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Temporal control of *Drosophila* central nervous system development

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A complex nervous system requires precise numbers of various neuronal types produced with exquisite spatiotemporal control. This striking diversity is generated by a limited number of neural stem cells (NSC), where spatial and temporal patterning intersect. *Drosophila* is a genetically tractable model system that has significant advantages for studying stem cell biology and neuronal fate specification. Here we review the latest findings in the rich literature of temporal patterning of neuronal identity in the *Drosophila* central nervous system. Rapidly changing consecutive transcription factors expressed in NSCs specify short series of neurons with considerable differences. More slowly progressing changes are orchestrated by NSC intrinsic temporal factor gradients which integrate extrinsic signals to coordinate nervous system and organismal development.

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Introduction

Neural development is choreographed over space and time to ensure that neurons form functional connections, circuits and networks. One critical mechanism to accomplish this is temporal patterning, in which NSC progeny change over time. Many essential genes discovered in the fruit fly have been found to have analogous roles in mammals; genes that confer neuronal temporal-fate specification are no exception [1–4]. Neuroblasts (NB), the *Drosophila* NSCs, asymmetrically divide to self-renewal and deposit intermediate precursors with more limited potential (see Figure 1a–b). While the fates of individual progeny are lineage-specific (due to spatial pattering [5]), temporal fating mechanisms are widely shared between lineages. The *Drosophila* central nervous system (CNS) consists of the ventral nerve cord (VNC), the central brain (CB) and the optic lobe (OL) (see Figure 1a). There are two phases of neurogenesis separated by a period of quiescence (see Figure 1c). In embryonic stages, VNC and CB NBs divide to produce the larval CNS. In post-embryonic stages, these NBs plus the OL generate over 90% of the adult CNS. NB proliferation ceases in a lineage-specific manner (see *Lineage termination and tumorigenesis*) and embryonic and postembryonic-born neurons reorganize to form the adult CNS. Here we present the current state of the temporal patterning field without going into much detail, as it has been recently thoroughly reviewed [6]. We emphasize recent studies with new mechanistic insights.

