type II NBs. Using 19H09 we show that Ase is upregulated before Dpn during INP maturation. Using both lines, we show that Prospero misexpression regulates proliferation but not identity within type II lineages. And using 9D11 we permanently label the majority of type II-derived neurons to show they are major contributors to the adult central complex brain region.

19H09 and 9D11 as tools to understand brain development and function

The 19H09-Gal4 and 9D11-Gal4 lines can also be used to monitor the development of type II NBs and INPs in different mutant backgrounds to help clarify the origin of a mutant phenotype. For example, early studies on tumor suppressor genes showed increases in global brain NB numbers; for some of these mutants (for example, *brat*, *numb*) we know now that the phenotype arises specifically within the type II lineages [5]. The 19H09-Gal4 and 9D11-Gal4 lines can also be used to drive UAS-RNAi, UAS-GFP constructs to test the role of any gene within these lineages. In addition, because these lines are made from defined enhancer fragments driving Gal4 placed into a specific attP site in the genome, it is easy to generate different transgenes with precisely the same expression pattern. Some future uses would be: using 19H09-FLPase to generate mutant clones or MARCM genetic screens in type II lineages; using 9D11 to drive expression of uracil phosphoribosyltranferase [32] to isolate RNA from INP sublineages; or using 9D11-grim to ablate specifically type II neurons to determine their role in larval or adult behavior.

The role of Prospero in type I and type II NB lineages

We have used the 19H09 and 9D11 lines to show that misexpression of Prospero can suppress proliferation within type II NBs and INPs without altering NB identity. As 19H09 is expressed only during the late larval stages, Prospero misexpression with 19H09 clearly distinguishes the effects of Prospero on NB proliferation from its effects on NB fate specification, which occurs in the embryonic stages. Misexpression with both

19H09 and 9D11 lead to a reduction in the number of INPs and neurons made by each type II NB. This reduction is unlikely to be due to an effect on the parental type II NBs, such as slowed down cell cycle or compromised NB survival, for the following reasons: first, low levels of ectopic Prospero are cytoplasmic in type II NBs, where Prospero has no known function; second, ectopic Prospero does not transform type II lineages to a type I identity based on the failure to upregulate *ase* expression; and third, misexpression of Prospero with both 9D11 and 19H09 give similar phenotypes, yet 9D11 is not expressed in type II NBs. We suggest that the reduction of clone size is due to an effect in the INP cell type. Possible mechanisms include INP apoptosis, INP cell cycle lengthening, premature cell cycle exit, or transforming INPs into central brain type II GMCs, which generate lineages with bifurcated axon fascicles. While we could not distinguish between these possibilities, we can tentatively exclude the mechanism of a transformation of INP to central brain type I GMC identity because the neurons still retained their ability to form bifurcated axon fascicles (Figure 4F), which are not a feature of central brain type I GMCs.

Type II NBs lack both Ase and Prospero, whereas type I NBs contain both proteins. Yet only misexpression of Ase can transform type II into type I NBs ([4] and this work), suggesting that Ase is sufficient to upregulate *prospero* expression in NBs. However, loss of Ase does not transform type I NBs into type II NBs [5], so there must be additional factors promoting the expression of Prospero in type I NBs. The analysis of gene expression differences between type I and II NBs would be one way of uncovering genes that control the difference between them.

The contribution of type II lineages to the adult brain

Lineage-tracing of INP-derived neurons shows that type II lineages make major contributions to all aspects of the central complex of the adult brain, as well as the BU and LAL accessory structures, including both small-field and large-field neurons [29]. Central complex neurons derived from type II lineages likely include several small-field types, such as VFS, pontine, fb-eb, fb-no, and pb-eb-no neurons, and, to a lesser extent, large-field types, such as F neurons, including F*m*, F*l* and ExF*l* subtypes and some extrinsic R neurons. A recent study found that type II NB clones in the pupal brain

projected to the PB, FB and NO regions, with some projections forming restricted arbors at the PB and innervating domains of the FB and NO, while others made widespread arborizations outside the central complex [23]. Our data showing labeling of the majority of type II neuronal progeny are consistent with those of [23], and complementary to these data: while we do not have the resolution to link cell bodies with axon projections, we are able to provide a more comprehensive view showing that type II lineages contribute to all central complex neuropils and accessory areas in the adult brain. Future studies that selectively ablate different spatial or temporal cohorts of type II neurons will be necessary to determine if all type II-derived neurons share a common function.

Although a large subset of central complex neurons derive from type II lineages, there are clearly some central complex neurons that originate from type I NBs or embryonic type II lineages. For example, we do not see projections that match those of the well-characterized large-field R neurons (R1 to R4) [29, 31]. It is not clear which small-field types are not derived from type II lineages as they are difficult to distinguish. However, it is clear that the type II lineages do not make up the entire central complex so there must be contributions from type I lineages as well.

Outside the central complex, we observed labeling of the region-specific staining of both the mushroom body and ALs; staining in the ALs was restricted to a subset of glomeruli. These could be novel connections from the central complex to the mushroom body and ALs formed by large-field or poorly understood extrinsic small-field neurons [29], or the projections of non-central complex neurons labeled by 9D11. Previous studies have revealed no direct connection between central complex and mushroom bodies or between LALs and ALs, and very few connections from LALs to mushroom bodies [29, 33]. The type II projection patterns from larval and pupal brains suggest that the lineages are not dedicated to a single neuropile center, which is consistent with type II lineages giving rise to non-central complex neurons as well. We also observed labeling of large regions in the protocerebrum outside the central complex. However, it was not possible to distinguish whether they were connected to the central complex or its accessory areas. Another caveat to our analysis is that 9D11 is also expressed in the larval optic lobes, and indeed we observed labeling in the adult optic lobes (Supplementary Figure 1, R-R’’’). We could not distinguish the projections from these cells from those of

the central brain cell bodies due to dense staining. Analysis of 1,200 Golgi-impregnated brains revealed direct connections between optic lobes and the BU neuropil, but not to the other central complex neuropils that we find labeled [29]. This suggests that most if not all central complex labeling is due to type II-derived neurons.

In addition to using 9D11 to lineage trace the contribution of larval-derived type II neurons to the adult brain, we also detected maintained expression of 9D11 in a small subset of adult neurons, which are likely to be P3 or P4 small-field pontine neurons, which are also detected by the Gal4 line NP2320 [30]. Thus, the 9D11 line, and others with similarly specific adult expression patterns, should be useful for future studies using TU-tagging to transcriptionally profile neuronal subsets [32], GRASP to identify pre/post-synaptic partners [34], or for expression of optogenetic modulators of neuronal activity to determine the role of specific neurons in behavior [35].

Our characterization of type II lineages suggests that as a group the type II NBs produce a wide variety of neuronal subtypes. This neural diversity can be achieved spatially if each type II NB generates just one or two types of neurons; this model is supported by clonal data showing that each type II NB produces neurons with distinct axon projection patterns [23]. In addition, temporal identity could generate further neuronal diversity as seen in type I NB lineages [36]. This model is supported by clonal analysis of a small central complex sublineage in the adult brain, which has revealed temporally distinct neuronal fates [37]. Finally, hemilineages could provide a final doubling of neuronal diversity, in which each sibling neuron derived from a single GMC takes either an ‘A’ or a ‘B’ cell fate [13]. The fact that bifurcating axon projections are seen even in the highly sparse type II lineages following Prospero overexpression is consistent with GMCs producing A/B neurons that have different fasciculation patterns. In the future, it will be important to determine the birth-order and identities of neurons in each type II lineage and the mechanisms that regulate spatial and temporal neural fate specification in these lineages.

MATERIALS AND METHODS

Fly stocks

Fly stocks were: *FRTG13*, *UAS-mcd8::GFP* (Bloomington Stock Center); *UAS-nls::GFP* (Bloomington Stock Center); *worniu-Gal4* [38]; *9D11-Gal4* [22]; *19H09-Gal4* (G Rubin, unpublished); *UAS-prosL* [39] (F Matsuzaki, unpublished); *Act[FRT-CD2-FRT]-Gal4*, *UAS-GFP* (gift from Bruce Edgar) crossed to *UAS-FLP/CyO* (Bloomington Stock Center).

Tissue preparation and immunohistochemistry

Larval brains were dissected in Schneider's medium (Sigma, St Louis, MO, USA); fixed in 100 mM Pipes (pH 6.9), 1 mM EGTA, 0.3% Triton X-100, and 1 mM $MgSO_4$ containing 4% formaldehyde for 25 minutes; washed 30 minutes in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (PBS-T); washed 30 minutes in PBS-T with 1% bovine serum albumin (PBS-BT); and incubated with primary antibodies in PBS-BT overnight at 4°C. Afterwards, brains were washed 1 h in PBS-BT, incubated with secondary antibodies for 2 h and washed 1 h in PBS-T.

Adult females 3 to 10 days old were anesthetized on ice and dissected immediately in ice-cold PBS (dissection time per brain approximately 4 minutes). Brains were fixed in PBS with 4% formaldehyde for 25 minutes; washed 10 minutes in PBS containing 1% Triton X-100 (PBT) three times and blocked with PBT containing 5% normal-goat serum (Vector Laboratories, Burlingame, CA, USA) prior to incubation with primary antibodies in PBT overnight at 4°C. Afterwards, brains were washed 10 minutes in PBT three times, incubated with secondary antibodies for 2 h and washed 10 minutes in PBT three times.

Primary antibodies were rat Dpn monoclonal (1:1), rabbit Ase (1:2,000), mouse Prospero monoclonal (purified MR1A, 1:1,000), rabbit GFP (1:500; Molecular Probes, Eugene, OR, USA), mouse GFP (1:500; Molecular Probes), chicken GFP (1:500; Aves Laboratories, Tigard, OR, USA), rat mCD8 (1:150; Invitrogen, Eugene, OR, USA), mouse Fasciclin II (1:100; Developmental Studies Hybridoma Bank), mouse nc82 (1:10; Developmental Studies Hybridoma Bank), and mouse Dlg (1:100). Secondary antibodies were from Molecular Probes (Eugene, OR, USA) and diluted at 1:500 in PBS-BT or PBT for larval and adult brains respectively.

Histology and imaging

Brains were mounted in Vectashield mounting medium (Vector Laboratories). Images were captured with a Biorad Radiance or Zeiss700 confocal microscope with a z-resolution of 1.0 (for three-dimensional reconstructions) or 1.5 microns and processed in ImageJ (NIH, Bethesda, MD, SUA) and Photoshop CS3 (Adobe, San Jose, CA, USA). Figures were made in Illustrator CS3 (Adobe). Three-dimensional brain reconstructions and movies were generated using Imaris software (Bitplane, Zurich, Switzerland).

Abbreviations

aEB, anterior ring of EB; Aimpr, anterior inferior medial protocerebrum; AL, antennal lobe; ALH, after larval hatching; Ase, Asense; Asmpr, anterior superior medial protocerebrum; Brat, Brain tumor; BU, bulb; CA, calx; CX, central complex; DM, dorsomedial type II lineage; DPC, dorsoposterior complex; Dpn, Deadpan; EB, ellipsoid body; eb-pb-lal, neuron connecting EB to PB to LAL; ExF, extrinsic fan-shaped neuron; ExR, extrinsic ring neuron; F, fan-shaped neuron; FB, fan-shaped body; fb-eb, neuron connecting FB to EB; fb-no, neuron connecting FB to NO; F*l*, fan-shaped lateral neuron; F*m*, fan-shaped medial neuron; GC, great commisure; GFP, green fluorescent protein; GMC, ganglion mother cell; HFS, horizontal fiber system; INP, intermediate neural progenitor; LAL, lateral accessory lobe; Milpr, middle inferior lateral protocerebrum; Mimpr, middle inferior medial protocerebrum; msmpr, middle superior medial protocerebrum; NB, neuroblast; NO, noduli; P, pontine; PB, protocerebral bridge; pb-eb-bu, neuron connecting PB to EB to BU; pb-eb-no, neuron connecting PB to EB to NO; PBS, phosphate-buffered saline; pEB, posterior ring of EB; psmpr, posterior superior medial protocerebrum; R, ring neuron; VBC, ventral body commisure; VFS, ventral fiber system; Vlpr, ventrolateral protocerebrum.

BRIDGE

The work presented in this chapter shows that Prospero represses proliferation in both type I and II NB lineages. The suppression of Prospero in type II NBs is required for the generation of INPs and expansion of proliferation in type II lineages. However, Prospero does not distinguish type I and II NB identity. Importantly, this work showed that type II NBs give rise to extraordinary neuronal diversity in the adult brain. Type II NBs make extensive contributions to all compartments of the adult CCX. In the next chapter, I characterize the temporal patterning mechanisms that expand the neural diversity in type II lineages.

CHAPTER III

COMBINATORIAL TEMPORAL PATTERNING IN PROGENITORS EXPANDS NEURAL DIVERSITY

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[1,2], but the temporal patterning cues used by individual progenitors to make different neural cell types over time remain poorly characterized. *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]. Thus, *Drosophila* embryonic and larval NBs are an excellent model system to study temporal identity.

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

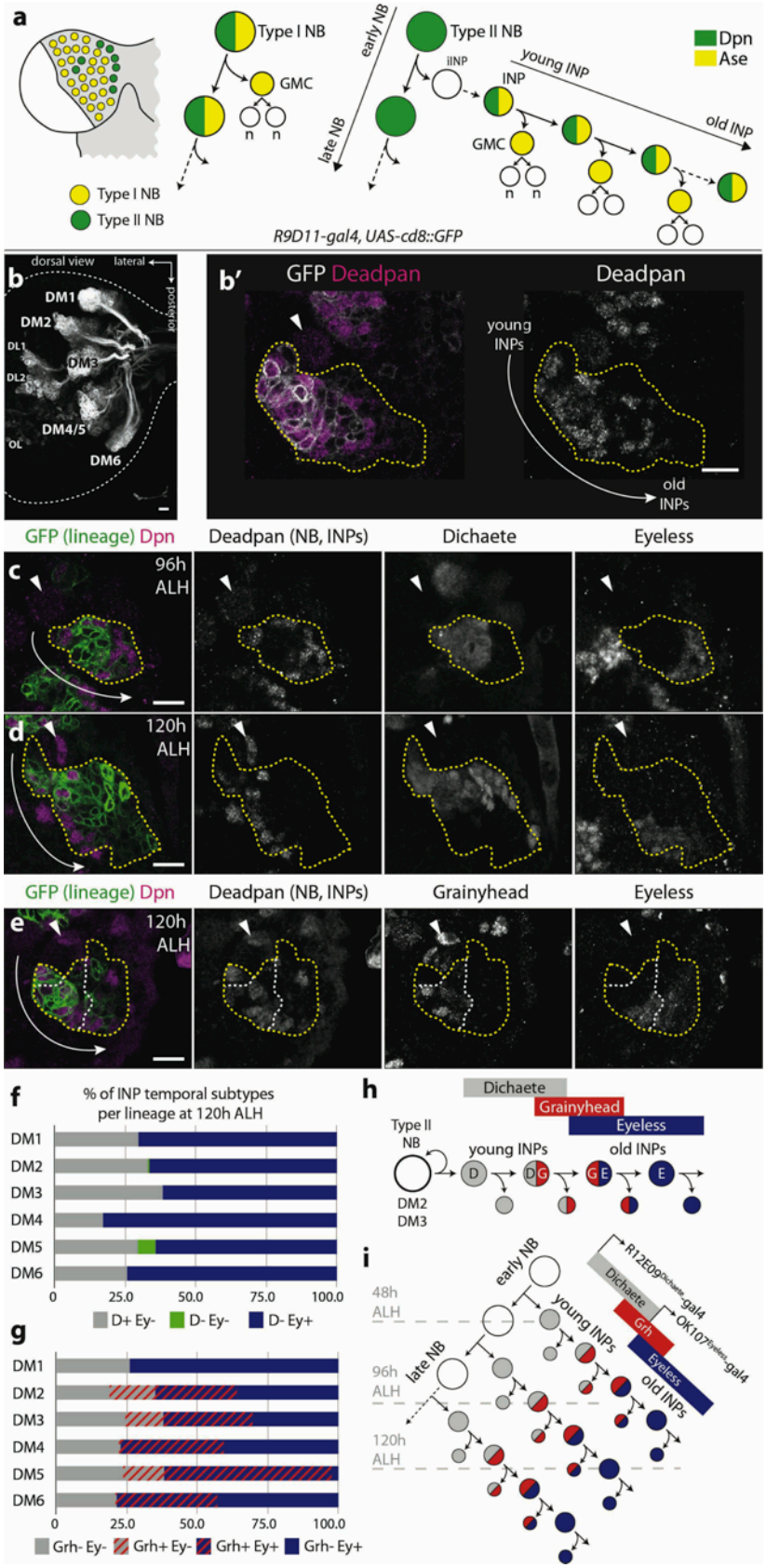
nothing about how parental NBs or INPs generate neural diversity. Do single INPs change over time to make different neural subtypes, or do they merely expand the numbers of a particular cell type? If INPs generate neural diversity, what are the mechanisms used? These are the questions we address in this paper.

RESULTS

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.

Fig 1 (next page). 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.


a
Type I NB
GMC
n n
early NB
late NB
Type II NB
iINP
INP
young INP
old INP
GMC
n n
Dpn
Ase
Type I NB
Type II NB
R9D11-gal4, UAS-cd8::GFP
b
dorsal view
lateral
posterior
DM1
DM2
DL1
DM3
DL2
DM4/5
OL
DM6
b′
GFP Deadpan
Deadpan
young INPs
old INPs
GFP (lineage) Dpn
Deadpan (NB, INPs)
Dichaete
Eyeless
c
96h ALH
d
120h ALH
GFP (lineage) Dpn
Deadpan (NB, INPs)
Grainyhead
Eyeless
e
120h ALH
f
% of INP temporal subtypes per lineage at 120h ALH
DM1
DM2
DM3
DM4
DM5
DM6
0
25.0
50.0
75.0
100.0
D+ Ey-
D- Ey-
D- Ey+
g
DM1
DM2
DM3
DM4
DM5
DM6
0
25.0
50.0
75.0
100.0
Grh- Ey-
Grh+ Ey-
Grh+ Ey+
Grh- Ey+
h
Dichaete
Grainyhead
Eyeless
Type II NB
young INPs
old INPs
D
D G
G E
E
DM2
DM3
i
early NB
48h ALH
young INPs
R12E09Dichaete-gal4
Dichaete
Grh
OK107Eyeless-gal4
Eyeless
late NB
96h ALH
old INPs
120h ALH

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 D Ey 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[28] (Fig. 1e). We conclude that most INPs progress through a stereotyped D > Grh > Ey transcription factor series (Fig. 1h-i).

