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Regulating neural proliferation in the *Drosophila* CNS

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Neural stem and progenitor cells generate the central nervous system (CNS) in organisms as diverse as insects and mammals. In *Drosophila*, multipotent asymmetrically dividing progenitors called neuroblasts produce neurons and glia throughout the developing CNS. Nevertheless, the time-windows of mitotic activity, the division modes, the termination mechanisms and the lineage sizes of individual neuroblasts all vary considerably from region-to-region. Recent studies shed light on some of the mechanisms underlying this neuroblast diversity and, in particular, how proliferation is boosted in two brain regions. In the central brain, some specialised neuroblasts generate intermediate neural progenitors that can each divide multiple times, thus increasing overall lineage size. In the optic lobe, an alternative expansion strategy involves symmetrically dividing neuroepithelial cells generating large numbers of asymmetrically dividing neuroblasts. Evidence is also emerging for a cell-intrinsic timer that alters the properties of each neuroblast with increasing developmental age. The core mechanism corresponds to a series of transcription factors that coordinates temporal changes in neuronal/glial identity with transitions in neuroblast cell-cycle speed, entry into quiescence and, ultimately, with termination.

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Introduction

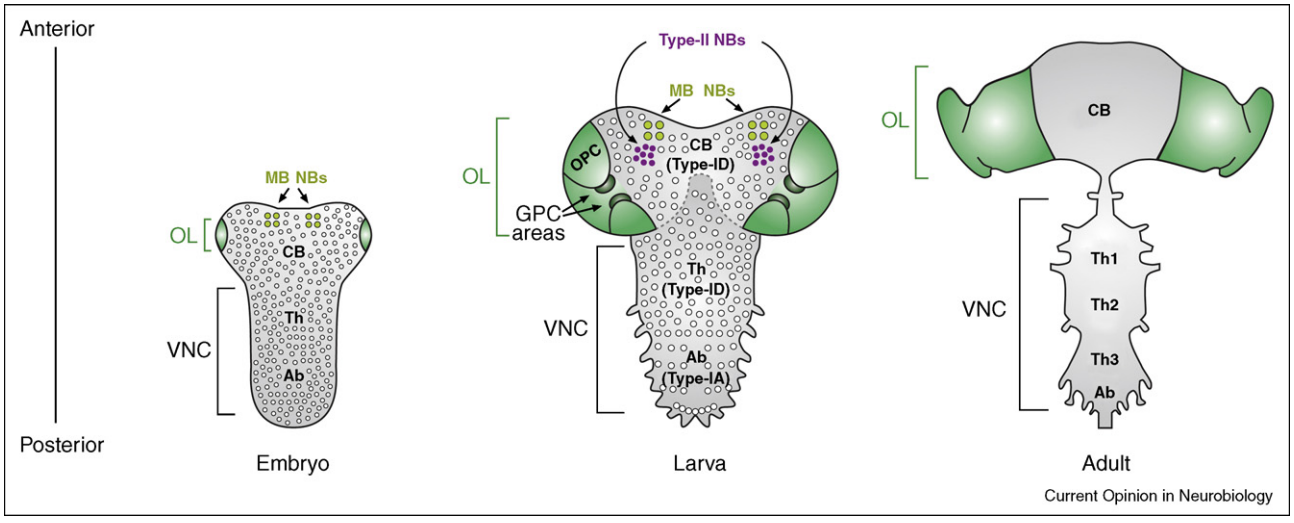
Understanding how the rich diversity of different neuronal and glial types is generated is a central theme in developmental neurobiology (*see reviews in this issue by T. Lee and H. Sawa*). An equally important question relates to how the numbers of each cell type are regulated and, more generally, how overall brain size is specified. Here we review recent progress in this second area, focusing on cell proliferation in the CNS of the fruit fly, *Drosophila*. This provides a powerful model system in which to observe neural stem cells in action and to screen for the genes underlying their remarkable properties.

