Seminars in Cell and Developmental Biology 142 (2023) 23–35

Contents lists available at ScienceDirect

Seminars in Cell and Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

The Drivers of Diversity: Integrated genetic and hormonal cues regulate neural diversity

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ARTICLE INFO

Keywords:
Drosophila
Temporal patterning
Neural stem cells
Neural diversity
Hormones
Central complex
Neural identity
Behavior

ABSTRACT

Proper functioning of the nervous system relies not only on the generation of a vast repertoire of distinct neural cell types but also on the precise neural circuitry within them. How the generation of highly diverse neural populations is regulated during development remains a topic of interest. Landmark studies in *Drosophila* have identified the genetic and temporal cues regulating neural diversity and thus have provided valuable insights into our understanding of temporal patterning of the central nervous system. The development of the *Drosophila* central complex, which is mostly derived from type II neural stem cell (NSC) lineages, showcases how a small pool of NSCs can give rise to vast and distinct progeny. Similar to the human outer subventricular zone (OSVZ) neural progenitors, type II NSCs generate intermediate neural progenitors (INPs) to expand and diversify lineages that populate higher brain centers. Each type II NSC has a distinct spatial identity and timely regulated expression of many transcription factors and mRNA binding proteins. Additionally, INPs derived from them show differential expression of genes depending on their birth order. Together type II NSCs and INPs display a combinatorial temporal patterning that expands neural diversity of the central brain lineages. We cover advances in current understanding of type II NSC temporal patterning and discuss similarities and differences in temporal patterning mechanisms of various NSCs with a focus on how cell-intrinsic and extrinsic hormonal cues regulate temporal transitions in NSCs during larval development. Cell extrinsic ligands activate conserved signaling pathways and extrinsic hormonal cues act as a temporal switch that regulate temporal progression of the NSCs. We conclude by elaborating on how a progenitor's temporal code regulates the fate specification and identity of distinct neural types. At the end, we also discuss open questions in linking developmental cues to neural identity, circuits, and underlying behaviors in the adult fly.

1. Introduction

The major function of the brain is to translate/integrate neuronal functions to produce behaviors, making the complexity of the central nervous system both mysterious and fascinating. The proper functioning of the brain relies not only on the generation of the vast repertoire of distinct neural (neurons and glia) types but also on its meticulous neural circuitry. However, how these neural populations are specified, differentiated, and organized into networks with various functions remains a topic of interest. Various cellular and molecular programs known to generate neural diversity may also play a major role in establishing appropriate neural connectivity.

Modern genetic and molecular techniques have allowed researchers to better understand generation of neural diversity; various invertebrate animal models, such as *D. melanogaster* and *C. elegans,* as well as vertebrate model systems, have provided mediums by which genetics of developmental processes can be tracked and observed. The ultimate goal of these animal model studies is to relate conserved neurodevelopmental mechanisms to human physiology and pathology [1–4]. Altogether, these scientific tools may be utilized to understand every detail of central nervous system development, starting from the earliest stem cell to the highly compartmentalized systems in the mature brain. Despite having fewer neurons (~100, 000) than the mammalian brain (86 billion in humans), the *Drosophila* brain supports a range of complex

Abbreviations: NSC, Neural Stem Cell; OSVZ, Outer Subventricular Zone; GMC, Ganglion Mother Cell; INP, Intermediate Neural Progenitor.

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https://doi.org/10.1016/j.semcdb.2022.07.007
Received 18 April 2022; Received in revised form 6 July 2022; Accepted 17 July 2022
Available online 29 July 2022

behaviors [5].

Understanding the development of neural diversity and its role in establishing complex circuits is essential for eliciting function, underlying behavior, and higher order cognition. The immense diversity of neurons and glia established during development is responsible for ensuring the proper functionality of the central nervous system (CNS). [6–10]. *Drosophila* neural stem cells (NSCs, also known as neuroblasts) divide asymmetrically to self-renew and produce differentiated progeny of diverse types [11,12]. Based on the division pattern, NSCs are categorized into type 0, type I, and type II lineages [11,13–16]. Type 0 NSCs self-renew to produce another NSC and two additional differentiated neurons and/or glia, and type I NSCs self-renew and produce one ganglion mother cell (GMC), which terminally divides to produce two neurons and/or glia [11]. In contrast, type II NSCs have evolved a special amplification division pattern: they divide to self-renew and produce a transit-amplifying intermediate neural progenitor (INP) [13,16,17]. Based on current knowledge, each INP divides approximately 4–6 times and generates a total of around 6 GMCs to expand and diversify the neural populations of the adult brain [16,18–22]. This unique division mode allows type II NSCs to amplify neural progeny populations 3–4 times more than type I NSCs (Fig. 1A). Interestingly, type II NSCs are similar to mammalian outer radial glial cells (oRGCs) in the outer subventricular zone (OSVZ) in their division pattern (Fig. 1B). The oRGCs are responsible for generating the diverse neural populations of the cortex via transit- amplifying INPs [6,8,9,16,22–26]. Furthermore, the considerable range of neural diversity found in both flies and mammals is only possible because of the intricate signaling mechanisms, hormonal cues, and tightly controlled spatiotemporal gene expression in neural progenitors during development [8,11,22,27–35].

Larval type II NSCs generate the neurons that lpredominantly populate the central complex, which makes up the bulk of the sensory and locomotion centers [19,36–38]. In the following sections, we will focus on the central brain NSCs of *D. melanogaster* larvae with an emphasis on type II NSCs (Fig. 2 A). Additionally, we will summarize recent findings relating to temporal patterning of type II lineages and the mechanisms that diversify the lineages which populate the adult central complex. Towards the end, we will discuss possible ways to link temporal patterning to neural identity, connectivity, circuit formation, and function.

2. Specification and temporal patterning of type II NSCs

During embryogenesis, type II NSCs are born and specified in the dorsal protocerebrum [12,39–41]. In comparison to other NSC lineages, type II NSCs make up a small proportion of the developing brain cell population. In the embryo, eight NSCs in each lobe divide into three distinct clusters [39] (Fig. 2 A): anterior dorsomedial (aDM), posterior dorsomedial (pDM), and dorsolateral (DL) [39,42]. However, type II NSC lineages form eight distinct clusters that occupy the dorsomedial (DM1-6) and the dorsolateral (DL1-2) regions in the larval brain.The EGFR signaling pathway and activation of the downstream genes *buttonhead* and *sp1* are essential for the specification of type II NSCs during this early time [39,43]. Recently, many other early specification genes, *tailless* (*tll*), *retinal homeobox* (*rx*), and *homeobrain* (*hbn*) have been shown to regulate the early formation of type II NSCs [41,44]. The NSC marker Deadpan (Dpn) is expressed in both type I and type II NSCs, while the proneural protein Asense (Ase) is expressed in type I NSCs and INPs only [22,42,45,46]. Additionally, the transcription factor pointed P1 (PntP1, which expresses specifically in type II NSCs, represses Ase via Tll activation [47,48]. In both embryos and larvae, the INPs derived from type II NSCs are born immature and undergo a maturation period before they enter the cell cycle upon which they express Earmuff (Erm) and Hamlet (Ham) [49–53]. Both Erm and Ham expression prevent INPs from reverting back to their parental type II NSC state via inhibition of Tll [50–52]. Prospero is an additional pro-neural factor that regulates type I NSC proliferation and differentiation and is found in GMCs derived from type II NSC [37]. All embryonic type II NSCs share the factors Dpn, Ase, PntP1, Tll, and Erm; however, Rx and Hbn are two factors found only in the pDM and DL clusters, suggesting transcriptional variation between clusters [39,44,54].

Throughout the embryogenic and larval stages, type II NSCs divide asymmetrically to produce INPs as described above; both type II NSCs and INPs stop dividing and enter a state of quiescence at the end of embryogenesis and remain in this state until the larva hatches and begins feeding [31,39,42]. Once the larval stage begins, type II NSCs and INPs reactivate and continue to divide to generate the lineages of the adult brain [13,16,17,20].