Table 1. Quantification of D and Ey expression in INPs in DM1-6 type II lineages at 96h ALH

96h ALH: average number of INPs (n=6 lobes)							
av#	**total INPs**	**high D+, Ey-**	**low D+, Ey-**	**total D+ Ey-**	**low D+ Ey+**	**D- Ey-**	**D- Ey+**
DM1	22.8 ± 2.2	8.2 ± 2.0	3.0 ± 0.9	11.2 ± 1.6	1.0 ± 1.3	0.0	10.7 ± 3.2
DM2	24.5 ± 1.9	12.8 ± 2.0	1.8 ± 1.2	14.7 ± 1.2	1.3 ± 1.4	0.0	8.5 ± 1.0
DM3	25.8 ± 2.5	13.7 ± 2.0	2.2 ± 1.5	15.8 ± 2.2	1.0 ± 0.9	0.0	9.0 ± 1.7
DM4	30.5 ± 2.4	7.8 ± 1.6	2.2 ± 0.8	10.0 ± 1.7	0.7 ± 0.8	0.0	19.8 ± 2.9
DM5	25.3 ± 2.9	4.0 ± 0.9	4.2 ± 1.2	8.2 ± 0.8	0.0	4.2 ± 1.9	13.0 ± 3.4
DM6	34.2 ± 1.9	6.7 ± 1.5	4.7 ± 0.5	11.3 ± 1.4	0.7 ± 0.8	0.2 ± 0.4	22.0 ± 2.8

Table 2. Quantification of D and Ey expression in INPs in DM1-6 type II lineages at 120h ALH

120h ALH: average number of INPs (n=6 lobes)							
av#	**total INPs**	**high D+, Ey-**	**low D+, Ey-**	**total D+ Ey-**	**low D+ Ey+**	**D- Ey-**	**D- Ey+**
DM1	29.2 ± 2.1	5.3 ± 1.2	3.3 ± 0.5	8.7 ± 1.4	1.2 ± 1.2	0.0	19.3 ± 1.4
DM2	31 ± 1.3	8.2 ± 1.6	2.0 ± 0.6	10.2 ± 1.5	0.2 ± 0.4	0.2 ± 0.4	20.5 ± 2.7
DM3	31.2 ± 2.3	9.2 ± 1.7	2.7 ± 1.6	11.8 ± 1.3	0.5 ± 0.5	0.0	18.8 ± 3.1
DM4	23.3 ± 1.5	2.7 ± 1.0	1.3 ± 0.8	4.0 ± 0.9	0.7 ± 0.8	0.0	18.7 ± 0.8
DM5	21.2 ± 2.4	4.2 ± 1.5	2.0 ± 1.3	6.2 ± 1.0	0.0	1.3 ± 1.4	13.7 ± 2.3
DM6	33.8 ± 3.2	6.0 ± 2.7	2.7 ± 1.4	8.7 ± 1.4	0.7 ± 0.8	0.0	24.5 ± 3.0

Table 3. Quantification of Grh and Ey expression in INPs in DM1-6 type II lineages at 96h ALH

96h ALH: average number of INPs (n=6 lobes)						
av #	**total INPs**	**Grh- Ey-**	**Grh+ Ey-**	**Grh+ Ey+**	**total Grh+**	**Grh- Ey+**
DM1	23.7 ± 3.7	11.3 ± 1.9	0.0	0.3 ± 0.5	0.3 ± 0.5	12.0 ± 3.7
DM2	24.8 ± 2.7	10.8 ± 1.2	3.7 ±1.0	6.0 ± 1.5	9.7 ± 2.0	4.3 ± 2.0
DM3	24.8 ± 4.4	9.8 ± 1.5	4.8 ± 2.6	6.0 ± 2.4	10.8 ± 3.6	4.2 ± 1.2
DM4	28.7 ± 2.7	9.5 ± 2.8	0.0	12.8 ± 1.9	12.8 ± 1.9	6.3 ± 3.3
DM5	26.3 ± 2.8	6.8 ± 0.8	5.5 ± 2.1	13.8 ± 3.1	19.3 ± 3.4	0.2 ± 0.4
DM6	35.5 ± 5.2	10.8 ± 0.8	0.5 ± 0.5	13.8 ± 4.3	14.3 ± 4.7	10.3 ± 3.3

Table 4. Quantification of Grh and Ey expression in INPs in DM1-6 type II lineages at 120h ALH

120h ALH: average number of INPs (n=6 lobes)						
av #	**total INPs**	**Grh- Ey-**	**Grh+ Ey-**	**Grh+ Ey+**	**total Grh+**	**Grh- Ey+**
DM1	26.8 ± 3.7	7.0 ± 1.5	0.0	0.0	0.0	19.8 ± 2.5
DM2	29.8 ± 2.3	5.7 ± 1.9	4.8 ± 1.5	8.7 ± 1.2	13.5 ± 2.3	10.7 ± 1.4
DM3	30 ± 1.9	7.3 ± 2.0	4.0 ± 0.9	9.5 ± 1.2	13.5 ± 1.0	9.2 ± 1.9
DM4	26.2 ± 1.7	5.8 ± 1.7	0.2 ± 0.4	9.5 ± 1.9	9.7 ± 2.0	10.7 ± 1.9
DM5	20.8 ± 2.8	5.0 ± 2.0	3.0 ± 0.6	12.3 ± 1.8	15.3 ± 2.3	0.5 ± 0.5
DM6	31.2 ± 4.1	6.3 ± 1.0	0.2 ± 0.4	11.2 ± 2.6	11.3 ± 2.8	13.5 ± 2.9

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 completely removed D expression 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 permanently expressed *UAS-Ey*^{RNAi} specifically within INPs (see Fig. 3a). 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 (Fig. 2m).

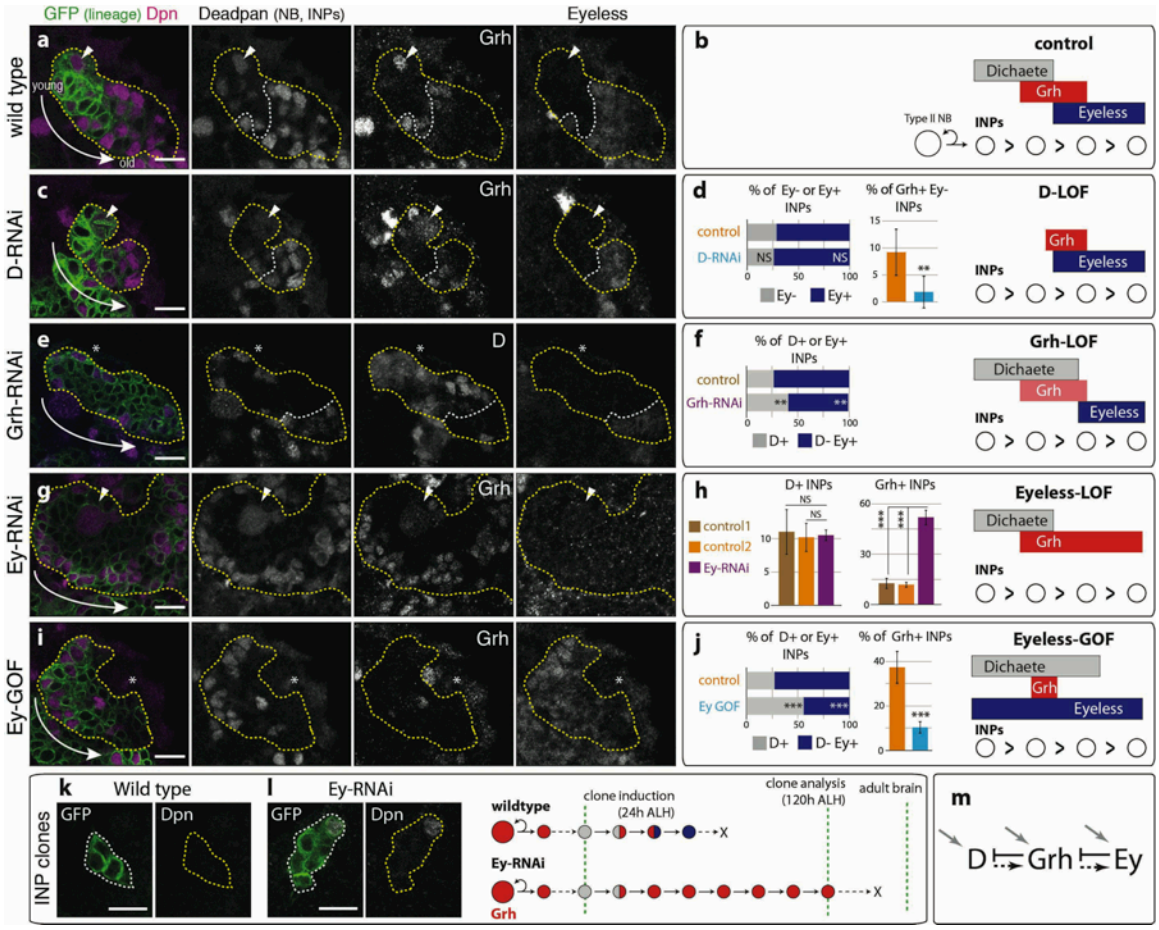


Fig 2. Cross-regulation between INP temporal transcription factors
INP temporal transcription factor expression in DM2 lineage at 120h ALH. INPs were marked with GFP (yellow outline) driven by: *wor-gal4 ase-gal80* (**a,c**), *R9D11-gal4* (**e,i**), or *R12E09^{D}-gal4* (**g**). See supplemental methods 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. (**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.

We noticed that Ey^{RNAi} not only extended the Grh expression window, but also 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. Taken together, our analysis of D, Grh, and Ey cross-regulation lead to a "feedforward activation / feedback repression" model (Fig 2m).

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 (Sup Fig 3; Fig 3b,e). 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; quantified in 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-i, quantified in 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.

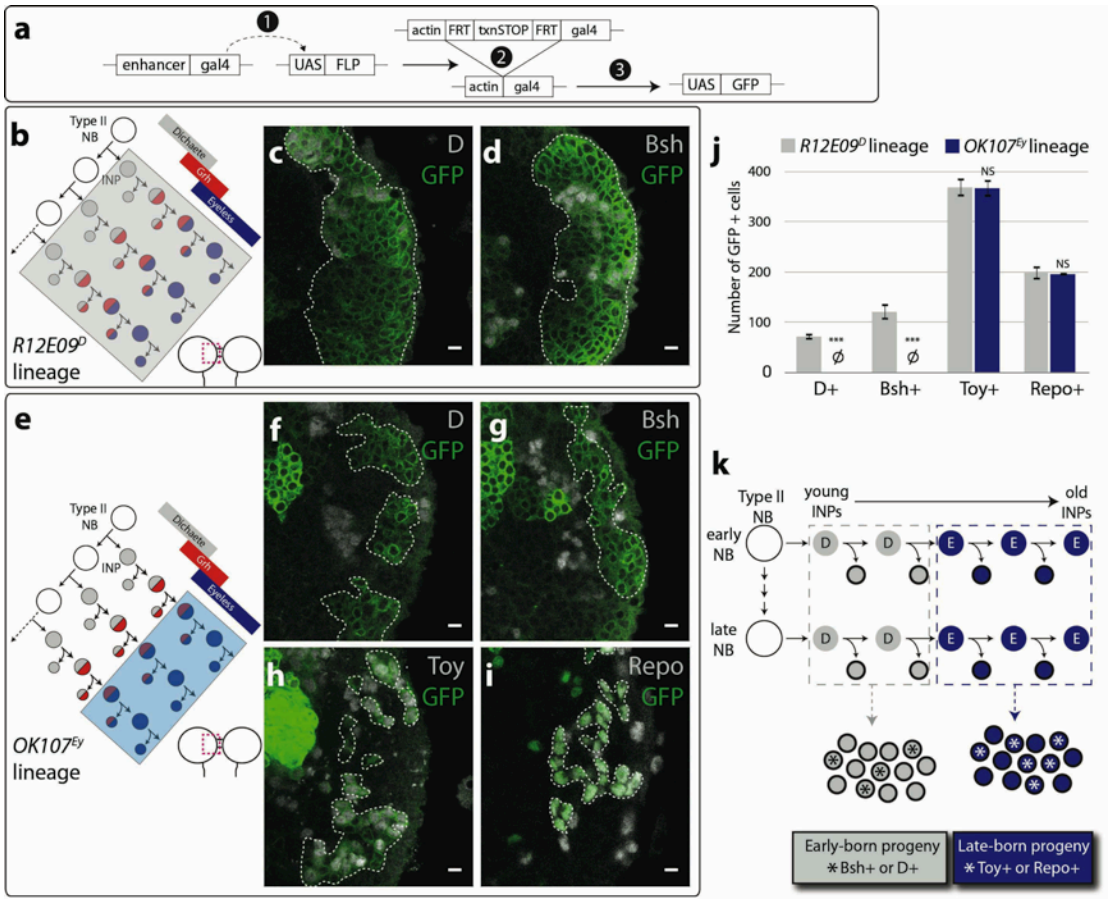


Fig 3. INPs sequentially generate distinct temporal identities
a, Genetics of permanent lineage tracing.
b-d, Permanent lineage tracing of all INP progeny using *R12E09^{D}*-gal4. Summary of GFP expression (**b**); expression of D and Bsh in the GFP+ INP progeny (**c,d**); dashed line surrounds GFP+ 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, $n \geq 3$ 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. ***$P<0.001$.

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 required for the formation of two classes of late INP progeny: Toy+ neurons and Repo+ glia. Conversely, permanent misexpression of Ey in early INPs led to an increase in the number of Toy+ neurons and a loss of early-born Bsh+ neurons (Fig. 4j-n), consistent with Ey specifying late INP temporal identity. Interestingly, ectopic Ey led to a reduction in 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-

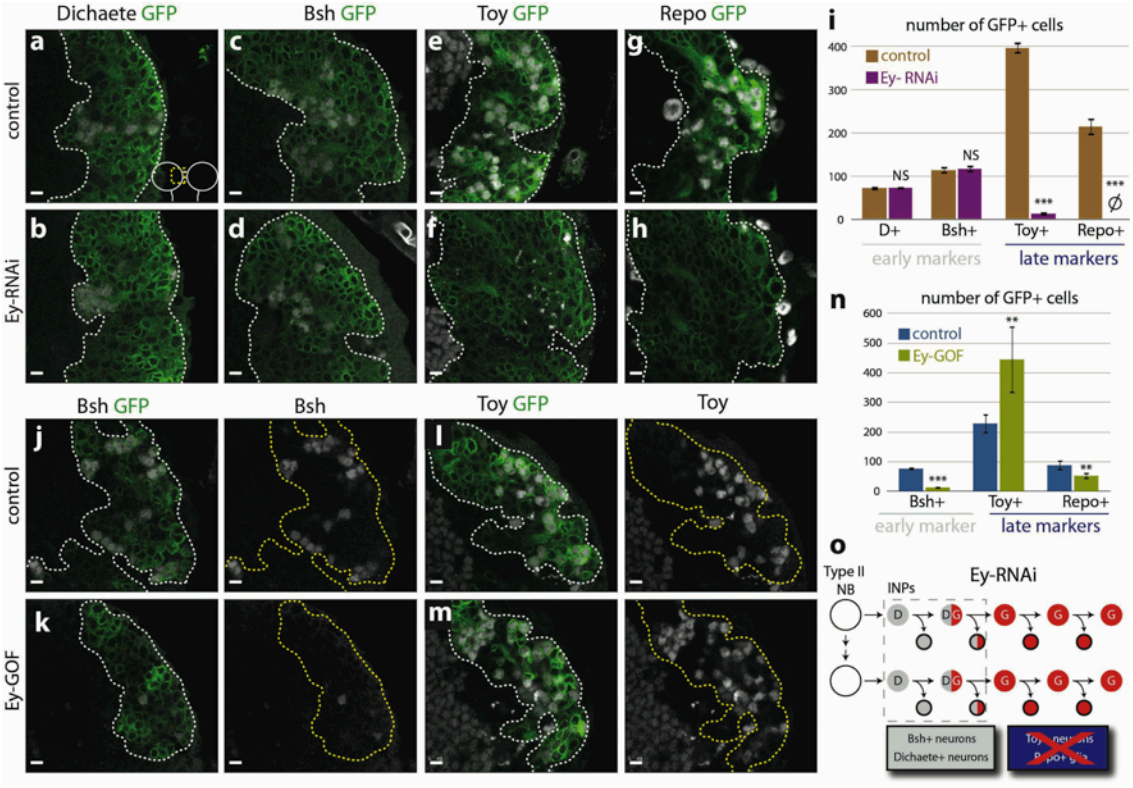


Fig 4. Eyeless is a temporal identity factor for late-born INP progeny
a-i, Ey^{RNAi} in INP lineages does not affect early-born INP progeny (**a-d**), but eliminates late-born Toy+ neurons (**e-f**) and Repo+ neuropil glia (**g-h**). (**i**) Quantification ($n \geq 4$ brain lobes).
j-n, Ey misexpression 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 ($n \geq 5$).
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. $^{**}P<0.01$, $^{***}P<0.001$.