Temporal transcription factors

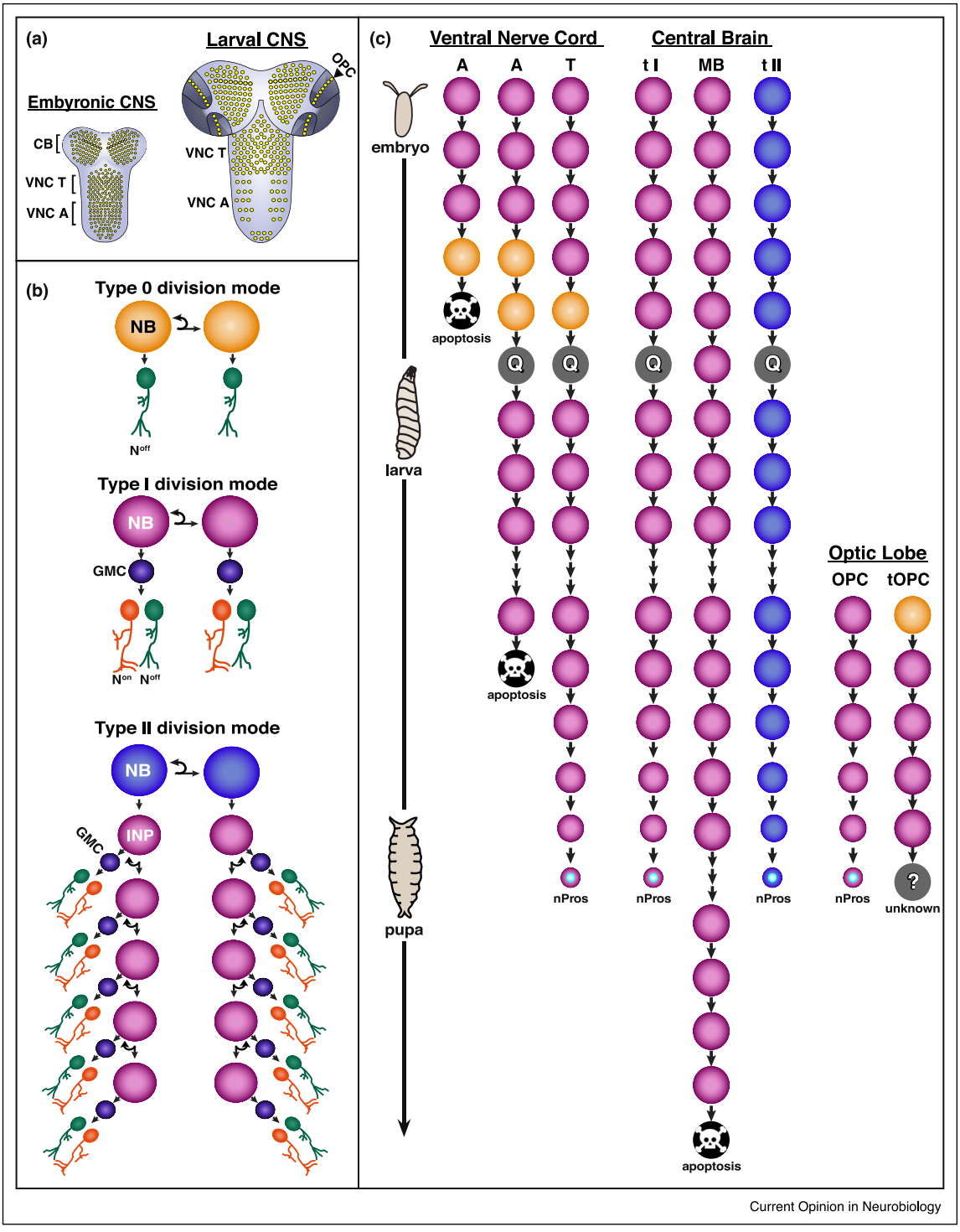
Temporal series of transcription factors are deployed to create remarkable diversity in short or early lineages. These so called temporal transcription factors (tTFs) have expression windows in the NBs, usually lasting 1–2 cell divisions. tTFs specify birth-order cell fate, independent of spatial cues. Embryonic VNC NBs, OL NBs and the intermediate neural progenitors (INPs) of type II NBs (mammalian-like NSCs) all utilize tTFs. As NBs age, they often lose competence to respond to early tTFs (reviewed in Ref. [7]).

Embryonic ventral nerve cord

The classic series of tTFs (see Figure 2a): Hunchback (Hb) → Krüppel (Kr) → Pdm2 and Nubbin (together called Pdm) → Castor (Cas) → Grainyhead (Grh) was discovered in the embryonic VNC [8–10]. Additionally, Seven-up (Svp) is described as a switching factor, turning off Hb expression [11]. Many studies have examined the ability of these tTFs to confer temporal fate in a large subset of embryonic VNC lineages (reviewed in Ref. [6]).

NBs express tTFs, often passing them to their progeny. A recent study suggests tTFs confer temporal fate within the NB/GMC rather than the progeny. Two VNC lineages require NB/GMC Hb to establish early temporal fates [12]. How then are multiple fates specified within the same tTF window, a process termed subtemporal patterning? Within a Hb window, differential post-mitotic levels of Hb can specify sequential neurons [13]. Additionally, the Thor lab describes subtemporal patterning of a large Cas window via opposing feed-forward loops (reviewed by Ref. [14]). Interestingly, Kr helps specify the first fate [15] and Grh helps specify the fourth fate [16].