The complexity of the brain functions emanates from distinct neural circuits comprising diverse neural cell types. Acquiring a unique identity and function requires each progeny to inherit specific molecular codes that set them apart from other neuron types. Whether it is the spatial position of the progenitor, intrinsic gene expression code, extrinsic

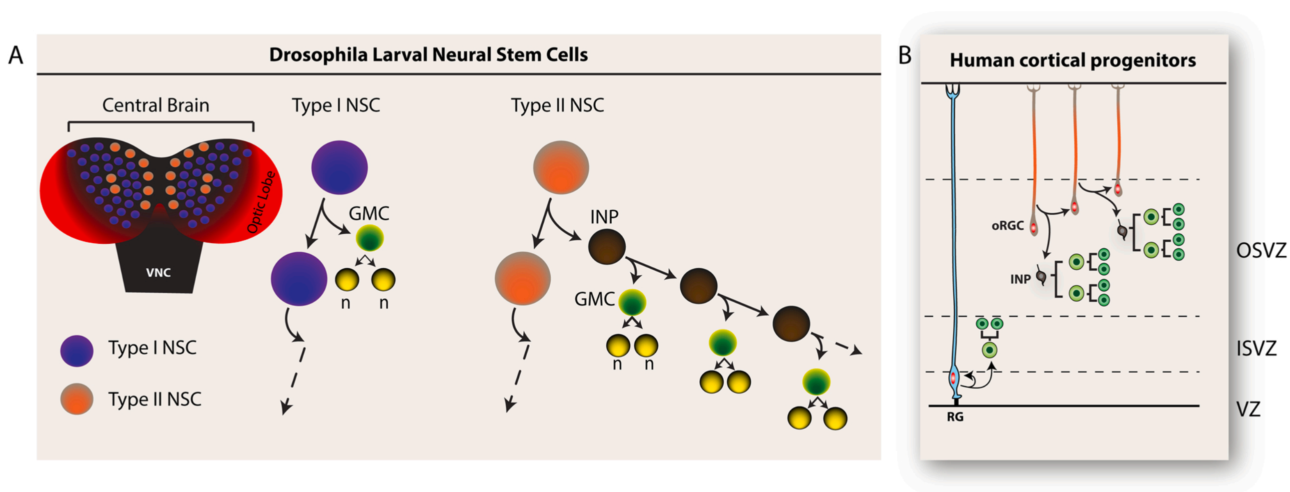

Fig. 1. Division pattern of Drosophila and Human cortical progenitors: A) Drosophila type I NSCs divide asymmetrically to self-renew and generate a GMC that terminally divides to make two neurons or glia. Type II NSCs divide to self-renew and generate INPs that also self-renew and generate GMCs, which ultimately produce two neurons or glia. B) Similar to type II NSCs, human ORGCs divide to generate INPs that expand the neural pool of the cortex. (Fig. 1.
B adapted from [26]).

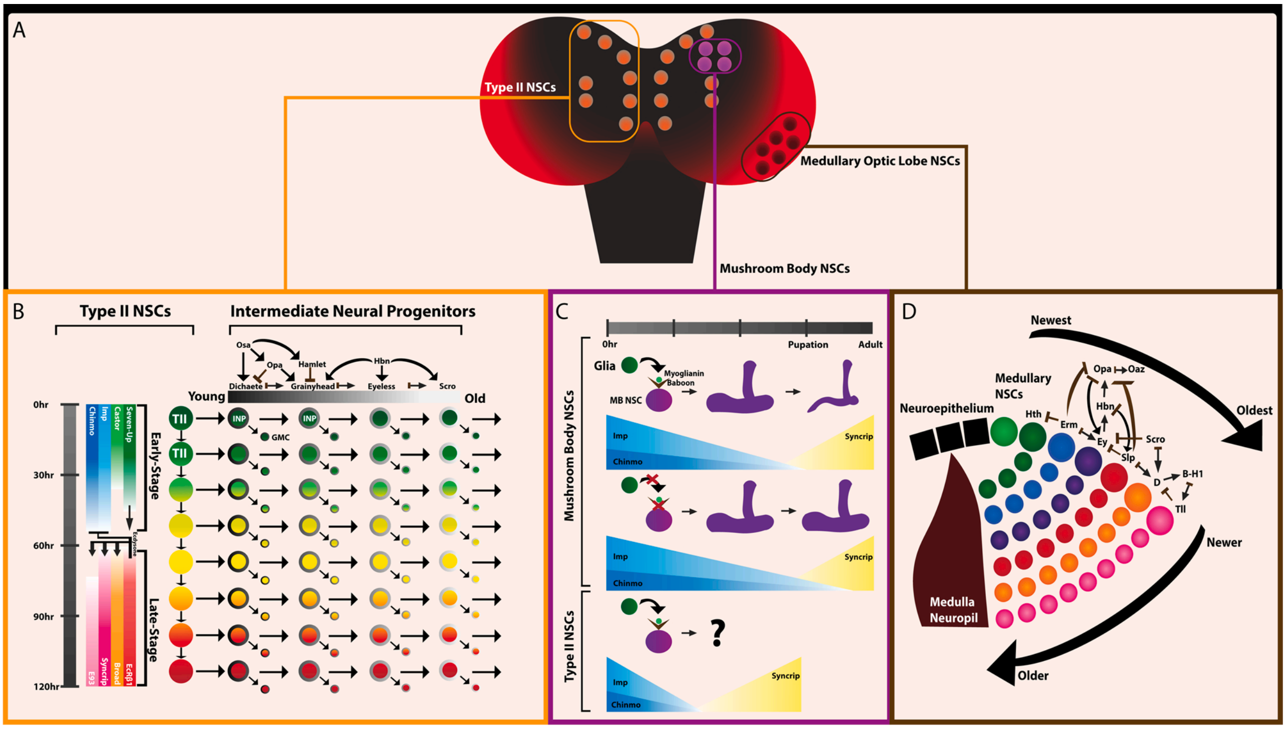

Fig. 2. Temporal Patterning of Larval NSCs: A) Shows a larval brain containing three types of NSCs: MB NSCs (purple), type II NSCs (orange), and medullary optic lobe NSCs (red). B) Schematic representing the Imp/Syp gradient, the effects of inhibiting components of the TGF- β pathway on the MB lobes which are shown as purple structures, and how the Imp/Syp gradient changes in MB NSCs as time progresses from 0h ALH until the end of pupation. The Imp/Syp gradient of type II NSCs is also shown for comparison to MB NSC gradient changes. C) Represents the temporal cascade of temporal genes expressed in type II NSCs and INPs as time progresses starting from 0h to 120h ALH. The colored circles changing from green to red located directly to the right of the type II NSC temporal cascade represent the changing composition of transcription factors in type II NSCs as time progresses while the outer rings of the INP-labeled circles changing from black to white represent the compositional changes in INP-derived transcription factors over time. The simultaneous transitioning of green to red circles and black to white rings represents the concept of combinatorial patterning. D) Depicts the direction of division and temporal patterning pathway of medullary optic lobe NSCs.

hormonal or metabolic cues, or the signaling pathways, such elaborate mechanisms require genes to be turned on or off temporally in the individual progenitors. Similar to the unique functions of a computer program, neural progenitors will generate unique cell types depending on the sequence of active genes present in the cell at a specific time and place [8,9,27,29,30,35,55–63]. A combination of internal and external cues are integrated in type II NSCs; the culmination of internal and external cues resulting in the distinct expression profile within the progenitor at a given time is known as "temporal patterning"; thus, birth order determines the temporal identity of the progeny derived from type II NSCs. [11,29,31,64–66]. Internal cues are signals within a progenitor that run off an internal clock to turn certain genes on and off. In contrast, external cues perform similar functions but are released from neighboring cells or hormonal centers found in different regions of the developing organism [30,66,67,72,73]. The integration of intrinsic and extrinsic cues thus regulates the differential gene expression in the NSC and INP derived from them, governing the formation of unique neural cell types (Fig. 2B) [18,22,31].

