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REVIEWS

Review

Playing Well with Others: Extrinsic Cues Regulate Neural Progenitor Temporal Identity to Generate Neuronal Diversity

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During neurogenesis, vertebrate and *Drosophila* progenitors change over time as they generate a diverse population of neurons and glia. Vertebrate neural progenitors have long been known to use both progenitor-intrinsic and progenitor-extrinsic cues to regulate temporal patterning. In contrast, virtually all temporal patterning mechanisms discovered in *Drosophila* neural progenitors (neuroblasts) involve progenitor-intrinsic temporal transcription factor cascades. Recent results, however, have revealed several extrinsic pathways that regulate *Drosophila* neuroblast temporal patterning: nutritional cues regulate the timing of neuroblast proliferation/quiescence and a steroid hormone cue that is required for temporal transcription factor expression. Here, we discuss newly discovered extrinsic cues regulating neural progenitor temporal identity in *Drosophila*, highlight conserved mechanisms, and raise open questions for the future.

Temporal Patterning Generates Neuronal Diversity

Neural diversity is essential for proper brain function including sensory perception, motor control, and consciousness. Neural diversity is generated by both spatial and temporal cues acting in combination on neural progenitors. Spatial cues assign progenitor regional identity, whereas temporal cues or **temporal patterning** (see Glossary) mechanisms allow single progenitors to make a sequence of different neurons and glia over time. Vertebrate neural progenitors have long been known to respond to changing environmental cues to undergo temporal patterning [1], although the molecular identity of these extrinsic cues is often still unknown. In contrast, *Drosophila* neural progenitors (neuroblasts) undergo a well-characterized, neuroblast-intrinsic transcription factor cascade that generates **temporal identity** [2–6]. Importantly, recent work has identified extrinsic cues that regulate *Drosophila* neuroblast temporal patterning, raising the possibility of conserved mechanisms used by vertebrate and *Drosophila* progenitors for the generation of neural diversity. Here, we briefly summarize what is known about temporal patterning in *Drosophila* and vertebrates, and then discuss newly discovered extrinsic signaling pathways that generate temporal patterning and increase neural diversity in *Drosophila* central brain neuroblasts.

Temporal Patterning in Mammalian and *Drosophila* Neural Progenitors

In mammals, most neural progenitors throughout the central nervous system (CNS) (cortex, retina, and spinal cord) can generate multiple neuronal subtypes over time, followed by a later

Trends

Temporal patterning in both vertebrates and Drosophila is regulated by extrinsic cues.

In mammals, TGFβ signaling regulates temporal patterning of neural progenitors in the midbrain and hindbrain.

In mammals, Wnt7 from early-born deep layer neurons induces cortical neural progenitors to switch to late-born superficial layer neuron production.

In Drosophila, nutrition (amino acids) initiate a signaling cascade leading to glial secretion of insulin-like peptides that induce timely neural stem cell exit from quiescence.

In Drosophila, the steroid hormone ecdysone is required to trigger a switch from larval neuroblast production of early-born Chinmo+ Imp+ progeny to late-born Broad$^+$ Syp$^+$ progeny. Ecdysone may synchronize the generation of neural diversity with non-neuronal tissue development.

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phase of gliogenesis [5,7–12]. Unlike early findings in *Drosophila*, temporal patterning mechanisms characterized in mammals primarily involve extrinsic signals – either feedback cues from previously generated neurons or cues from unknown sources – although some evidence from *in vitro* culture suggests the presence of intrinsic cues [13,14]. Nevertheless, the best characterized temporal patterning mechanisms involve extrinsic cues, as briefly summarized in the following four examples (Figure 1). First, early cortical progenitors transplanted into older hosts switch to making late-born neurons [15]. Second, ablation of early-born deep-layer neurons leads to prolonged early-born neuron production and a delay in generating late-born upper layer neurons, suggesting a negative feedback signal from early-born neurons to ventricular zone progenitors [16]. Third, transforming growth factor (TGF)-β signaling is required in three different brain regions to trigger a switch from early-born to late-born neural subtypes [17]. Fourth, the Wnt7 and Ntf3 ligands provide feedback signals from newly born neurons to cortical progenitors to trigger a switch from deep layer to upper layer neurogenesis, and Wnt7 also triggers the subsequent neurogenesis to gliogenesis switch [18,72]. Additional examples are reviewed elsewhere [19–22]. Thus, many aspects of vertebrate neural temporal patterning are regulated by extrinsic signals.