Figure 1



An overview of *Drosophila* CNS development. (**a**) Diagram of embryonic and larval CNS progenitors, neuroblasts (small yellow circles). The optic lobe is dark gray. CB = central brain, VNC T = thoracic VNC, VNC A = abdominal VNC, OPC = outer proliferation center of the OL. (**b**) Proliferation modes of neuroblast daughters. Type 0 division directly produces a single neuron, which is either Notch independent [32] or Notch^{off} (N^{off}) [64]. Type I division produces ganglion mother cells (GMC) which asymmetrically deposit the Notch inhibitor Numb, creating a Notch^{off} and a Notch^{on} (N^{on}) neuron/glial progeny. Type II NBs produce intermediate neural progenitors (INP) which undergo 4–6 self-renewing divisions, generating GMCs that produce two daughters. The type II mode yields roughly 5-fold more progeny per NB division than type I. (**c**) Time-course of NB

Together these studies demonstrate that tTFs can be utilized for both temporal and subtemporal patterning.

The question of how the same terminal phenotype can be specified by different temporal windows was recently elegantly examined with embryonic Nplp1 neurons (Figure 2b). These neurons, born from two different NBs, are specified by different temporal windows and spatial cues. In these lineages, the same terminal-selector cascade is activated by distinct combinations of temporal and spatial patterning cues [17•]. Analysis of the cis-regulatory information controlling the initial gene in the cascade revealed that separate, cell type-specific enhancers are utilized, each containing binding sites for temporal and spatial transcription factors [18•].

Regulation of the classic tTF series is actively researched. Cross regulation features sequential activation and complicated repression (see Figure 2a). tTF binding sites within enhancers support some direct cross regulation [19–21]. Robustness of the system can be seen within tTF enhancers. For example, multiple unique activation elements were found within a temporally regulated core sequence of a *nubbin* enhancer [21]. Similarly, no single conserved sequence block (CSB) could control temporal activity of a *grh* enhancer [20]. Intriguingly, embryonic CB and VNC NBs frequently utilize different enhancers or CSBs within an enhancer [20–22], suggesting that VNC and CB regulation of tTF expression is not comparable.

Embryonic central brain

While many of the tTFs are expressed sequentially in CB NBs [10], little evidence supports critical roles in CB temporal fate. tTF function has only been comprehensively examined in a single CB lineage, ALad1, where only Kr plays a role in fate-dependent morphology, being required for the 11th of 18 distinct embryonic fates [23]. Another recent study examined the NB that produces lateral horn leucokinergic (LHLK) neurons. Pdm, Cas and Svp (not Hb, Kr or Grh) are expressed in the NB, yet it is unclear whether they exist as a temporal series [24]. Reported data implies specification of LHLK by Kr or Cas, though more precise manipulations and phenotypic analysis are needed [24].

The embryonic origin of the type II NBs was recently determined by two labs [25,26]. The NBs delaminate from the ectoderm in late embryonic stages expressing the latter tTFs: Pdm → Cas → Grh. Studies have yet to confirm whether the tTFs indeed confer temporal fate.

Intermediate neural progenitors of the type II lineage

Type II NBs divide to produce intermediate neural progenitors (INP), which themselves act similar to type I NBs (Figure 1b). Initiated by the SWI/SNF complex [27], larval born INPs express a series of transcription factors Dichaete (D) → Grh → Eyeless (Ey), which specify the temporal fates of their progeny [28] (Figure 2c). In embryonic born INPs, one group found only D expression [25], while another reported D and Ey [26]. It is unclear whether the tTFs operate alike in embryonic and postembryonic INPs.

