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Temporal Patterning in the *Drosophila* CNS

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Abstract

A small pool of neural progenitors generates the vast diversity of cell types in the CNS. Spatial patterning specifies progenitor identity, followed by temporal patterning within progenitor lineages to expand neural diversity. Recent work has shown that in *Drosophila*, all neural progenitors (neuroblasts) sequentially express temporal transcription factors (TTFs) that generate molecular and cellular diversity. Embryonic neuroblasts use a lineage-intrinsic cascade of five TTFs that switch nearly every neuroblast cell division; larval optic lobe neuroblasts also use a rapid cascade of five TTFs, but the factors are completely different. In contrast, larval central brain neuroblasts undergo a major molecular transition midway through larval life, and this transition is regulated by a lineage-extrinsic cue (ecdysone hormone signaling). Overall, every neuroblast lineage uses a TTF cascade to generate diversity, illustrating the widespread importance of temporal patterning.

Contents

INTRODUCTION

In this review I focus on temporal patterning in *Drosophila*, for which there has been rapid progress over the past few years. In fact, the last Annual Reviews article on this topic (Pearson & Doe 2004) covered only 5 *Drosophila* temporal identity papers describing two embryonic neuroblast lineages; the current review covers more than 50 *Drosophila* temporal identity papers on neuroblast lineages in every region of the CNS and every stage of neurogenesis.

TYPES OF NEURAL STEM CELLS (NEUROBLASTS)

Drosophila neural stem cells [neuroblasts (NBs)] form in several regions of the CNS: Thoracic and abdominal NBs delaminate from embryonic neuroectoderm of the ventral nerve cord (VNC), central brain NBs delaminate from the embryonic procephalic neuroectoderm, and optic lobe NBs segregate from the edge of an optic lobe epithelium during larval stages—the only NBs

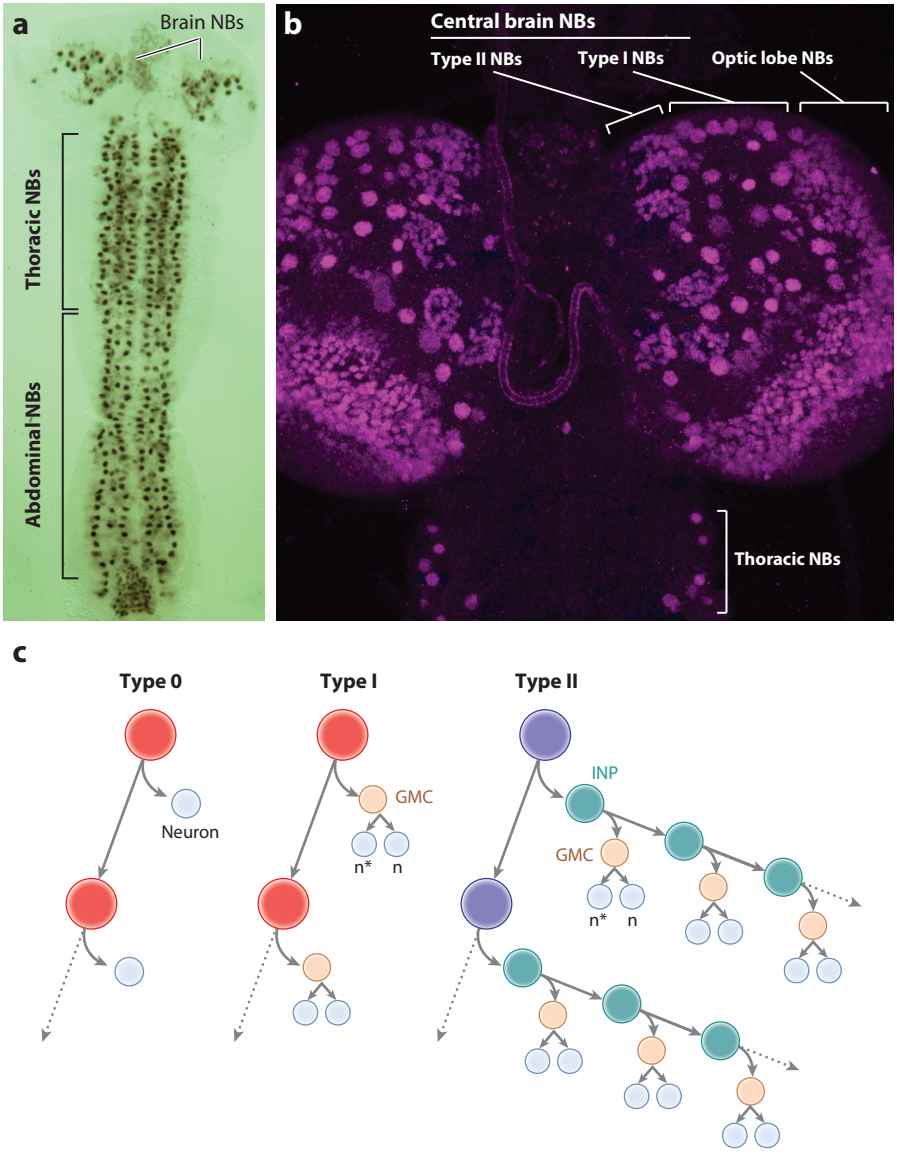

Figure 1

(*a*) Stage 10 embryo flat mounted and stained for Snail protein to identify brain, thorax, and abdominal neuroblasts (NBs). (*b*) Third-instar larval brain and thoracic CNS stained for Deadpan to identify the indicated pools of NBs. (*c*) Three modes of NB cell division. The largest cells are NBs. Abbreviations: GMC, ganglion mother cell; INP, intermediate neural progenitor; n, neurons [all sibling neurons are either $Notch^{ON}$ (n*) or $Notch^{OFF}$ (n)].

formed postembryonically (**Figure 1*a*,*b***). In the VNC there are 30 NBs per bilateral hemisegment, arranged in rows and columns, giving rise to their row/column naming scheme (e.g., NB7-1 is in row 7, column 1) (Broadus et al. 1995, Hartenstein et al. 1994). There are ~105 central brain NBs per brain lobe (Ito et al. 2013, Urbach & Technau 2003, Yu et al. 2013) and more than 800 optic lobe NBs per brain lobe (Bertet et al. 2014, Li et al. 2013, Yasugi et al. 2008). Each NB

contributes a stereotyped family of neurons and glia to the CNS (Bossing et al. 1996, Ito et al. 2013, Schmid et al. 1999, Schmidt et al. 1997, Yu et al. 2013).

All *Drosophila* NBs undergo molecularly asymmetric cell divisions to generate a series of smaller progeny, using three modes of division (**Figure 1*c***). Type 0 NBs produce progeny that directly differentiate as a neuron; this division mode is seen in late-embryonic NB lineages (Baumgardt et al. 2014) and early optic lobe NB lineages (Bertet et al. 2014). Type I NBs produce ganglion mother cells (GMCs) that divide once to form a pair of sibling neurons; this is the most common division mode, and it is seen in early-embryonic NBs, most central brain NBs, and late optic lobe NB lineages. Type II NBs produce intermediate neural progenitors (INPs), each of which undergoes a series of molecularly asymmetric cell divisions to produce 4–6 GMCs and subsequent sibling neurons (Bello et al. 2008, Boone & Doe 2008, Bowman et al. 2008). This division mode is seen in just six dorsal medial (DM1–6) and two dorsal lateral (DL1 and DL2) central brain NBs.

