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Birth time/order-dependent neuron type specification

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Abstract

Neurons derived from the same progenitor may acquire different fates according to their birth timing/order. To reveal temporally guided cell fates, we must determine neuron types as well as their lineage relationships and times of birth. Recent advances in genetic lineage analysis and fate mapping are facilitating such studies. For example, high-resolution lineage analysis can identify each sequentially derived neuron of a lineage and has revealed abrupt temporal identity changes in diverse *Drosophila* neuronal lineages. In addition, fate mapping of mouse neurons made from the same pool of precursors shows production of specific neuron types in specific temporal patterns. The tools used in these analyses are helping to further our understanding of the genetics of neuronal temporal identity.

Introduction

During the course of neurogenesis, exceptionally diverse neuronal cell types, as characterized by various morphological, electrophysiological, and molecular features, are generated by a limited number of neural progenitors. These neural progenitors undergo spatial patterning, during which they respond to various inductive signals and express unique combinations of transcription factors [1,2,3,4,5]. Such organization allows the progenitors to produce characteristic sets of progeny in distinct regions of the developing nervous system, a process that has been highly conserved from invertebrates to vertebrates.

In *Drosophila*, neural progenitors, called neuroblasts (Nbs) repeatedly undergo asymmetric cell divisions to deposit intermediate precursors while regenerating the progenitors (Figure 1). In *Drosophila*, neural progenitors, called neuroblasts (Nbs) deposit intermediate precursors via repeated asymmetric divisions (Figure 1). In the embryonic ventral ganglion, DiI labeling of single Nbs and their progenies allowed the Technau and Doe labs to discover families of neurons and glia produced by each Nb [6,7,8]. Specific Nbs deposit neurons in an invariant sequence [9,10]. Consequently, within a given neuronal lineage, the identity of each neuron might depend chiefly on its birth time/order via a process of temporal cell fate specification. These observations have led to the identification of a cascade of transcription factors that act in precursors to specify multiple neuron types in sequence [9,11,12]. Further investigations of timing mechanism(s) in *Drosophila melanogaster* have followed [13,14,15,16,17••].

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In the vertebrate nervous system, birthdating of post-mitotic neurons by pulse labeling of proliferating cells provided initial evidence for such temporally guided neuron type specification [18,19]. For example, cortical neurons exhibiting distinct properties reside in different laminas of the cortex [20]. Birthdating of post-mitotic neurons by transplantation and pulse labeling with [H3]thymidine or 5-bromo-2'-deoxyuridine (BrdU) has established the correlation between the laminar position of cortical neurons and their birth date [20,21,22, 23]. Specific cortical neurons are thus made at specific times of development. The orderly production of distinct neurons has also been observed in the developing retina, spinal cord, and olfactory bulb [2,24,25].

Elucidating how neurons acquire different fates based on birth order or time is essential for understanding how the complex brain develops. However, our lack of a complete description of the cell lineages prevents detailed mechanistic study on the temporal origin of the vertebrate neuronal diversity. This problem is further confounded by the fact that many neural progenitors yield intermediate precursors, which themselves divide multiple times before differentiating into post-mitotic neurons. Recent evidence shows self-renewing intermediate precursors in *Drosophila* as well. In the classical view, intermediate precursors called ganglion mother cells (GMCs), divide only once to make two post-mitotic neurons (Figure 1A). In contrast, the recently found transit-amplifying precursors undergo a limited run of asymmetric cell divisions to deposit multiple pairs of post-mitotic neurons (Figure 1B) [26, 27, 28••].

A comprehensive study on neuronal temporal identity and its underlying mechanisms depends on thorough lineage analysis to identify the offspring of a progenitor and determine the order in which they arise. Here we review how novel tools of genetic lineage analysis and fate mapping have facilitated study of neuronal temporal identity. An improved system of mosaic analysis with repressible cell markers, called twin-spot MARCM, permits high-resolution lineage analysis [29••]. It allows one to determine each sequentially derived post-mitotic neurons in a protracted *Drosophila* neuronal lineage. A similar system is available in mice, and its application to lineage analysis is an exciting area of technical development [30,31]. Recent in-vivo studies of vertebrate neuronal temporal identity have primarily employed an inducible genetic marking technique to fate map specific precursors at different times of development [25,32,33••,34•]. All these genetic tools further permit conditional manipulation of genes in the precursors making specific neuron types, and have helped elucidate the intrinsic mechanisms controlling neuronal temporal identity.