Optic lobe

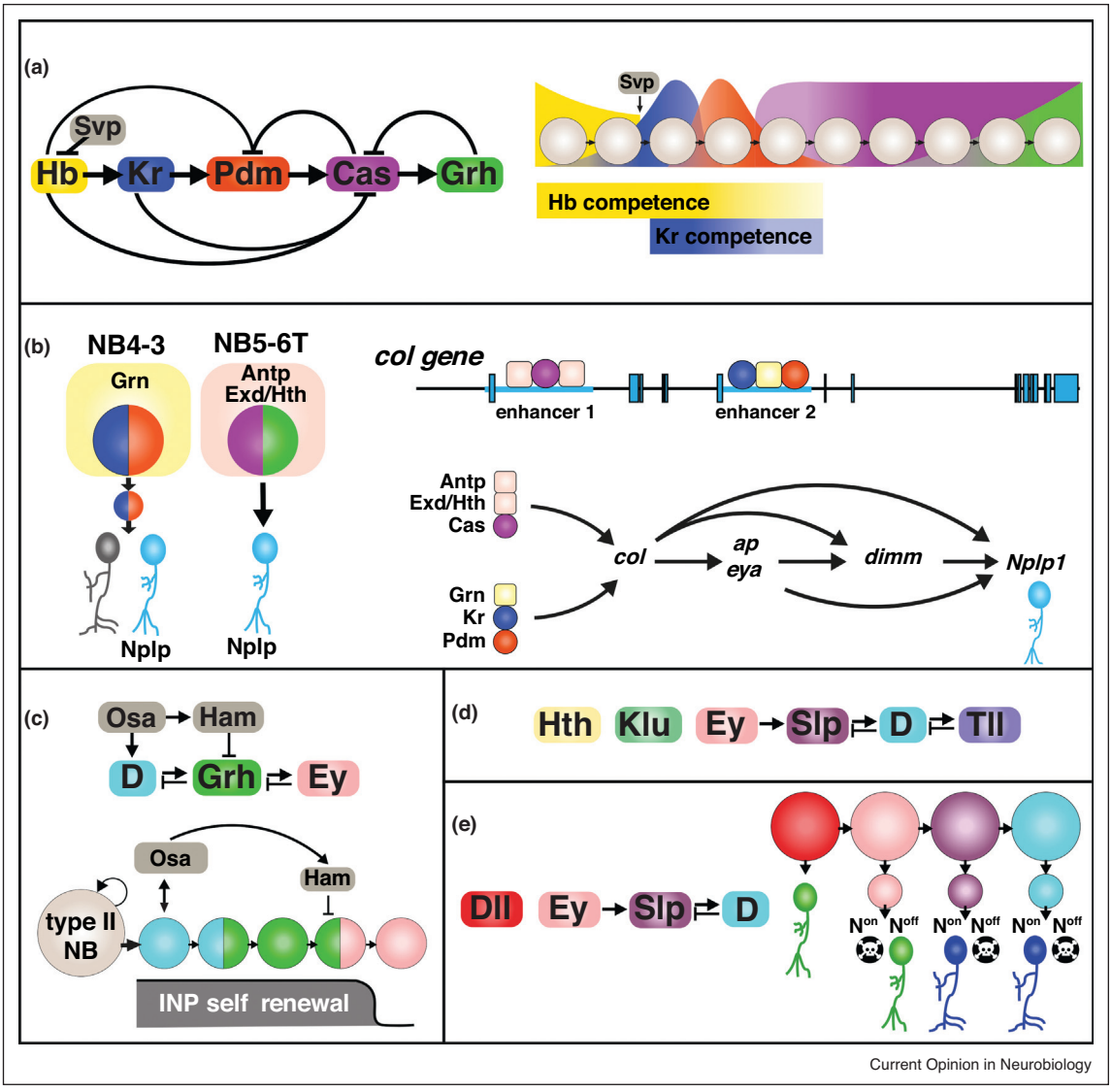
The OL has two main proliferation centers, the inner proliferation center (IPC) and outer proliferation center (OPC) each with their own temporal regulation (30–34). tTFs have been described in the main OPC and tips of the OPC (tOPC). The main OPC NBs express Homothorax (Hth) → Klumpfuss (Klu) → Eyeless (Ey) → Sloppy paired 1 and 2 (Slp) → Dichaete (D) → Tailless (Tll) (Figure 2d) [29,30]. The Desplan lab elegantly described the integration of temporal and spatial patterning [31•]. Six spatial domains are defined by transcription factor expression. Following each NB division, neurons undergo binary sister fate decisions of Notch^{ON} or Notch^{OFF} fates. In Notch^{ON} neurons, the spatial domains are apparently ignored and each NB produces the same temporal series of unicolumnar neurons. In the Notch^{OFF} neurons, the spatial information is used to create diverse, region-specific, multicolumnar neurons. The tOPC NBs express the tTFs Distalless (Dll) → Ey → Slp → D (Figure 2e) [32]. IPC NBs express D → Tll, but these function as switching factors for NB gene expression rather than as tTFs [33].