The anatomy of the developing *Drosophila* CNS can be divided into the optic lobes (OL), the central brain (CB) and the ventral nerve cord (VNC). The CB contains olfactory learning and memory centres known as the mushroom bodies (MB) and the VNC is further subdivided into 3 thoracic (Th) and 9 abdominal (Ab) neuromeres (Figure 1). The cells that generate the neurons and glia in all of these CNS regions are neural stem-cell-like progenitors called neuroblasts. These multipotent cells are specified from ectodermal epithelia by a process involving proneural genes and Notch signalling. The CB and VNC neuroblasts derive from the ventral neuroectoderm of the early embryo, whereas OL neuroblasts form later on, during larval life, from neuroepithelial placodes (reviewed by [1,2]). In the forming VNC, there are ~30 neuroblasts per hemi-neuromere, which delaminate internally in a stereotypical pattern. Neuroblasts then go through numerous asymmetric self-renewing divisions, each time producing an intermediate progenitor called a ganglion mother cell (GMC). In turn, GMCs divide only once to yield two postmitotic cells that can be neurons or glia. Each of the ~30 VNC neuroblasts is unique in that it expresses different dorsoventral and anteroposterior patterning genes and generates a unique embryonic lineage of neurons and glia [3–7]. Although the short embryonic phase of neuroblast divisions is sufficient to form the functioning CNS of the larva, it only contributes ~10% of the neurons in the adult CNS. A long postembryonic phase of progenitor divisions, involving the continued activity of most (but not all) of the original embryonic neuroblasts accounts for the remaining ~90% of adult neurons [8–12]. Postembryonic neuroblast divisions are highly region specific, with a general bias for anterior domains to retain a larger fraction of the original number of embryonic neuroblasts and for each one to divide more times than in posterior domains (reviewed by [13]). This leads to dramatic anterior expansion of the CNS during larval/pupal stages, in line with the anterior locations of major adult-specific sensorimotor structures such as eyes, wings and legs.

The nuts and bolts of a neuroblast

Neuroblasts utilise two different molecular machines that endow them with stem-cell-like properties. The first of these corresponds to asymmetrically localised protein complexes that direct one daughter cell to self-renew whereas the other becomes a GMC. The second molecular machine comprises a set of sequentially expressed transcription factors that allow each neuroblast to make an