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 to map the contribution of the late-born Ey+ INP progeny to the adult CCX (*$OK107^{Ey}$>>act-gal4 UAS-cd8:GFP*). 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). 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 (Figure 6a,b). Although we never detected D in all type II NBs at 24h, permanent lineage tracing with the *R12E09^{D}>>act-Gal4* labels all type II NBs (Sup Fig 3), indicating that all type II NBs transiently express D. The third transcription factor, Seven-up (Svp),

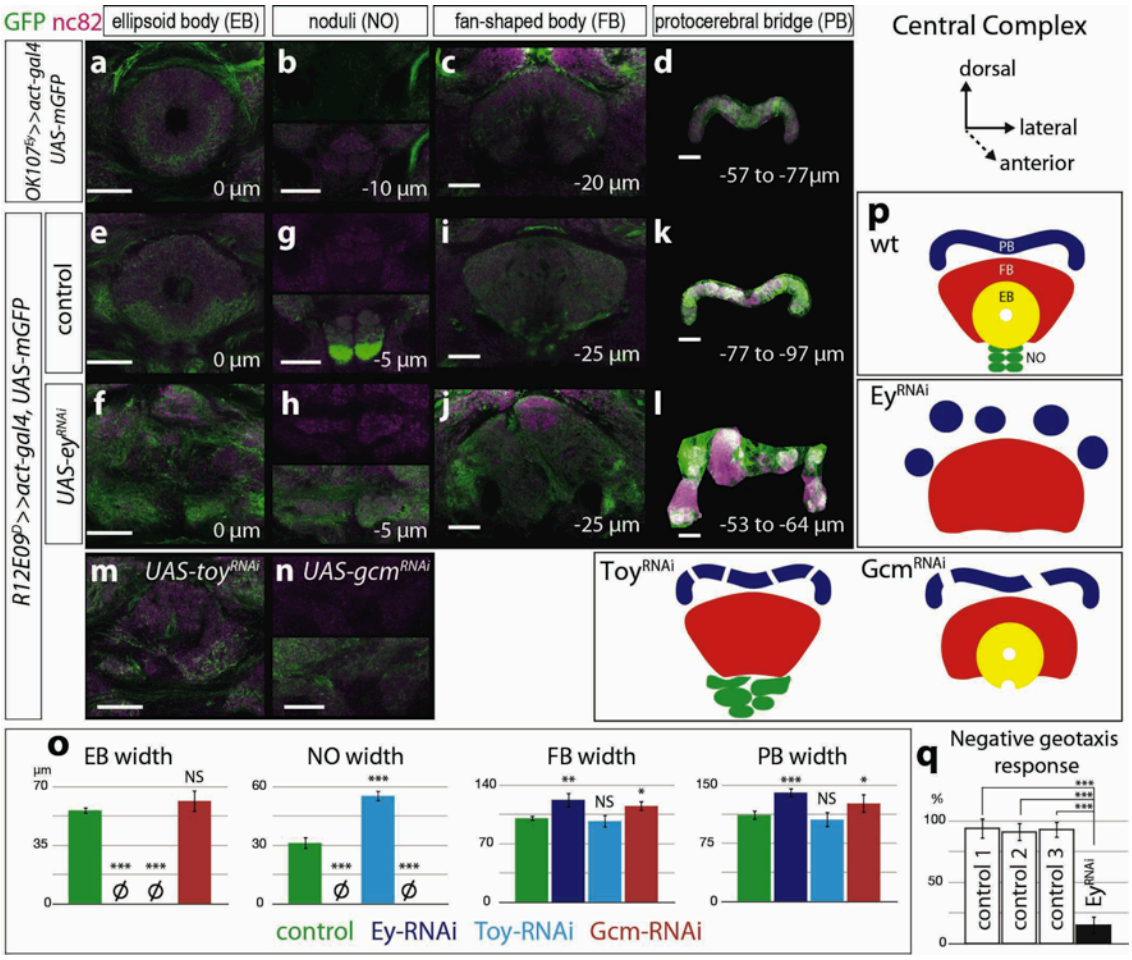


Fig 5. Eyeless is required for adult brain central complex morphology and behavior
a-d, Permanent lineage tracing of old INPs and their progeny (*OK107^{Ey} >>act-gal4)* extensively labels the adult central complex.
e-n, Ey^{RNAi} (**f-l**), Toy^{RNAi} (**m**), or Gcm^{RNAi} (**n**) in INPs lineages 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 ($n \geq 5$).
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.

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 and 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 in clones induced at the latest timepoint (Fig. 6e,g,i), showing that Bsh+ neurons are generated quite 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), thus 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 that NB temporal patterning acts combinatorially with INP temporal patterning to generate increased neural diversity in the adult brain (Fig. 6k).

Fig 6 (next page). 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 *R12E09^{D}-gal4* induced at early (**c**) or late (**d**) larval stages; all timepoints analyzed at 120h ALH. Gray shading, labeled INP and progeny.

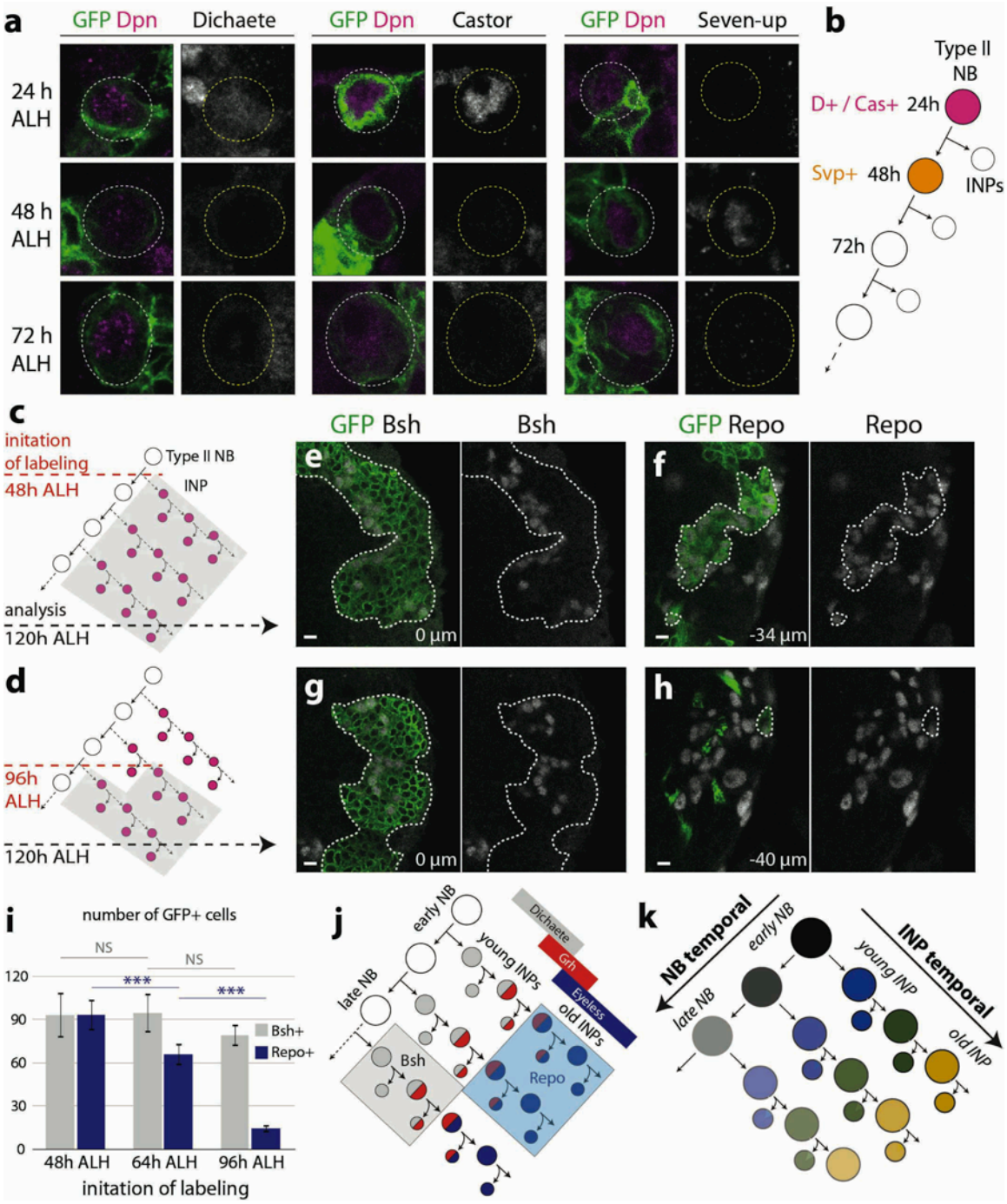
e-f, Bsh+ neurons and Repo+ glia are both marked by permanent labeling early in type II NB lineages. Focal planes: Bsh, near NB; Repo, further from the NB (-34 μm).

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.


a
GFP Dpn
Dichaete
GFP Dpn
Castor
GFP Dpn
Seven-up
24 h ALH
48 h ALH
72 h ALH
b
Type II NB
D+ / Cas+ 24h
Svp+ 48h
INPs
72h
c
initation of labeling
48h ALH
Type II NB
INP
analysis
120h ALH
GFP Bsh
Bsh
GFP Repo
Repo
e
0 µm
f
-34 µm
d
96h ALH
120h ALH
g
0 µm
h
-40 µm
i
number of GFP+ cells
NS
NS
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120
90
60
30
0
Bsh+
Repo+
48h ALH
64h ALH
96h ALH
initation of labeling
j
early NB
young INPs
late NB
Dichaete
Grh
Eyeless
old INPs
Bsh
Repo
k
NB temporal
INP temporal
early NB
young INP
late NB
old INP

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 three temporal factors that we have identified in INPs – D, Grh, and Ey – are all used in other contexts during *Drosophila* development. Embryonic NBs sequentially express D and Grh[3]. Ey is expressed in mushroom body NBs through larval life[39], where it is required for proper 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 the type II NBs and INPs change over time to make different neurons and glia. Our findings show that INPs are used to increase both the size and diversity of neural stem cell progeny. 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

Fly stocks

The chromosomes and insertion sites of transgenes (if known) are shown next to genotypes. Unless indicated, lines were obtained from Bloomington stock center (FlyBase IDs shown).

- Enhancer gal4 lines and reporters: *R9D11-gal4* (*III, attP2*)[25]. *R9D11-gal4* (*II, attP40*)[43]. *R12E09^{D}-gal4* (*III, attP2*)[25]. *OK107^{Ey}-gal4* (*IV*)[26]. *R9D11-CD4-tdTom* (*III, attP2*)[44]. *10XUAS-IVS-mCD8::GFP* (*III, su(Hw)attP2*)[9] (referred to as *UAS-GFP*).
- Mutant stocks: *D^{87},FRT2A/Tm3,Sb^{23}*. *grh^{370}/CyO,actGFP*[45].
- Transgenic RNAi: *UAS-D^{RNAi}* (II; VDRC, 107194). Lines from the TRiP collection (*III, attP2*): *UAS-grh^{RNAi}* (FBst0028820). *UAS-ey^{RNAi}* (FBst0032486). *UAS-toy^{RNAi}* (FBst0029346). *UAS-gcm^{RNAi}* (FBst0031518). TRiP RNAi controls: *y v; attP2* and *y sc v; UAS-mCherry^{RNAi}*. Other controls: *y w*, *w^{118}*, or *UAS-His2A::mRFP*.
- Lineage tracing transgenes: *UAS-FLP* (*I*; FBst0008208 and *III*; FBst0008209). *actin-FRT-stop-FRT-gal4* (*I*; FBst0004779 and *III*; FBst0004780). *tub-gal80^{ts}* (*II*; FBst0007108).
- Other: *UAS-D* (*II*) (FBst0008861). *UAS-grh* (*II*)[9]. *UAS-ey* (*II*) (FBst0006294).
- Recombinant chromosomes generated in this study: *R9D11-gal4, UAS-GFP* (*III*). *R12E09^{D}-gal4, UAS-GFP* (*III*). *UAS-FLP, actin-FRT-stop-FRT-gal4* (*both I and III*).

Fly genetics

Permanent lineage tracing, which involves the FLP-mediated removal of a transcriptional stop cassette between the constitutive *actin* promoter and the *gal4* open reading frame, is summarized in Fig 3a. For lineage tracing of young or old INP progeny (Fig 3 and 5), the *R12E09^{D}* or *OK107^{Ey}* gal4 lines were either crossed to *UAS-FLP, actin-FRT-stop-FRT-gal4;;UAS-GFP* (*I;;III*) for labeling with membrane localized GFP or to *UAS-FLP, ubi-FRT-stop-FRT-nGFP* (*II*) for labeling with nuclear GFP (G-TRACE)[46].

For driving expression of *UAS-RNAi* or misexpression transgenes, following lines were used: *UAS-dcr2; wor-gal4, ase-gal80; UAS-mCD8::GFP*[29]. *R9D11-gal4; R9D11-gal4, UAS-GFP (II, III)*. *R12E09^{D}>>act-gal4* [*UAS-FLP, actin-FRT-stop-FRT-gal4;;*

R12E09^{D}-gal4, UAS-GFP/Tm6B (*I;;III*)]. *R9D11>>act-gal4* [*UAS-FLP, actin-FRT-stop-FRT-gal4;; R9D11-gal4, UAS-GFP* (*I;;III*)]. Below are the genotypes used in RNAi and misexpression experiments:

- D^{RNAi} was driven by *wor-gal4 ase-gal80 UAS-dcr2* in *D^{87}*/+; control was *w^{1118}*.
- Grh^{RNAi} was driven by *R9D11-gal4, R9D11-gal4* in *grh^{370}*/+; control was *attP2* (empty transgene docking site).
- Ey^{RNAi} was driven by *R12E09^{D}>>act-gal4*; controls were (1) *attP2* and (2) *UAS-mCherry^{RNAi}*.
- Ey^{GOF} was driven by *R9D11-gal4 >>act-gal4*; control was *yw* or *UAS-His2A::*mRFP (for quantification of INP progeny).
- Toy^{RNAi} and Gcm^{RNAi} were driven by *R12E09^{D}>>act-gal4.*

For inducible lineage tracing (Fig 6), *R12E09^{D}>>act-gal4* was combined with the ubiquitously expressed *tub-gal80^{ts}* so that temperature shifts were used to turn on labeling by *R12E09^{D}* at different points in the type II NB lineages. *R12E09^{D}-gal4, UAS-GFP* flies were crossed to *tub-gal80^{ts}; UAS-FLP, actin-FRT-stop-FRT-gal4* (*II;III*). The newly hatched 0-6 h ALH larvae were reared at restrictive temperature at 18^{o}C for 72, 96, or 144 hours (which correspond to 48, 64, and 96 hours of development at 23^{o}C respectively), then shifted to permissive temperature at 30^{o}C to induce labeling.

To generate wild type or *D^{87}* type II NB MARCM clones, *hsFLP; tub-gal4, UAS-mCD8::GFP/CyO; tub-gal80, FRT2A/TM6C,Sb* flies were crossed to *FRT2A* or *D^{87},FRT2A*/*Tm3,Sb flies respectively.* To induce clones, 24h ALH larvae were heat shocked at 37^{o}C for 30 min, and reared to 120h ALH. To generate wild type or Ey^{RNAi} INP MARCM clones, *hsFLP; FRT40A, tub-gal80/CyO,actGFP; tub-gal4, UAS-mCD8::GFP/TM6B* flies were crossed to *FRT40A* or *FRT40A; UAS-ey^{RNAi}* flies respectively. To induce clones, 24h ALH larvae were heat shocked at 37^{o}C for an hour, and reared to 120h ALH. INP clones were identified in the dorsomedial brain as multicellular clones (n > 3 cells) without a NB.

Unless indicated otherwise, larvae were staged to 120h ALH based on age and morphology (late wandering larvae near pupariation) for dissections. For other timepoints, newly hatched 0-4h ALH larvae were picked and reared accordingly. Adult females were aged to 3-5 days for dissections.

Immunohistochemistry

Primary antibodies were rat anti-Dpn (1:50, Doe lab), guinea pig anti-Dpn (1:2000, Jim Skeath), chicken anti-GFP (1:2000, Aves Laboratories, Tigard, OR), guinea pig anti-D (1:500, John Nambu), rabbit anti-D (1:500, John Nambu), rabbit anti-Ey (1:3500, Uwe Walldorf), rat anti-Grh (1:1000, Stefan Thor), guinea pig anti-Bsh (1:250, Makato Sato), guinea pig anti-Toy (1:500, Uwe Walldorf), mouse anti-Repo (1:4, DHSB), mouse anti-nc82 (1:100, DHSB, Iowa City, IO, USA), rabbit anti-DsRed (1:500, Clontech Laboratories, Mountain View, CA, USA), rabbit anti-Ase (1:2000, Cheng-Yu Lee), mouse anti-Pros (MR1A, 1:1000, Doe lab), rat anti-Elav (1:50, DHSB), rabbit anti-Cas (1:1000, Ward Odenwald), rat anti-Svp (1:500, Takako Isshiki). Secondary antibodies were from Molecular Probes (Eugene, OR, USA) or Jackson Immunoresearch (West Grove, PA, USA).

Dissection and immunostaining were performed as described previously[15] with few modifications: Larval brains were fixed in 4% formaldehyde in PBST (1X PBS with 0.3% Triton X-100) for 25 min, rinsed, and blocked in PBST with 5% normal goat and donkey serum mix (Vector Laboratories, Burlingame, CA, USA) for 30 min. Adult brains were fixed in 4% formaldehyde in PBT (1X PBS with 1% Triton X-100), rinsed, and blocked in PBT + 5% serum. Adult brains were incubated in primary antibodies for two days at 4^{o}C, then in secondary antibodies for two days at 4^{o}C. Brains were stored in Vectashield (Vector Laboratories). For EdU incorporation, dissected larval brains were incubated in S2 medium (Sigma, St. Louis, MO) containing 100 mg/mL EdU (Molecular Probes, Eugene, OR) at 25^{o}C for 2 hours. After completing standard fixation and antibody staining procedures, EdU was detected by following manufacturers protocols (Molecular Probes, Eugene, OR).

Imaging

Brains were mounted in Vectashield (Vector Laboratories). Images were captured with a ZeissLSM700 or LSM710 confocal microscope with a z-resolution of 1.0 micron, and processed in the open source software FIJI (http://fiji.sc) and Photoshop CS5 (Adobe, San Jose, CA, USA). Figures were made in Illustrator CS5 (Adobe). Three-dimensional brain

reconstructions were generated using Imaris software (Bitplane, Zurich, Switzerland).

Quantification of INPs and progeny

INPs were labeled with cell-type specific gal4 driven UAS-mCD8::GFP and distinguished from their GMC/neuronal progeny by Deadpan (Dpn) staining. For the quantification of Grh expression in middle-aged INPs, newly mature INPs that show weak levels of Grh (inherited from immature INPs) were excluded. For the quantification of temporal identities, INP progeny were marked with permanent lineage tracing. GFP+ INP progeny in DM1-6 lineages were counted. GFP+ Repo+ glia in the lateral brain were also counted, see Supplementary Figure 10. For better labeling of glia, nuclear localized GFP (nGFP) was used.

Negative geotaxis assays were performed as described previously[42]. Ten adults of each genotype (3 day old virgin females) were placed in a vial at room temperature. Flies were allowed to acclimate for 1 minute, and then gently tapped to the bottom of the vial. The number of flies that climbed above the vertical distance of 8 cm by 10 seconds after the tap was recorded as a percentage of total flies. Ten trials were conducted for each genotype, with 1 min rest period between each trial. The results of ten trials were averaged and plotted as the negative geotaxis response. Ey^{RNAi} genotype was *R12E09^{D}>>act-gal4 UAS-ey^{RNAi}*. Controls were (1) *R12E09^{D}>>act-gal4 attP2*, (2) *R12E09^{D}>>act-gal4 UAS-mCherry^{RNAi}* (3*) no gal4>>act-gal4 UAS-ey^{RNAi}*.