2.1. Type II NSC transcription factor cascade

Genomic and transcriptomic signals modulate internal transcription factor compositions that are responsible for determining temporal identity, and these compositions differ based on the developmental stage. NSCs in the fly embryonic stage utilize the sequentially expressed factors Hunchback (Hb), Kruppel (Kr), Pdm (POU-domain protein), Castor (Cas), and Grainyhead (Grh) to generate neural diversity, while larval NSCs use a completely different set of factors [11,27,30,65,68–70]. Another difference between embryonic and larval NSCs is that the embryonic NSCs mainly utilize intrinsic temporal factors with no extrinsic factor or signal identified thus far; however, this view may change as better genetic tools are developed for studies in the embryo. Larval NSCs also divide for extended time before exiting the cell cycle in the pupal stage [53,63,71,72]. Furthermore, unlike the larval stage, where every type II NSC begins and progresses with the same set of temporal factors, the set of factors present within an embryonic type II NSC differs from one type II NSC to another, with the defining parameter being the birth time: the early born embryonic type II NSCs start the temporal cascade from Pdm, while the latter born type II NSCs start from Cas and Grh [42].

Although the field has gained valuable insights into temporal patterning from embryonic neurogenesis studies, the bulk of adult brain formation occurs during the larval stage, which utilizes a series of developmental triggers that induce neuroblast division, self-renewal, and differentiation [11,30,31,70]. Also, many other environmental and systemic cues such as nutrition and hormones guide larval growth. All these complex signaling systems have allowed larval NSCs to evolve sophisticated ways of generating diverse neural types via integratingtemporal extrinsic and systemic hormonal cues with intrinsic gene expression programs. This sophisticated complexity has led to the development of a highly coordinated temporal signaling mechanism within larval type II NSCs which does not follow the sequential activation method exhibited in embryonic NSCs. Larval type II NSCs develop highly diverse progeny with variable molecular and morphological states exceeding that observed in the progeny of embryonic NSCs. Thus, a more extensive array of regulatory temporal patterning programs must govern neural identity in these lineages. The following section ,will focus on the mechanisms that dictate larval type II NSC temporal patterning.

2.2. Discovery of the novel type II temporal identity factors

Do larval type II NSCs undergo temporal patterning ? To address this question, two recent pioneering studies performed transcriptomic analysis of type II NSCs at the major phases of larval development [30,73]. Together, both studies identified over a dozen temporally expressed genes in type II NSCs that fall into two broad phases of gene expression signatures: early and late-stage genes (Fig. 2B). In addition to the transcription factors, many RNA binding proteins (RBPs) were also found to be expressed temporally in type II NSCs. Early expressed genes include transcription factors Seven-Up (Svp), Cas, Chinmo, and RBPs Lin-28, and IGF-II mRNA-binding protein (Imp) [22,68,74–76,30,70,31,33,65,77,78]. Among these early factors, Chinmo, Lin28, and Imp are expressed for longer periodof up to 56h ALH (after larval hatching), while Cas and Svp are expressed in pulses until 36hrs ALH [30,70]. The older type II NSCs express late transcription factors, Ecdysone receptor (EcR), Broad (Br), Ecdysone-induced protein 93 (E93), and the RBP Syncrip (Syp) (Fig. 2B, Fig. 3) [11,30,31,68,70]. Interestingly, type II NSCs express steroid hormone nuclear receptor isoform B1 (EcRB1) at approximately 56 h ALH and promote early to late gene transition via the ecdysone

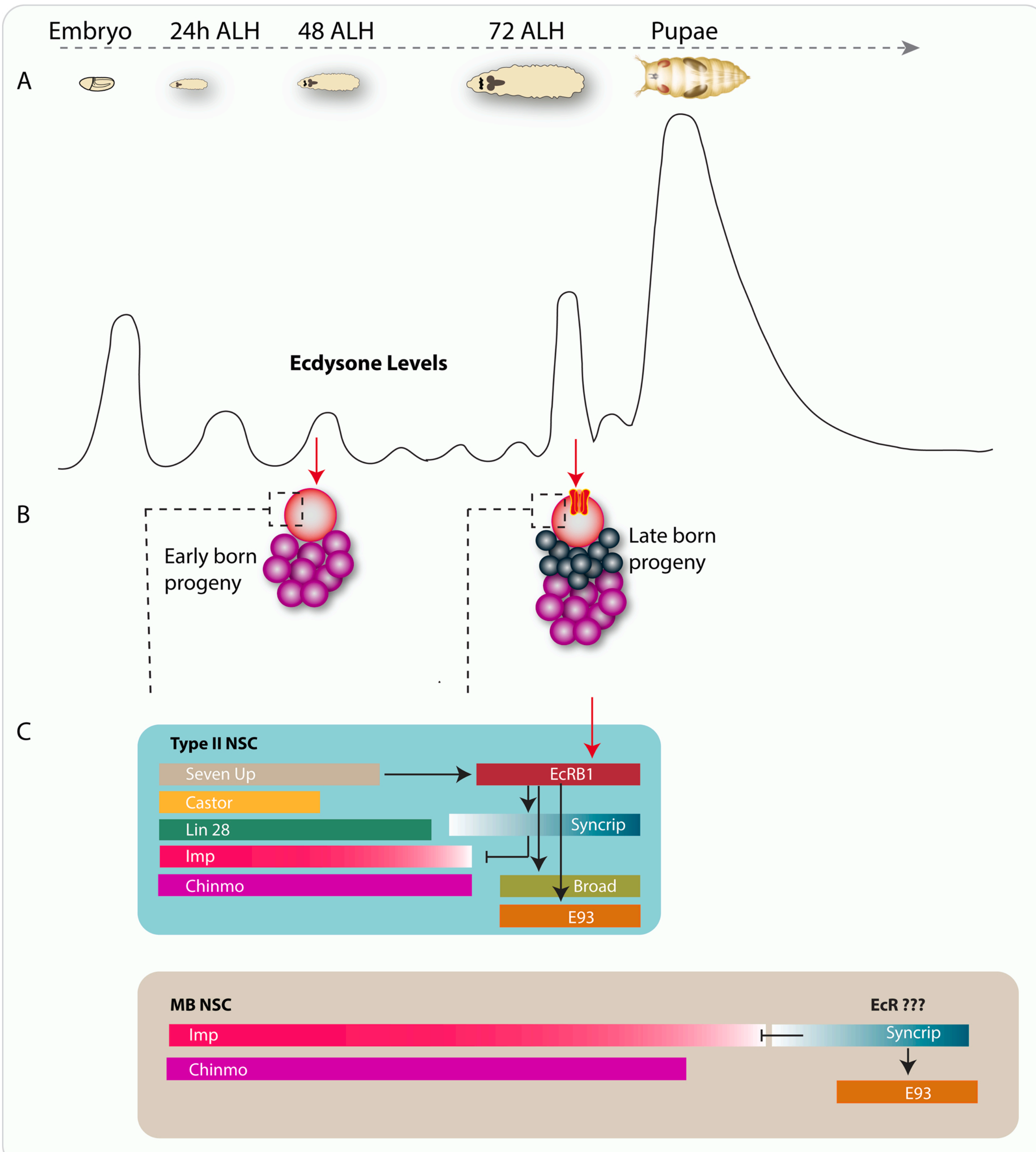

Fig. 3. Temporal hormonal cues regulate timely gene transitions within larval NSCs: A) Ecdysone pulses at various developmental time points from embryo to pupae. Early NSCs lack the receptor for ecdysone, EcR. B) Ecdysone signaling via EcR mediates early to late gene transition in late type II NSCs. C) Late pulse of ecdysone in MB NSCs mediate temporal gene expression and NSC termination via E93.

signaling pathway [30]. Taken together, the temporal expression of EcR mediates the timely transition from early to late-stage genes. However, the mechanism regulating timely EcR expression at 56hALH is currently unknown.