Figure 1



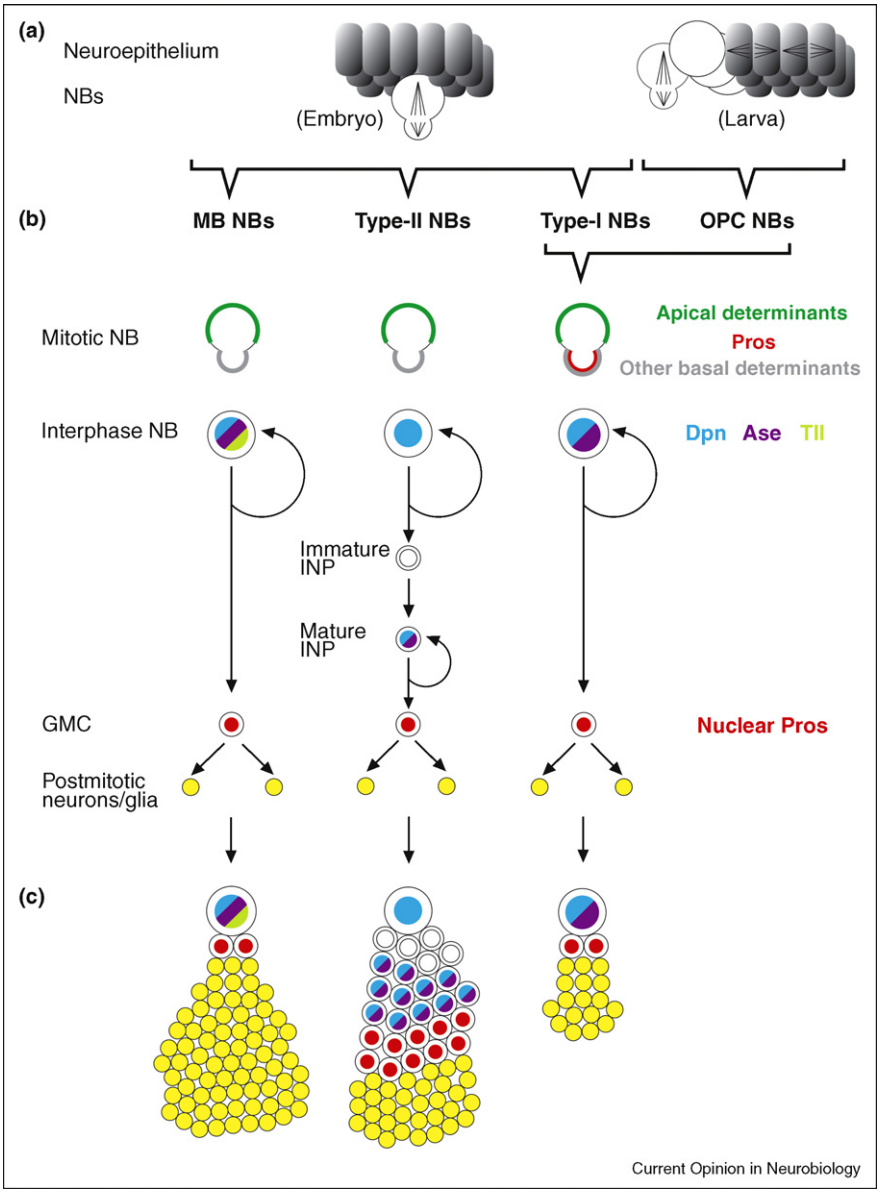
Distribution of neuroblasts in the developing *Drosophila* CNS.
Schematic representations of the embryonic, late-larval and adult CNS, highlighting neuroblasts (NBs, circles). The Optic Lobe (OL, green) is subdivided into the Glial Precursor Cell (GPC) areas and the Outer Proliferation Centre (OPC), which generates OPC neuroblasts. For clarity, the Inner Proliferation Centre (IPC) is not shown. The Central Brain (CB) contains numerous Type-ID neuroblasts, four Mushroom Body (MB) neuroblasts and eight Type-II neuroblasts. The Ventral Nerve Cord (VNC) is subdivided into Thoracic (Th) neuromeres and Abdominal (Ab) neuromeres containing Type-ID and Type-IA neuroblasts respectively. See text and Table 1 for descriptions of neuroblast types. Note that no identifiable neuroblasts are present in the adult CNS.

invariant series of different GMC identities, and thus different types of neurons/glia.

Many molecular components required for asymmetric neuroblast divisions have now been identified (reviewed by [14–16]). In brief, each asymmetric division involves the renewed neuroblast inheriting apical protein complexes and the GMC inheriting basal protein complexes (Figure 2). The latter contain fate determinants that contribute to the differentiative division of the GMC. One key basal component is the homeodomain protein Prospero (Pros), which is prevented from regulating transcription in the neuroblast by tethering to the basal cortex via the adaptor protein Miranda (Mira). Once inherited by the daughter GMC, Mira is degraded and Pros enters the nucleus. Genome-wide expression profiling and DNA-binding studies indicate that Pros regulates hundreds of transcriptional targets in GMCs: suppressing neuroblast genes and triggering cell-cycle exit by switching off Cyclin E, Cdc25/String and the transcription factor E2F as well as by activating a differentiative transcriptional programme [17••]. Like most transcription factors, the effect of Pros is context-dependent and in certain glial precursors it promotes rather than inhibits cell proliferation [18]. In some but not all neuroblasts, loss of Pros or other asymmetric components (such as Lethal-giant-larvae, Miranda, Brain Tumour or Numb) can lead to GMC transformation into neuroblast-like cells that form large and metastatic tumours [19,20••,21–27].

Work over the past decade has identified a timing mechanism that allows a single neuroblast to generate an invariant temporal sequence of distinct GMCs and thus different neuronal and glial fates. At the core of this mechanism is a series of transcription factors that are expressed within each neuroblast in a characteristic developmental sequence, known as the temporal series. Thus far, a series of four of these transcription factors has been identified: Hunchback (Hb) → Kruppel (Kr) → Pdm → Castor (Cas), although not every neuroblast utilises all four factors and additional members of the series probably remain to be identified [28–33]. In addition neuroblasts express at least two bursts of a fifth transcription factor, Seven up (Svp). The first Svp burst occurs in the embryo and provides a switching function required to downregulate Hb [33,34]. A later Svp pulse is expressed during larval stages, some time after the Cas burst [35••]. This larval Svp burst is required for neuroblasts to generate late neuronal temporal identities and is therefore likely to promote switching through additional, as yet unidentified, larval/pupal temporal transcription factors. Interestingly, although Hb protein is present in early-born neurons as well as in their progenitors, expression in postmitotic neurons is not sufficient to impart early temporal identity [36]. This implies the existence of, as yet unknown, relay mechanisms for passing temporal identity status from neuroblast to GMC to neuron (reviewed by [37]).