Technical advances: high-resolution cell lineage analysis in fly and temporally inducible genetic fate mapping in mouse

Classical lineage tracing in the *Drosophila* ventral ganglion depends on DiI labeling of an identifiable Nb, and determining neuronal birth order further requires pulse labeling with BrdU or analysis of temporally induced clones. These approaches are rather tedious and only work for birthdating of embryonic neurons that express known markers [8,9]. Cell lineage analysis in the *Drosophila* brain, especially during post-embryonic development, was made easier by various genetic techniques that permit positive labeling of clones, including MARCM [35], G-TRACE [36] and Twin-Spot Generator [37]. However, high-resolution lineage analysis to determine the neurons derived from each sequentially born intermediate precursor remained challenging until the development of twin-spot MARCM (Figure 2A and 2B) [29••].

Twin-spot MARCM permits labeling of the two homozygous daughter cells derived from a heterozygous precursor in distinct colors in an otherwise unstained background. With minimal neuronal migration in the *Drosophila* brain, neurons are labeled in clones and the differentially marked sister clones reside in pair. The neuron(s) derived from an intermediate precursor would thus pair with a multicellular Nb clone that consists of neurons born afterwards. Analysis of

the associated Nb clone reveals where (lineage origin) and when (birth order) the labeled neuron (s) has derived. This allows identification of single neurons and determination of their developmental origin at the same time. It can identify, in principle, each sequentially derived neuron in any protracted neuronal lineage to reveal birth time/order-dependent neuron type specification. Either the marked single neuron(s) or the associated Nb clone can be made homozygous for any mutation distal to the site of mitotic recombination [29••,35]. The prospective cell fate or neuron type composition of the mutant clone can be determined from its wild-type sister clone. Twin-spot MARCM offers high-resolution cell lineage analysis and greatly facilitates genetic mosaic analysis of neuronal temporal identity.

In vertebrates, lineage tracing by retroviral library injection to mark clonally related cells has nicely demonstrated that multiple neuron types could derive from a common progenitor. Pulse labeling of dividing precursors has further revealed different neuron types are made in distinct temporal patterns. However, it was challenging to target specific precursors for detailed lineage analysis until the development of recombinase-based genetic fate mapping [32,33••,38], which permits genetic fate mapping of specific precursors at specific times of development. This involves targeted expression of a chimeric recombinase whose activity can be temporally controlled (Figure 2C and 2D) [33••] and requires a promoter that can drive the expression of the recombinase continuously and specifically in the pool of the intermediate precursors of one's interest. Transient activation of the recombinase mediates excision of a premature stop from the reporter gene in the intermediate precursors that exist during the time of recombinase activation. This turns on reporter gene expression selectively in the progeny derived from the intermediate precursors present at a particular time of development [33••]. Thus, we can mark neurons born at different times, supposedly from the same precursors, for analysis of neuronal temporal identity. The recombinase can simultaneously excise a pre-engineered endogenous gene to conditionally knock out gene function in the precursors making specific neurons [39]. Such temporally inducible genetic fate mapping techniques have extended the study of neuronal temporal identity by molecular genetics into the complex mouse brain.

Insights from cell lineage analysis of *Drosophila* brain

Cell lineage analysis in the *Drosophila* brain shows that each Nb is programmed to make specific neurons in an invariant sequence [40,41,42,43•,44•]. Notably, timing and duration of specific neuronal subtype generation differ from lineage to lineage, suggesting individual Nbs alters its temporal identity in distinct tempos. For example, the Nbs that yield neurons constituting the olfactory learning and memory center, the mushroom bodies (MBs), produce only two types of neurons through larval development [40,43•]. In contrast, the Nbs of antennal lobe (AL) projection neurons (PNs) make many more neuron types in a shorter period of time [41,45]. Twin-spot MARCM analysis of a group of six neurons that innervate a subset of midline neuropils of the central complex (CX) has further revealed that these CX neurons are individually unique in neurite trajectory and that their common progenitor makes them in an invariant contiguous order [29••]. These observations indicate that some Nbs may “track” every division through the protracted process of neurogenesis from embryos to larvae. It would be interesting to determine the cell number of each AL PN type by twin-spot MARCM to see if *Drosophila* Nbs really count cell divisions and not only produce multiple neuron types in an invariant sequence but also deposit a fixed number of neurons for each neuron type.