2.3. Conserved gene Svp facilitates early to late gene transition

The COUP-family gene *svp* is expressed in pulses and acts as a switching factor for tTFs in embryonic and larval NSCs [6,27,30,68,69,70]. In larval type II NSCs, Svp is expressed asynchronously from 17 h to 36 h ALH, whereas the embryonic type II NSCs start the temporal cascade late and lack Svp expression [39,42]. This suggests that Svp may be acting as a switching factor specifically in larval type II NSCs. The early larval type II NSCs make early-born Chinmo+ , Brain-specific homeobox (Bsh)+ neurons and Repo+ glia, while the late larval NSCs produce Br+ neurons and no glia; however, Svp mutant NSCs fail to produce Br+ neurons and continue to generate early-born neurons and glia [30,65]. Recent studies suggest ecdysone signaling plays a role in inducing early to late transition within NSCs, and the temporal expression of ecdysone receptor EcRB1 is regulated by *svp* (Fig. 2B) [30]. This conclusion is further supported by experiments in which Svp mutant type II NSCs fail to express EcRB1, resulting in a stalled early to late gene transition [30,70]. Svp mutant NSCs also prolong the expression of early factors, which leads to the generation of more early-born but fewer late-born progeny [30,31,65]. Moreover, the Svp mutant type II NSCs continued to proliferate even into the adult stages, suggesting Svp is a necessary for NSC decommissioning (i.e., cell cycle exit) [76]. Svp expression is downregulated approximately 20 h before EcR begins to appear at 56 h ALH in NSCs, which suggests the presence of multiple unknown regulatory steps between Svp downregulation and EcR expression. Additionally, how the temporal expression of Svp is regulated in NSCs remains unknown.

2.4. Opposing RNA binding protein gradients and cross talk

Despite the vast body of knowledge in the field, the regulatory mechanisms behind temporal patterning and fate determination are not entirely understood. How could the temporally expressed type II NSC factors govern the establishment of diverse cell fates? Do the larval temporal transcription factors (tTFs) govern fate in the same way as they do in the well-studied embryonic NSCs? With the recent discovery of opposing temporal Imp/Syp RBP gradients and tTFs, it seems the temporal identity mechanisms are more complex and may require a cross-regulation of both RBP gradients and tTFs in post-embryonic NSCs [11,30,33,65,70,79]. Early type II NSCs express Imp at high levels, and its expression gradually decreases over time. Conversely, Syp expression starts in mid-aged NSCs and increases with time until the pupal stages [30,70]. In early NSCs, Imp promotes proliferation via regulation of *myc* [80], while Syp plays a role in decommissioning of most late NSCs [77,81,82]. Notably, ecdysone signaling promotes decommissioning in most NSCs except MB NSCs, which utilize Imp and Syp to regulate the decommissioning process [77].

The early temporal factor Svp mediates timely establishment of opposing Imp and Syp gradients, which in turn regulate gene expression post-transcriptionally; however, Svp activity is only necessary for Syp expression but not induction of Imp. Chinmo and the RBPs, Imp, and Syp, have been the center of focus for many studies relating to RBP gradient and tTF interactions [30,77,83,84]. As mentioned previously, Chinmo and Imp are early-stage temporal factors; their expression is high at the onset of the larval stage and both Chinmo and Imp steadily decline in concentration until their loss of expression at approximately 56h ALH when EcR is activated and Syp levels increase [30,70]. Since Imp is an RBP and both Imp and Chinmo follow a similar expression/-downregulation pattern, Imp is a suspected post-transcriptional regulator of Chinmo mRNA.

How could the Imp/Syp RBPs regulate the temporal expression of tTFs? Notably, RBPs have been known to regulate essential cellular processes via protein-protein interactions between other regulatory proteins and post-transcriptional associations with mRNA [80,81,82]. Although there are several possibilities for how Imp regulates Chinmo, one likely explanation is Imp sequesters Chinmo mRNA and prevents its degradation via protein-mRNA binding. As time progresses, the late genes EcR and Syp are turned on, which decreases the concentration of Imp. Throughout this process, the progeny contain declining levels of Chinmo and other early factors, which may establish a unique identity. Although this explanation holds theoretical promise, these claims have yet to be substantiated in type II NSCs; future studies investigating protein-mRNA interactions utilizing biochemical and molecular methods may determine how much support we can gain for these hypotheses. Additionally, the molecular component(s) which activates Chinmo and Imp expression is currently unknown; there are many unanswered questions regarding how the Chinmo and Imp gradients are first formed and whether Imp plays a role in regulating translation and/or sequestration of Chinmo mRNA in type II NSCs. Another conserved RBP, Lin28, is also expressed in early type II NSCs, and its role in temporal patterning is currently unclear.

3. INP temporal patterning

Unlike type II NSCs which undergo complex temporal gene expression mechanisms utilizing both intrinsic and extrinsic cues, INPs have long been known to sequentially express three transcription factors: Dichaete (D), Grh, and Eyeless (Ey) [22]. Notably, there are some gaps in INP studies, such that temporal patterning mechanisms of INPs derived from dorsolateral 1 and 2 (DL1 and DL2) type II NSCs are yet to be determined. Moreover, not all INPs express the same combination of transcription factors, and this difference depends on the origin of the individual type II NSCs they are derived from. For example, the dorsomedial 1 (DM1) NSC generates INPs lacking Grh but expressing D and Ey, unlike the INPs of the other type II NSCs which express all three transcription factors [22,81]. Studies focusing on INP temporal factor progression have shown that D activates Grh, which ultimately leads to Ey expression. In turn, Grh activation inhibits D while Ey inhibits Grh; however, Ey activity does not directly inhibit D and requires Grh for feed-backward inhibition of D. If the presence of these three factors is necessary for appropriate sequential fate determination, how do DM1 NSC derived INPs skip the middle factor (Grh) and progress normally? Are there other regulatory factors responsible for regulating the tTF expression?

As it turns out, recent studies discovered additional regulatory components that influence the sequential expression of the three initially described INP factors. Transcriptomic analyses have identified new young INP factors Osa and Odd Paired (Opa), which may either inhibit or promote D and/or Grh, while an old INP factor, Hbn, promotes induction of Grh and Ey (Fig. 2B) [85,86]. In turn, these INP patterning genes indirectly control the feedback and feedforward mechanisms of the three main temporal factors. Additional studies have also supported the involvement of yet another temporal factor named Scarecrow (Scro), which is present within the INP temporal pathway and is upregulated following Ey downregulation (Fig. 2B) [86].

Furthermore, type II derived INPs utilize a sequential transcription factor cascade pattern similar to the optic lobe NSCs (Fig. 2D). This suggests that temporal pathways may differ from parental NSCs and can also share temporal pathway similarities with unrelated lineages. It is important to note that individual transcription factors may be shared across various NSC lineages and function differently from one lineage to another. For example, optic lobe NSCs undergo a completely different transcription factor cascade to produce the medulla of the optic lobe. These NSCs undergo a sequential activation and subsequent deactivation of the transcription factors Homothorax (Hth), Erm, Ey, Hbn, Sloppy paired 1 and 2 (Slp), D, and Tll to produce diverse neural types (Fig. 2D) [79,87,88,89,90,91,92,93]; therefore, both the transcription factors utilized and the regulatory mechanisms responsible for guiding temporal progression may differ from one NSC type to another. The tTFs, Opa, Hbn, Ey, and Scro are found in both INPs and medullary optic lobe NSCs [85,86,88,93]. Opa expression in both INPs and medullary optic lobe NSCs promotes the transition from earlier to later stage transcription factors, but the factors they promote differ between the two lineages. To elaborate, Opa promotes Grh expression in INPs, which in turn activates Ey [85,86]; but it promotes Ey directly in the medullary optic lobe NSC (Fig. 2D) [92].

The fascinating mysteries of INP temporal patterning are gradually being revealed, yet many questions remain. What additional mechanisms are at work within INPs and type II NSCs that allow certain INPs to express transcription factors in a lineage-specific manner? Are there any interactions between type II NSC and INP temporal factors? Do parental type II NSC temporal factors modify INP chromatin as time progresses? Additionally, only early, mid, and late larval time points have been assayed for gene expression; but are there finer temporal subdivisions? Besides intrinsic cues, how do the extrinsic cues impact tTF progression? Although the steroid hormone ecdysone is the best characterized external signaling molecule acting on larval type II NSCs, other molecules and signaling cascades have been suggested to play a role in influencing temporal identity and neural fate with most of these examples identified in MB NSCs [74,94,95,96]. An interesting open question is whethere these extrinsic cues also affect type II lineages?