Temporal control of proliferation state

In addition to neuronal fate specification, tTFs can influence the proliferation state. As embryogenesis ends, VNC NBs switch proliferation modes from Type I (producing progeny that divide once) to Type 0 (producing progeny that differentiate directly) (see Figure 1b) [34]. The Type 1 → 0 switch is temporally and spatially coordinated (Figure 1c) [35–37]. Also, spatiotemporally regulated is the NB's destiny to die or enter quiescence at the end of embryogenesis (Figure 1c) (reviewed by Refs. [38,39]). Further, Type II INPs divide only 4–6 times due to tTF progression; the final tTF, Ey, diminishes the ability to self-renew (Figure 2c) (reviewed in Ref. [7]). Additionally, tTFs in the tOPC confer a Type 0 → 1 proliferation mode transition and temporal shifts in progeny apoptosis (Figure 2e) [32]. Moreover, two phases of neuronal production in the OL IPC are correlated with temporal expression of proneural genes Asense and Atonal [40]. Although these genes do not confer neuronal fates, they

(Figure 1 Legend Continued) divisions. Proliferation mode is color coded (colors from **b**). A subset of abdominal VNC NBs undergo apoptosis at the end of embryogenesis, while most other CB and VNC lineages undergo quiescence. The mushroom body neuroblasts (MB) are an exception. NBs terminate by apoptosis or shrinking followed by nuclear entry of Prospero leading to cell cycle exit. It is unknown how the tOPC lineage terminates.