In *Drosophila*, most neuroblasts in the ventral nerve cord (VNC) and brain produce a type I lineage (**Type I neuroblasts**) and undergo several important **temporal transitions**: they switch from proliferation to quiescence at the embryo/larval transition; resume proliferation in early larvae; and terminate their lineage soon after pupariation (Figure 2). They also make a stereotyped sequence of neural subtypes during each phase of proliferation [4]. Temporal patterning has been best characterized in the embryonic VNC neuroblasts. Embryonic neuroblasts sequentially express a series of five **temporal transcription factors (TTFs)** – Hunchback, Krüppel, Pdm (the redundant Nubbin and Pdm2 proteins), Castor, and Grainy head – that are each necessary and sufficient to specify the unique temporal identity of neurons born during each expression window [23–25] (Figure 2A). The **TTF cascade** can occur in single cultured neuroblasts and thus is regulated by a lineage-intrinsic mechanism [26,27]. Interestingly, the early TTF Hunchback and the late TTF Castor have mammalian orthologs (Ikaros and CasZ1, respectively); Ikaros specifies early-born neuronal identity in the cortex and retina, while Casz1 specifies late-born neuronal identity in the retina [28,29]. Recent work has uncovered similar TTF cascades in optic lobe neuroblasts and the intermediate neural progenitors (INPs) within larval **type II neuroblast** lineages; all of these TTF cascades are characterized by feed-forward transcriptional activation and feedback repression [30–32], conceptually similar to extrinsic feedback repression that triggers early–late cortical neuron switching and neuron–glial switching [16,18]. Thus, the initial characterization of *Drosophila* temporal patterning revealed primarily lineage-intrinsic mechanisms. Below, we review recent work showing that extrinsic cues are used to generate temporal patterning throughout the prolonged stages of postembryonic neurogenesis.

Glial-Derived Cues Regulate the Timing of Neuroblast Quiescence

All *Drosophila* neuroblasts undergo quiescence in the late embryo/early larvae, with the exception of five central brain neuroblasts (four mushroom body neuroblasts and one ventrolateral neuroblast) [33]. Entry and exit from quiescence occurs in a stereotyped sequence: embryonic neuroblast proliferation, neuroblast size reduction, neuroblast quiescence, neuroblast enlargement and proliferation in the young larva, and finally neuroblast size reduction and terminal differentiation in the early pupa [34–36]. Here, we discuss new findings showing that these temporal transitions are regulated, in part, by extrinsic signals that reflect organismal nutritional status (Figure 2).

Neuroblast entry into quiescence involves cell size reduction and an internal timer (completion of the TTF cascade) followed by nuclear import of the Prospero transcription factor, which represses cell cycle gene expression and initiates quiescence [37,38]. In contrast, neuroblast

Glossary

Ecdysone signaling pathway: the steroid hormone ecdysone, made in the prothoracic gland, binds the ecdysone receptor (A, B1, and B2 isoforms) which dimerizes with the common co-receptor Ultraspiracle to regulate gene expression in many or all embryonic, larval, and pupal tissues. Pulses of ecdysone trigger a diverse spectrum of developmental events and coordinate development between tissues.

Temporal identity: a cell fate that is specified by a temporal patterning mechanism. Temporal identity combined with spatial identity (e.g., the spatial location of the progenitor) can generate a highly specific or even unique neuronal identity.

Temporal patterning: specification of cell fate by temporally restricted intrinsic or extrinsic cues.

Temporal transcription factor: a transcription factor that is transiently expressed in a progenitor and specifies progeny temporal identity.

Temporal transitions: changes in gene expression or proliferation mode occurring at stereotyped times of development.

TTF cascade: temporal transcription factor cascade, typically regulated by feed-forward activation and feedback repression acting intrinsically within the progenitor.

Type I neuroblast: canonical *Drosophila* neuroblast present in throughout the CNS that divides asymmetrically to produce a smaller ganglion mother cell (GMC) that typically produces two postmitotic neurons. There are ~100 type I neuroblasts in each brain lobe, each producing 30–150 progeny [70].

Type II neuroblast and INPs: there are eight type II neuroblasts per brain lobe that divide asymmetrically to produce an INP with each division. Each INP divides asymmetrically to generate 4–6 GMCs, and thus type II neuroblasts each produce larger clones with 400–500 progeny [71]. Both type II neuroblasts and INPs exhibit temporal patterning.