Statistics

Data represent mean ± s.d. Two-tailed Student's t-tests were used to assess statistical significance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

CHAPTER IV

CONCLUSIONS

In this dissertation I show that (i) Prospero regulates proliferation in type II lineages but does not distinguish type I and II NB identity, (ii) type II NB lineages give rise to a diverse collections of neurons and make significant contributions to the adult brain central complex, (iii) INPs undergo temporal patterning and are used to increase both the size and diversity of type II NB progeny, (iv) INP temporal patterning is regulated by sequentially expressed transcription factors, and (v) parental NB and INP temporal patterning are combined to generate a diverse collections of neurons and glia in type II lineages. I discuss the implications of these results as well as future directions of research and more recent findings in type II NB lineages below.

The regulation of type I/II NB proliferation pattern and identity

My results show that Prospero suppresses INP proliferation and the repression of Prospero in type II NBs is necessary for the generation of INPs in type II lineages. However, misexpression of Prospero in type II NBs does not transform them into a type I NB-like identity. Upon Prospero misexpression, type II NBs are still Ase- and generate neuronal progeny with bifurcating axons. The misexpression of Ase in type II NBs also suppresses INP proliferation [1], however it is unknown whether ectopic Ase can repress Prospero expression in type II NBs. In addition, loss of Ase or Pros from type I NBs alone does not transform into type II NBs [1, 2]. What are the "master" factors upstream of Prospero and Ase that distinguish type I and II NB identity? A recent elegant study suggested that the Ets transcription factor Pointed (Pnt) is a master regulator of type II NB identity [3]. Pointed is specifically expressed in type II NBs, immature and young INPs in the larval brain. Loss of Pointed expression from type II lineages suppresses the generation of INPs and upregulates Ase expression in type II NBs. Furthermore, misexpression of Pointed in type I NBs leads to the generation of ectopic INP-like cells and renders type I lineages susceptible to tumor formation upon loss of translational repressor Brat. While Prospero expression in NBs has not been examined upon the

manipulation of Pointed expression, it is likely that Pointed represses Prospero in type II NBs. Alternatively, Prospero might be repressed by another factor that acts together with Pointed to specify type II NB identity.

The neuronal and glial progeny of type II NBs in the adult brain

The lineage tracing experiments presented here showed that type II NB lineages give rise to a diverse collection of neurons and glia in the adult brain. Recent studies confirmed that type II NB lineages give rise to extraordinary neural diversity in the adult brain [4, 5]. In these studies, the neural progeny of each larval central brain NB was distinguished in the adult brain with genetic clones. This comprehensive analysis showed that individual type I NBs generally contribute neurons to one or two distinct brain regions, "home" neuropils, in the adult brain. In contrast, the neuronal and glial progeny of individual type II NBs are not restricted to a few brain regions and contribute to many brain areas. While most type II NB lineages contribute to the CCX (see below), a common pattern to their contributions in other brain regions is not clear. These results confirm that type II NBs generate increased neural diversity than type I NBs.

The contribution of type II NBs to the adult brain central complex

Type II NBs contribute neurons to all compartments of the adult CCX. What are the CCX neuronal subtypes generated by type II NBs? Do type I NBs also contribute to the CCX? The clonal studies mentioned above found that 15 larval NB lineages in each brain lobe contribute to the CCX [4-6]. Seven type II NB lineages, DM1-6 and DL1, and eight type I NB lineages give rise to neurons that innervate the CCX. The DM1-4 lineages generate all the small-field neurons of the CCX. Small-field neurons are intrinsic to the CCX and organized into more than 30 isomorphic sets (i.e. a group of neurons with stereotyped morphologies that connect different CCX subdivisions in a repetitive manner) [7, 8]. DM1-4 type II NB lineages contribute to largely non-overlapping but equivalent subdivisions of all CCX compartments, implicating that each lineage generates all 30 small-field neuronal subtypes [6]. The remaining type II NB lineages, DM5-6 and DL1, give rise to unique sets of large-field neurons that connect CCX to its accessory areas or other brain regions: DM6 contributes large-field neurons to all CCX

compartments while the contributions of DM5 and DL1 are restricted to the PB and FB respectively [6]. These observations confirm that type II NB lineages make significant and diverse neuronal contributions to the CCX. Interestingly, large NB lineages that contain transit-amplifying progenitors also give rise to CCX neurons in grasshoppers [9]. Taken together, these observations show that the generation of the CCX neurons by type II NB-like progenitors is an evolutionarily conserved feature of insect brain development and suggest that transit-amplifying INPs are commonly used to increase neuron size and complexity in the insect brain.

In addition to extensive neuronal contributions to the CCX, most DM type II NB lineages make neuropile glia that closely associate with the CCX [4, 6, 10]. In the grasshopper brain, neuropile glia also associate with the CCX [11]; it is likely that these glia are generated from type II NB-like lineages mentioned above. Despite these anatomical observations in both insects, the functional roles of CCX-associated glia were not clear. The glial ablation experiments in type II lineages presented in Chapter III (i.e. Gcm-RNAi) reveal that these neuropile glia are non-cell autonomously required for the proper innervation of distinct CCX compartments by type II lineage derived neurons. In addition to DM-lineage derived CCX glia, the DL1 type II NB gives rise to glial cells of the optic lobe [12]. Taken together, these results show that type II NBs are multipotent progenitors that give rise to diverse neurons and glia in the adult brain.

The characterization of the diverse neuronal and glial cells formed in each type II NB lineage leads to many interesting questions about the spatial and temporal patterning mechanisms that generate these neural subtypes, these are discussed below.

Temporal patterning in INPs: a new source of neural diversity

Here I provide the first evidence in any organism that transit-amplifying INPs undergo temporal patterning to generate diverse cell types. These initial findings pose many questions for future research.

First, INPs sequentially express three transcription factors (D > Grh > Ey) and generate different neural subtypes during the early and late transcription factor windows. How many distinct temporal neural subtypes (i.e. temporal identities) are generated from each INP in type II lineages? INPs transition through four transcription factor windows

(D+Grh-, D+Grh+, Grh+Ey+, Grh-Ey+). However, the exact number of GMCs generated during each of these windows from early or late-born INPs in multiple type II NB lineages is unknown. It is likely that multiple GMCs are born during each transcription factor window; these GMCs might have the same neural identity or might be distinguished by subtemporal patterning cues as in embryonic type I neuroblasts [13].

Second, INP temporal identity transitions are partially regulated by feedforward activation and feedback repression in the D > Grh > Ey series. What are the external factors that regulate INP temporal identity transitions (i.e. the INP "timer" genes)? Are these transitions dependent on cell cycle progression, similar to the Hb > Kr switch in the embryonic neuroblasts [14]? In addition, are post-transcriptional mechanisms also involved in the regulation of INP temporal identity transitions?

Third, INP transcription factors are required for the generation of temporally distinct neural subtypes. Do D and Grh specify other early and mid-born INP progeny than Bsh+ neurons? Does Grh co-operate with D or Ey in the specification of some mid-born INP progeny? What are the other late-born INP progeny specified by Ey? To answer these questions, we need more molecular and anatomical markers of INP progeny.

Fourth, Ey is an INP temporal identity factor that specifies late-born Toy+ neurons and Repo+ glia. What is the fate of late-born INP progeny upon loss of Ey? These cells might be missing if loss of Ey causes late INPs to skip divisions or late INP progeny to undergo cell death. However, our results show that late INPs undergo extended lineages upon loss of Ey and generate many neurons, thus some late-born INP progeny are still present. Late INP progeny might adopt mid-born identities upon loss of Ey, as Grh expression extends into all aging INPs. Alternatively, their identities might be "partially" specified by NB temporal and spatial patterning cues (discussed below).

Last, the majority of INPs –from different type II NBs and from early or late NBs – sequentially express D > Grh > Ey and generate temporal subtypes, strongly suggesting that INP temporal patterning is largely cell-intrinsic. Are the cell-intrinsic mechanisms that regulate type II NB identity and INP temporal patterning inherently linked? In other words, do the mechanisms that generate INPs from type II NBs also trigger the D > Grh > Ey mediated temporal patterning in type II NB progeny? It will be important to test if the transcription factor Pointed (see above) that is expressed in type II NB induces INPs

to undergo the D > Grh > E series and generate distinct progeny over time. Additionally, it will be interesting to see if the INP-like cells that are derived from type I NBs upon Pointed misexpression express D and other INP transcription factors. Alternatively, the mechanisms that regulate the type II NB proliferation pattern and INP temporal patterning could be independent, and the latter could be regulated by other factors in type II NBs.

Temporal patterning in type II NBs

Here I also show for the first time that type II NBs undergo temporal patterning: they sequentially generate the INPs that make the Repo+ glia and Bsh+ neurons respectively. Most of the questions posed for INPs in the previous section also apply to type II NB temporal patterning e.g. how many temporal identities are generated from each type II NB? An important future research goal is the identification of type II NB temporal identity factors. The transcription factors D, Cas and Svp that are expressed in early type II NBs are good candidate temporal identity factors. Cas and Svp regulate the switch from Chinmo+ to Broad+ neuron production in several larval neuroblast lineages [15]. Both Chinmo and Broad are expressed in subsets of neurons in type II lineages, it will be interesting to see if they are sequentially specified from type II NBs by Cas > Svp. Another related mechanism that could regulate type II NB temporal patterning is Hedhehog (Hh) signaling. It is known that in the majority of larval central brain NBs including type II NBs, the early Cas expression triggers an increasing temporal gradient of Hh signaling [16]; this gradient could regulate the specification of early and late born temporal identities from type II NBs. Moreover, the increasing gradient of Hh signaling might regulate the temporal patterning of type I NBs as well, providing a global temporal cue to larval NBs. As a Shh signaling gradient regulates spatial patterning in the mammalian spinal cord [17], it will be important to test the role of Hh signaling in NB temporal patterning.

Another interesting question is whether all type II NBs progress through the same temporal series. My findings suggest that all type II NBs transiently express D, and at least the DM1-3 NBs temporally express Cas and Svp. If all type II NB progress through the same temporal series, type II NB temporal patterning could be induced by the cell-

intrinsic factors that regulate type II NB identity. Alternatively, the mechanisms that specify type II NBs in early development (i.e. delamination from the precursor neuroectoderm) could initiate type II NB temporal patterning.

My studies identify three genes that are expressed in early type II NBs, however the factors that are specifically expressed in late type II NBs are unknown. In addition, in-depth studies of type II NB and INP temporal patterning require comprehensive molecular markers of their neuronal progeny. In the future, whole genome expression analysis can provide ample candidate temporal identity factors and molecular markers in type II lineages. These experiments will require the isolation of RNA from type II NBs, INPs or their neuronal progeny at different timepoints; fluorescent activated cell sorting (FACS) [18] or TU-tagging [19] can be used for this purpose.

Spatial patterning in type II NBs

Recent studies showed that the eight type II NBs give rise to distinct lineages of neurons and glia in the adult brain (see above) [4-6, 12]. Consistently, I found several molecular markers that distinguish the neuronal progeny of the DM type II NBs in the larval brain (Appendix 2, Sup Table 1). For example, Bsh is expressed in a subset of neuronal progeny only in the DM2 and DM3 type II NB lineages (Appendix 3, Sup Fig 9). These observations show that spatial patterning acts together with NB and INP temporal patterning to generate neural diversity in type II lineages.

An interesting aspect of spatial patterning in type II lineages is the generation of intrinsic neurons of the CCX. Four type II NBs, DM1-4, contribute intrinsic small-field neurons to largely non-overlapping subdivisions of each CCX compartment [6]. Yet, these subdivisions are thought to be equivalent and contain small-field neurons of each 30 isomorphic sets [6, 7]. Thus, the DM1-4 type II NBs generate equivalent sets of small-field neurons that must be topographically ordered in the CCX; it will be fascinating to understand the spatial patterning mechanisms that give rise to this complex neuronal organization.

Currently, the spatial patterning mechanisms that distinguish type II NBs are unknown. It is likely that spatial patterning cues are provided to type II NBs in early development. In the embryonic brain, each newly formed central brain NB expresses a

unique combination of transcription factors [20] suggesting that spatial patterning of type II NBs might be regulated by a combinatorial code. In the future, the identification of the embryonic origin of type II NBs, and characterization of type II NB lineage specific gal4 lines and additional molecular markers will be useful for studying spatial patterning mechanisms in these lineages.

Combinatorial patterning in type II NB lineages

My findings reveal a combinatorial program that expands neural diversity in type II NB lineages. Spatial and temporal patterning in type II NBs act together with INP temporal patterning to generate an increased neural diversity compared to type I NB lineages. The generation of Bsh+ neurons exemplifies combinatorial patterning in type II NB lineages: the formation of Bsh+ neurons is restricted to DM2 and DM3 lineages by spatial patterning, then further restricted to late type II NBs and young INPs by NB and INP temporal patterning respectively. The combinatorial nature of temporal patterning explains the extraordinary neural diversity generated in type II NB lineages. I discuss important directions for future research on combinatorial temporal patterning, grouped under descriptive and functional studies, below.

Descriptive studies of combinatorial temporal patterning: the CCX as a model

An important step towards fully understanding the combinatorial temporal patterning program is the complete morphological characterization and birthdating of all type II NB lineage derived neurons and glia in the adult brain. Such a comprehensive study would identify every single neuron or glia generated by each INP division, and provide a complete developmental sequence of all type II NB lineages. This task is technically feasible in *Drosophila* as the fly CNS is highly stereotyped and powerful genetic tools such as MARCM based clonal-analysis and cell-type specific enhancer gal4 lines allow extensive and precisely controlled lineage tracing [21]. Recent studies have utilized these strengths to characterize the entire developmental sequence of larval type I NB lineages [22] or map the neuronal connectivity of entire adult brain regions such as the protocerebral bridge [23]; similar approaches can be undertaken to examine neuronal diversity in type II NB lineages.

What would the complete developmental history of type II NB lineages reveal about combinatorial temporal patterning? As emphasized in the previous sections, a comprehensive morphological description of temporal identities will greatly aid the study of temporal patterning mechanisms. Importantly, it will fully reveal the role of combinatorial temporal patterning in the generation of neural diversity in type II lineages and its contribution to brain organization. For example, each type II NB contributes neurons to several adult brain regions. Do type II NBs sequentially make INPs that contribute to distinct brain areas? If the answer is yes, then each INP lineage would be restricted to a distinct brain region and INP temporal patterning could generate neuronal diversity within each brain structure. Alternatively, each INP might contribute neurons to various brain regions. Perhaps all the neurons that are made in type II lineages during a defined developmental window (e.g. 72 to 80 hours after larval hatching) contribute to the same brain region; this would imply a complex temporal patterning scheme in which early type II NB-derived old INPs and late type II NB-derived young INPs provide neurons to the same brain structure. Finally, given the enormous number of neurons generated in type II NB lineages, their morphological characterization will uncover a significant portion of the neuronal connectivity in the adult brain.

The complete characterization of neuronal diversity in type II NB lineages is technically feasible, yet a massive undertaking. The adult brain CCX provides a more manageable model for initial studies as it has a higher degree of neuronal architecture than other brain regions and the morphologies of CCX neurons have been extensively characterized [7, 8]. Moreover, the contributions of each type II NB to the CCX has already been described at a lineage level [6]. As noted before, the DM1-4 type II NBs give rise to the most interesting CCX innervation pattern as they each contribute more than thirty small-field neuron subtypes to all CCX compartments in a topographically ordered fashion [6]. Do these type II NBs contribute to the CCX and other brain regions in a sequential manner? Within the CCX, do these type II NBs sequentially contribute neurons to different compartments (e.g. PB > FB > EB > NO)? Does a single INP contribute several subtypes of small-field neurons to each CCX compartment? Alternatively, does each INP contribute to several different CCX compartments? The CCX also provides an opportunity to link combinatorial temporal patterning in type II

NB lineages to functional studies of neural circuitry as the role of CCX neurons in the modulation of various locomotor behaviors has been studied to some extent [24]. In the future, it will be interesting to see whether INP lineages give rise to distinct functional circuits and how combinatorial temporal patterning generates the complex neuronal circuitry of the CCX.

Functional studies of combinatorial temporal patterning: the enigma of integration

NB and INP temporal patterning act together to expand neural diversity in type II lineages. However, it is largely unknown how NB and INP temporal inputs are integrated to specify diverse neural cell fates. Similarly, spatial and temporal inputs are integrated in type I NBs in the embryonic VNC but the mechanistic basis of this integration is poorly understood. Understanding the mechanisms that underlie the integration of patterning inputs will be a major future direction for functional studies of combinatorial patterning.

First of all, the integration of NB and INP patterning does not necessarily imply that these inputs are dependent on each other. NB and INP patterning programs can be induced independently. As stated above, NB spatial patterning can be induced during NB delamination in the embryonic brain, while an independent program that specifies type II NB identity or an extrinsic signal could initiate NB temporal patterning. Then, INPs could undergo D > Grh > Ey based temporal series independent of NB patterning. Alternatively, these patterning programs could be interdependent, for example spatial patterning cues could initiate temporal patterning in type II NBs. The integration of NB and INP patterning indicates the convergence of these inputs in INPs and their progeny to specify downstream neural cell fates.

An important issue during the integration of NB and INP patterning is the inheritance of NB spatial/temporal patterning information to INPs. It is possible that NB temporal identity factors are passively inherited to INPs during NB divisions, where they activate their own expression or directly interact with INP temporal identity factors to specify cell fate. Alternatively, NB temporal identity factors could define the epigenetic state of the cell that is inherited to INPs during cell division (i.e. epigenetic memory), which then regulates the INP temporal patterning program. To distinguish between these possibilities, first we need to identify type II NB temporal identity factors and determine

whether they act in NBs or INPs to specify cell fate. For the latter scenario, it will be important to examine the roles of the Polycomb-group (PcG) proteins which are required for inheritance of epigenetic memory during cell division across multiple organisms [25] [26], although the mechanisms underlying PcG-mediated epigenetic memory are not fully understood either.