Figure 2



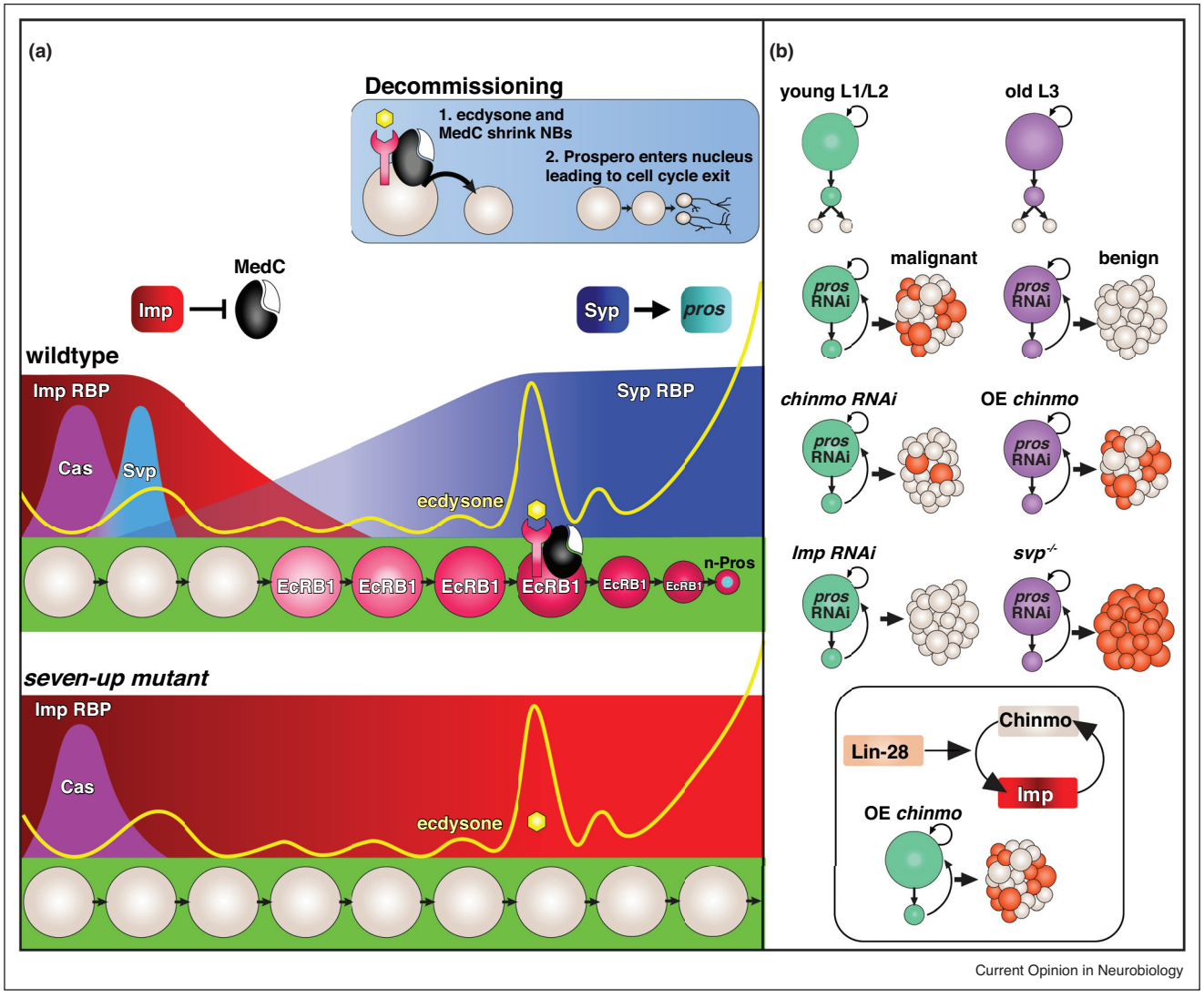
Temporal transcription factors. (**a**) Left: Cross regulation of VNC tTFs Hunchback (Hb), Krüppel (Kr), Pdm2 and Nubbin (Pdm), Castor (Cas) and Grainyhead (Grh). Seven-up (Svp) inhibits Hb. Right: Diagram of changes in tTF expression as NBs age. As the series progresses NBs loose competence to early tTFs Hb [65] and Krüppel [66]. (**b**) Summary of Gabilondo *et al.* [17•] and Stratmann and Thor [18•]. Different NBs, expressing different spatial (squares) and temporal (circles) code can produce similar Nplp neuron types. Spatiotemporal transcription factors bind to NB-specific enhancers on the *col* gene, initiating a cascade leading to terminal selector gene *Nplp1*. (**c**) Cross-regulation of Type II INP tTFs: Dichate (D), Grh and Eyeless (Ey). Initiation of D expression is prompted by SWI/SNF member Osa. Osa also activates Hamlet (Ham), which in turn inhibits Grh, allowing Ey expression and the loss of INP self-renewal capability. (**d**) The tTFs of the optic lobe outer proliferation center (OPC) and their cross regulation. Homothorax (Hth), Klumpfuss (Klu), Ey → Sloppy paired 1 and 2 (Slp), D, and Tailless (Tll). (**e**) Left: the tTFs of the tips of the OPC (tOPC) and their cross regulation. Right: tTF transitions alter the progeny division mode from type 0 to type 1, and modes of apoptosis from $Notch^{on}$ to $Notch^{off}$.

alter the proliferation state of the NBs from asymmetric to symmetric divisions [40].