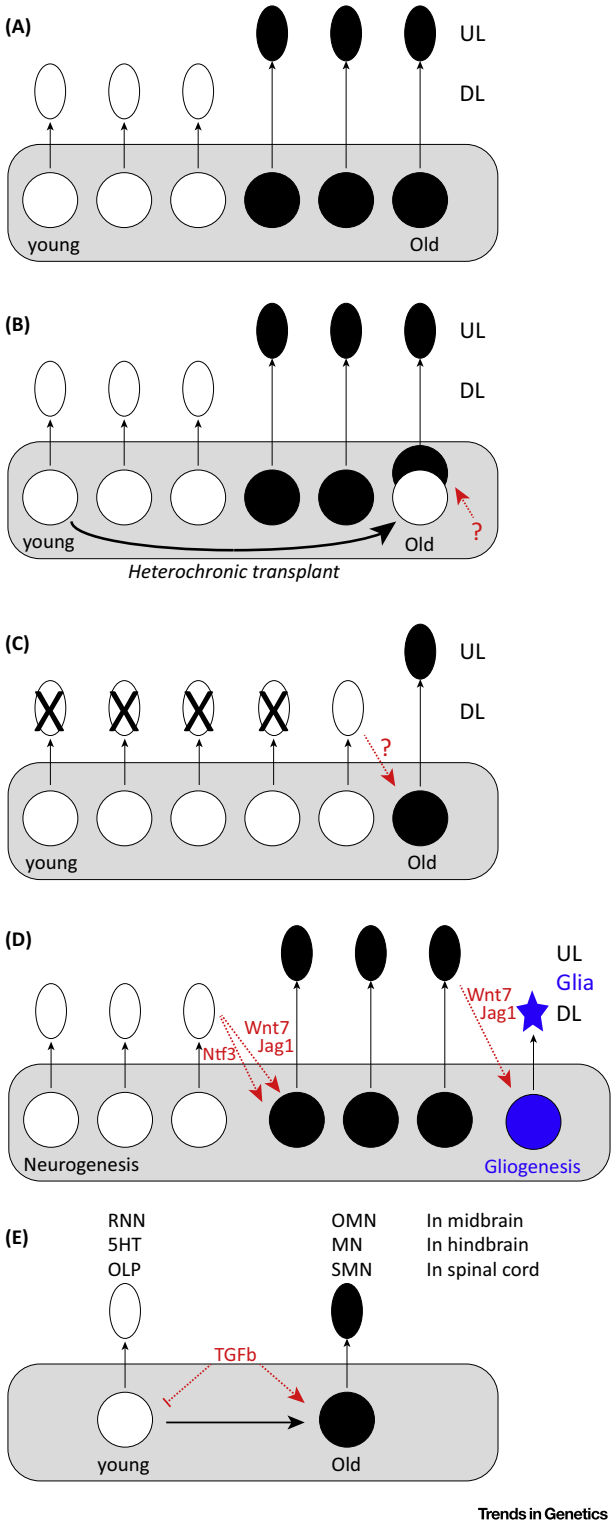