4. Extrinsic and shared temporal cues in larval NSCs

Although MB NSCs follow a different temporally regulated pattern than type II NSCs, studies have shown that different NSC lineages may use similar mechanisms for differential regulation of temporal gene expression [11,41,79,97,98,99,100]. To put this concept into context, MB NSCs are a separate lineage of neural progenitors which generate Kenyon cells. Kenyon cells are divided into four subtypes (γ, α'/β', pioneer α/β, α/β) and populate circuits of the central brain involved in olfactory sensation and memory [94,101]. Similar to type II NSCs, MB NSCs are born in the embryonic stage and undergo temporal patterning throughout larval life. This designates the type of neuron their progeny will become and the circuits they will be a part of during the development of MBs. Various studies have demonstrated that MB NSCs utilize both cell intrinsic and extrinsic factors to generate the appropriate neural types of the MB neuropil (Fig. 2 C) [30,34,94,101,102,103,104,105]. Interestingly, type II and MB NSCs share some common intrinsic and extrinsic cues in their developmental pathways despite having different lifespans, modes and the number of divisions, and variable temporal gradient fluctuations; however, type II NSCs exhibit more pronounced changes between the Imp/Syp gradient (Fig. 3C) [11,30,34,77,81,94]. Both RBPs help define developmental stages via their respective opposing gradients they produce, which are affected by cues taken within and outside the NSC. Like type II NSCs, Imp and Syp act in an opposing manner in MB NSCs. Early-stage MB NSCs have high Imp levels and generate γ neurons, while high Syp levels are characteristic of late-stage MB NSCs, which generate α/β neurons. Intermediate α'/β' neuron fate is generated via the overlapping Imp and Syp gradient [11,94]. As previously described, Imp activity supports self-renewal and continuous proliferation while Syp reduces cell size and induces cessation of self-renewal in both type II and MB NSCs [80,63,81]. Although the Imp/Syp gradient works in similar ways between the two NSC types, the timeframe in which the Imp/Syp gradient triggers early to late stage transition differs: Imp expression spans 24–80hrs ALH while Syp expression extends from 80hr ALH to 6h after pupal formation in MB NSCs (Fig. 3) [77]. Both Imp and Syp expression durations are shorter in type II NSCs landing between 24 and 60 hr ALH and 60–120 hr ALH, respectively [30,69,70,88]. In this way, type II and MB lineages have many morphological and molecular pathway differences which set them apart from each other in order to establish distinct neural types; but they also contain highly conserved pathways that function across lineages such as the Imp/Syp gradient as well as the ecdysone hormone pathway. In both lineages, the Imp/Syp gradient and ecdysone hormone work similarly in that they control early to late temporal identity changes; however, unlike type II NSCs where ecdysone is utilized in the NSC itself to control temporal fate transitions, ecdysone is only utilized in middle-born neurons to promote definition of the α'/β' fate in MB NSCs (Fig. 2 A) [11,30,94,105].

4.1. Intrinsic and extrinsic temporal factor and function similarities between type II and MB NSC lineages

The highly conserved transforming growth factor-β (TGF-β) signaling pathway has been shown to regulate the identity of early- to late-born MB neural types [95]. Recent studies have identified that inhibition of the TGF-β pathway components during larval development leads to defects in the morphology of the adult MB lobes and neural identity [94,95,96,105,106,107]. Experiments inhibiting the TGF-β receptor *Drosophila* homolog, Baboon, showed an absence of α'/β' neurons with higher numbers of both γ and α/β neurons [94]; furthermore, the MB structures composed of these neurons exhibited morphological deformities [95,96,108]. This work suggests that the TGF-β pathway is responsible for integrating the effects of intrinsic RBP Imp and Syp signaling to produce the intermediate α'/β' neural types [94]. These studies propose that activating the TGF-β pathway somehow reduces Imp expression for timely downregulation of Chinmo [77,81,83,94,109]. In turn, lower Chinmo levels increase the expression of Mamo—the effector molecule utilized to define the temporal identity of α'/β' neurons in MB NSCs [94]. Additionally, the TGF-β pathway utilizes an external signaling molecule, Myoglianin, which is produced and secreted by glial cells [96,110]. Lack of Myoglianin signaling leads to similar outcomes as those observed from Baboon inhibition [96]. Therefore, external signals released from developing neighboring cells, such as glia, may influence temporal differentiation and the development of nearby NSCs. This regulation happens via the modulation of the NSC's internal tTF regulatory pathways—specifically those processes involving the Imp/Syp gradient [94,96,105,107].

Ecdysone signaling also plays a role in the fate specification of MB lineages; however, unlike in type II NSCs, it acts on the post-mitotic neurons [102,108]. In EcR loss of function lineages, prospective α'/β' neuronal progeny adopt the later-born pioneer α/β identity [105]. Moreover, EcR expression within post-mitotic MB neurons requires TGF-β pathway activity in MB NSCs; how the TGF-β pathway induces EcR expression is not clearly understood. Despite emerging evidence of paracrine signaling influences on MB NSCs from glial cells, the effects of paracrine signaling pathways, including the TGF-β pathway, have yet to be substantiated in the type II NSC lineages. Overlapping temporal patterning mechanisms between MB NSCs and type II NSCs, such as ecdysone signaling and the Imp/Syp gradient, pose the possibility that the TGF-β pathway may be another common component utilized to govern temporal fate by both NSC types. Future studies in this area will be essential for understanding whether the TGF-β pathway plays any role in patterning type II NSCs and their lineages. Furthermore, investigations into conserved pathways may shed some light on how type II NSCs establish neural diversity of the adult central complex.

4.2. Hormonal regulation of temporal brain patterning

Although the endocrine effects of hormonal signaling on physiological processes have been extensively described in many different animal models, the influences of these signaling molecules on brain development have only recently been explored. As previously mentioned, ecdysone signaling is essential for the induction of late-stage genes and inhibition of early-stage genes in type II NSCs. Additionally, ecdysone signaling mediates NSC decommissioning at the onset of pupation [75]. Furthermore, ecdysone is the only hormone researched to date known to play a direct role in type II NSC temporal patterning and neural diversity

[30,31].

EcRB1 is expressed temporally at ~56 hr ALH in the central brain NSCs and drives their early to late gene transition (Fig. 3) [30]. In the Svp mutant type II NSCs, the ecdysone-induced proteins Br and E93 are not produced; and the expression of the late factor Syp is delayed. On the contrary, in Svp mutants, the early factors Imp and Chinmo persist into later instar stages [30,70]. Interestingly, in Svp mutant type II NSCs, where EcRB1 expression is lost, the NSCs are stalled in the early gene expression compositions and never transition to the expression of late-stage genes. This is likely because Svp mediates timely expression of EcRB1 in type II NSCs whereupon ecdysone binding induces subsequent signaling and changes the fate competence of the early NSCs. In support of this claim, blocking ecdysone signaling in type II NSCs resulted in ectopic early-born glia at the expense of late-born Bsh+ neurons in the late larval brain [30].

Interestingly, thyroid hormone signaling was shown to regulate early to late-born cone cells in human retinal organoids [111], suggesting that steroid growth hormones have conserved mechanisms across species. The S cone neural types are specified early, followed by the L/M cone subtypes; interestingly, the temporal transition from the S to the L/M subtype requires thyroid hormone signaling [[111]. Additionally, thyroid hormone signaling is essential for proper cortical development in humans and other mammals [112,111,113]. Whether the Svp homolog COUP-TF regulates the expression of the thyroid hormone receptor in mammalian neural progenitors similarly to how Svp regulates EcRB1 expression in type II NSCs remains unknown. Furthermore, COUP-TF mediates neurogenic to gliogenic switch in the oRGCs and also mediates the transition from early-born to late-born cortical neurons [114, 115].