In sum, neighboring Nbs may produce multiple neuron types in distinct tempos. This suggests involvement of lineage-autonomous mechanisms in the birth order-dependent neuron type specification [17••,42]. Most larval-born neurons, regardless of the birth order, undergo differential morphogenesis during metamorphosis, further arguing pre-determination of the distinct sibling fates [46]. These phenomena support the model that *Drosophila* Nbs are

intrinsically programmed to express distinct temporal codes to confer distinct fates on the neurons born during different temporal identity windows.

In addition, molecular asymmetries during the neuron-producing mitoses can provide different fates to sister neurons [47,48]. Such binary sibling fate decision underlies the production of PNs and local interneurons simultaneously in the *Drosophila* lateral AL neuronal lineage [49]. Notably, the same precursors make many distinct PNs while yielding few types of local interneurons [45]. This suggests the presence of dual sets of temporal codes that alter in distinct tempos through the production of the sequentially derived intermediate precursors. Finally, *Drosophila* PAN lineages, like vertebrate neural progenitors, produce post-mitotic neurons through transit amplifying precursors [26,27,28••]. Studying neuronal temporal identity in the PAN lineages may shed more light on the mechanisms of temporal patterning of neuron fates in higher brains.

Insights from genetic fate mapping of mouse neurons

Temporally inducible genetic fate mapping of mouse neurons made from the same pool of precursors has been recently applied to demonstrate the production of specific neuron types proceeds in specific temporal signatures [25,34•,39,50]. For example, ten distinct subtypes of cortical interneurons that derive from *Olig2*-positive precursors arise at different embryonic time points [34•]. Depending on the time point of generation, each type of Olig2-positive cortical interneuron has its unique physiological property. Another example is the generation of olfactory bulb (OB) interneurons. At least seven identifiable subtypes of OB interneurons are produced from *Dlx1/2*-expressing precursors [25]. Tyrosine hydroxylase (TH)-positive interneurons, Blanes cells, and calbindin interneurons are largely derived during embryonic stage. In contrast, calretinin (CR) cell and CR-positive granule cell productions are low during embryogenesis and increase postnatally. Parvalbumin interneurons are produced just around the birth. Interestingly, the generation of 5T4-positive granule cells does not change significantly during neurogenesis. In both examples, some neuron types are preferentially born during early development, others predominantly derive during late development, while certain neuron types can be made throughout the development. Moreover, significant overlapping exists in the orderly production of different neuron types, and multiple neuron types can derive from the same pool of precursors at the same time.

Current genetic fate mapping in mice deals with pools of possibly heterogeneous precursors, which is in great contrast with the analysis of one Nb at one time in *Drosophila*. The overlapping production of discrete mouse neurons could simply result from presence of distinct precursors that make different neuron types at the same time. Besides, the control of recombinase activity by acute delivery of chemicals may not provide enough temporal resolution to target neurons born in a narrow window. Nonetheless, distinct mechanisms could govern the stage-specific production of different neuron types in fly versus mouse. For instance, both intrinsic mechanisms and extrinsic cues have been implicated in the proper specification of neuronal temporal cell fates in mice [2,24,51,52]. In contrast, *Drosophila* Nbs are probably intrinsically programmed to make multiple neuron types in invariant sequences (see below).

Molecular mechanisms of neuronal temporal cell fate specification

Mosaic analysis of gene functions utilizing the above genetic tools is expected to expedite the discovery of genes controlling neuronal temporal cell fates. At present, we have not moved far beyond the Hb→Kr→Pdm→Cas cascade of temporal factors governing neuronal temporal identity. They are expressed in a non-overlapping sequence in diverse lineages of the *Drosophila* embryonic ventral ganglion [42]. Hb and Kr act in neural precursors to define the first and second temporal identity in both Nb 7–1 and Nb 3–1 lineages [11,16]. Intriguingly, Pdm and Cas determine the third and fourth temporal identity in the Nb 7–1 lineage, but regulate

the expression of the preceding temporal factor in the Nb 3–1 lineage [12,16]. Loss of Pdm or Cas delays, but not abolishes, the production of next neuron types, and ectopic Pdm or Cas is not sufficient to specify the third or fourth temporal cell fate in the Nb3–1 lineage [12,16]. In addition, dynamic expression of Svp promotes the transition from Hb to Kr during early lineage development and may govern another temporal factor expression later in development [14, 15,53]. So, a temporal factor may directly dictate a temporal cell fate or acts to regulate the expression of temporal fate determinants, and its mechanism of action may even vary from one lineage to another (Figure 3).