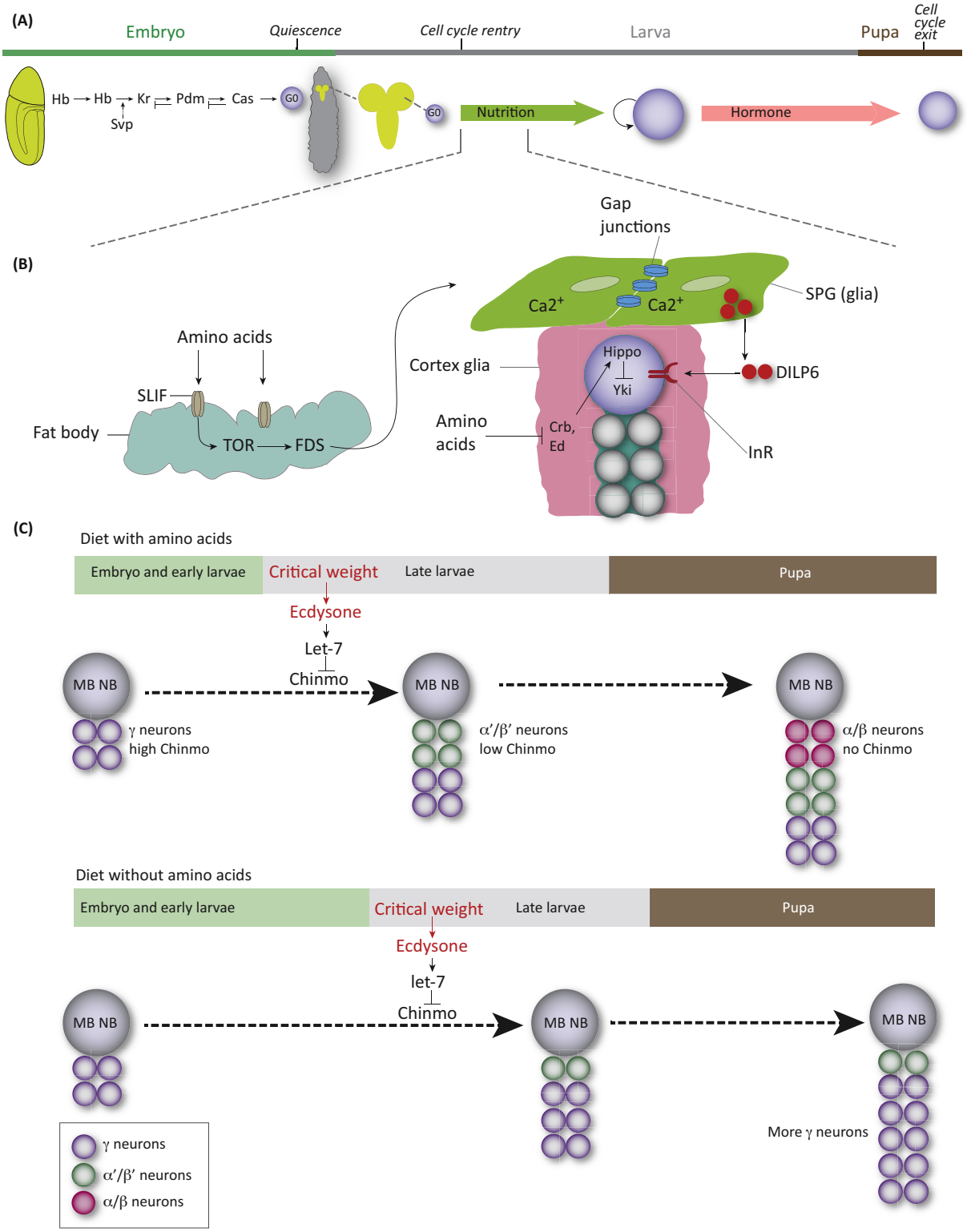