Besides the issue of inheritance, how are NB and INP temporal patterning inputs integrated in INPs? It is useful to consider two distinct mechanisms in their extreme form. (a) NB and INP patterning programs could largely intersect. For example, NB and INP temporal identity factors could act as transcriptional co-factors. They could independently bind to an overlapping set of enhancers, or form transcriptional complexes that recognize different enhancers than either factor alone. If the NB temporal identity factors regulate epigenetic memory, INP temporal identity factors could recognize different enhancers in early versus late-born INPs due to distinct epigenetic states of the cells. The NB and INP patterning programs can also intersect further downstream of temporal identity factors. In order to test these models, one could initially compare the binding sites of late INP temporal identity factor Eyeless in early and late born INPs by chromatin immunoprecipitation (ChIP), and then analyze gene expression from of Eyeless-bound enhancers in early and late born INPs with TU-tagging and sequencing. (b) NB and INP patterning programs could be largely independent. NB and INP temporal identity factors could regulate non-overlapping transcriptional programs that could collectively specify cell fate. As mentioned above, these are extreme cases and combinatorial temporal patterning could be composed of both intersecting and overlapping NB-INP patterning programs that regulate the expression of a multitude of cell fate genes.

My findings in type II NB lineages suggest that NB and INP temporal patterning programs intersect at least for some temporal identities. For example, Bsh is only expressed in the progeny of late NB-derived young INP progeny. If NB and INP temporal patterning programs did not intersect at all, Bsh expression could not be confined to the progeny of late born INPs. Aforementioned, NB and INP temporal patterning could be independent to some extent. For example, upon Ey loss-of-function type II lineage derived neurons still innervate the midline brain area where CCX

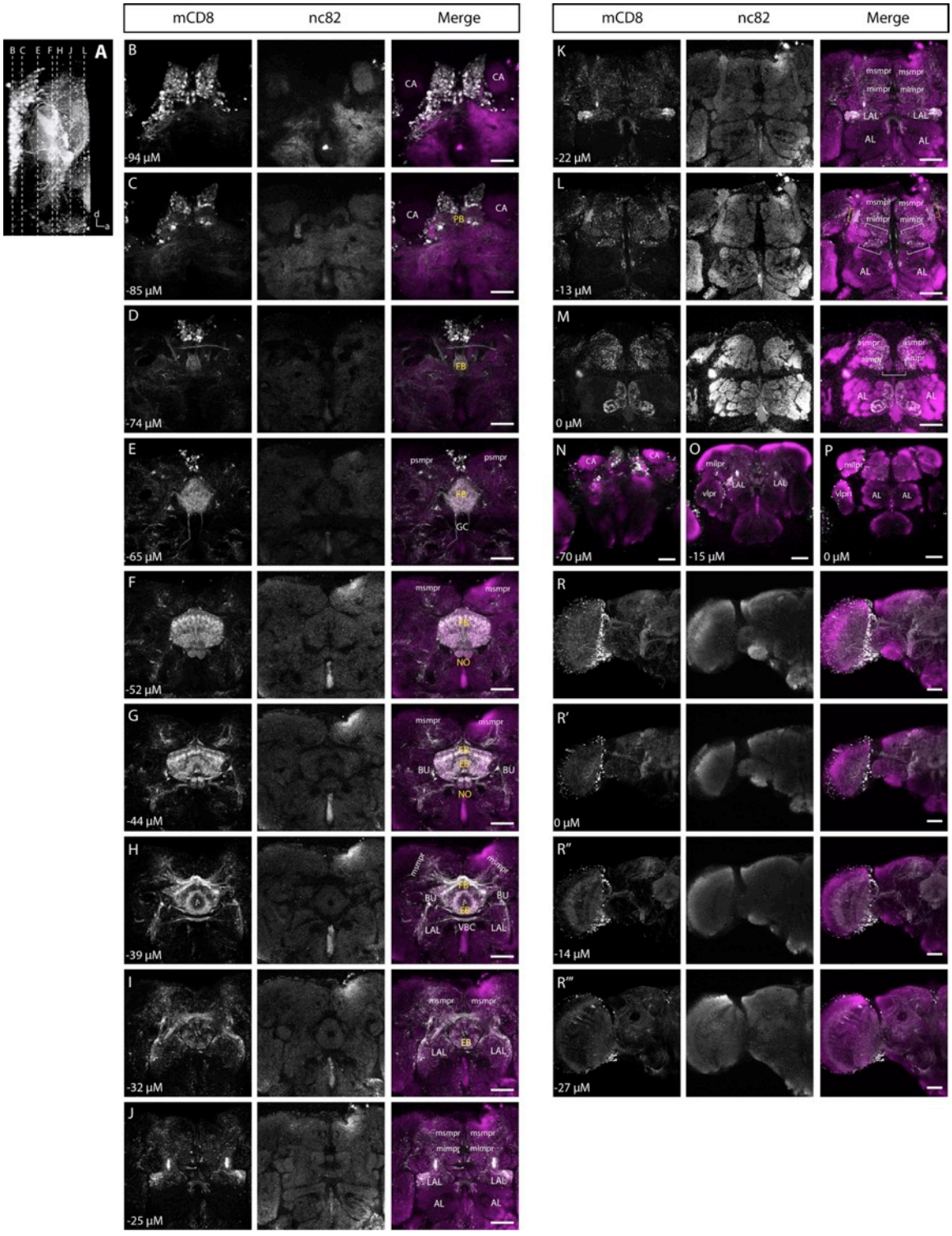
normally resides, this could indicate that some aspects of NB spatial/temporal patterning are still intact and direct late born INP progeny to target their axons to the midline. In the future, the identification of additional molecular markers of cell fates in type II lineages and their relationships to NB and INP patterning inputs will allow a more comprehensive assessment of the mechanistic basis of combinatorial patterning. It will be important to ask similar questions across multiple model system (e.g. embryonic type I NBs, mouse neural progenitors) to understand if there are general rules governing the integration of different patterning inputs during neural cell fate specification.

Here I have shown are two axes of temporal patterning within type II NB lineages: both the type II NBs and INPs change over time to make different neurons and glia. These findings open a new field for the study of temporal patterning. In addition, it will be fascinating to investigate whether INPs undergo temporal patterning in other organisms, and whether combinatorial temporal patterning contributes to the neuronal complexity of the human neocortex.

APPENDIX A

SUPPLEMENTARY FIGURES FOR CHAPTER II

Supplementary Figure 1 (next page). Lineage tracing with 9D11 labels the adult central complex and associated regions.
(A-M) Three-dimensional reconstruction of the brain presented in Figure 5C-G is shown in sagittal view (A) and serial frontal confocal sections through the same brain are shown from posterior to anterior (B-M). The positions of several sections are indicated in (A), the z-position of each confocal section relative to M are also shown in the right panels. (B) The majority of cell bodies can be seen in the DPC medial to mushroom body calyces. (K) Connections between the ALs are labeled. (L,M) Specific glomeruli of the ALs are labeled. White and yellow brackets indicate labeling at the mushroom body medial and vertical lobes, respectively. The latter was innervated more heavily. The dorsal parts of mushroom body vertical lobes, which were innervated sparsely, are indicated with asterisks. **(N-P)** Serial low magnification frontal confocal sections through another brain of the same genotype are shown with their relative z-positions to (P) showing the locations of labeled cell bodies. Cell bodies were found in the posterior cortex (N), including the DPC and areas ventral and ventrolateral to mushroom body calyces, middle inferior lateral protocerebrum (milpr) and ventrolateral protocerebrum (vlpr) regions (O,P), the latter lateral to anterior LAL, regions next to the mushroom body vertical lobes (P), and around the optical tubercule (not shown).
(R) Z-projection image of serial low magnification frontal confocal sections through the anterior brain of the same genotype showing labeling in optic lobes. **(R'-R''')** Single confocal sections with their relative z-positions to (R'). Abbreviations are listed in the Abbreviations section. Scale bars: 40 μM.


mCD8
nc82
Merge
A
B C E F H J L
d
a
B
-94 µM
CA
C
-85 µM
PB
D
-74 µM
FB
E
-65 µM
psmpr
GC
F
-52 µM
msmpr
NO
G
-44 µM
BU
H
-39 µM
mimpr
VBC
LAL
I
-32 µM
EB
J
-25 µM
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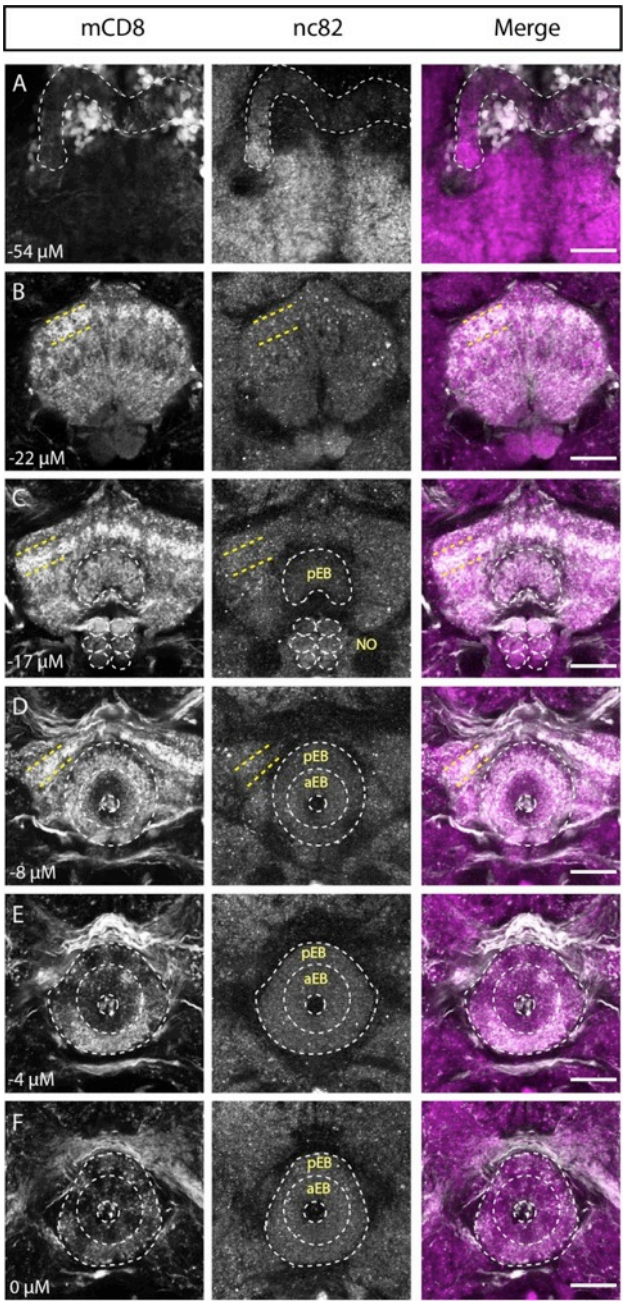


Supplementary Figure 2. High magnification images of the labeling at the central complex.

(A-F) Serial high magnification frontal confocal sections of the central complex of the adult brain presented in Figure 3C-G and Additional file 6 from posterior to anterior. The z-position of each confocal section relative to (F) are also indicated. White outlines represent neuropils visualized by nc82 staining. Yellow dashed lines indicate the dense layer of innervations at the dorsal FB. See text for details. Abbreviations are listed in the Abbreviations section. Scale bars: 20 µM.

APPENDIX B

SUPPLEMENTARY TABLE FOR CHAPTER III

Supplementary Table 1: Antibody based screening of gene expression in type II lineages. The expression pattern of neural transcription factors in DM1-6 type II neuroblast lineages at 120h ALH.

Gene	Symbol	Ab[1]	NB expression		INP expression		Progeny expression
				spatial		spatial	
Cut	**Ct**	Ms	yes	DM1-6	yes	DM1-6	*
Distal Antenna	**Dan**	Rt	yes	DM1-6	yes	DM1-6	DM1-6
Distalless	**Dll**	Gp	yes	DM1-6	yes	DM1-6	DM1-6
Optix	**Optix**	Rb	yes	DM1,2, 3,6	yes	DM1,2,3, 6	DM1-6
Tailless	**Tll**	GFP	yes	DM1-6	-	-	-
Castor	**Cas**	Rb	no	-	yes	DM1-6	DM1-6
Dichaete	**D**	Gp	no	-	yes	DM1-6	*
Eyeless	**Ey**	Rb	no	-	yes	DM1-6	DM1-6
Grainyhead	**Grh**	Rt	no	-	yes	DM2-6	DM2-5
Runt	**Runt**	Gp	no	-	yes	DM1,2,3, 4,6	all-DM5
Drifter	**Drf**	Rt	?	?	-	-	*
Visual System Homeobox 1 ortholog	**Vsx1**	Gp	?	?	yes	DM1	*
Broad Complex	**Brc**	Ms	no	-	no	-	DM1-6
Brain-specific homeobox	**Bsh**	Gp	no	-	no	-	DM2-3
Dachsund	**Dac**	Ms	no	-	no	-	DM2,3,6*
Extradenticle	**Exd**	Rb	no	-	no		DM4-6*
Homeothorax	**Hth**	Rb	no	-	no	-	DM4-6*

Supplementary Table 1 (continued): Antibody based screening of gene expression in type II lineages. The expression pattern of neural transcription factors in DM1-6 type II neuroblast lineages at 120h ALH.

Gene	Symbol	Ab[1]	NB expression		INP expression		Progeny expression
				spatial		spatial	
Kruppel	**Kr**	Gp	no	-	no	-	*
Lim1	**Lim1**	Gp	no	-	no	-	DM1-6
Nervy	**Nvy**	Rb	no	-	no	-	DM3,4,6*
Retinal Homeobox	**Rx**	Rb	no	-	no	-	DM1-6
Orthodenticle	**Otd**	Gp	no	-	no	-	DM5
Sloppy paired	**Slp1**	Rb	no	-	no		DM5,6*
Seven-up	**Svp**	Rt	no	-	no	-	*
Twin of Eyeless	**Toy**	Gp	no	-	no	-	DM1-6
Zinc finger Homeodomain 2	**Zfh2**	Rt	no	-	no	-	*

[1]Antibody species: Ch (Chicken), Gp (Guinea Pig), Ms (Mouse), Rb (Rabbit), Rt (rat).
GFP: Recombineered GFP fusion.
*Lineages-of-origin could not be identified or ambiguous

Supplementary Table 1 (continued): Antibody based screening of gene expression in type II lineages. The expression pattern of neural transcription factors in DM1-6 type II neuroblast lineages at 120h ALH.

No expression in type II lineages at 120h ALH, expression found elsewhere in the brain		
Gene	**Symbol**	**Antibody**
Antennapedia	**Antp**	Ms
Brother	**Bro**	GFP
Deformed	**Dfd**	GFP
Engrailed	**En**	Ms
Eyes Absent	**Eya**	Ms
Fmrfamide-Related	**Fmrfa**	Rb
Gooseberry	**Gsb-D**	Rt
Hairless	**H**	GFP
Hunchback	**Hb**	Rb
HB9/Extra Extra	**HB9/Ex-Ex**	Gp
Ladybird Early	**Lbe**	Gal4
Drop	**Msh**	Rb
POU Domain Protein 2	**Pdm2**	Rt

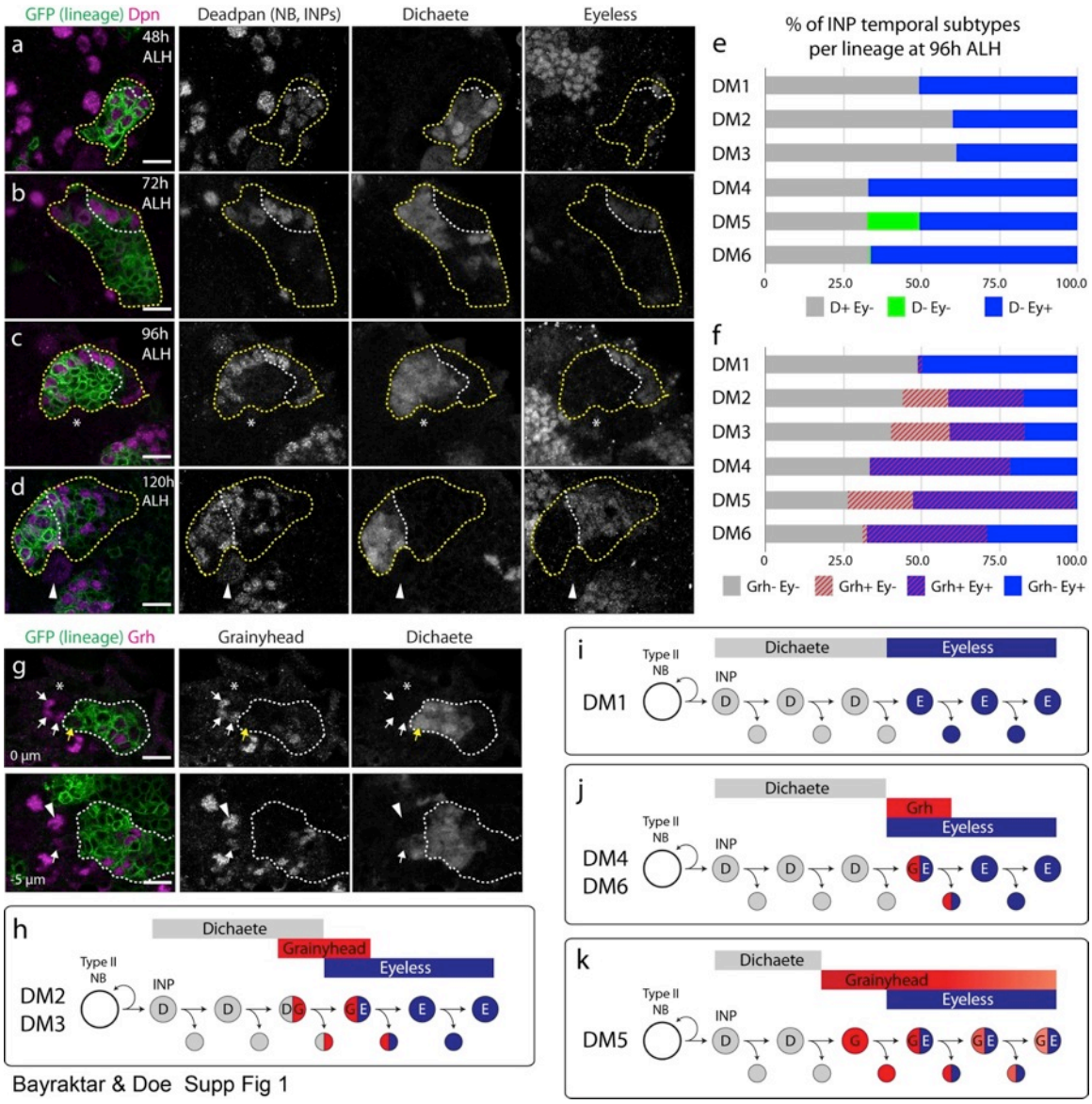
Supplementary Table 1 (continued): Antibody based screening of gene expression in type II lineages. The expression pattern of neural transcription factors in DM1-6 type II neuroblast lineages at 120h ALH.