While lineage analysis of *Drosophila* post-embryonic neurogenesis reveals more and more temporal cell fates, we observe no evidence for reuse of those embryonic temporal identity factors. Novel temporal factors remain to be identified. Interestingly, Chinmo, a BTB-zinc finger nuclear protein, may govern multiple neuronal temporal cell fates in diverse lineages [43•]. Levels of Chinmo proteins in newborn post-mitotic cells dictate multiple temporal cell fates in the MB lineages. It also governs multiple temporal fates in the rapidly changing anterodorsal PN lineage [43•; Kao & Lee, unpublished results]. However, loss of Chinmo selectively affects the third temporal identity among six contiguously derived CX neurons [29••]. In addition, Chinmo, unlike Hb, Kr, Pdm, or Cas, acts in post-mitotic neurons to specify their temporal cell fates [43•]. These observations implicate Chinmo as a temporal fate master gene. Identifying the temporal factors that regulate *chinmo* expression should shed new light on the temporal patterning of neural development.

Could similar intrinsic machinery operate in vertebrates? Two winged helix transcription factors, Foxg1 [54,55] and Foxn4 [56], have been implicated in regulating cortical and retinal progenitors' abilities to sequentially produce different types of progeny, respectively. Foxg1 is not expressed in cortical progenitors until the first cortical layer of neurons are born, and has been shown to suppress early temporal identity during late cortical neurogenesis [54]. In contrast, Foxn4 is selectively expressed in young retinoblasts to promote the production of early neuron types in the retinal lineages [56]. Further, the mouse homolog of *Drosophila* Hb, Ikaros, acts to confer early temporal competence to mouse retinal progenitor cells [57••]. Ikaros expression in retinal progenitors is necessary and sufficient for the generation of early-born retinal cell types, suggesting a conserved mechanism of temporal cell fate specification in diverse organisms. However, it remains undetermined if these vertebrate temporal factors govern neuronal temporal cell fates in distinct lineages. Besides, the environment may regulate the temporal identity of neural progenitors. Both instructive (e.g. BDNF signaling during cerebral cortex neurogensis) [58] and negative feedback (e.g. SHH expressed in retinal ganglion cells) [59] environmental signals have been implicated in governing neuronal temporal cell fates in mice [2,24,52]. Dynamic changes in the extrinsic cues as well as the competence of the progenitors may jointly govern the sequential derivation of distinct neuron types at correct times of development.

Conclusion

Neurons are born with certain identity based on their developmental origin. The genetic tools that mark neurons transiently derived from specific precursors unambiguously demonstrate birth time/order-dependent neuron type specification. Furthermore, such tools permit targeted manipulation of genes, guaranteeing identification of many more genes controlling neuronal temporal identity. Determining the requirement and sufficiency as well as where and when they act to govern neuronal temporal identity should continue to provide mechanistic insights into how a neural progenitor can keep track of time to deposit specific neurons at specific times of development.