Figure 1. Temporal Patterning by Extrinsic Cues in Mammalian Neural Progenitors. Grey box represents the progenitor population at the ventricular zone. Time flows from left to right. Young progenitors, white circles; old progenitors, black circles. DL, deep layer early-born neurons (white ovals); UL, upper layer late-born neurons (black ovals). Dashed red arrows or T-bars represent the extrinsic cues that regulate temporal patterning. (A) Wild-type cortical development. (B) Transplantation of young cortical progenitors into an older VZ shows that environmental cues can transform progenitor temporal identity to an older state [15]. (C) Ablation of early-born neurons reduces the number of deep-layer early-born neurons and delays production of late-born upper layer neurons [16]. (D) Cortical Wnt7 induces a switch from DL to UL neurogenesis, as well as a switch from neurogenesis to gliogenesis [72]. (E) Transforming growth factor-β signaling terminates production of early-born fates and induces production of late born fates in three brain regions [17]. Abbreviations: 5HTN, serotonergic neuron; MN, motor neuron; OLP, oligodendrocyte precursor cell; OMN, oculomotor neuron; RNN, red nucleus neurons; SMN, somatic motor neuron.



Trends in Genetics

Figure 2. Temporal Patterning by Nutrition Cues in *Drosophila*. (A) Summary of neuroblast temporal patterning events from embryogenesis through the end of the neuroblast lineage in the early pupal stage. In the embryo, a neuroblast-intrinsic TTF cascade generates temporal patterning. In the early larvae, extrinsic nutritional

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enlargement and exit from quiescence requires multiple extrinsic signals; at least one activated by larval feeding. As the newly hatched larvae begin feeding on protein-rich food, amino acids are detected by the liver-like fat body via the amino acid transporter Slimfast (Slif), which in turn leads to a currently unknown fat-body-derived signal to the subperineurial glia that contact each neuroblast [34,35,39]. Consequently, glia secrete insulin/insulin-like growth factor (IGF)-like peptides (ILPs) that activate the neuroblast insulin receptor (InR), driving them out of quiescence [34,35]. More recently it has been shown that the subperineurial glial require calcium waves and the gap junction proteins Innexin 1 and 2 to trigger neuroblast reactivation [40,41], although the mechanism remains undefined.

In addition, nutritional cues and glia promote neuroblast enlargement and re-entry into the cell cycle by suppressing the Hippo pathway; a conserved pathway for inhibiting cell growth [42]. Both glia and neuroblasts express the cell surface proteins Crumbs and Echinoid, which are required to activate the Hippo pathway thereby keeping the Yorkie effector in the neuroblast cytoplasm and preventing neuroblast enlargement. Loss of Crumbs, Echinoid, or Hippo results in premature translocation of Yorkie into the neuroblast nucleus where it stimulates neuroblast growth and proliferation [43,44]. Finally, glia also secrete the Anachronism (Ana) protein, which is required to maintain neuroblast quiescence by an unknown mechanism [45]. Thus, multiple extrinsic cues converge on neuroblasts to regulate the precise timing of enlargement and exit from quiescence; it will be interesting to see if these pathways also regulate exit from quiescence of mammalian neural stem cells. In the future, it will be important to identify the fat-body-derived signal, and understand how glial calcium dynamics, gap junctions, and IGF, Hippo, and Ana signaling pathways are all integrated to regulate neuroblast quiescence.

Mushroom body neuroblasts generate three major classes of neurons: early-born γ neurons, middle-born α′/β′ neurons, and late-born α/β neurons [46]. Larvae fed a sucrose diet (no amino acids) show severe growth deficits, and as described above, most neuroblasts fail to exit quiescence. Yet, the four mushroom body neuroblasts continue dividing under these conditions; perhaps because they never entered quiescence in the embryo. Interestingly, under these conditions, the mushroom body neuroblasts continue making first-born γ neurons, make fewer later-born α′/β′ neurons, and fail to make the last-born α/β neurons [46]. Thus, nutritional cues are required for temporal patterning in larval mushroom body neuroblast lineages. It remains unknown how amino acid uptake is communicated to the mushroom body neuroblasts to trigger the early-late switch in neuronal identity.

Hormonal Cues Regulate Larval Neuroblast Temporal Identity

Embryonic neuroblasts use an intrinsic TTF cascade to generate neuronal diversity – this mechanism is ideally suited for rapid, invariant, short cell lineages of just 3–10 progenitor divisions [4]. In contrast, larval neuroblasts can divide >50 times over 120 h to generate hundreds of neurons and glia [47] – this likely requires a completely different temporal patterning mechanism, particularly to coordinate the timing of neuron production between different lineages, which might be important for neural circuit assembly. Indeed, work over the past decade has identified several genes with broad expression domains in early-born or late-born neurons [48–50], but few candidate TTFs expressed for just one or two cell divisions [51], as in the embryonic TTF cascade. For example, the Chinmo transcription factor, Lin-28 and Imp RNA-binding proteins are expressed in all early-born neurons (0–60 h after larval hatching,

cues generate the properly timed exit from quiescence. In the late larvae, extrinsic hormonal cues generate a switch from early candidate TTFs to late candidate TTFs). (B) The nutritional extrinsic signaling pathway that triggers neuroblast exit from quiescence. (C) Comparison of MB NB temporal pattering in the presence of dietary amino acids (top) or in the absence of dietary amino acids (bottom). Note the failure to undergo proper temporal patterning in the absence of extrinsic nutritional cues. Abbreviations: Crb, ; DILP6, Drosophila insulin-like peptide 6; Ed, Echinoid; FDS, Fat body Derived Signal; InR, insulin receptor; MB NB, mushroom body neuroblast; SLIF, Slimfast; SPG, Sub-perineurial glia; TOR, Target of Rapamycin; TTF, temporal transcription factor; Yki, Yorkie.