EMBRYONIC NEUROBLASTS

Temporal transcription factors (TTFs) were first characterized in embryonic NBs of the VNC, and it remains the system in which the most is known about TTF regulation and function. To date, temporal patterning has been studied in 8 of the 30 VNC NBs (**Figure 2**).

Discovery of Temporal Transcription Factors

Embryonic VNC NBs sequentially express the transcription factors Hunchback (Hb) (zinc finger Ikaros family), Nubbin/Pdm2 (Pdm) (POU domain family), and Castor (Cas) (zinc finger Casz1 family) (Kambadur et al. 1998). These three candidate TTFs are detected in layers within the postmitotic neurons of the late embryonic CNS—Hb in the deepest, first-born neurons; Cas in the most superficial, late-born neurons; and Pdm in between—suggesting that neurons maintained the TF present at their birth. The discovery of molecularly distinct layers of neurons within the *Drosophila* CNS was surprisingly reminiscent of laminar gene expression in the mammalian cortex (Greig et al. 2013). Subsequently, two additional candidate TTFs were identified: Krüppel (Kr) (zinc finger Krüppel-related family), which shows high-level expression following Hb (Isshiki et al. 2001), and Grainy head (Grh) (CP2 domain family), which follows Cas (Brody & Odenwald 2000) and persists in all larval NBs, but not in their progeny (Almeida & Bray 2005, Cenci & Gould 2005). These pioneering descriptive studies were shortly followed by functional studies showing that Hb and Kr are necessary and sufficient for the specification of temporal identity in the NB7-1 and NB7-3 lineages and that Hb specified first-born temporal identity in three other lineages (Isshiki et al. 2001), identifying the first TTFs.

Temporal Transcription Factors

Hb is the first TTF expressed by most VNC NBs and specifies early-born temporal identity in all lineages assayed to date (NB7-1, NB7-3, NB1-1, NB4-2, NB6-4, NB3-1, NB3-5) (Isshiki et al. 2001, Moris-Sanz et al. 2014, Novotny et al. 2002, Pearson & Doe 2003, Tran & Doe 2008) (**Figure 2**). Importantly, in all these lineages, Hb misexpression results in ectopic neurons with first-born identity, although with varying degrees of penetrance, e.g., ≥5 ectopic U1 motor neurons in the NB7-1 lineage but only 1–2 ectopic aCC/pCC (first-born) neurons in the NB1-1 lineage (Isshiki et al. 2001, Novotny et al. 2002). These differences in response to ectopic Hb highlight the role of spatial identity in sculpting the response to TTFs in each lineage (see below). Some NB lineages generate two or three GMCs during the Hb expression window (**Figure 2**),

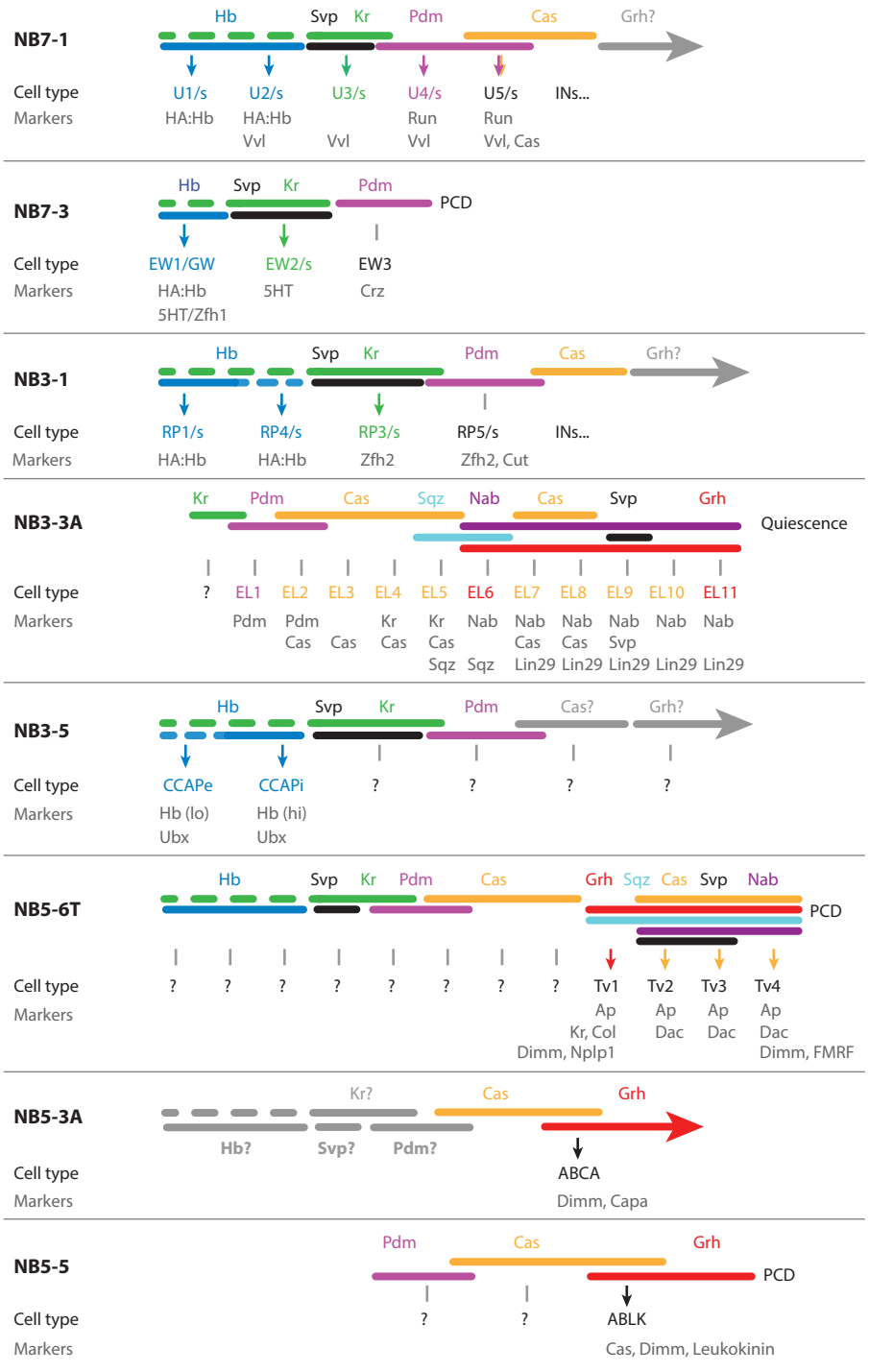

Figure 2

Temporal patterning in embryonic neuroblasts. The horizontal lines represent gene expression over time (early to late from *left* to *right*); the dashed lines represent lower-level expression. The "Cell type" rows indicate the neuron(s) born from each ganglion mother cell (GMC). The "Markers" rows indicate the molecular markers that distinguish neurons within a lineage. Abbreviations: INs, interneurons of unknown identity; PCD, programmed cell death; s, sibling cell of unknown identity. Citations to expression data are given in the text.

and the level of Hb distinguishes GMC identity (Baumgardt et al. 2009, Isshiki et al. 2001, Moris-Sanz et al. 2014, Novotny et al. 2002, Pearson & Doe 2003, Tran & Doe 2008). In contrast, a few late-forming NBs never express Hb, starting their TTF cascade at Kr (NB3-3), Pdm (NB5-5), or Cas (NB6-1) (Benito-Sipos et al. 2010, Cui & Doe 1992, Tsuji et al. 2008).