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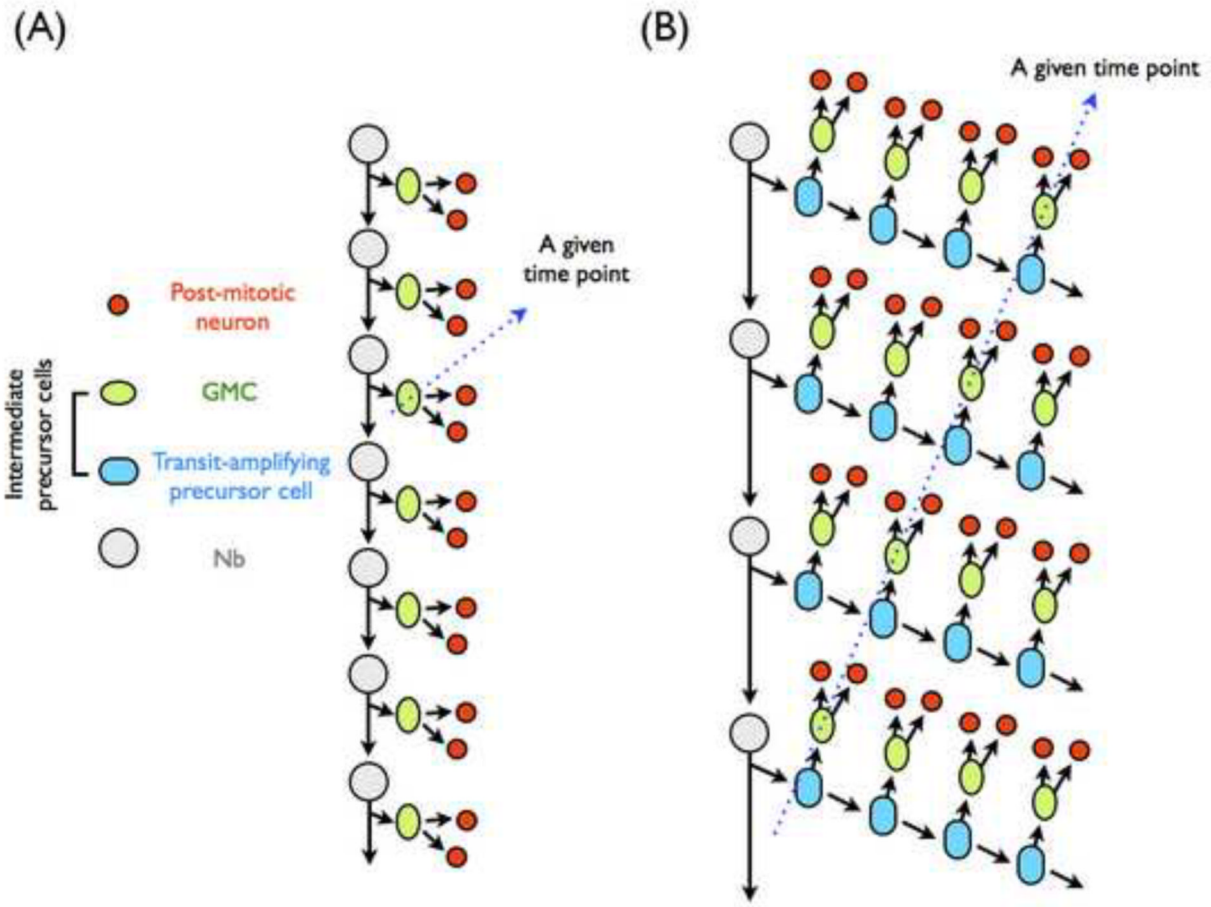

Figure 1.

Patterns of neural progenitor proliferation in *Drosophila*. (A) The classic mode of neural proliferation involves the generation of an intermediate precursor, GMC, from a neural progenitor. A GMC divides only once to yield two post-mitotic neurons. The serially derived GMCs may make post-mitotic neurons sequentially, such that only one GMC per lineage divides at any given developmental time (as indicated by a blue stroke). (B) Distinctive from classical GMC, specialized intermediate precursors, known as transit-amplifying precursors, are generated by those posterior Asense-negative Nbs in the *Drosophila* central brain. This type of intermediate precursors can undergo a limited number of cell divisions, not only regenerating itself but also giving rise to a GMC for each cell cycle. Consequently, multiple pairs of post-mitotic neurons can be generated at a given developmental time from precursors of distinct temporal origins (as indicated by a blue stroke).