No expression detected in the brain		
Gene	**Symbol**	**Antibody**
Abdominal B	**Abd-B**	Ms
Achaete	**Ac**	Ms
Acj6**	**Acj6**	Ms
Collier	**Col**	Gp
Eagle	**Eg**	Rb
Even Skipped**	**Eve**	Ms
Fushi Tarazu	**Ftz**	Ftz
Huckebein	**Hkb**	Rt
Islet/Tailup**	**Tup**	Ms
Klumpfuss**	**Klu**	Rb
Late Bloomer	**Lbm**	Ms
Lim3**	**Lim3**	Gp
Mirror	**Mirror**	Mrr
Hgtx	**Nkx6**	Rt
Neuropeptide-Like Precursor 1	**Nplp1**	Ch
Odd Skipped	**Odd**	Gp
POU Domain Protein 1	**Pdm1**	Rb
Spalt**	**Salm**	Rb
Tango	**Tag**	Ms
Ultrabithorax	**Ubx**	Ms
Ventral Nervous System Defective**	**Vnd**	Rb
Wingless**	**Wg**	Ms
Zinc Finger Homeodomain 1**	**Zfh1**	Rb

** Bad antibody staining (failure to replicate prior brain expression patterns).

APPENDIX C

SUPPLEMENTARY FIGURES FOR CHAPTER III



Supplementary Figure 1: The D, Grh, and Ey series is observed in INPs born early or late in multiple type II lineages.

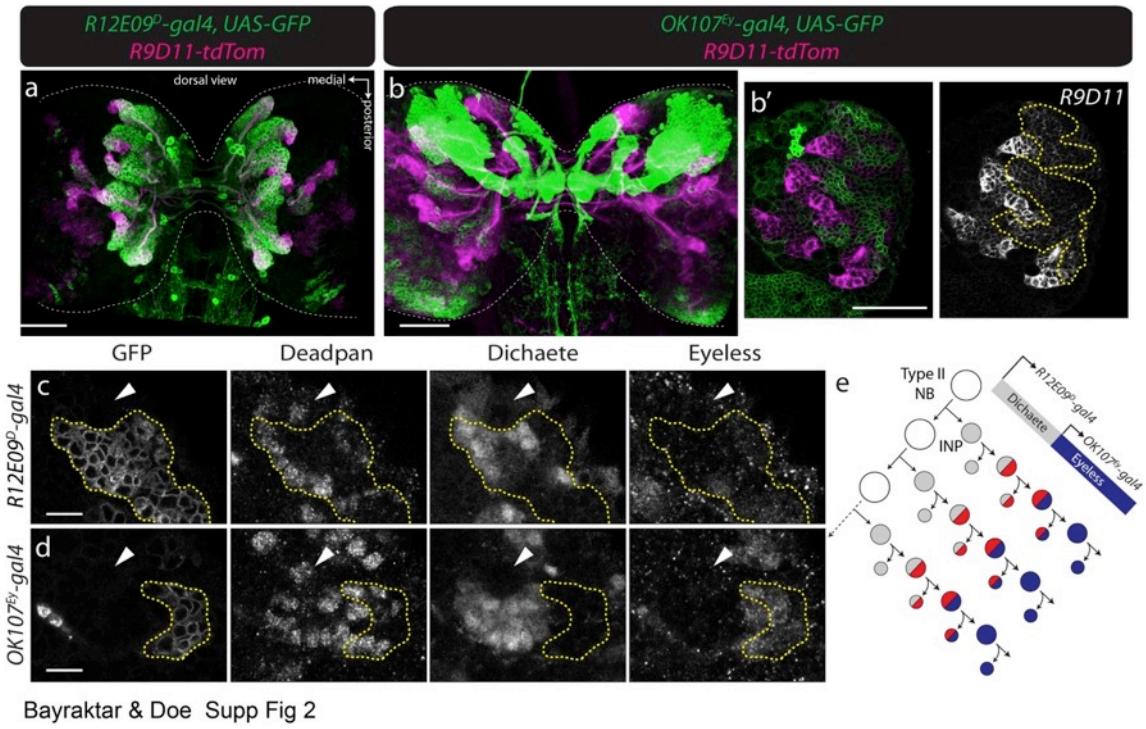
a-d, D and Ey have complementary expression in INPs all larval stages; during early larval stages (48h ALH) there are more D+ INPs possibly because the INPs are not old enough to be Ey+ yet. DM2 lineage shown in **c,d**.

e-f, Quantification of INP gene expression in DM1-DM6 lineages at 96h ALH (n = 6 brains, percentages per each lineage were averaged). Similar results are observed at 120h ALH (Fig 1).

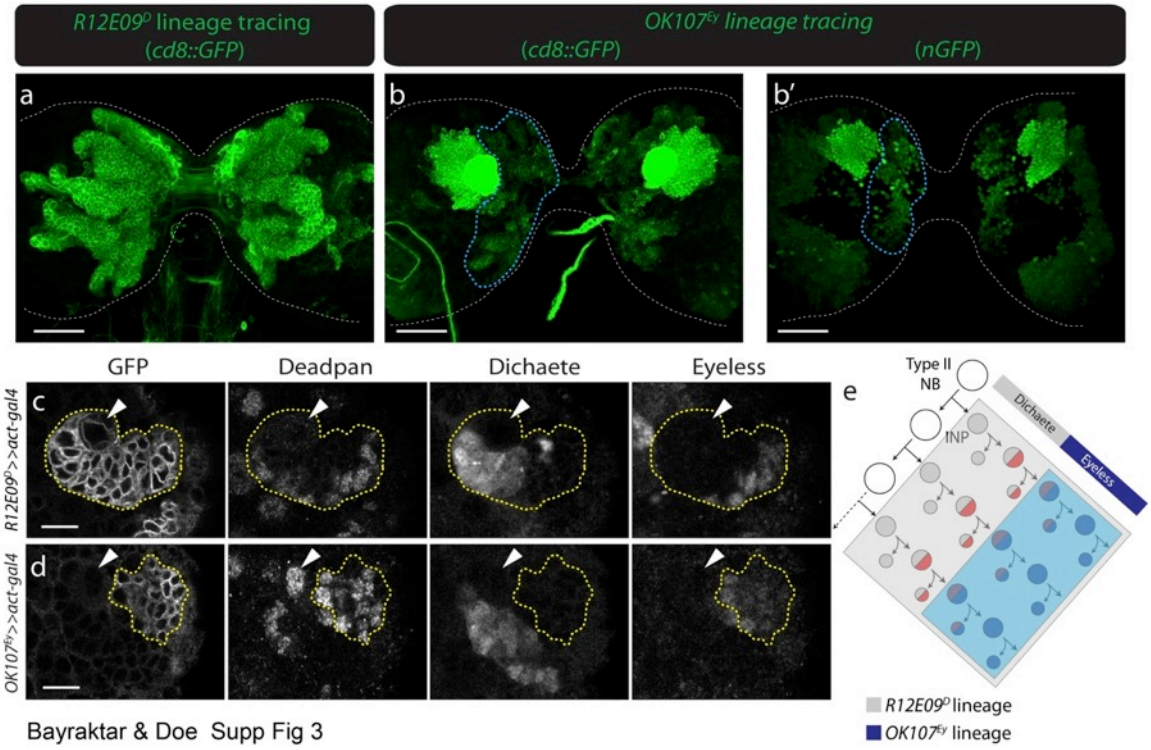
g, Grh is transiently expressed in immature INPs. DM2 lineage shown at two focal planes. Immature INPs (white arrows) are small R9D11- cells next to the Grh+ type II NB (arrowhead). Most immature INPs are Grh+ D-. Grh is also found weakly in newly mature R9D11+ INPs (yellow arrow), these are excluded from mature INP quantification to avoid confusion with the strong Grh expression in middle-aged INPs.

h-k, Summary of D > Grh > Ey expression patterns observed in INPs in all dorsomedial type II lineages. Variations among DM lineages are largely due to differences in Grh expression. Grh levels decline over time in INPs in the DM5 lineage (**k**).

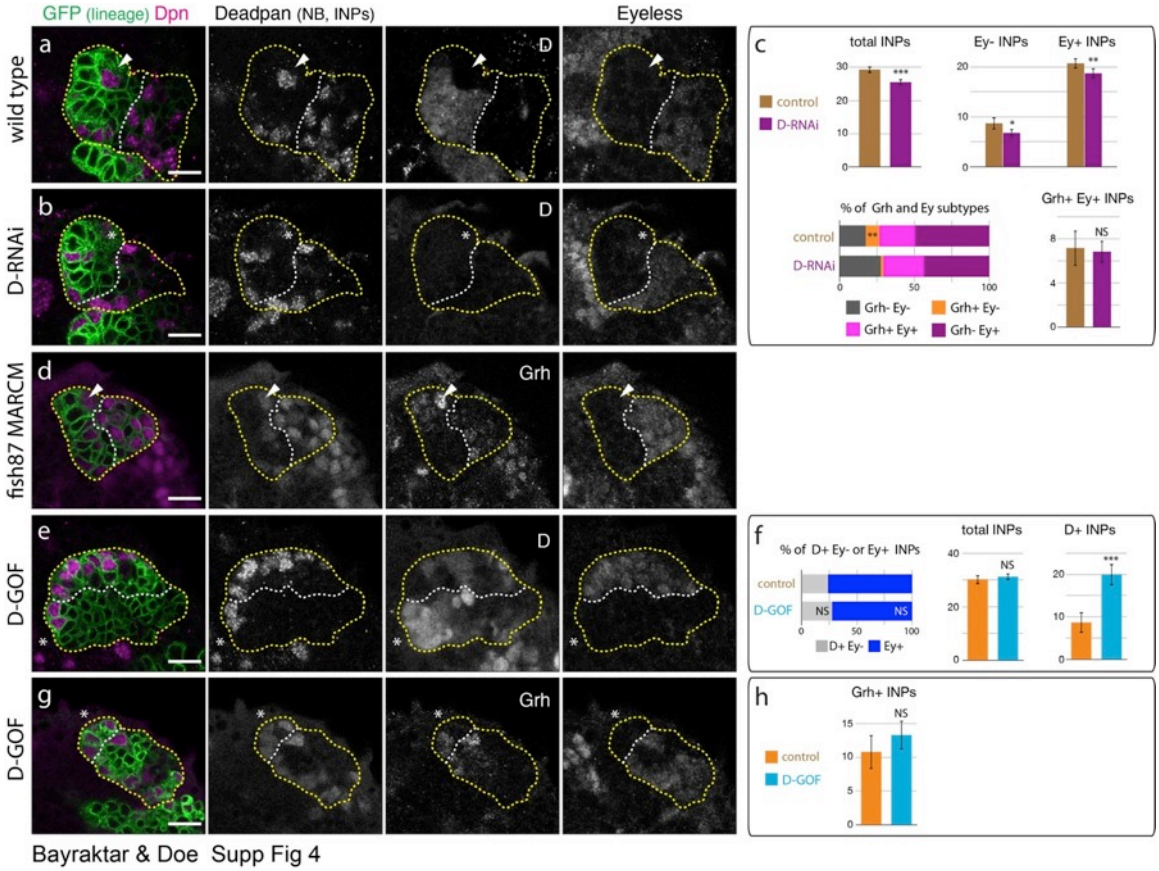
Type II NBs, arrowheads. Asterisk, type II NBs in different focal planes. Yellow outline indicates INPs and progeny labeled by *R9D11-Gal4 UAS-GFP*. White dashed lines, D-Ey expression. Scale bars, 10 µm.



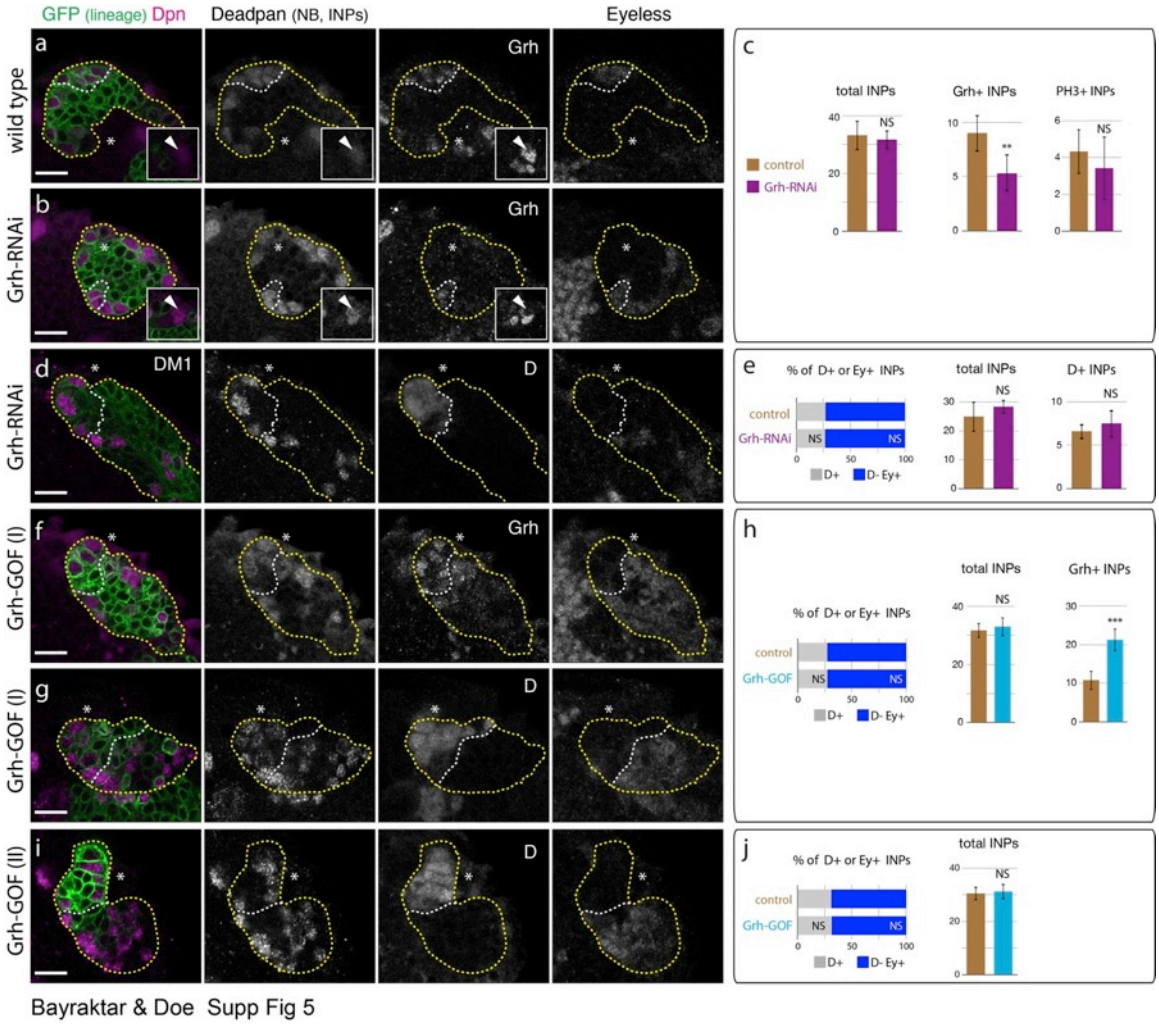
Supplementary Figure 2: *R12E09^{D}* and *OK107^{Ey}* Gal4 lines mark young and old INPs respectively.
a-d, *R12E09^{D}-gal4* and *OK107^{Ey}-gal4* driving *UAS-GFP* (green) in the late larval brain at 120h ALH. 3D reconstructions of the dorsal brain are shown in (**a,b**) where type II NB lineages were marked with R9D11-tdTomato fluorescent protein (magenta). Anterior is up.
a, *R12E09^{D}-gal4* expression is specific to type II NB lineages in the entire central brain, where it extensively overlaps with R9D11-tdTomato. It is also sparsely expressed in the optic lobes.
b, *OK107^{Ey}-gal4* expression is largely specific to type II NB lineages in the dorsomedial brain, where it partially overlaps with R9D11-tdTomato (yellow outline in **b'**, single confocal section). It is also expressed extensively outside type II NB lineages in the central brain and optic lobes.
c, *R12E09^{D}-gal4* marks all young D+ INPs, and is also found in old Ey+ INPs (likely due to perdurance of gal4/GFP).
d, *OK107^{Ey}-gal4* only marks old Ey+ INPs. The presence of few GFP- Ey+ INPs next to young INPs is likely due to late onset of GFP labeling.
e, Summary of *R12E09^{D}-gal4* and *OK107^{Ey}-gal4* expression.
Type II NBs, arrowheads. Scale bars, 50 μm (**a,b,f,g**), 10 μm (**c,d,h,i**)



Supplementary Figure 3: Permanent lineage tracing with *R12E09^{D}* and *OK107^{Ey}* gal4 lines allows identification of young and old INP progeny.
a-d, *R12E09^{D}* and *OK107^{Ey}* permanent lineage tracing driven GFP expression (green) in the late larval brain at 120h ALH. 3D reconstructions of the dorsal brain are shown in (**a,b**). Anterior is up.
a, *R12E09^{D}>>act-gal4* permanent lineage tracing labels primarily type II NB lineages in the central brain. It also labels few type I NB lineages and parts of the optic lobes.
b, *OK107^{Ey} >>act-gal4* permanent lineage tracing with cd8:GFP (**b**) or nuclear GFP (**b'**) labels primarily type II NB lineages in the dorsomedial brain (magenta outlines).
c, *R12E09^{D}>>act-gal4* marks all INPs and their progeny. While *R12E09^{D}-gal4* alone does not express in type II NBs at 120h ALH, *R12E09^{D}>>act-gal4* labels all six DM type II NBs suggesting that *R12E09^{D}* is transiently expressed in type II NBs earlier in development.
d, *OK107^{Ey} >>act-gal4* only marks old Ey+ INPs and their progeny.
e, Summary of *R12E09^{D}* and *OK107^{Ey}* lineage tracing.
Type II NBs, arrowheads. Scale bars, 50 μm (**a,b,f,g**), 10 μm (**c,d,h,i**).