Kr is the second TTF expressed by most VNC NBs and specifies temporal identity in all three lineages assayed to date (NB7-1, NB7-3, NB3-1) (Cleary & Doe 2006, Isshiki et al. 2001, Tran & Doe 2008) (**Figure 2**). NBs lacking Kr fail to make neurons normally born from the Kr expression window (U3/sib, EW2, and RP3), and misexpression of Kr in these lineages generates ectopic U3/sib, EW2, and RP3 neurons (Cleary & Doe 2006, Isshiki et al. 2001, Kanai et al. 2005, Tran & Doe 2008). Notably, misexpression of Kr does not alter the first-born Hb^+ cell fates. Kr is also required in the brain anterodorsal (AD, also termed ALad1) NB during the embryonic portion of its lineage. The AD NB generates 18 different projection neuronal subtypes during its embryonic lineage and an additional 22 neuronal subtypes during its larval lineage (Kao et al. 2012) (**Figure 3**). *Kr* mutant clones generated in the AD NB result in a highly specific loss of the eleventh fate—the VA7l neuron—whereas clones generated in the GMC precursor to this neuron show a transformation of the eleventh fate to the twelfth fate (VA7l to VA2) (Kao et al. 2012). These data are consistent with Kr acting as a NB temporal identity factor required specifically for the eleventh fate in the embryonic AD NB lineage and lead to the prediction that Kr should be transiently expressed in the AD NB at the time the eleventh GMC is produced.

Pdm is the third candidate TTF expressed by most VNC NBs (Pdm refers to the redundant and coexpressed Nubbin and Pdm2 proteins). In the NB7-1 lineage, the Pdm NB expression window gives rise to the Pdm^+ U4 motor neuron and the Pdm^+ Cas^+ U5 motor neuron (Grosskortenhaus et al. 2006). Loss of Pdm eliminates U4/U5 cell fates, and misexpression of Pdm generates ectopic U5 fates due to a prolonged Pdm/Cas coexpression window (Grosskortenhaus et al. 2006), consistent with Pdm acting as a TTF in this lineage. In contrast, NB3-1 sequentially generates the Hb^+ RP1 and RP4, Kr^+ RP3, and Pdm^+ Cut^+ RP5 ventral-projecting motor neurons (Tran & Doe 2008) (**Figure 2**). In this lineage, Pdm is not required for specifying the Cut^+ RP5 identity, nor can misexpression of Pdm generate ectopic Cut^+ RP5 motor neurons (Tran & Doe 2008). Thus, Pdm has TTF function in NB7-1, but not in NB3-1. Analysis of Pdm function in additional lineages will be necessary to determine whether it is a true multilineage TTF.

Cas and Grh regulate temporal identity in six different NBs. The best characterized is NB5-6T, which has two Cas expression windows; immediately after the first Cas window, NB5-6T switches to a type 0 division mode and undergoes four divisions to produce the four Apterous $(Ap)^+$ neurons Tv1–Tv4, also termed Ap1–Ap4 (Baumgardt et al. 2009, Stratmann et al. 2016). Tv1 is made between the two Cas windows, with Tv2–Tv4 made during the second Cas window (which is Cas^+ Grh^+) (**Figure 2**). Loss of Cas eliminates Grh expression and misspecifies all four Tv neurons (the loss of the Cas^- Tv1 neuron is likely due to the loss of the preceding Cas window and extended Pdm expression). Loss of Grh results in persistent Cas expression, in the production of ectopic Tv2/Tv3 neurons, and in failure to specify Tv4 identity; conversely, Grh misexpression produces ectopic Tv4 neurons (Baumgardt et al. 2009). Thus, both Cas and Grh specify late-born neuronal identity in the NB5-6T lineage. Additional subtemporal factors are required to distinguish Tv1 from Tv2/3 neurons (see the next section titled Subtemporal Transcription Factors).

Cas and Grh also specify temporal identity in several other partially characterized NB lineages. NB5-5 generates a single ABLK (abdominal leukokinin) neuron. *pdm* mutants have normal ABLK neuron specification, whereas *cas* or *grh* mutants lack the ABLK neuron, and Cas misexpression produces ectopic ABLK neurons (Benito-Sipos et al. 2010). Thus, ABLK likely forms during the Cas/Grh expression window (**Figure 2**). Similar results have been reported for the NB3-3, NB7-1,