ALH), whereas the Broad transcription factor and Syncrip RNA-binding protein are expressed in all late-born neurons (60–120 h ALH) [48–50]. Two temporal windows are not sufficient to generate the known diversity of neurons made by each larval neuroblast [52–54]. To identify additional, novel candidate temporal factors expressed by larval neuroblasts, unbiased transcriptomic screens were performed [55,56]. Our screen identified the above-mentioned factors, plus additional factors including the ecdysone receptor, raising the possibility that the steroid hormone ecdysone may be used to generate temporal identity within larval neuroblasts (Figure 3).

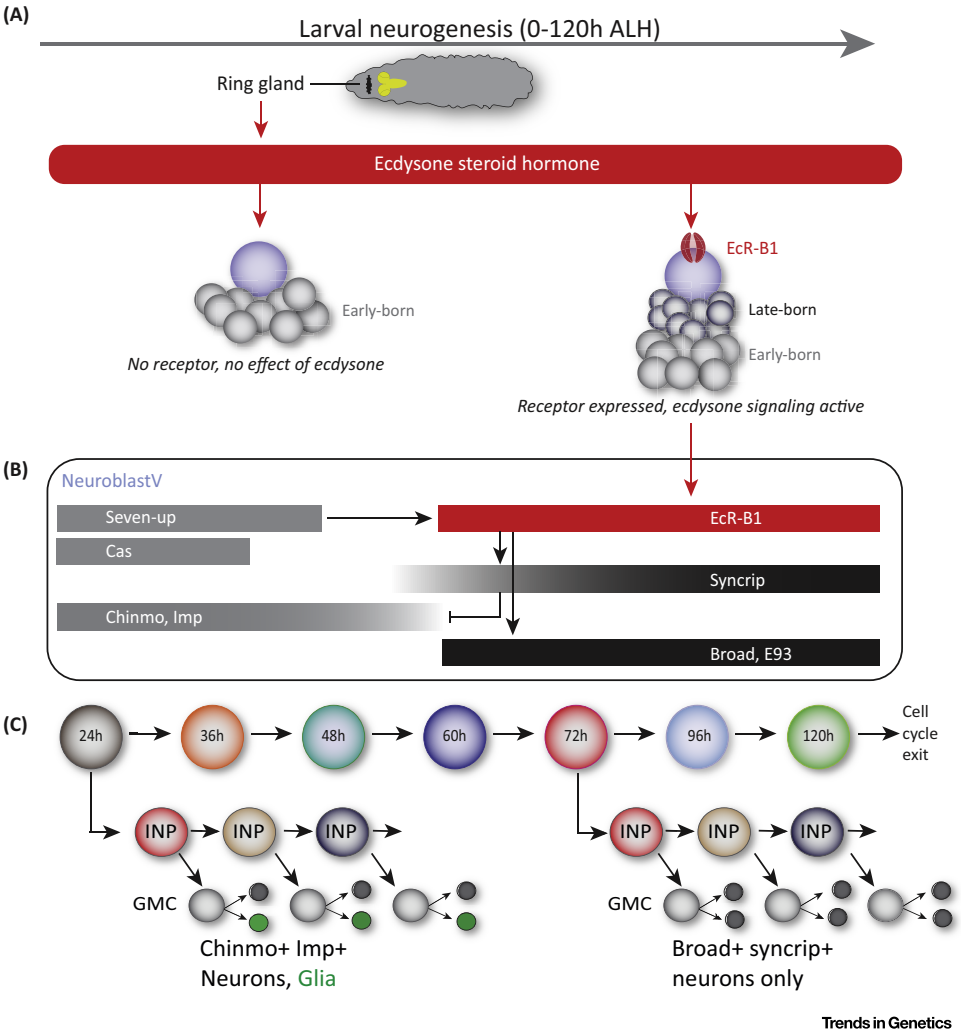

Figure 3. Temporal Patterning of Larval Neuroblasts by Hormonal Cues in *Drosophila*. Larval stages from 0 to 120 h ALH shown from left to right. (A) The non-neuronal ring gland releases the hormone ecdysone at all stages of larval life, but neuroblasts only respond to ecdysone after they express EcR-B1 at ~60 h ALH. (B) Simplified diagram of candidate TTFs expressed by larval central brain neuroblasts. Grey and red, TTFs not requiring ecdysone for their expression. Black, TTFs requiring ecdysone for their expression. (C) Type II neuroblasts at the beginning of their larval lineages make INPs that produce both neurons and glia and express Chinmo and Imp; type II neuroblasts later in their lineage produce INPs that generate neurons only and express Broad and Syncrip – this transition illustrates how the type II neuroblasts change over time to generate neural diversity. INPs undergo temporal patterning over time (color coded). Abbreviations: ALH, after larval hatching; EcR-B1, ecdysone receptor B1; GMC, ganglion mother cell; INP, intermediate neural progenitor; TTF, temporal transcription factor.