Ecdysone signaling also plays a role in early neuroepithelial to NSC transition in the optic lobe [116] by repressing Chinmo [84] and activating Br [117]. Recent studies have also identified the roles of ecdysone signaling in proper brain wiring [118]. While many other ecdysone-induced genes and nuclear hormone receptors are expressed in the developing brain, their role in temporal patterning remains largely unknown. How steroid hormone signaling and temporally expressed genes regulate neural identity and behavior is an interesting open area of research. In the next portion of the review, we will attempt to provide a framework for future studies in linking development with neural identity and function, focusing on the central complex lineages.

5. Linking stem cells to generation of neural diversity in the adult fly brain

In this section, we address how the identity of type II NSCs plays a role in generating neural diversity within the central complex, and how temporal patterning in type II NSCs alongside birth order-dependent protein expression in INPs combinatorially generates distinct neural types.

5.1. The adult brain central complex

Primarily consisting of unpaired structures, the central complex is a highly conserved brain structure across insect species [119,120,121] that integrates signals from the right and left sides of the brain and generates motor output [122]. In *Drosophila* and other arthropods, the central complex is well studied to be involved in higher-order cognition, memory, and behavior. Itplays an important role in integrating sensory and motor inputs. These, in turn, confer appropriate responses to the environmental stimulus in a context-dependent manner [123,124,125, 126]. The major neuropils of the central complex are (a) fan shaped body (FB): the largest of the neuropils in the central complex; (b) ellipsoid body (EB): partially embedded in the FB and is shaped like a torus; (c) protocerebral bridge (PB): handlebar shaped, conserved in various arthropods and vertically divided into distinct glomeruli; (d) the paired noduli (NO) and (e) asymmetrical body (AB): a bilateral structure consisting of left and right sides that are asymmetric in size. The AB has been recently added as fifth independent neuropil of the central complex [127].

The neuropils of the central complex are majorly populated by the progeny derived from type II NSCs (Fig. 4). The progeny includes diverse neural types generated from different type II lineages (DM1–6 and DL1–2), at different times during development [19,70]. Each lineage generates progeny that is packed together and innervate central complex neuropils in a unique pattern; thus, the contribution of each lineage is distinct [19]. Molecularly distinct domains generated via patterning in the neuroepithelium early in development produce NSCs with differential spatial identity; and these, in turn, give rise to heterogenous pools of progeny (discussed in a separate review by Sonia Sen in this issue). In addition to spatial patterning, temporal patterning in these progenitors gives rise to distinct progeny and expands diversity. The neurons from all the lineages are eventually connected via extensive inter-neuropil networking [19] (Fig. 5A-G). Furthermore, INPs derived from type II NSCs are known to expand neural diversity. It has been shown that each INP serially gives rise to neural types that are morphologically similar as modulated by the parent type II NSCs but differ based on the INP birth order. Neurons derived from an INP at different times of their life have different neurite trajectories and thus belong to distinct neuronal classes (Fig. 5H) [18]. In an earlier study, using Twin Spot MARCM sister clones derived from a common progenitor were differentially labeled [128].

Each type II NSC generates ~400 progeny; in a recent study following extensive analysis of the connectome, a 'cell type' was defined as a single cell or group of cells based on similar cell body location, size of the cell population, similar morphology, and pattern of synaptic connectivity. A total of 22,594 neurons with 5229 morphology types and 5609 connectivity types were identified and named in the fly hemibrain, of which the central complex neuropil included a total of 2826 cells with 224 morphology types and 262 connectivity types [5]. One hypothesis for generating such diversity is that temporal expression of transcription factors and RBPs make different combinations in NSCs. Additionally, as INPs bud off from the parent type II NSCs, they inherit the temporal factors that are further diluted out with each INP division resulting in each INP inheriting a unique combination of parental temporal factors. For example, young INPs born from the early type II NSC will inherit RBP Imp; and that Imp could be expressed as a gradient in the INP window as well. The simultaneous expression of the INP temporal factors together with the NSC tTF gradient might be responsible for generating diverse neural types combinatorially. Another hypothesis yet to be tested is that tTFs and RBPs inherited from the parental type II NSC combined with the INP tTFs form distinct tTF codes that might be responsible for making each neuron unique (Fig. 2B) [18,22,31,41,87].

Thus, the combined activity of the transcription factor gradient defines each INP's unique neural fate; moreover, the simultaneous expression of the parental type II NSC factors at the time of the INP's generation is also responsible for defining fate. It is possible that each INP produced has a unique combinatorial molecular code that defines a unique neural identity. Additionally, type II NSC temporal factors might be influencing the temporal patterning of INPs. Despite these observed connections, the molecular mechanisms behind combinatorial temporal patterning remain unknown.

5.2. Generation of Neural Diversity

The identification of dozens of new temporally expressed genes in type II NSCs has laid a foundation to address the long-standing question of how adult central complex lineages acquire their identity and function during development. We discussed in detail (In Section 2) the temporal patterning in larval type II NSCs of *Drosophila* [30], [70]; their division pattern is analogous to the neural progenitors in the mammalian OSVZ region, and they produce INPs [25]. These type II NSCs and INPs express a distinct set of temporal factors over time and eventually give rise to the diverse progeny that majorly populate the central

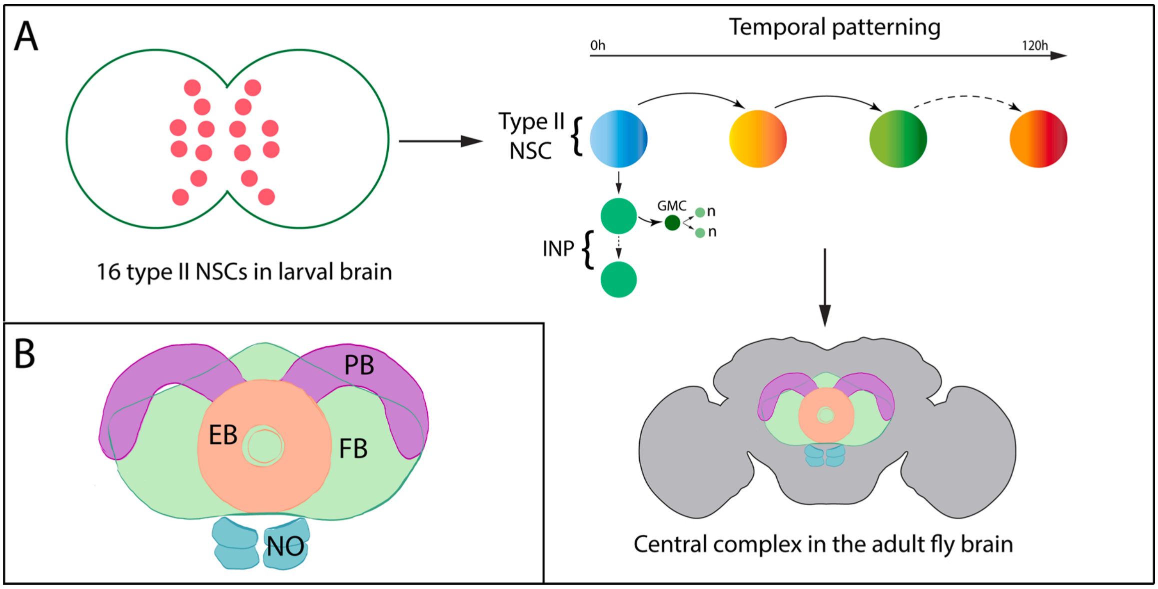

Fig. 4. The progeny from type II NSCs majorly populates the central complex: A) 8 distinct type II NSCs per larval brain lobe undergo asymmetric cell division and temporal patterning. The progeny derived from these type II NSCs innervate the central complex. b) The major neuropils of the central complex are the protocerebral bridge (PB), ellipsoid body (EB), fan shaped body (FB), and the noduli (NO).

complex [30]. It is imperative that this temporal patterning in progenitor cells plays a role in (a) the fate specification of the diverse neuronal progeny that arise from them, thus generating neural diversity, (b) governing and maintaining the neuronal identity in the post-mitotic neurons, (c) establishing proper neural connectivity and (d) regulating function and behavior.