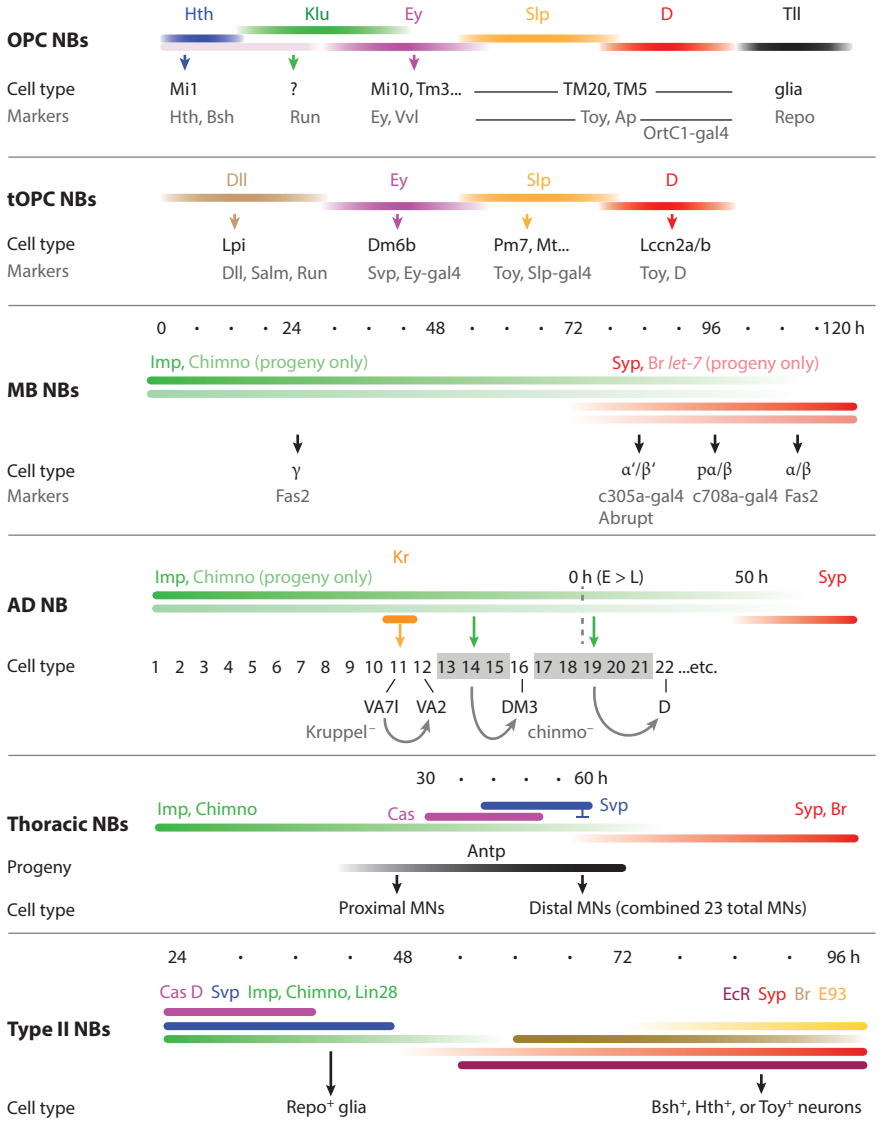

Figure 3

Temporal patterning in larval neuroblasts (NBs). Where known, hours after larval hatching are shown at the top. The lines at the top of each panel represent gene expression over time (early to late from *left* to *right*). The identity of each line is color coded with the gene name above [for example, in the mushroom body (MB) NB panel, Imp is shown in *dark green*, whereas Chinmo is shown in *light green*]. In the indicated NB population, line gradients reflect approximate expression gradients. Downward arrows indicate that the NB temporal factor is required to specify a particular neuronal identity, lack of an arrow indicates a correlation only between NB factor expression and a specific neuronal identity, a T bar indicates an inhibitory relationship, and curved upward arrows in the anterodorsal (AD) NB panel indicate the cell fate transformations occurring in the absence of Kr or Chinmo. In the AD NB panel, E denotes embryo, and L denotes larva. The "Cell type" row indicates the neuron(s) born during each expression window. The "Markers" row indicates the molecular markers that distinguish neurons within a lineage. The "Progeny" row in the thoracic NB panel indicates temporal expression in NB progeny. Abbreviations: MNs, motor neurons; OPC, outer proliferation center; tOPC, tip OPC.

and NB5-3 lineages (Gabilondo et al. 2011, Grosskortenhaus et al. 2006, Lai & Doe 2014, Tsuji et al. 2008) (**Figure 2**). In conclusion, Cas and Grh are multilineage TTFs that specify late-born neuronal identity within the VNC and brain. This is not the only CNS function of Grh, however, as Grh expression is maintained throughout larval NB life, during which it promotes cell cycle progression and survival (Cenci & Gould 2005).

Subtemporal Transcription Factors

The experiments described above for Cas and Grh in the NB5-6 lineage fail to explain how Tv1 and Tv2/3 are distinguished. The Squeeze and Nab subtemporal transcription factors further partition the Cas expression window to distinguish Tv1 from Tv2/3 (Baumgardt et al. 2009). Squeeze is expressed during the production of all four Tv neurons, but its binding partner Nab is expressed only during production of Tv2–Tv4. The Nab/Squeeze complex is required to repress Collier, which is a Tv1 determinant, thereby limiting production of Col^+ Tv1 neurons (Baumgardt et al. 2009). Similarly, *nab* or *squeeze* mutants fail to specify neuronal fates during the Cas expression window in the NB3-3 lineage (Tsuji et al. 2008). The role of Nab and Squeeze in other NBs remains to be tested, but they are widely expressed during the Cas expression window (Clements et al. 2003) and are likely to function widely as subtemporal transcription factors in many NB lineages.

Temporal Transcription Factor Timing

Most embryonic NBs express the full series of Hb → Kr → Pdm → Cas → Grh TTFs. What drives the unidirectional cascade of TTF gene expression? Analysis of TTF single or double mutants shows that the absence of one or two TTFs does not prevent progression of the cascade (Benito-Sipos et al. 2010, 2011; Cleary & Doe 2006; Grosskortenhaus et al. 2005, 2006; Isshiki et al. 2001; Kanai et al. 2005; Mettler et al. 2006; Novotny et al. 2002; Tran & Doe 2008; Urban & Mettler 2006). The essential transcriptional activators for each gene remain unknown. In contrast, misexpression studies reveal robust feedback repression: Kr is repressed by Pdm in some but not all lineages (Grosskortenhaus et al. 2006, Tran & Doe 2008), Pdm is directly repressed by Cas (Grosskortenhaus et al. 2006, Kambadur et al. 1998, Tran & Doe 2008, Tsuji et al. 2008), and Cas is repressed by Grh (Baumgardt et al. 2009). This leads to a model in which general activation coupled with feedback repression generates the observed TTF cascade. In the optic lobe NB lineages, however, removing most TTFs causes the cascade to remain stuck in the previous temporal window (Li et al. 2013), described in more detail below.

Downstream of Temporal Transcription Factors

Recent work has shown that the TTF Cas is required for expression of the Kr transcription factor in the postmitotic Tv1 neuron, whereas the subtemporal transcription factors Squeeze and Nab prevent Kr expression in postmitotic Tv2–Tv4 neurons; *cas* mutants also have a broader loss of Kr in postmitotic neurons of unknown lineages (Stratmann et al. 2016). Moreover, Kr is necessary for Tv1 specification, and ectopic Kr can transform Tv2–Tv4 neurons into a Tv1 identity (Stratmann et al. 2016). Thus, Kr has dual roles: In its early NB expression window, it functions as a classical TTF, and in the postmitotic Tv1 neuron, it specifies neuronal identity. In addition, the Thor lab has described a feed-forward transcriptional regulatory cascade linking Cas and Grh TTFs to the expression of the neuropeptides Nplp1 and FMRFamide (reviewed in Allan & Thor 2015). Although many other genes are expressed in neurons of a known birth order (see **Figure 2**),

the transcriptional regulatory logic from TTF to postmitotic neuron expression remains mostly unknown. Detailed studies of TTF-to-neuronal regulatory logic, similar to those of the Thor lab on NB5-6, are needed in most other lineages.

Neuroblast Competence

NBs gradually lose competence to generate early-born neurons in response to Hb or Kr in the NB. I only briefly cover this topic because it was recently reviewed (Kohwi & Doe 2013). Early in NB lineages, a pulse of Hb or Kr generates ectopic early-born neurons, but competence to respond is lost in older NBs (Cleary & Doe 2006, Kohwi et al. 2013, Pearson & Doe 2003, Touma et al. 2012, Tran & Doe 2008). Loss of competence to respond to Hb is limited by movement of the *hb* locus to the nuclear lamina (Kohwi et al. 2013), whereas loss of competence to respond to Kr is due to Polycomb repressive complex 1/2 function (Touma et al. 2012). Loss of competence to respond to an early TTF has also been reported in optic lobe NBs (Li et al. 2013).