The steroid hormone ecdysone signaling pathway governs many developmental transitions in the *Drosophila* life cycle: three small pulses of ecdysone direct each of the larval molts (L1, L2, and L3) and a large pulse during the pupal stage is required for metamorphosis [57]. Ecdysone is produced by the prothoracic gland (outside the CNS) and acts systemically throughout the animal, via binding cell type specific Ecdysone receptor (EcR) isoforms. We found that the EcR-B1 isoform was temporally expressed in most or all larval central brain neuroblasts from ~60 h ALH onwards [56]. Interestingly, most other temporal factors were expressed fully before or after this midlarval timepoint: young neuroblasts expressed Castor, Seven-up, Chinmo, Imp, and Lin-28, whereas old neuroblasts expressed EcRB1, Broad, E93, and Syncrip; this raises the hypothesis that ecdysone signaling could induce a major temporal gene expression transition in larval neuroblasts. To test this hypothesis, we took three different approaches. First, we found that ecd^{ts} mutant larvae, lacking the ability to synthesize ecdysone, had neuroblasts with defects in the early-late gene expression switch: they maintained early factors Chinmo and Imp and showed a delay or absence of late factors Broad, Syp, and E93. Second, we cultured larval brains from 48 to 72 h ALH with and without exogenously added ecdysone: brains cultured without ecdysone failed to make the early–late temporal factor switch, but adding ecdysone allowed normal switching to occur in these cultures. Importantly, EcR-B1 was expressed normally at 60 h ALH, ruling out a general developmental delay. Third, we expressed an EcR dominant negative protein specifically in larval neuroblasts, which delayed the early–late gene expression switch. This experiment also shows that failure to make the neuroblast gene expression switch is not due to an indirect effect on organismal growth or development, but rather a neuroblast-autonomous effect of ecdysone signaling [56]. As expected, loss of ecdysone signaling had no effect on the timing of EcR expression. Rather, EcR expression was fully dependent on the prior expression of the early temporal factor, Seven-up; an orphan nuclear hormone receptor [56]. Loss of Seven-up not only completely prevented EcR expression, but all late temporal factor expression, with continued expression of early genes such as Imp, Lin-28, and Chinmo [49,55,56]. It is likely that the difference in phenotypes between loss of Seven-up and manipulation of ecdysone is due to the fact that we were unable to remove all ecdysone signaling. Even in the case of the dominant-negative EcR, if enough ecdysone is present, it is possible that the early to late switch could eventually occur. Because Seven-up is required for EcR expression, the only manipulation that definitively removes all ecdysone signaling from neuroblasts is loss of Seven-up. It is also possible, however, that redundant pathways exist that induce the early to late gene switch, and these pathways are both Seven-up dependent and ecdysone independent. Cell-type-specific removal of EcR would be a good way to distinguish between these possibilities.

Ecdysone is required for a distinct late temporal step: the termination of larval neuroblast lineages in early pupal stages. Ecdysone acts together with the Mediator complex and Syncrip to promote neuroblast size reduction, nuclear Prospero localization, and terminal differentiation [36,58]. This late event also requires Hedgehog signaling [59], and downregulation of Imp [60]. The detailed mechanism of ecdysone, Mediator, and Hedgehog signaling remains to be determined.