Supplementary Figure 4: D is necessary but not sufficient for the timely activation of Grh in INPs.
INP temporal transcription factor expression in DM2 lineage at 120h ALH. INPs were marked with GFP (yellow outline) driven by the following Gal4 lines: *wor-gal4 ase-gal80* (**a,b**), *tub-gal4* (MARCM)(**d**), or *R9D11-gal4* (**e,g**). Ey border, white line. Type II NB, arrowhead, or asterisk when out of focal plane.
a, Wild type expression of D and Ey in INPs. Type II lineage marked with *wor-gal4 ase-gal80 UAS-GFP*.
b-c, D^{RNAi} completely removes D expression from INP lineages (**b**). D^{RNAi} leads to a small reduction in the number of Ey+ and Ey- INPs (**c**, top). D^{RNAi} results in a loss of Grh+ Ey- INPs without altering the number of Grh+ Ey+ INPs (**c**, bottom). Quantification in **c** ($n = 6$). D^{RNAi} driven by *wor-gal4 ase-gal80 UAS-dcr2* in D^{87}/+; control is w^{1118}.
d, Removal of D from INP lineages with MARCM delays Grh expression. Consistent with D^{RNAi}, D^{87} MARCM clones induced early in the DM2 type II neuroblast contain Grh+ Ey+ INPs, but no Grh+ Ey- INPs are observed.
e-h, D misexpression does not lead to ectopic Grh expression. D^{GOF} gives rise to ectopic D expression in Ey+ INPs (**e**), but does not alter the number of total INPs or young Ey- INPs. (**f**). D^{GOF} does not significant increase Grh expression in INPs (**g,h**). Quantification in **f,h** ($n \geq 5$). D^{GOF} genotype was *R9D11-gal4 >>act-gal4 UAS-D*; control is *yw*.
Scale bars, 10 μm. All data represent mean ± s.d. NS, not significant. $^{**}P < 0.01$, $^{***}P < 0.001$.



Supplementary Figure 5: Grh is necessary but not sufficient for D repression and Ey activation in INP lineages

INP temporal transcription factor expression in DM2 lineage (unless indicated otherwise) at 120h ALH. INPs were marked with GFP (yellow outline) driven by the following Gal4 lines: *R9D11-gal4* (**a-g**) or *wor-gal4 ase-gal80* (**i**). Grh border, white line (**a,b**). Ey border, white line (**d-i**). Note that Grh is detected in the type II NB (arrowhead, or asterisk when out of focal plane).

a, Wild type expression of Grh and Ey in INPs. Grh expression in type II neuroblasts and immature INPs shown in side panels. Type II lineage marked with *R9D11-gal4 UAS-GFP*.

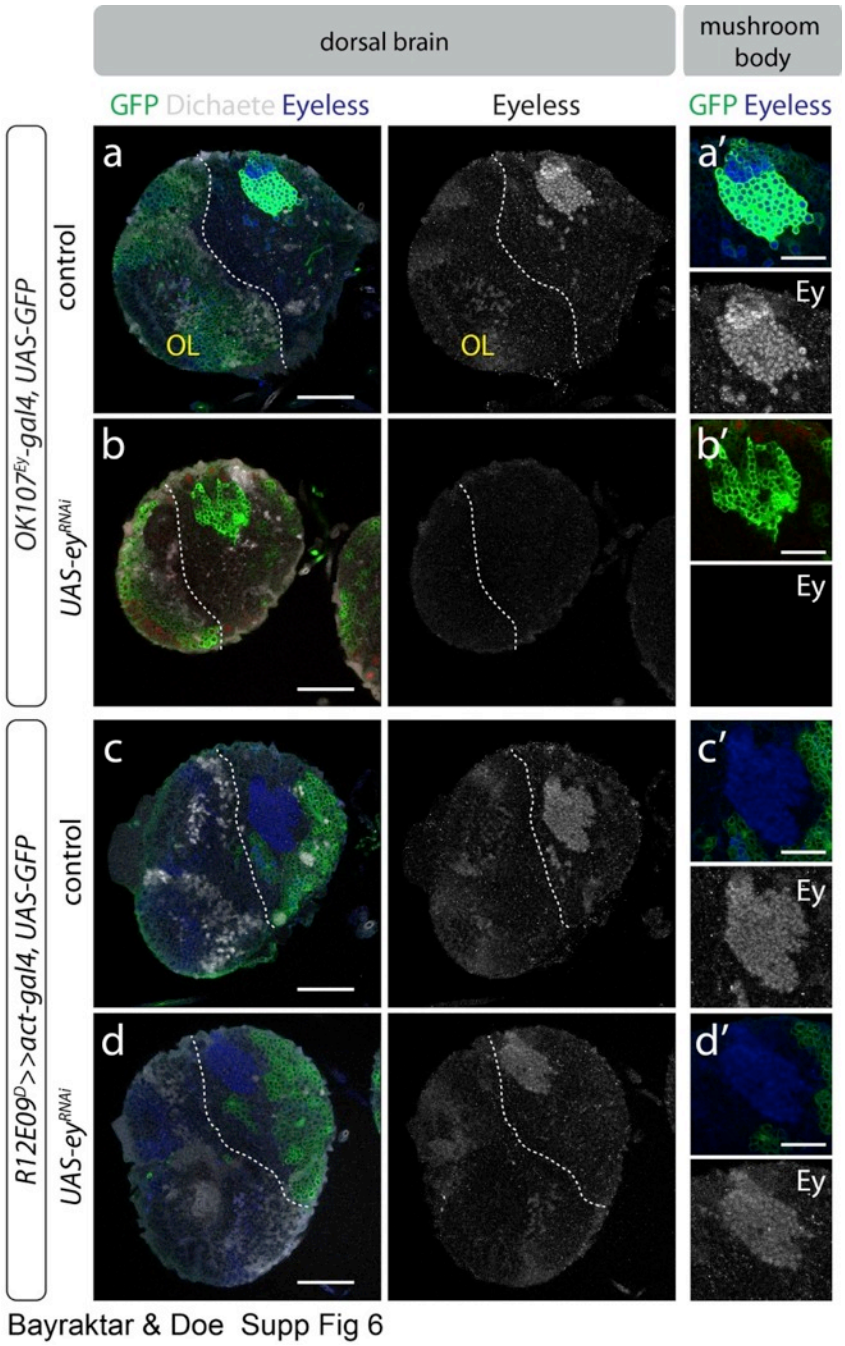
b-c, Grh^{RNAi} significantly reduces Grh staining in middle aged INPs (**b**), but leaves Grh expression in type II neuroblasts and immature INPs intact (**b**, side panels). Grh^{RNAi} does not alter the total number of INPs (**c**), or reduce the number of PH3+ mitotic INPs (**c**). Quantification in **c** ($n \geq 5$). Grh^{RNAi} driven by *R9D11-gal4, R9D11-gal4* in grh^{370}/+; control is *attP2*.

d-e, Grh^{RNAi} does not increase the number of D+ INPs in the DM1 lineage. (**e**) Quantification ($n = 5$).

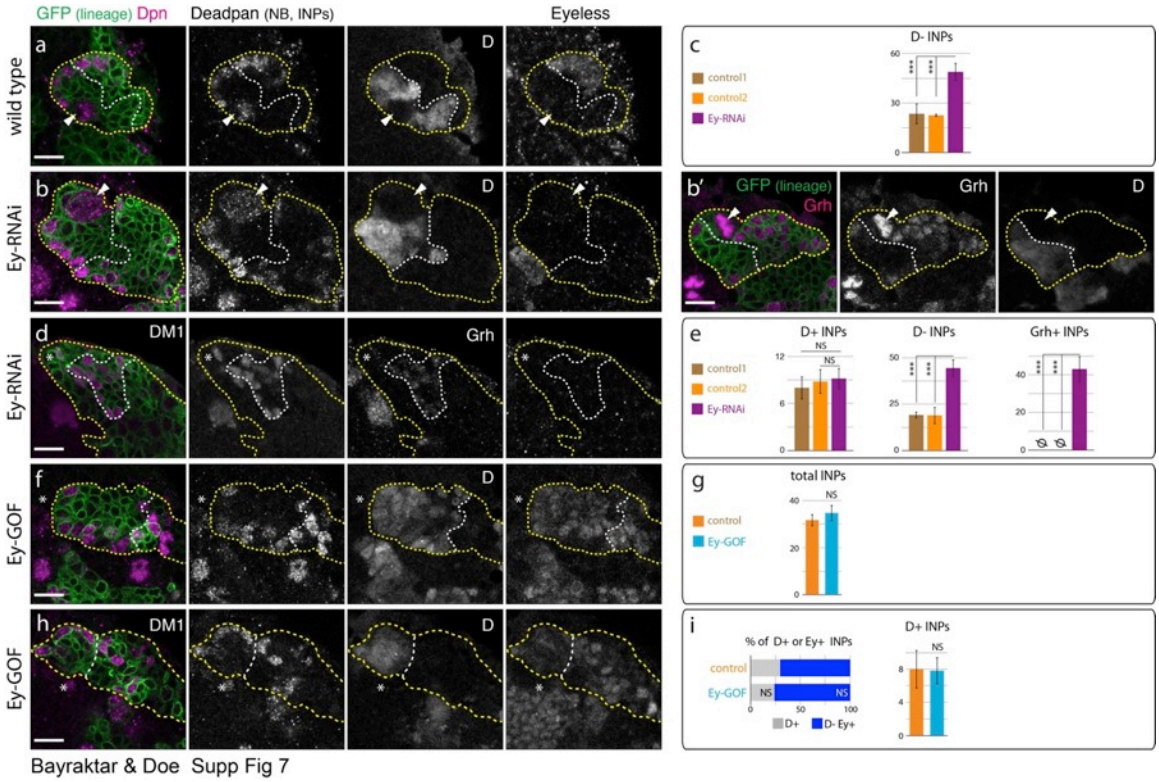
f-h, Grh misexpression does not lead to ectopic Ey+ INPs. Grh^{GOF} gives rise to ectopic Grh expression in early and middle-aged INPs (**f**), but does not alter the number of early D+ and late Ey+ INPs (**g**). Quantification in **h** ($n \geq 5$). Grh^{GOF} genotype was *R9D11-gal4 >>act-gal4 UAS-Grh*; control is *y w*.

i-j, Grh misexpression does not lead to ectopic Ey+ INPs. (**h**) Quantification ($n \geq 4$). Grh^{GOF} genotype was *wor-gal4 ase-gal80 UAS-Grh;* control is *attP2*.

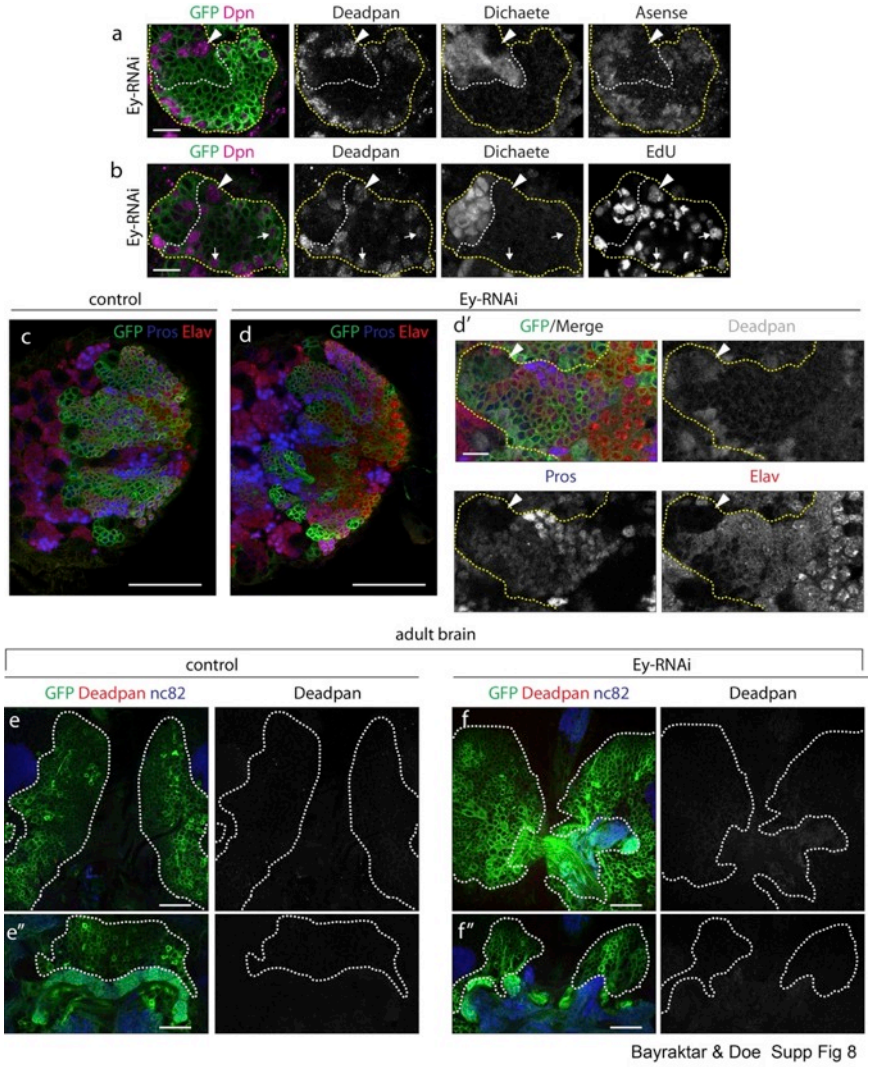
Scale bars, 10 μm. All data represent mean ± s.d. NS, not significant. $^{**}P < 0.01$, $^{***}P < 0.001$.



Supplementary Figure 6: Transgenic RNAi driven by R12E09 lineage tracing eliminates Ey expression specifically in INP lineages.
a-b, Validation of the *UAS-ey^{RNAi}* transgene. *OK107^{Ey}-gal4 UAS-ey^{RNAi}* eliminates Ey staining in the optic lobe and reduces its size (**b**) and eliminates Ey in the mushroom body (**b'**).
c-d, *R12E09^{D} >> act-Gal4 UAS-ey^{RNAi}* does not affect Ey levels in the optic lobe or its size (**d**) nor the levels of Ey in the mushroom body (**d'**).
All images show larval brains at 120h ALH. Controls are *attP2* (empty RNAi transgene docking site). OL, optic lobes. White dashed line, OLs. Scale bars, 50 μm (**a-d**), 25 μm (**a'-d'**).



Supplementary Figure 7: Ey is necessary and sufficient to terminate the Grh expression window in INPs
INP temporal transcription factor expression in DM2 lineage (unless indicated otherwise) at 120h ALH. INPs were marked with GFP (yellow outline) driven by the following Gal4 lines: *R12E09^{D}-gal4* (**a-d**) or *R9D11-gal4* (**f-h**). Ey border, white line (**a,b,f,h**). Grh border, white line (**d**). Type II NB, arrowhead, or asterisk when out of focal plane.
a, Wild type expression of D and Ey in INPs. Type II lineage marked with *R12E09^{D}>>act-gal4 UAS-GFP*.
b-c, Ey^{RNAi} gives rise to ectopic late D- Grh+ INPs. Ey^{RNAi} removes all Ey expression from INP lineages (**b**). Ey^{RNAi} leads to the accumulation of late D- Grh+ INPs (**b'**). (**c**) Quantification ($n \geq 4$). Ey^{RNAi} driven by *R12E09^{D}>>act-gal4*; controls are (1) *attP2* and (2) *UAS-mCherry^{RNAi}*.
d-e, Ey^{RNAi} leads to ectopic Grh expression in INPs in the DM1 lineage, which normally does not have detectable Grh. (**e**) Quantification ($n \geq 4$).
f-g, Ey misexpression increases the number of D+ INPs (**f**, quantified in Fig. 2k), without altering the total number of INPs (**g**). Quantification in **g** ($n \geq 4$). Ey^{GOF} genotype was *R9D11-gal4 >>act-gal4 UAS-Ey*; control is *yw*.
h-i, Ey misexpression does not affect the number of D+ INPs in the DM1 lineage which normally has no detectable Grh in INPs. (e) Quantification ($n \geq 4$).
Scale bars, 10 μm. All data represent mean ± s.d. NS, not significant. $^{**}P < 0.01$, $^{***}P < 0.001$.



Supplementary Figure 8: Loss of Ey extends INP lineages without any sign of de-differentiation or tumor formation.

a-f, Ey^{RNAi} in INP lineages with *R12E09^{D}>>act-gal4* in the late larval brain at 120h ALH (**a-d**) and adult brain (**e-f**).