Open Questions

- What activates embryonic TTF expression? The identification of *cis*-regulatory modules that confer proper temporal expression has begun (Hirono et al. 2012, Kuzin et al. 2012, Ross et al. 2015) and should help answer this question.
- What are the TTF target genes? Are they different in each NB due to prior activity of spatial factors, or are they the same in all NBs but act combinatorially with spatial factors to generate lineage-specific cell types?
- Do the TTFs function in the NB, GMC, or postmitotic neuron? Selective removal of the Hb TTF from postmitotic neurons has no effect (Hirono et al. 2017), and Hb misexpression in postmitotic neurons has no effect (Pearson & Doe 2003). These findings suggest that TTFs may act transiently in NBs or GMCs to specify heritable temporal identity.

LARVAL OPTIC LOBE NEUROBLASTS

Optic lobe NBs segregate from the optic lobe epithelium in waves beginning in second-instar larvae; thus, they undergo their lineages asynchronously, with young NBs adjacent to the epithelium and older NBs further distant (Egger et al. 2007, Li et al. 2013, Yasugi et al. 2008). There are two regions of the optic lobe: the outer proliferation center (OPC), which gives rise to the lamina and medulla, and the inner proliferation center (IPC), which gives rise to the lobula and lobula plate (Apitz & Salecker 2016, Meinertzhagen & Hanson 1993, Yasugi et al. 2008). Here I focus on the optic lobe NBs of the OPC.

Temporal Transcription Factors

To identify candidate TTFs in optic lobe NBs, the Desplan and Sato labs exploited the fact that all stages of the NB lineage can be observed in a single brain. Each lab identified candidate TTFs with sequential but overlapping expression in NBs as they age from young to old (Li et al. 2013, Suzuki et al. 2013): Homothorax (Hth) → Klumpfuss (Klu) → Eyeless (Ey) → Sloppy paired 1 and 2 (Slp1 and Slp2) → Dichaete (D) → Tailless (Tll) (**Figure 3**). Each of these transcription factors is sequentially expressed in all OPC NBs, except those at the tips of the OPC (see below). Cross-regulation of these factors shows that Hth and Klu are regulated independently of the other factors, whereas the remaining factors exhibit feed-forward activation with feedback repression at

each step of the cascade. Thus, single-gene mutants for the latter four factors result in a stall in the progression of the cascade (Li et al. 2013, Suzuki et al. 2013). Loss-of-function and misexpression experiments confirm that Hth, Ey, and Slp are true TTFs. First, brain-specific homeobox (Bsh)$^+$ neurons born during the Hth expression window are reduced in *hth* mutant clones and are increased by continuous Hth expression in the lineage (Li et al. 2013, Suzuki et al. 2013). Second, extending Klu expression generates ectopic Runt$^+$ neurons, which are likely to derive from the Klu NB expression window (Suzuki et al. 2013), but *Klu* mutant clones have an NB tumor phenotype, precluding analysis of the role of Klu in temporal patterning. Third, Drifter/Vvl$^+$ neurons, born during the Ey window of expression, are reduced following Ey RNAi and are increased following extension of Ey NB expression in *slp* mutants (Li et al. 2013). Fourth, OrtC1-gal4$^+$ neurons born during the Slp expression window are lost in *slp* mutant clones and are increased following continuous Slp expression in the lineage (Li et al. 2013).

It was recently reported that this TTF cascade is modified at the lateral tips of the OPC. The tip OPC (tOPC) is defined by the expression of Wingless in the neuroepithelial cells, and in this region the NBs perform a Distalless (Dll) → Ey → Slp → D cascade, using Dll instead of Hth as the first factor and dropping the Tll expression window (Bertet et al. 2014) (**Figure 3**). Cross-regulation studies match that of the main OPC cascade, with the first factor Dll being independently regulated and the final three factors undergoing feed-forward activation with feedback repression at each step (Bertet et al. 2014).

Downstream of Temporal Transcription Factors

Medulla NBs give rise to the well-characterized neurons of the adult visual system, such as the medulla intrinsic neuron Mi1 and the transmedulla neuron Tm3, that process visual input (Maisak et al. 2013, Meier et al. 2014, Serbe et al. 2016). Recent work has identified a number of transcription factors acting downstream of the central optic lobe NB TTFs (Li et al. 2013). For example, the early TTF Hth is required to generate Bsh$^+$ Ap$^+$ neurons, and Bsh is necessary to produce the adult Mi1 neuron. Similarly, the late TTF Ey is required to produce Drifter/Vvl$^+$ Ap$^+$ neurons, and Vvl is required for the formation of 10 different neuronal subtypes, including Tm3. Finally, the TTFs Slp and D are required to generate Toy$^+$ Ap$^+$ OrtC1-gal4$^+$ neurons that include Tm20 and Tm5. Taken together, this body of work identifies several transcriptional targets of the optic lobe NB TTF cascade and links neuronal birth order to the specification of physiologically and functionally defined adult neurons.

To identify the mature neurons born from the tOPC NBs, Bertet et al. (2014) used a clever genetic trick, termed FLEXAMP, to permanently mark all wingless-gal4$^+$ neuroepithelial cells in the tOPC domains. They then used hundreds of antibodies to transcription factors to subdivide these neurons and discovered just four neural subtypes, each produced during one of the four TTF windows (Bertet et al. 2014). The Dll window generated Salm$^+$ Runt$^+$ neurons, the Ey window generated Seven-up (Svp)$^+$ neurons, the Slp window produced Toy$^+$ neurons (these were labeled with Slp-gal4 driving GFP expression, which persists longer than native Slp protein), and the D window also produced neurons that were Toy$^+$ but lacking Slp-gal4 expression (Bertet et al. 2014). Interestingly, the candidate TTFs do not appear to be required for specifying the identity of neurons born during their own window of expression, as expected, but rather they are required for the production of neurons in the subsequent TTF expression window. For example, *dll* mutants have no effect on the Salm$^+$ Runt$^+$ neurons made during the Dll expression window but instead show a decrease in the number of Svp$^+$ neurons made in the subsequent Ey window. Similarly, Ey is required not for Svp$^+$ neuron specification, but for the Toy$^+$ neurons born during the subsequent Slp expression window (Bertet et al. 2014). These unexpected results may be due

to persistent expression of each TTF into the subsequent TTF expression domain—e.g., low levels of Dll are coexpressed with Ey, and low levels of Ey are coexpressed with Slp—and perhaps these combinations are what specify neuronal identity. Nevertheless, each tested TTF (with the exception of D) is required for proper neuronal specification, thereby authenticating Dll, Ey, and Slp as true multilineage TTFs.

Open Questions

- How does spatial patterning of the OPC lead to two different TTF cascades: one starting with Hth in the main OPC and one starting with Dll in the tOPC? The answer is not as simple as Dll repressing Hth, because *dll* mutants do not upregulate Hth expression (Bertet et al. 2014).
- Is the medulla TTF cascade NB intrinsic (can it occur in isolated, cultured NBs), similar to the VNC NB cascade (Grosskortenhaus et al. 2005), or does it require NB-, lineage-, or brain-extrinsic cues, similar to late-larval type II NBs (Syed et al. 2017)?
- What are the direct targets of the optic lobe NB TTFs? Do these targets directly regulate mature neuronal properties such as arbor morphology and neurotransmitter choice, or do they act through an intermediate tier of transcription factors, such as the morphology TFs that regulate aspects of leg motor neuron identity (Enriquez et al. 2015)?