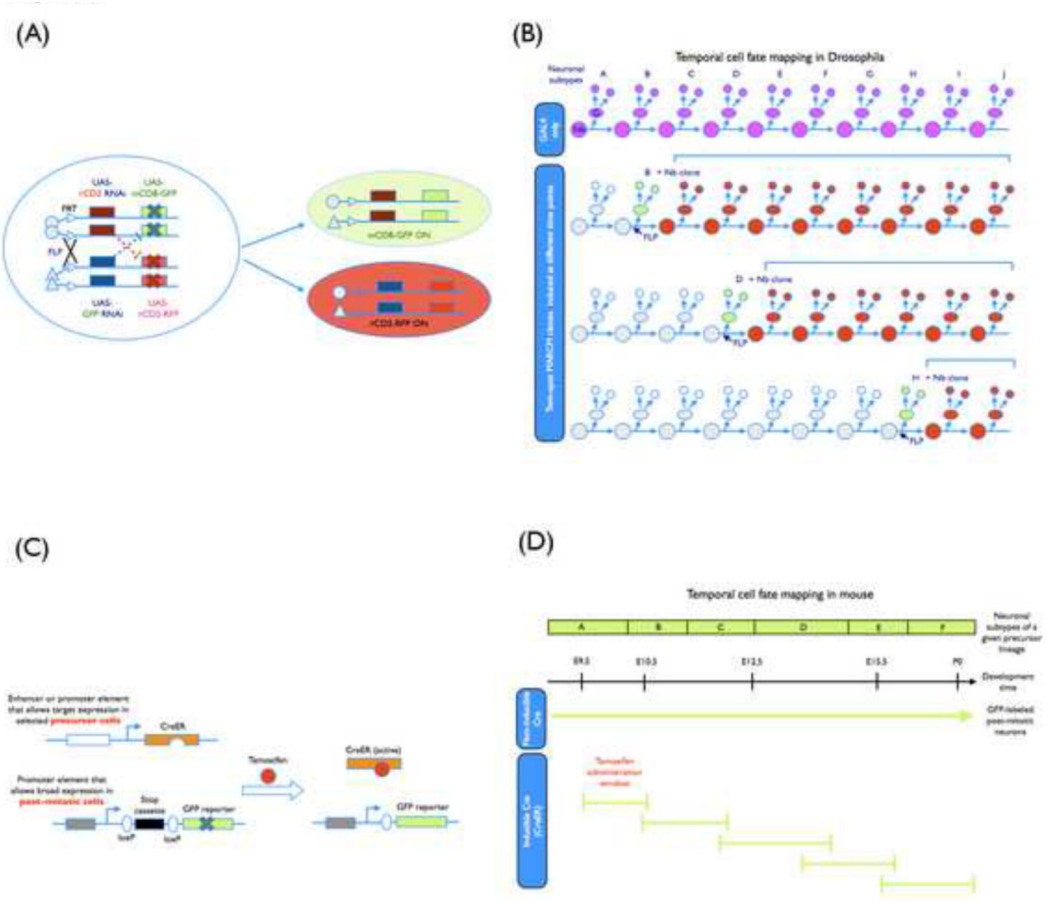

Figure 2.

(A) A schematic representation of genetic elements used in the twin-spot MARCM system. With the adoption of two sets of reporters and corresponding silencers (miRNA-based suppressors against two reporter genes in the current design) that have been placed on opposing homologous chromosome arms and distal to the recombination site, the paired Nb and two-cell clones can be labeled differentially at the same time in a mosaic brain after FRT/FLP-mediated mitotic recombination. Symbol "X" indicates the suppression of reporter gene expression. (B) Temporal cell fate mapping with twin-spot MARCM analysis. For a given neuronal lineage, a selected GAL4 driver was used to label the sequentially produced neural subtypes (A–J subtypes shown in the figure). By including this GAL4 driver in twin-spot MARCM, the temporal identity of individual marked two-cell clones generated at different developmental time points can be unambiguously determined based on the size and composition of their sister Nb clones, allowing high-resolution birth order mapping. (C) A schematic representation of genetic elements used in the inducible genetic fate mapping. Two transgenes are involved. One encodes an inducible site-specific recombinase CreER, which is a fusion protein of Cre recombinase with a tamoxifen-responsive Estrogen Receptor ligand-binding domain. Only after tamoxifen administration, CreER activity is turned on, providing a temporal control over the recombination event. Moreover, CreER expression under the control of a distinct promoter (or enhancer) element is restricted to specific precursors. The other transgenic element encodes an inheritable reporter whose expression depends on Cre-mediated excision of a stop signal and persistently marks the progeny of CreER-positive precursors. (D) Temporal cell fate mapping with the inducible genetic fate mapping in mouse. For a given neuronal precursor lineage, the sequentially derived neural subtypes (A–F types

labeled by a non-inducible Cre activity) can be selectively labeled by the CreER activity depending on the time window of tamoxifen administration. Their birth order could be subsequently deduced.