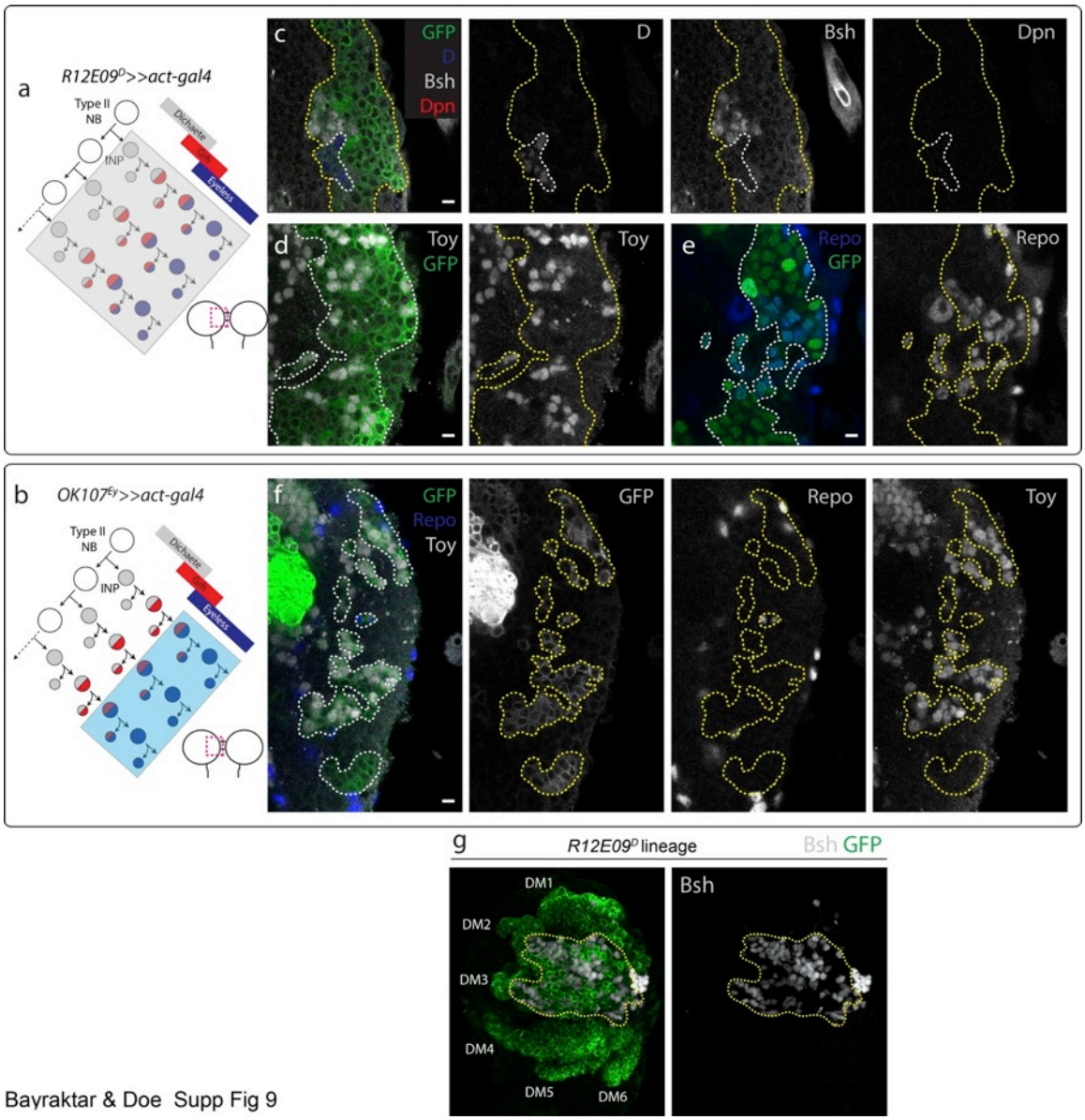
a, Ectopic D- old INPs all express the mature differentiated INP marker Asense (Ase). Type II NBs and immature INPs are Ase-; de-differentiation of INPs in tumorigenic backgrounds like *brat* or *erm* result in the downregulation of Ase.

b, Ectopic D- old INPs proliferate and incorporate EdU upon a short 2-hour pulse (arrows). Consistent with this, some Dpn- D- progeny found next to old INPs are also EdU+.

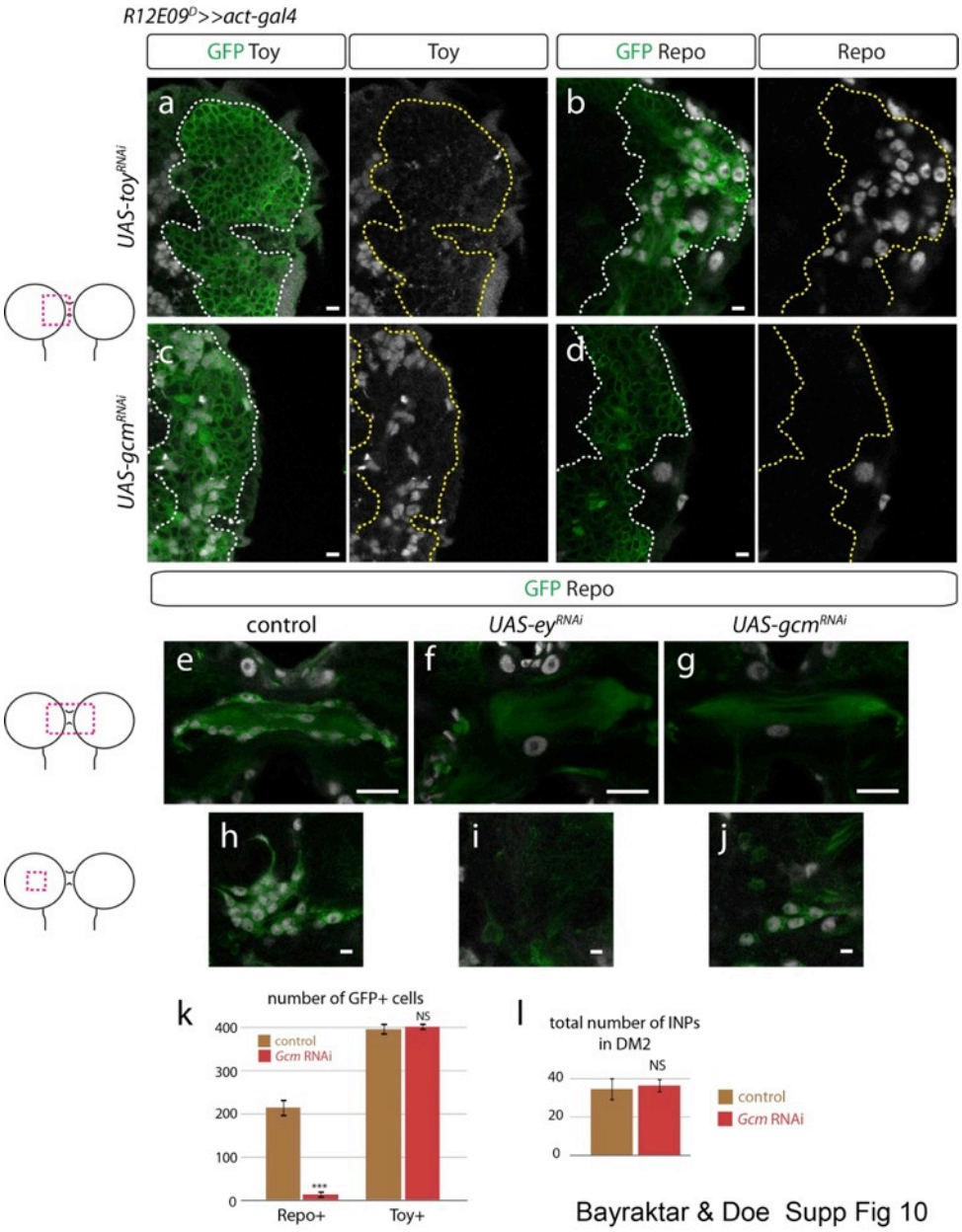
c-d, Upon Ey^{RNAi} the majority of cells in DM type II lineages still show expression of the neuronal markers Prospero (Pros, blue) and Elav (red), indicating that differentiation is not significantly compromised and that ectopic old INPs generate neurons. Low magnification of dorsomedial brain shown in (**c,d**), DM2 shown in (**d'**).

e-f, Upon Ey^{RNAi}, no Dpn+ cells are found in type II lineages the adult brain (**f**) showing that INP divisions have eventually terminated. GFP labeled cell bodies of type II NB progeny in the dorsoposterior cortex (white outline) shown in (**e,f**), more cell bodies shown in a more anterior section above the protocerebral bridge in (**e',f'**).

Images show the DM2 lineage in (**a,b,d'**). Type II NBs, arrowheads. Outlines indicate GFP+ permanent lineage tracing from *R12E09^{D}>>act-gal4*. Scale bars, 10 μm (**a,b,d'**), 50 μm (**c,d**), 25 μm (**e,e'**).



Supplementary Figure 9: Old INPs give rise to Toy+ and Toy- neurons, and Repo+ neuropil glia.
a-b, Schematic of permanent lineage tracing experiments. (**a**) *R12E09^{D}>>act-gal4* labels young and old INPs and their progeny; (**b**) *OK107^{Ey}>>act-gal4* labels only old Ey+ INPs and their progeny. Region of dorsomedial brain imaged at 120h ALH in **c-f** shown as dashed box within brain lobe cartoon.
c-e, Permanent lineage tracing of all INP progeny using the early INP *R12E09^{D}*-gal4 line. Non-overlapping expression of D and Bsh (**c**), expression of Toy (**d**) and Repo (**e**) in subsets of GFP+ INP progeny. Yellow dashed line surrounds GFP+ cells, white dashed line surrounds D+ progeny. Repo+ neuropil glia are labeled with *R12E09^{D}* lineage tracing (*G-TRACE with nuclear GFP*). Neuropil glia in the medial brain are shown.
f, Permanent lineage tracing of old INP progeny using the late INP *OK107^{Ey}*-gal4 line. Toy+ neurons, Toy- neurons, and Repo+ glia are among the late-born INP progeny. Dashed line surrounds GFP+ cells.
g, Bsh+ neurons are only made in DM2 and DM3 lineages. Three-dimensional reconstruction of DM lineages at 120h ALH is shown.
Scale bars, 5 μm (**c-f**), 20 μm (**g**).



Supplementary Figure 10: Toy+ neurons and Repo+ glia do not require each other for their formation.

a-b, Transgenic RNAi against Toy driven by *R12E09^{D}>>act-gal4* removes Toy expression (**a**) but Repo+ neuropil glia are still generated (**b**).

c-d, Transgenic RNAi against the glial fate determinant Glial cells missing (Gcm) driven by *R12E09^{D}>>act-gal4* eliminates Repo+ glia (**c**) but Toy+ neurons are still generated (**d**).

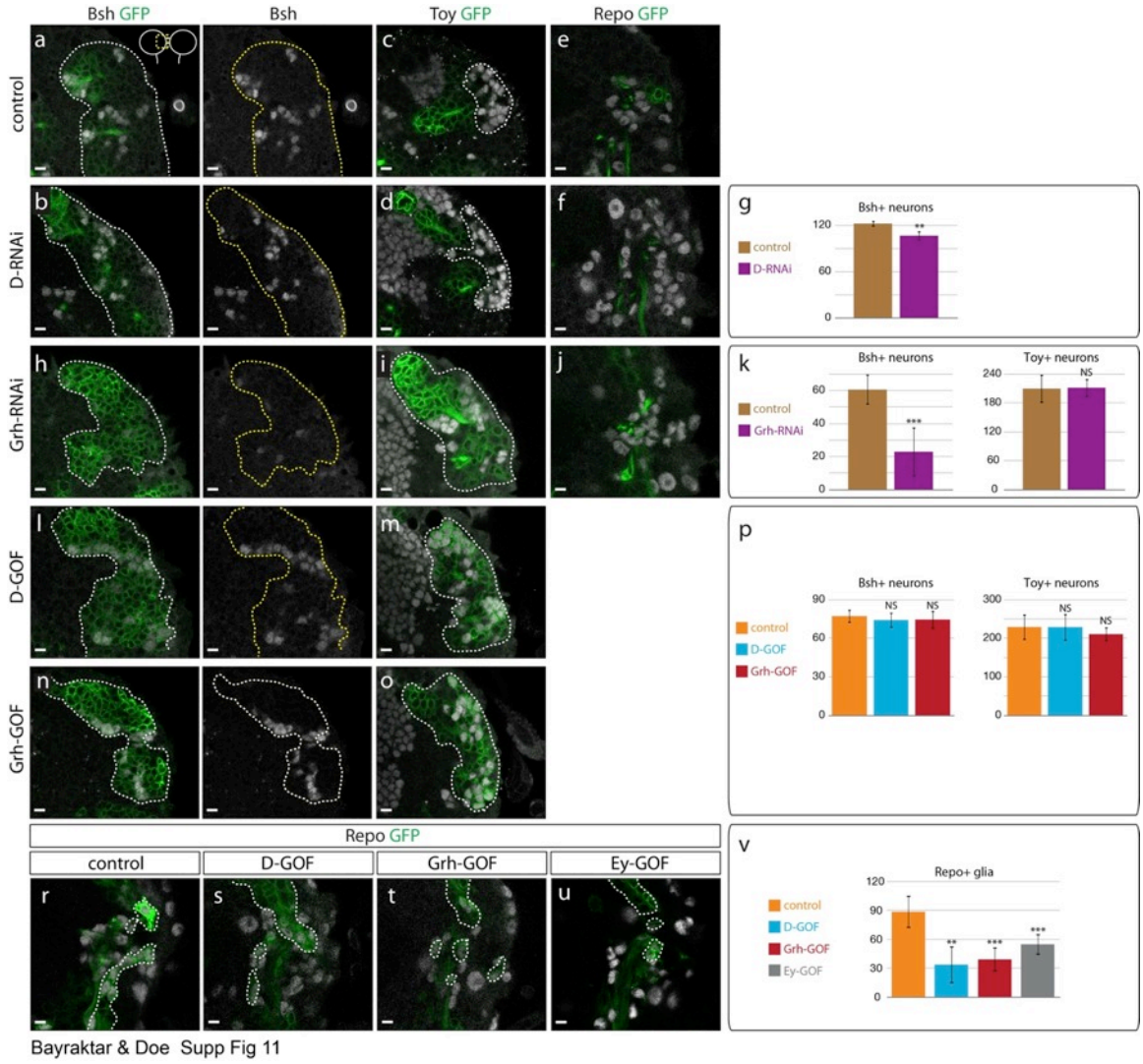
e-f, Repo+ neuropil glia at the interhemispheric junction (**e**) are eliminated upon Ey^{RNAi} (**d**) or Gcm^{RNAi} (**e**).

h-j, Repo+ neuropil glia located in the lateral brain (**f**) are eliminated upon Ey^{RNAi} (**i**) or Gcm^{RNAi} (**j**).

k, Quantification of Gcm^{RNAi} phenotypes ($n \geq 3$ single brain lobes each). GFP+ cells in all three brain locations shown above were scored for Repo and Toy expression.

l, Gcm^{RNAi} does not affect the number of INPs in DM2 ($n = 4$).

Region of brain imaged at 120h ALH are shown as dashed box within brain lobe cartoons. Control is *attP2*. Scale bars, 5 μm (**a,b,f-h**), 20 μm (**c-e**). All data represent mean ± s.d. NS, not significant. ***$P <$ 0.001.



Supplementary Figure 11: D and Grh are required, but not sufficient, for the production of early-born Bsh+ neurons.

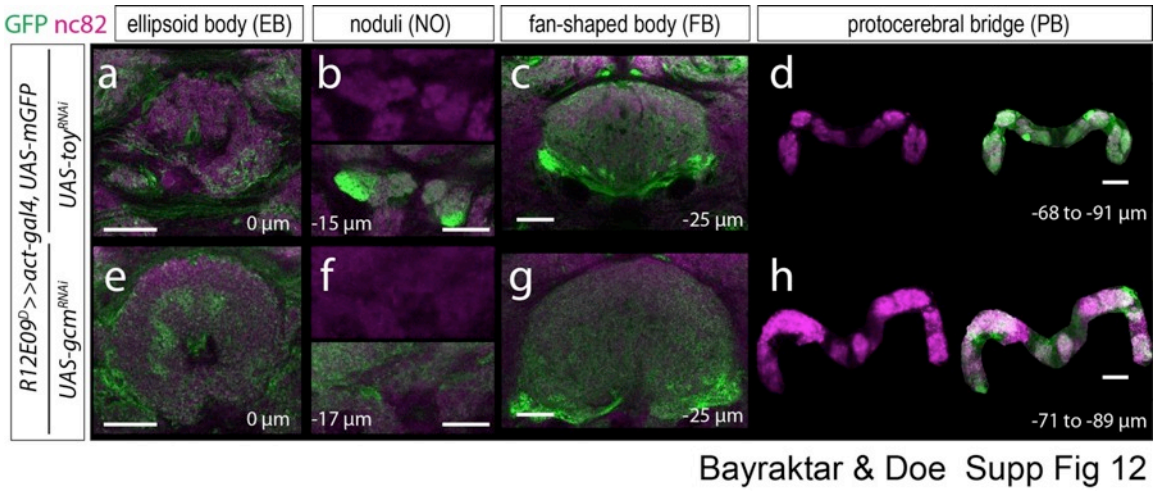
a-f, D^{RNAi} in INP lineages leads to a small reduction in early-born Bsh+ neurons (**a,b**), but shows no obvious change in late-born Toy+ (**c,d**) and Repo+ (**e,f**). Late-born progeny are largely unlabeled without lineage tracing in D^{RNAi}; dashed line surrounds Toy+ progeny in the dorsomedial region. (**g**) Quantification of Bsh+ neurons ($n \geq 5$ brain lobes each). D^{RNAi} driven by *wor-gal4 ase-gal80 UAS-dcr2* in D^{87}/+; control is w^{1118}.

h-k, Grh^{RNAi} in INP lineages leads to severe loss of early-born Bsh+ neurons (**h**), but does not affect the number of late-born Toy+ neurons (**i**). Grh^{RNAi} shows no obvious defects in late-born Repo+ progeny, which are largely unlabeled without lineage tracing (**j**). Quantification of Bsh+ and Toy+ neurons in **k** ($n \geq 5$). Grh^{RNAi} driven by *R9D11-gal4, R9D11-gal4* in grh^{370}/+; control is *attP2*.

l-p, D and Grh misexpression do not alter the number of early Bsh+ neurons (**l,n**) or late Toy+ neurons (**m,o**). Quantification in **p** ($n \geq 5$). D^{GOF} and Grh^{GOF} driven by *R9D11-gal4>>act-gal4*; control is *UAS-His2A::mRFP*.

r-v, Misexpression of D, Grh, or Ey all lead to reductions in the number of Repo+ glia. This effect might be due to permanent expression of these transcription factors in INP progeny with *R9D11-gal4>>act-gal4*which could potentially interfere with glial differentiation and local proliferation. (**v**) Quantification ($n \geq 5$).

Region of dorsomedial brain imaged at 120h ALH shown as dashed box within brain lobe cartoon. Scale bars, 5 μm. All data represent mean ± s.d. NS, not significant. $^{**}P < 0.01$, $^{***}P < 0.001$.



Supplementary Figure 12: Toy+ neurons and Repo+ glia are required for distinct aspects of adult CCX morphology.
a-d, Toy^{RNAi} leads to the absence of a discernible EB (**a**), disorganization and expansion of the NO (**b**), minor defects in the FB (**c**), and fragmentation of the PB (**d**).
e-h, Gcm^{RNAi} leads to minor defects in the EB (**e**), absence of a discernible NO (**f**), an expanded FB (**g**), and minor fragmentation of the PB (**h**). While the fragmentation of the PB was less severe than in Toy^{RNAi}, the PB fragments were more dispersed in the posterior brain.
Adult brains shown in frontal view in all images**.** The z-coordinates of single confocal sections are shown relative to the site of the EB (immediately posterior to the dorsal mushroom body lobes). The PB was cropped out of the brain and displayed as a projection of indicated z-coordinates in (**d, h**). Scale bars, 20 µm.

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CHAPTER IV

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