Temporal patterning in extended linages

The postembryonic NBs (pNB) of the VNC and CB divide ~50 times and over 4 days to produce the adult CNS. Such protracted postembryonic lineages raise doubts that a series of tTFs could control temporal fating. Indeed, while Grh, Cas and Svp are expressed in a majority of pNBs, they seem to have functions other than defining temporal windows (see *Seven-up promotes early to late transition* and Figure 3a) [28,41,42•,43–45]. Instead,

Figure 3



Lineage progression, termination and tumorigenesis. (**a**) Lineage progression and decommissioning in type II postembryonic neuroblasts. Summary of findings from Syed *et al.* [49], Ren *et al.* [42•] and Yang *et al.* [46••]. Each illustrated NB division represents the passing of approximately 12 h. The diagram starts after NBs reactivate from quiescence (approximately 12 h after larval hatching). In the *seven-up* mutant, the Imp/Syp gradient does not proceed and there is no decommissioning; high Imp inhibits the mediator complex (MedC), the ecdysone receptor EcRB1 is not expressed, NBs do not shrink, and Prospero does not accumulate due to lack of Syp. (**b**) Summary of findings from Narbonne-Reveau *et al.* [48••]. Knocking down *prospero* (*pros* RNAi) results in dedifferentiation of GMCs. Inducing *pros* RNAi at early stages (young L1/L2, teal) leads to malignancy (orange cells), whereas the tumorigenic potential decreases with age (benign tumors form with *pros* RNAi in old L3, purple). Early factors Imp and Chinmo promote malignancy; also arresting the temporal progressing by mutating *svp* promotes malignancy. Inset: Chinmo and Imp produce a feedforward loop that together with Lin-28 act as an oncogenic module to drive tumor growth. Chinmo overexpression alone in young NBs leads to malignancy.

slowly progressing, hierarchical gradients lead to two major temporal windows, subdivision of which has not been described.

RNA binding protein gradients

At the top of the hierarchy of postembryonic temporal factors are a pair of RNA-binding proteins (RBP) that regulate mRNA stability/translation. IGF-II mRNA-binding protein (Imp) and Syncrip (Syp) are expressed in opposite temporal gradients in pNBs (high-to-low and low-to-high, respectively) (Figure 3a). Opposing Imp/Syp temporal gradients may be universal in postembryonic CB and VNC NBs [42•,46••,47,48••]. In MB NBs, Imp and Syp are reciprocally regulated; eliminating one causes upregulation of the other [47]. Imp inhibition of Syp may be NB-specific or Syp may be independently regulated in some NBs, as a subset of

Imp$^{-/-}$ type II NBs (DM1–DM6) did not precociously express Syp [49] and DL1 NBs expressing Imp RNAi had increased rather than precocious Syp expression [42•].

The opposing Imp/Syp gradients govern the timing of temporal fate transitions and/or expression of temporal factors. Imp loss or Syp overexpression results in expansion of late temporal fates at the expense of early fates; the opposite is true for Imp overexpression or Syp loss. The clearest example is the MB, which encompasses three main sequentially generated neuron types, γ → α'β' → αβ [50]. Imp defines γ, Imp and Syp together denote α'β' and Syp designates αβ [47]. Imp/Syp together help form the descending gradient of Chinmo [47], which is essential for γ and α'β' specification [51]. Chinmo also specifies early fates in type II and ALad1 lineages [23,42•]. However, Imp/Syp exert broader effects on temporal fates [23,42•], suggesting that Imp and Syp regulate additional factors. Notably, perturbing Imp/Syp gradients in the rapidly changing ALad1 lineage can alter the ratio of young and old temporal fates without losing gross diversity [47].

Seven-up promotes early to late transition

Svp and Cas are expressed early in many pNBs [41,49]. The transition of VNC neurons from early-born to late-born requires Svp and Cas in ~70% and ~20% of lineages, respectively [41]. Both the Doe and Lee labs recently examined the role of Svp in the temporal progression of type II pNBs (Figure 3a). Ren *et al.* found that Svp is required to initiate Imp/Syp gradient progression; *svp*$^{-/-}$ NBs maintained high Imp levels and kept Syp levels essentially nonexistent [42•]. Syed *et al.* showed that Svp is required for ecdysone receptor (EcR-B1) expression in pNBs around the early-to-late temporal transition and confirmed persistent expression of early factors and loss of late factors in *svp*$^{-/-}$ pNBs [49]. Neither group demonstrated a requirement for Cas, however Cas overexpression delayed progression of the Imp/Syp gradients [42•], suggesting closure of the Cas expression window is essential for proper temporal progression.