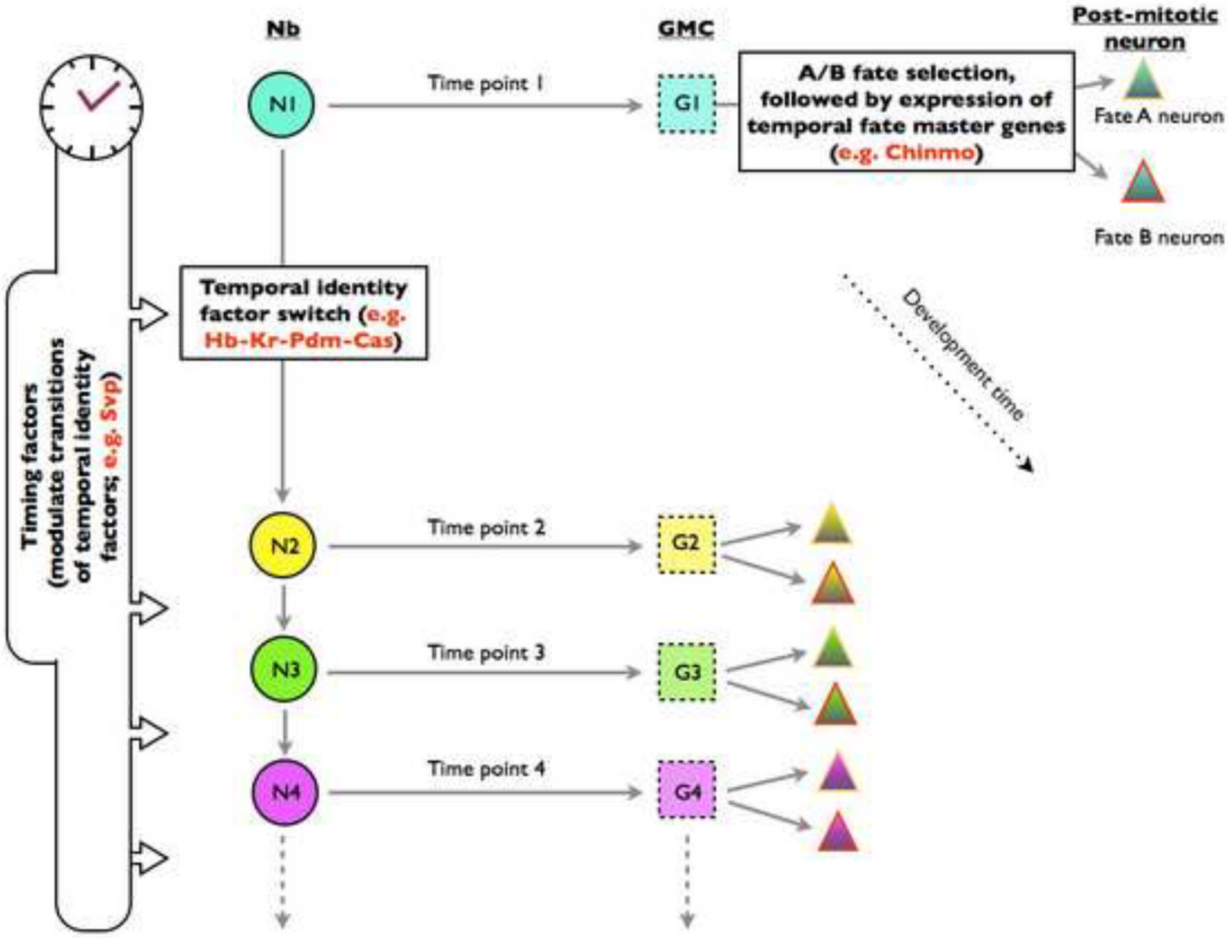

Figure 3.

A model of birth time/order-dependent neuron type specification in *Drosophila* CNS. As neurogenesis proceeds, a distinct set of temporal identity factors (as indicated with different colors), which sequentially express in the Nb (circle) and its GMC progeny (square), determines their individual temporal identities at specific time points. On top of the temporal identity factors, certain timing factors are used to regulate their temporal expression profiles, controlling the switch of temporal identity windows. Upon birth of post-mitotic neurons (triangle), temporal fate master genes start to express, dictating the terminal cell fates. The molecular asymmetries during neuron-producing mitoses may confer different fates on sister neurons born from the same GMCs. Together, multiple layers of temporal modulations underlie the production of many distinct neurons from a common progenitor.