Figure 2



Different modes of neuroblast division.
(a) Schematic representations of neuroblasts (NBs, white) forming via delamination from the neuroepithelium (grey). For most NBs this process happens in the early embryo but in the Outer Proliferation Centre (OPC) it occurs in the larva. The OPC neuroepithelium proliferates by symmetric divisions (represented by symmetric spindles) whereas NBs divide asymmetrically (represented by asymmetric spindles and cleavage furrow position). Note that the origin of Type-II neuroblasts and whether or not they derive from embryonic Type-I neuroblasts is not yet clear. **(b)** Three different molecular signatures and division modes in mitotic and interphase postembryonic NBs. Mushroom Body (MB), Type-I, Type-II and OPC neuroblasts are shown. All NB types and the INPs of Type-II lineages self-renew (curved arrow) but the ganglion mother cell (GMC) does not. The distribution of Deadpan (Dpn), Asense (Ase), Tailless (Tll), Prospero (Pros) and other basal and apical determinants are shown. **(c)** The lineage sizes of MB and Type-II neuroblasts are larger than those of Type-I neuroblasts. This reflects, at least in part, the absence of a quiescent period in MB neuroblasts and the presence of INPs in Type-II lineages. The distributions of the key regulatory molecules depicted in B are shown at the late-larval stage.

How many different types of postembryonic neuroblasts are there?

In contrast to embryonic stages, the repertoire of neuronal/glial types generated by each neuroblast during postembryonic development has yet to be clearly defined for most CNS regions. Exceptions are MB and antennal-lobe neuroblasts where multiple different neuronal types produced in a stereotypical order have been defined systematically [38,39]. In addition, a systematic study of Th neuroblasts has identified 24 different lineages

per hemisegment based on their position and the morphology of their larval neurite bundles [40]. Although molecular markers that define each of these 24 neuroblast lineages have yet to be identified, a recent study shows that only 7 contain Delta-expressing neurons [41]. A current challenge in the field is to identify the complete (embryonic plus postembryonic) lineages for each one of the unique neuroblasts that have been identified in the embryo. An important step towards this goal is the ability to trace a single identified neuroblast from embryo to larva, which was recently achieved for one neuroblast (Neuroblast 3-3, see next section) [42••]. For other neuroblasts, however, this is proving more difficult because many of the molecular markers used to identify them in the embryo do not persist into larval stages. The available data suggest to us that most, if not all, postembryonic neuroblasts in a hemi-neuromere are likely to be distinct, each generating a unique set of neuronal/glial fates in the larva/pupa just as they are known to do in the embryo. Nevertheless, there is now enough information about modes of division and termination mechanisms (rather than progeny repertoires) to group postembryonic neuroblasts into five distinct types (Table 1). Future analyses, especially in complex regions such as the OL and CB, are likely to uncover further types of neuroblasts and, even for some of those listed here, the termination mechanisms remain to be determined. We now review progress in understanding how the division patterns of different neuroblast types are developmentally regulated and how this impacts on lineage size. In particular, we focus on several recent studies that have shed light on how the postembryonic progenitor pool is expanded and how neuroblast divisions are switched on and off at various times during development.

Most neuroblasts undergo reversible quiescence

Late in embryogenesis, the majority of neuroblasts in the CB and VNC enter a reversible G1 arrest known as quiescence [9]. This separates the embryonic and postembryonic phases of neurogenesis. The only postembryonic neuroblasts that do not undergo quiescence are the four MB neuroblasts, which generate very large lineages of ~500 neurons each, and one less-well characterised ventrolateral CB neuroblast [11,12,39].