Ecdysone signaling facilitates proper temporal progression

One critical question is how intrinsic clocks can be tuned by extrinsic signals which control developmental timing. Removing ecdysone signaling from developing larval brains maintained early factors (Imp and Chinmo) while the late factor, Syp, was expressed in fewer pNBs (from 100% to 50–90%) at 72 hours after larval hatching [49]. While ecdysone may direct the early-to-late transition, it is possible that the transition was simply delayed as Dillard *et al.* show with Chinmo downregulation following similar manipulations of ecdysone signaling [52]. The relationship between Chinmo and ecdysone signaling was recently examined in MB neurons [53], revealing a feedback loop where ecdysone initiates *let-7* expression to downregulate *chinmo* [53]. The *let-7* microRNA is a conserved hetereochronic gene that coordinates hormone signaling and temporal transitions (reviewed by Ref. [54]). Intriguingly, in mouse NSCs, *let-7b* downregulates IMP1 expression [4].

Lineage termination and tumorigenesis: two sides of the same coin

The same temporal factors that control lineage progression also guide lineage-specific pNB termination (Figure 3a). Toward the end of neurogenesis, pNBs are actively decommissioned; they undergo an ecdysone and mediator directed metabolic switch that results in pNB shrinking, nuclear Prospero accumulation and cell cycle exit (reviewed in Ref. [55]). Temporal progression is essential for decommissioning, as mutating *svp*, knocking down *syp*, or overexpressing *imp* all result in NBs lasting into adulthood [41,46••]. Early Imp inhibits mediator complex components and late Syp promotes Prospero accumulation, thus enabling lineage-specific NB termination. Indeed, high Imp levels in MB NBs allow them to avoid the otherwise universal early pupal decommissioning [46••].

Predictably, the same temporal factors are implicated in tumorigenic potential (Figure 3b). NB tumors can be induced by dedifferentiating INPs or GMCs by a number of manipulations including loss of Prospero, induction of Notch signaling, or loss of Brain tumor (Brat). As type II INPs age, they lose competence to be dedifferentiated by Notch signaling [56]. Similarly, the potential for dedifferentiated NBs to become malignant depends upon the expression of early temporal factors (Figure 3b). Investigating the features that cause dedifferentiated cells to become malignant revealed the continued expression of early factors Chinmo [48••] and Imp [48••,57]. Curiously, in *brat* RNAi dedifferentiated NBs, the late factor Syp was still expressed, but it was sequestered to the cell cortex [57].

Conclusions

Two mechanisms of neuronal temporal patterning are described. First, rapidly changing series of tTFs specify embryonic VNC lineages, OL lineages, and type II INP sublineages. Second, slower progressing hierarchical gradients pattern postembryonic VNC and CB lineages. It is probable that tTF cascades are used to specify discrete temporal fates, whereas slowly progressing gradients generate more closely related cell types. The idea that postembryonic cell types are closely related is supported by single-cell RNAseq of olfactory projection neurons [58]. Nevertheless, multiple cell types can be specified within a tTF window. Despite reports of tTF expression increasing over time and differentially specifying cell fates [13,16], tTF gradients have largely been ignored.

tTF cascades are intrinsically controlled with little to no plasticity in response to organismal development, a feature which ensures that all neuron types are created [59]. Postembryonic VNC and CB NBs, on the other hand, coordinate NB proliferation and temporal transitions with organismal development—particularly with the hormone signal that helps initiate NB decommissioning.

Neuronal phenotypes are specified in a combinatorial and context-dependent manner, merging spatial and temporal-fating mechanisms as well as binary-fate decisions. Together these mechanisms serve not only to enrich the number of neuron types, but to do so in a particular order. Recently, a number of studies examined associations between birth-order and circuit assembly [60–63], helping paint a picture where relatively simple spatiotemporal codes can lead to development of complex circuitry.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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