CENTRAL BRAIN TYPE I NEUROBLASTS

Central brain and VNC larval NBs are born in the embryo and persist into larval stages (Truman & Bate 1988). They can divide more than 50 times to generate >100 neurons during the 120 h of larval life (Ito et al. 2013, Yu et al. 2013). All larval NBs generate stereotyped clones of morphologically distinct neurons (Awasaki et al. 2014, Ito et al. 2013, Lee & Luo 1999, Yu et al. 2013) that arise, when tested, in an invariant birth order (Baek & Mann 2009, Jefferis et al. 2001, Kao et al. 2012). Thus, there must be a mechanism to reliably specify neuronal temporal identity over the length of these extended NB lineages. Here I discuss three larval NB lineages: the mushroom body (MB) NBs, which produce the Kenyon cells of the adult MB; the single AD (ALad1) NB, which generates olfactory projection neurons; and thoracic VNC NBs, including NB2-3T (also termed linA or lin15), which generate many of the adult leg motor neurons. All these larval NBs are characterized by two broad windows of TTF expression, subdividing the lineages into early and late components; a few more narrowly expressed factors create diversity within early and late neuronal populations (**Figure 3**).

Temporal Transcription Factors

Pcd6 is an embryonic brain NB located in the center of the brain NB array (Urbach & Technau 2003). It generates a pool of Cas$^+$ lateral horn leukokinin (LHLK) neurons. Although the LHLK neurons have not been lineage mapped, they require Cas, but not Hb or Grh, for their specification (Herrero et al. 2014). Some LHLK neurons may also derive from a Kr$^+$ window because they are decreased in *Kr* mutants and increased in *pdm* mutants (Herrero et al. 2014), consistent with *pdm* mutants having extended Kr expression in many lineages (Isshiki et al. 2001).

The best-characterized central brain larval TTFs are not transcription factors, but RNA-binding proteins that regulate gene expression posttranscriptionally. IGF-II mRNA-binding protein (Imp) and Syncrip (Syp) are expressed in complementary gradients: Young NBs have high Imp and low Syp expression, middle-aged NBs express both, and old NBs have low Imp and high Syp expression (Liu et al. 2015) (**Figure 3**). The timing of the Imp-to-Syp transition varies among

NBs (Liu et al. 2015, Ren & Lee 2017), suggesting that NB-intrinsic cues regulate the timing of the transition. The roles of Imp and Syp have been best characterized in MB NB lineages. MB NBs produce the Kenyon cells required for olfactory learning; there are at least four neural subtypes sequentially generated: γ, α′/β′, pioneer α/β, and α/β. *Imp* mutants show precocious Syp and an early-to-late transformation of neuronal identity, whereas *Syp* mutants have the reciprocal transformation (Liu et al. 2015). For example, Imp is required for specification of the early-born γ neuron identity, Syp is required for specification of the late-born α/β identity, and coexpression of Imp and Syp is required for intermediate α′/β′ neuronal identity (Liu et al. 2015). Imp and Syp have a related function within the AD NB lineage, which contains ~60 neurons of 22 different subtypes. *Imp* RNAi leads to precocious formation of late neuronal identities, whereas *Syp* RNAi extends the production of early temporal identities; this is consistent with a role in temporal patterning, although 21 of the 22 neuronal subtypes are still produced (Liu et al. 2015). Thus, in this lineage Imp and Syp act more like timers to control the pace of temporal identity specification. The transcription factors D and Cas are also expressed in many larval type I NB lineages (Maurange et al. 2008), but their role in specifying temporal identity remains unknown.

Downstream of Temporal Transcription Factors

Two transcription factors are temporally expressed in larval type I NB progeny: Chronologically inappropriate morphogenesis (Chinmo) is detected in neurons born from young NBs, whereas Broad is expressed in neurons born from old NBs; the patterns are virtually complementary (Maurange et al. 2008, Zhou et al. 2009). Despite their complementary expression in early-born versus late-born neurons, Chinmo and Broad do not cross-repress each other (Maurange et al. 2008, Syed et al. 2017, Zhou et al. 2009). What establishes the Chinmo/Broad expression windows? The early TTF Imp is required for Chinmo expression in early-born progeny, whereas the *let-7* complex of microRNAs blocks Chinmo translation in late-born neurons (Chawla & Sokol 2012; Kucherenko & Shcherbata 2013; Liu et al. 2015; Sempere et al. 2002, 2003; Wu et al. 2012). In addition, *let-7* restricts expression of the Abrupt transcription factor to the α′/β′ neurons, and Abrupt is required to specify α′/β′ neuronal identity (Kucherenko & Shcherbata 2013) (**Figure 3**).

What is the function of Chinmo in specifying neuronal temporal identity? Chinmo function has been tested in the MB and AD NB lineages. In the MB lineage, *chinmo* mutant clones lack the early-born γ and α′/β′ neuronal identity (Zhu et al. 2006). Chinmo is also required in the AD NB lineage, which generates 40 different projection neuron subtypes characterized by their unique innervation of antennal lobe glomeruli (Jefferis et al. 2001, Yu et al. 2010). *chinmo* mutant clones transform the thirteenth–fifteenth fates to the sixteenth fate (DM3 neuron) and transform the seventeenth–twenty-first fates to the twenty-second fate (D neuron) (Kao et al. 2012) (**Figure 3**). This phenotype may arise from the failure of graded Chinmo to specify distinct neuronal identities within two temporal windows, collapsing each of them into a single fate. For example, a DM3 identity factor could be expressed across the seventeenth–twenty-second divisions, with the Chinmo gradient being required to subdivide this window into six neuronal identities; in the absence of Chinmo, all neurons adopt the default DM3 fate. In this manner, each of the many successive multidivision temporal windows may use the Chinmo gradient to produce a diversity of neuronal cell types.

Although there are no proven TTFs in the thoracic NB2-3 lineage, I am including it here because of the elegant work on temporal expression of the HOX protein Antennapedia (Antp) in postmitotic motor neurons in this lineage. NB2-3T (Lacin & Truman 2016)—also termed linA or lin15 (Baek & Mann 2009, Brierley et al. 2012)—generates 29 morphologically distinct

motor neurons, each of which innervates a specific domain of adult leg muscles. These motor neurons have a stereotyped birth order between 22 and 66 h after larval hatching (ALH), probably falling into the Chinmo$^+$ neuronal window (**Figure 3**). Early-born motor neurons innervate more proximal muscles in each leg, and late-born motor neurons innervate more distal domains (Baek & Mann 2009, Brierley et al. 2012). What specifies these 29 motor neuron identities? The Mann lab showed that Antp is detected in a temporal gradient within the postmitotic motor neuron population: Early-born proximal-projecting neurons have low Antp levels, whereas late-born distal-projecting neurons have high Antp levels (early- and late-born neurons were identified by their distance away from the NB) (Baek et al. 2013). Misexpression of high levels of Antp in all motor neurons generated neurons innervating distal targets at the expense of neurons innervating proximal targets, and Antp RNAi in all motor neurons gave the opposite phenotype (Baek et al. 2013). Thus, Antp is a presumed target of NB TTFs and is essential for generating adult motor neuron diversity.