Hormonal Cues Regulate Neuronal Temporal Identity

Drosophila mushroom body neuroblasts display the longest phase of neurogenesis, beginning their lineage during embryogenesis and continuing until late pupal stages. They sequentially produce three types of neurons; γ, α′/β′, and α/β neurons, each with a unique projection pattern [61,62]. Early studies showed that the transcription factor Chinmo is detected in a high to low gradient in early-born to late-born neurons, and is required for specification of early-born γ and α′/β′ neuron identity [63]. Thus, establishing the gradient of Chinmo is essential to generate neuronal diversity in mushroom body neurons – how is the Chinmo gradient established? Recent studies from the Lee laboratory have shown that mushroom body neuroblasts

generate opposing temporal gradients of Imp and Syncrip RNA-binding proteins – Imp highest early and Syncrip highest late, and Syncrip is required to repress Chinmo expression at the post-transcriptional level in late-born neurons [48,55]. In addition, the miRNA let-7 is also expressed in a temporal gradient, with the highest levels in late-born neurons, where it represses Chinmo expression [64]; thus, both let-7 and Syncrip are required to keep Chinmo levels low in late-born neurons. Pushing back the question, what creates the late temporally restricted let-7 expression? Recent work has shown that it is the steroid hormone ecdysone: reduced ecdysone levels eliminate let-7 expression in late-born mushroom body neurons, and the α'/β' to α/β fate transition is abrogated [64–66]. Ecdysone regulates α/β identity cell autonomously, as blocking of ecdysone signaling by expressing an EcR dominant negative transgene or an EcR RNAi transgene in these neurons generated a similar failure in α'/β' to α/β fate transition [65]. Thus, ecdysone can act to regulate neuroblast temporal patterning (see above) as well as directly regulating postmitotic neuronal temporal identity.

Concluding Remarks

Interestingly, all known extrinsic cues regulating temporal transitions in proliferation or neuronal identity occur in larvae, and not embryos. The short length of embryogenesis, less than 1 day, may require neuroblast-intrinsic 'hard-wired' temporal patterning mechanisms to produce the correct number and type of neurons in a short time interval. In contrast, the relatively long length of larval stages (5 days) and complexity of developmental events (larval molts, initiation of metamorphosis) may require an organismal cue, such as the steroid hormone ecdysone or nutrition, to coordinate neurogenesis with growth and remodeling of non-neural tissues. This may explain why the first examples of extrinsic control of neuronal temporal patterning in *Drosophila* are active during larval neurogenesis.

It has long been known that some aspects of vertebrate temporal patterning rely on extrinsic cues, but only recently are these cues being elucidated. In contrast, it has long been thought that *Drosophila* temporal patterning programs are cell intrinsic. The papers covered here demonstrate a shift in our understanding of *Drosophila* temporal patterning, and offer exciting parallels to vertebrate temporal patterning. There are many interesting questions remaining, however: see Outstanding Questions. For example, it will be interesting to explore the similarities between hormonal regulation of temporal identity in *Drosophila* neural progenitors and thyroid hormone regulation of temporal patterning in vertebrates. Both ecdysone and thyroid hormone pathways are known to regulate neural progenitor proliferation and neuronal specification [57,67–69], and it would be exciting to test whether thyroid hormone also regulates neural progenitor temporal patterning.

Outstanding Questions

Do all temporally expressed transcription factors act as true TTFs to specify neuronal identity based on birth-order? To date nearly every candidate TTF has been shown to specify neuronal temporal identity when assayed, but many new candidate TTFs remain to be tested.

How do TTFs specify neuronal identity? In some cases they may act as simple transcription factors that regulate gene expression (perhaps for neuroblast-intrinsic cascades); in other cases they may regulate the competence to respond to an extrinsic cue, such as the role of the EcR in allowing a mid-larval response to ecdysone. We note that the late candidate TTF E93 has been show to regulate cell competence to in other cell types [73,74], perhaps it acts in this way in neuroblasts as well.

What are the downstream targets of the TTFs? This has begun to be dissected in the late embryonic neuroblast cascade, in elegant work from the Thor lab [75–77], and in optic lobe neuroblasts in recent work from the Desplan lab [31,32]. But in most cases the downstream effectors of TTFs remain uncharted.

How are spatial and temporal factors integrated to specify unique neuronal identity? For example, the TTF Hunchback specifies a first-born motor neuron in the embryonic neuroblast 7–1 lineage, but a first-born serotonergic interneuron in the adjacent neuroblast 7–3 lineage [23]. Similarly, each of the eight type II neuroblasts in the larval brain generates a distinct clone of neurons, despite progressing through the same TTF cascade.

To what degree does cell cycle progression regulate TTF expression? In embryonic neuroblasts, most of the TTF transitions can occur in G2-arrested neuroblasts [26]. Is this a general property of neuroblast TTF cascades or do larval neuroblasts in the VNC, central brain, or optic lobe use a different mechanism?

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