With the availability of sophisticated genetic tools, we are now in a position to manipulate a temporal factor specifically in the type II NSCs and assay the neural identity change in the adult brain. Also, the hemibrain connectome of the adult fly is now publicly available, including browser interfaces such as NeuPrint. These tools allow us to dissect the functional circuits in the adult fly brain. Another resource, the Virtual Fly Brain, allows the study of *Drosophila* neuroanatomy and provides access to databases detailing brain structure, connectivity, and gene expression [129,130]. NeuronBridge accesses color depth search results for Light Microscopy (LM) and Electron Microscopy (EM) data published by the FlyLight and FlyEM projects [131,132]. With all these publicly available resources, we can decipher the morphology, identity, and connectivity of individual neurons and add on to our current understanding of how these networks are established during early development. Understanding the cues that regulate the formation of these functional, intricate neural connections will help us to discern the parallels in the development of the adult mammalian brain. It will also help us understand what can go wrong during neurodevelopmental disorders.

Alongside all the discoveries relating developmental cues to the generation of neural diversity, there are many studies establishing neural circuitry and connectome of the central complex. Recently, the central complex EM connectome of *Drosophila* has been extensively studied at a synaptic resolution [133]. In this study, new neuron types of the central complex have been identified and assigned to established circuit pathways. Additionally, novel circuit pathways have been elucidated. It is yet to be determined how the progeny derived from different NSCs at different times come together and form functional circuits. Also, with the advanced genetic tools available, we can now track a neuron back to its lineage and birth time. A functional neural circuit is made up of interconnected neurons, which are derived from the same or distinct lineages.

Several studies have implicated distinct neuronal classes, which are part of diverse circuits that innervate multiple neuropils of the central complex and regulate distinct behaviors. For example, ring neurons innervate the EB; dFB neurons in dorsal layers of the FB; vFB neurons in the ventral layers of the FB; E-PG, P-EN, and P-FN neurons innervate the PB and FB or EB [133,134] (Fig. 6). Additionally, type II NSCs also give rise to diverse glial cells via region specific, and birth order regulated gliogenesis. Glial cells are also generated from type II NSCs via INPs, and different type II NSCs produce distinct glial networks of the adult fly brain. Many of them give rise to both ensheathing glia and astrocytes-[135]. It was reported that only the early born INPs produce glia; thus type II NSCs lose the gliogenic potential as the NSC ages [22].

6. Temporal patterning in the stem cells establish neuronal diversity

Now that we link temporal patterning to the generation of neural diversity, the question of interest is how the components of different neural circuits develop and form precise connections. Are the neurons that target the same neuropils or the neural types in the same circuit generated from the same progenitors, or are the neuron morphology and identity lineage independent? An earlier report describes that the progeny from each NSC exhibit common trajectories and innervate specific neuropils of the central complex [70]. While tremendous progress has been made in defining distinct neural types, neural circuits, and underlying behaviors; however, what regulates the generation of such neural diversity during development is not entirely understood. One hypothesis is that the temporally expressed factors in the NSCs regulate fate determination, identity, and function of the distinct neural types. Studies from the Doe lab have shown that sequential expression of temporally regulated expression of transcription factors in *Drosophila* embryonic NSCs, Hb > Kr > Pdm > Cas, and this patterning determines the cell fate wherein the progeny inherits the transcription factors temporally [27], Expression of early factor Hb specifies early-born neuronal and glial fate while another factor Kr specifies the second born neurons (Discussed in a separate review in this issue, Pollington et al.). As discussed in detail earlier, temporal patterning in larval NSCs has been determined [30]; however, how this patterning regulates generation of neural diversity in the adult fly brain is an open area of investigation. In type II NSCs, Imp/Syp temporal gradients are shown to regulate fate determination where Imp promotes early, and Syp promotes late temporal fates [70]. It was recently reported that a homeodomain transcription factor in the ventral nerve cord (VNC) known as

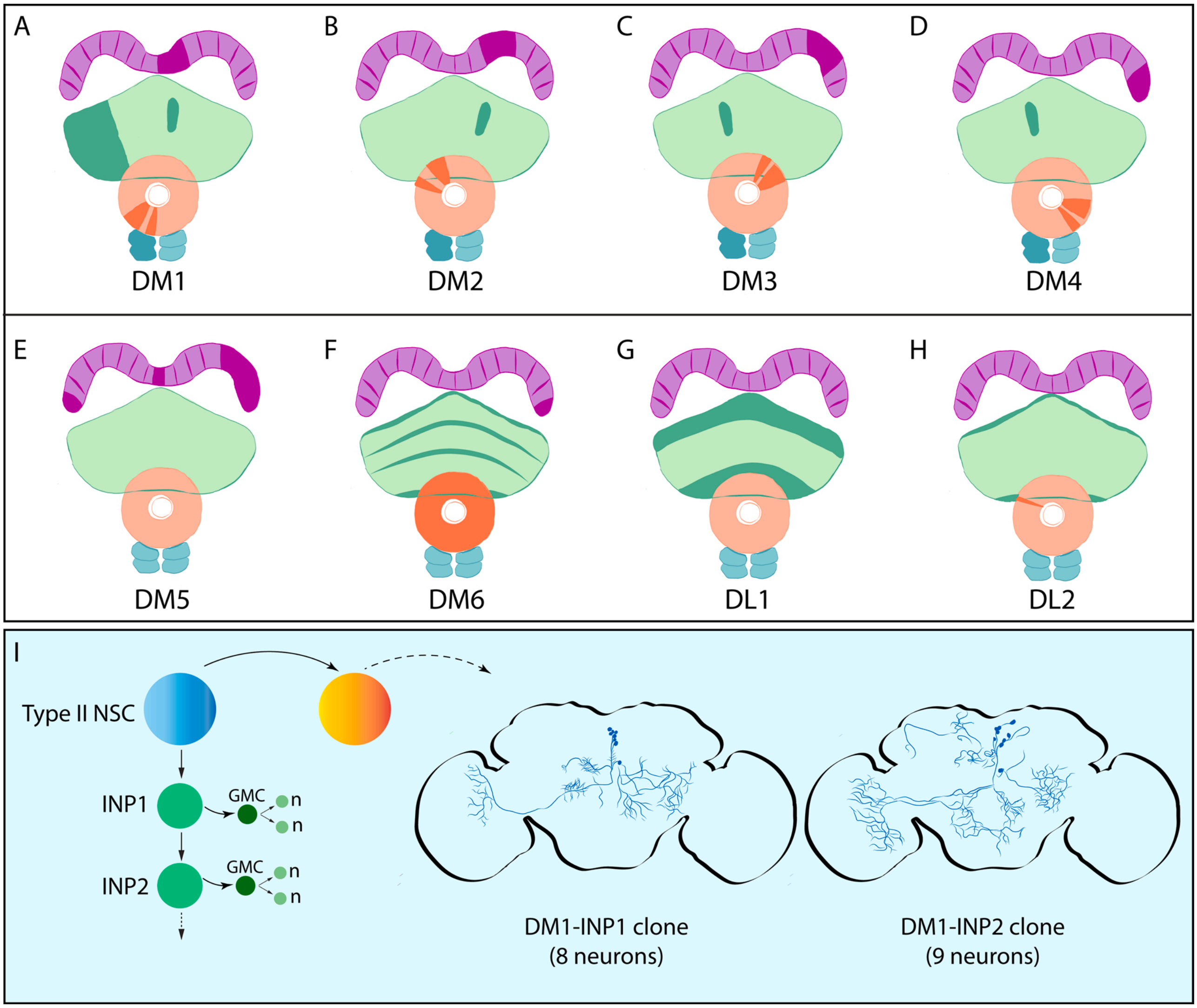

Fig. 5. A-H) The dark shaded areas represent the innervated subdomain of the neuropils by the progeny of A-F) DM1-DM6, G-H) DL1-DM2 (Adapted from [19]). Schematic representing sister clones at 8 hr ALH derived from DM1-INP1 and DM1-INP2 exhibiting similar as well as some distinct trajectories (Adapted from [18]).