Open Questions

- What is the relationship between Imp, Chinmo, and Antp in specifying motor neuron identity?
- Does Antp specify neuronal temporal identity in other larval NB lineages (whether they make motor neurons or interneurons)?
- What are the Imp and Syp target genes in addition to Chinmo? How do these target genes generate neuronal diversity?

TYPE II NEUROBLASTS AND INTERMEDIATE NEURAL PROGENITORS

Clonal analysis of early-larval type II NBs shows that each produces a diverse array of neural progeny and that each contributes a distinct family of neurons and glia to the adult brain (Ito et al. 2013; Izergina et al. 2009; Riebli et al. 2013; Viktorin et al. 2011, 2013; Yang et al. 2013; Yu et al. 2013). Notably, the DM1–DM4 type II NBs produce many of the small field-intrinsic neurons that populate the adult central complex (Hanesch et al. 1989, Yang et al. 2013, Young & Armstrong 2010), a brain region required for navigation, locomotion, and sensorimotor integration (Boyan & Reichert 2011, Koniszewski et al. 2016, Pfeiffer & Homberg 2014, Strauss 2002).

Neuroblast Temporal Transcription Factors

To identify candidate TTFs in larval type II NBs, both our lab and the Lee lab performed transcriptomic analysis of type II NBs at several stages of larval development (Ren & Lee 2017, Syed et al. 2017). Similar to other larval NBs, the type II NBs have two broad phases of gene expression: (*a*) an early phase from 0 to 60 h ALH characterized by expression of Imp, Lin28, and Chinmo and (*b*) a late phase from ~60 to ~120-h ALH characterized by expression of Syp and the Ecdysone receptor B1 (EcRB1) (Ren & Lee 2017, Syed et al. 2017). Each of these phases is subdivided by additional candidate TTFs: D (a Sox family member), Cas, and Svp in the early phase and Broad and E93 (Eip93: Flybase) during the late phase (Syed et al. 2017) (**Figure 3**). The observation that type II NBs express the ecdysone receptor (EcR) at 56 h ALH prompted examination of the role of the extrinsic steroid hormone ecdysone in temporal pattering. Ecdysone is made outside the CNS and acts as an endocrine timer in many tissues (Thummel 2001, Yamanaka et al. 2013). Loss of ecdysone (using a temperature-sensitive mutant in the ecdysone biosynthetic pathway) or type II lineage–specific expression of a dominant negative EcR resulted in the loss

of all late TTF expression, in extension of selected early TTFs, and in corresponding changes in neuronal and glial identity (Syed et al. 2017).

Intermediate Neural Progenitor Temporal Transcription Factors

Type II NBs are unique in producing INPs rather than GMCs during their lineage. INPs are small—the size of a GMC—but they resemble a type I NB in marker expression and cell lineage. This raises the question of whether INPs undergo temporal patterning to generate a diverse population of neurons or whether INPs merely expand the numbers of a single type of neuron. Recent work shows that the former is the case. Clonal analysis of a single INP clearly shows that INPs make morphologically diverse neurons (Wang et al. 2014), and molecular marker analysis reveals that young INPs produce D^+ or Bsh^+ neurons, whereas old INPs produce Toy^+ neurons or $Repo^+$ glia (Bayraktar & Doe 2013). Thus, INPs generate neuronal diversity, but do they use TTFs to produce this diversity? The answer is yes. INPs express D during the first half of their lineage, the Pax6 family member Ey during the last half of their lineage, and Grh during the middle part of the lineage overlapping the D/Ey border; this is observed in INPs born at all larval stages and for DM2–6 NBs; DM1 lacks Grh, and DL1 and DL2 were not assayed (Bayraktar & Doe 2013). Reducing levels of D or Grh (both of which are early TTFs) leads to a loss of early-born Bsh^+ neurons. Similarly, removing the late TTF Ey from INP lineages leads to reduction in the late-born Toy^+ neurons and $Repo^+$ glia without altering early-born neuron identity, whereas precocious expression of Ey in INP lineages increases Toy^+ neurons and decreases Bsh^+ neurons (Bayraktar & Doe 2013). Thus, D, Grh, and Ey appear to act as TTFs to generate neuronal and glial diversity within INP lineages.

The regulation of the D → Grh → Ey cascade is similar in several ways to the embryonic VNC TTF cascade discussed above. First, each TTF represses the preceding TTF, revealing a feedback repression motif. Second, loss of one TTF only slightly delays expression of the next factor, showing that additional transcriptional activators must exist for each gene. One of these activators may be the PRDM zinc finger transcription factor Hamlet, which is expressed throughout INP lineages and is required for Ey expression (Eroglu et al. 2014); what prevents Hamlet from activating Ey earlier in the INP lineage is unknown.

Downstream of Temporal Transcription Factors

There are many markers for neuronal progeny of type II lineages, but few have been lineage mapped to early or late in the NB lineage, and thus there are few assays for the role of type II TTFs in specifying neuronal identity. At least four type II NBs (DM1–DM4) generate many of the intrinsic neurons of the adult central complex. Although there is a rapidly growing collection of markers for specific intrinsic neurons of the central complex (Jenett et al. 2012, Kahsai & Winther 2011, Wolff et al. 2015), it remains unclear how NB and INP TTFs generate neuronal diversity in the central complex. Progress will require mapping the birth order of each neuronal subtype and identifying intermediate tiers of transcription factors that translate progenitor TTF codes to single-neuron identity.

Open Questions

- What are the progeny of the type II NBs? What is the role of candidate TTFs in specifying their identity?
- How are NB and INP TTFs integrated to produce specific cell types? Are NB TTFs maintained in their INP progeny?

- How are spatial factors integrated with NB and INP TTFs to generate lineage-specific cell types? For example, we know that Bsh$^+$ neurons are produced only by the DM1–DM3 NBs, and in these lineages they arise late in the NB lineage but early in the INP lineage (Bayraktar & Doe 2013). Thus, these Bsh$^+$ neurons must integrate three types of developmental information: spatial identity, NB temporal identity, and INP temporal identity. How does such integration occur?