Unc-4 regulates neurotransmitter identity and locomotion behavior in the adult fly in a lineage specific manner [136]. Another study shows that INPs sequentially express different factors and give rise to distinct neuronal types during specific transcription factor windows; young INPs generate Bsh+ neurons, and old INPs generate Toy+ neurons [22]. It has also been established that distinct subtypes of central complex neurons regulating navigation in *Drosophila* are derived from different temporal windows in the INP cell lineage [134], suggesting that the neurons whose axons and dendrites target the same neuropil have similar developmental origins. It is demonstrated that specific late INP factor Ey is involved in the fate determination of these neurons, wherein Ey promotes the development of late-born E-PG and PF-R neuron types and represses early -born P-EN and P-FN neurons. Recently, it was discovered that the unique identity of a neuron is not only determined by the distinct profile of inherited transcription factors but the post-mitotic combinatorial transcription factor code. It was observed that the morphology of the motor neurons from the Lin B lineage is cell-intrinsically dictated by the combinatorial expression of morphology TFs (mTFs), and alteration of this mTF code transformed the morphology of the neurons in a predictable manner [137]. Neuron type-specific temporal transitions have been reported in post-mitotic neurons in *C.elegans* as well [138]. Given all these discoveries, it lays the groundwork to elaborate on what specific cell types are formed from each NSC and how the functional circuits are established.

7. Conclusions and future directions

Despite the tremendous advances in identifying new cell types and genes via transcriptomics and genetic screens, we currently have limited information about how NSC factors govern neural identity, connectivity, and function at the molecular level. With the identification of many new temporally expressed genes in type II NSCs, their roles in specifying neural diversity of the central complex lineages are only beginning to start. Despite expressing similar temporal factors, each type II NSC gives rise to a distinct progeny indicating the involvement of unknown spatial factors or signaling pathways. An interesting future question is how intrinsic and extrinsic signals integrate to coordinate the timely gene transitions within NSCs. Furthermore, how conserved and common transcription factors and signals modulate the temporal patterning is unknown. For example, the RBPs Imp and Syp are highly conserved across species, but their mechanistic behavior and involvement in establishing neural diversity has yet to be defined. Although both RBPs are essential for closing NSC competence windows and guiding temporal transitions from early to late transcription factor compositions, how they perform these functions is still debated: do Imp and Syp bind tTF mRNAs (e.g., *chinmo*) to modulate gradient production of temporal factors to

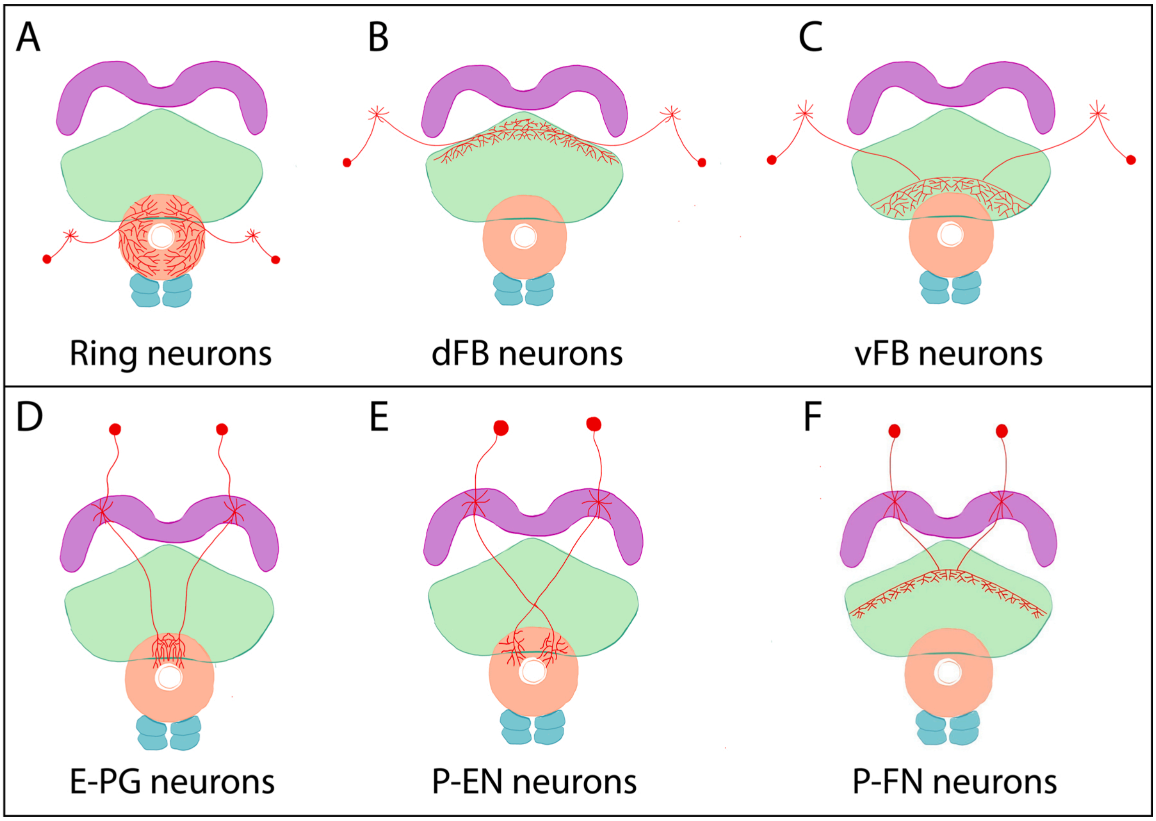

Fig. 6. Neuron class innervating different neuropils in the central complex: A) Ring neurons mainly innervate EB, B) dFB neurons innervate the dorsal layers of FB C) vFB neurons innervate the ventral layers of FB, D) E-PG neurons and E) P-EN neurons innervate the PB and EB and F) P-FN neurons innervate the PB and FB.

establish neural diversity, or is this phenomenon guided by another RBP-driven mechanism? Besides RBPs, how are the overlapping signaling mechanisms and factors related between different NSC lineages? For example, Opa is found in both INPs derived from type II NSCs and optic lobe NSCs; however, the downstream factors they activate differ. Is a universal signaling mechanism shared between all lineages with slight variations that set each NSC type apart from the rest to establish highly diverse neural populations? Also, how type II temporal programs integrate with the INP temporal axis to define the distinct fate of the progeny through combinatorial patterning remains unknown. The information gained will help understand the combinatorial temporal patterning of the human cortical lineages. The ecdysone hormone plays a significant role in temporal patterning transition in type II NSCs and MB neurons. Still, the specific molecular mechanisms and the downstream targets of EcR are not properly understood. Many ecdysone-induced factors such as Br and E93 are expressed in the late NSCs. Do these genes play any role in the timely cell cycle exit, and the fate specification of the central complex lineages remains to be addressed?

Spatio-temporal patterning in *Drosophila* type II NSCs establishes the groundwork to ask how the molecular cues regulate temporal patterning during development. An existing question is if and how the NSC identity regulates the fate specification of distinct neural types and to what extent the factors inherited from the progenitor cell play a role in maintaining the morphology, connectivity, and identity of the individual neurons. It is possible that the inherited temporal code from type II NSCs and INPs together govern the neuropeptide identity of these lineages like in the VNC lineages [139]. With the advanced genetic tools, and EM connectome data available, we can answer what lineage gives rise to each circuit component and if manipulating early developmental code affects connectivity and behavior.

Unlocking the processes that facilitate control of the neural identity code via temporal patterning is not only essential for understanding the fundamental principles of central nervous system development but is also necessary for exploring pathophysiologic mechanisms behind neurodevelopmental disorders. With the advent of stem cell reprogramming and brain organoid technologies, studies on fruit fly temporal patterning might take us further towards re-generating the lost or damaged neural types.

Funding

This work was supported by the National Science Foundation under CAREER award IOS-2047020 to MH Syed.

Conflict of interests

None.

Acknowledgments

We thank Chris Q. Doe, Asif Bakshi and Ashley Medina and for providing critical feedback on the manuscript. We thank Lindsey Thomas for proofreading the manuscript.

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