SEVEN-UP AND COUP-TF1/2: CONSERVED TEMPORAL SWITCHING FACTORS

The orphan nuclear hormone receptor Svp (a COUP-TF family member) is required for temporal TF switching in multiple lineages. In embryonic NBs, Svp is first expressed immediately after Hb, and it is required for timely switching from Hb to Kr expression in the well-characterized NBs NB7-1, NB7-3, NB3-1, and NB5-6 as well as many other NBs (Benito-Sipos et al. 2011, Kanai et al. 2005, Mettler et al. 2006, Tran & Doe 2008). Svp also switches off later TTFs: NB5-6 has two pulses of Svp, an early post-Hb pulse and a later pulse following Tv1 neuron birth (**Figure 2**). Loss of Svp leads to a failure to switch from Tv1 to Tv2/3 production, consistent with failed TTF switching (Benito-Sipos et al. 2011). Thus, the first Svp expression window represses Hb, and the second Svp expression window represses Cas (Stratmann et al. 2016). In addition, Svp acts as a switching factor in larval NBs, in which it is required to terminate expression of the early TTFs Imp and Chinmo and to promote the expression of the late temporal factors Syp and Broad in both type I and II NBs (Maurange et al. 2008, Ren & Lee 2017, Syed et al. 2017). Interestingly, mammalian Svp homologs COUP-TF1 and COUP-TF2 also function as switching factors to regulate temporal identity transitions in the developing CNS (Naka et al. 2008), suggesting an ancient, conserved role for this family of proteins in regulating temporal patterning.

TEMPORAL PATTERNING AND TUMORIGENESIS

One output of the temporal patterning cascade is to terminate NB proliferation at specific times. Embryonic NBs lacking Pdm undergo premature quiescence, whereas loss of Cas leads to prolonged NB proliferation (Lai & Doe 2014, Tsuji et al. 2008). Perhaps more dramatically, larval NBs lacking the switching factor Svp fail to terminate expression of the early TTFs Imp and Chinmo, resulting in abnormal extension of NB proliferation into the adult brain, where proliferation is normally never observed (Narbonne-Reveau et al. 2016).

In addition, temporal patterning regulates tumorigenic potential. NBs and INPs divide asymmetrically, segregating the differentiation factors Prospero (a transcription factor that represses cell cycle genes) and Numb (which inhibits Notch signaling) into their GMC progeny (Knoblich 2008). Interestingly, removal of the Prospero differentiation factor during the early Chinmo$^+$ temporal window produces malignant NB tumors that persist into adulthood and invade other tissues, whereas removal of Prospero from older Chinmo$^-$ NBs gives fewer, smaller tumors (Narbonne-Reveau et al. 2016). To distinguish NB temporal patterning from larval age, Narbonne-Reveau et al. removed the Svp switching factor to maintain a young temporal identity into late-larval stages and found that temporally young Chinmo$^+$ NBs within old larvae could still form malignant tumors. Malignant Chinmo$^+$ tumors also upregulated the early temporal factors Imp and Lin28, which act together as an oncogenic module (Narbonne-Reveau et al. 2016). Thus, temporal patterning not only specifies neuronal and glial identity but also regulates susceptibility to malignant tumor formation.

Similar results have been observed within INP lineages, in which the late TTF Ey limits Notch-induced tumorigenesis. Forced expression of the constitutively activated Notch intracellular

domain (N^{intra}) in newly born INPs results in tumor formation, whereas expression of N^{intra} in old Ey^+ INPs surprisingly has no effect (Farnsworth et al. 2015). Importantly, removing Ey allows old INPs to form N^{intra}-induced tumors (Farnsworth et al. 2015). How Ey prevents Notch-induced tumor formation remains to be determined.

INTEGRATION OF SPATIAL AND TEMPORAL IDENTITY

Every NB described in this review forms from a specific region of neuroectoderm, and every NB produces a unique, stereotyped lineage (Bossing et al. 1996, Ito et al. 2013, Schmid et al. 1999, Schmidt et al. 1997, Urbach & Technau 2003, Yu et al. 2013), with the exception of the four apparently identical MB NBs (Ito et al. 1997). This suggests that spatial patterning within the neuroectoderm is used to generate NB-specific cell lineages. Indeed, some of these spatial cues have been identified: Wingless specifies row 4 NB identity (Chu-LaGraff & Doe 1993), Gooseberry specifies row 5 NB identity (Skeath et al. 1995, Zhang et al. 1994), homeotic genes generate segment-specific differences in NB identity and lineage (Karlsson et al. 2010, Rogulja-Ortmann & Technau 2008, Tsuji et al. 2008), and Orthodenticle specifies LALv1 brain NB identity (Sen et al. 2014). Spatial cues act in the neuroectoderm (Chu-LaGraff & Doe 1993), prior to TTF expression, and can persist in embryonic NBs transplanted to a new location (Berger et al. 2001, Prokop & Technau 1994). Each of these spatial factors is combined with TTF expression to generate lineage-specific neurons.

Recent work has expanded this concept into the optic lobe neuroepithelium (Erclik et al. 2017). The optic lobe neuroepithelium can be subdivided into six domains on the basis of the expression of three transcription factors (Retinal homeobox, Optix, and Vsx1) and two signaling pathways (Hedgehog and Dpp) (Erclik et al. 2017). Late optic lobe NBs have a type I lineage (NB makes GMCs, which undergo a terminal division to make $Notch^{ON}$ and $Notch^{OFF}$ sibling neurons; **Figure 1*c***). Interestingly, all $Notch^{ON}$ neurons use only NB temporal information to generate regionally similar unicolumnar neurons; in contrast, the $Notch^{OFF}$ neurons also integrate spatial information to generate region-specific multicolumnar neurons. In this way, optic lobe NBs can generate two very different classes of neurons: (*a*) an even distribution of unicolumnar neurons that establish retinotopy specified by temporal cues alone and (*b*) several smaller pools of region-specific multicolumnar neurons that require both spatial and temporal patterning (Erclik et al. 2017). The decision as to whether to ignore or respond to spatial cues is made not by the NB, but rather by the GMC-derived sibling neurons (the $Notch^{ON}$ sibling ignores spatial cues, and the $Notch^{OFF}$ sibling responds to spatial cues). It is tempting to speculate that active Notch signaling erases the spatial information that persists from neuroepithelium to neuron.

For both the embryonic neuroepithelia and optic lobe neuroepithelia, it remains unknown how spatial factors act transiently to generate long-lasting differences in neuronal identity. One model is that spatial factors generate epigenetic changes that modify target access for TTFs. A second model is that spatial factors initiate a transcriptional cascade that acts combinatorially with TTFs. A third model is that such factors change the palette of TTFs expressed, as observed for the OPC/tOPC NBs (Bertet et al. 2014). Testing these and other models is a major challenge for understanding the generation of neuronal diversity.

CONCLUDING REMARKS

Do mammalian orthologs of the fly TTFs have similar functions? Recent work has shown that the Hb-related gene *Ikaros* has a role in specifying early-born neuron identity in the mammalian retina and CNS (Alsio et al. 2013, Elliott et al. 2008) and that the Cas-related gene *Casz1* specifies

late-born identity in the mammalian retina (Mattar et al. 2015). Grh is related to mammalian CP2 domain proteins Grhl1–3, with this family having a conserved function in epithelial biology rather than in temporal patterning (Boglev et al. 2011, Senga et al. 2012). Less is currently known about the mammalian homologs of Kr, Pdm, or the many larval TTFs. Nevertheless, work in *Drosophila* shows that the use of TTFs to specify temporal identity is widespread, even though the factors can vary from region to region, and thus TTFs are very likely used to generate neuronal diversity in both flies and mammals.

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