Neuroblast quiescence is known to be regulated by several different intrinsic factors but the precise sequences of molecular and cellular events involved during entry into and exit from this poorly defined state are far from clear. The timing of entry and exit are strongly influenced by antero-posterior position and thus must be regulated, at least at some level, by Hox genes [10]. A recent study of Neuroblast 3-3 (NB3-3) elegantly demonstrates that the Hox protein Antennapaedia is required for NB3-3 to enter a period of quiescence in thoracic segments [42••]. By contrast, the Hox protein Abdominal-A appears to function in preventing NB3-3 from undergoing quiescence in abdominal segments. Importantly, this study also reveals that the time at which NB3-3 enters quiescence is regulated by the temporal transcription factor series [42••]. NB3-3 is not Hb^+ at birth but does sequentially express Kr, Pdm and two bursts of Cas as it generates its embryonic lineage. In thoracic but not abdominal neuromeres, NB3-3 then enters quiescence in the late embryo while Cas^+ for the second time. When NB3-3 subsequently resumes postembryonic divisions one day later it remains Cas^+, suggesting that quiescence suspends progression through the temporal series. Loss and gain-of-function experiments indicate that Pdm must be switched off in order for NB3-3 to enter quiescence. This requires repression of Pdm by Cas, the next member of the temporal series. In turn, loss of Pdm derepresses the transcription factor Nab and its cofactor Squeeze, both of which are required for timely quiescence. Thus, neuroblast entry into quiescence is regulated intrinsically by inputs from Hox genes and the temporal transcription factor series: with the former a likely regulator of the latter. It will be interesting to explore the generality of these findings in neuroblasts other than NB3-3.

Table 1

Distinct types of postembryonic neuroblasts. Currently, at least five different postembryonic neuroblast (pNB) types can be distinguished, based on modes of formation, division and termination (temporal series dependent, Pros-dependent, cell-cycle exit). Outer Proliferation Centre (OPC), Mushroom body (MB), Type-II, Type-ID and Type-IA neuroblasts, their approximate lineage sizes (number of progeny) and locations within the CNS are shown. They express distinct molecular signatures, comprising Asense (Ase), Prospero (Pros) and/or Tailless (Tll). The presence (+), absence (−) or indeterminate nature (n.d.) of each neuroblast property is indicated.

pNB type	Progeny number	Location	Molecular signature			Termination mechanism			
			Ase	Pros	Tll	Temporal series	Pros	Cell-cycle exit	Apoptosis
OPC	n.d.	OL	+	+	n.d.	n.d.	n.d.	n.d.	n.d.
Type-II	~400	CB	−	−	−	n.d.	n.d.	n.d.	n.d.
MB	~500	CB	+	−	+	n.d.	−	n.d.	n.d.
Type-ID	~100	CB/Th	+	+	−	+	+	+	−
Type-IA	4–12	Ab	+	+	−	+	−	−	+

Reactivation of neuroblast divisions following quiescence is also known to be regulated by extrinsic influences including nutrition, a glial-cell niche and several mitogenic signals (reviewed by [2,13]). Regarding nutrition, larval dietary restriction blocks exit from quiescence and a landmark study using various drop-out media indicated that amino-acids are the crucial component required for neuroblast reactivation [43]. The same study also showed, using co-culture experiments, that neuroblast reactivation can be triggered by a diffusible mitogenic signal made by the fat body, a *Drosophila* nutrient-sensing organ with adipose and liver-like functions [43,44]. Glial cells appear to provide both proliferative and anti-proliferative signals to neuroblasts. Evidence for the former comes from work showing that blocking the homotypic cell adhesion molecule E-Cadherin, either in glia or in neuroblast lineages, reduces neural proliferation in the CB [45]. In principle, this proliferative role for E-Cadherin could result from effects on quiescence exit and/or later neuroblast divisions. Glia also act negatively, preventing CB neuroblasts from undergoing premature quiescence exit, by secreting the glycoprotein Anachronism (Ana) [46]. This anti-proliferative signal is counteracted by Terribly Reduced Optic Lobes (Trol), an extracellular matrix molecule of the Perlecan family [47,48]. Recently it has also been shown that a positive feedback loop between Branchless/FGF and Hedgehog regulates the exit of CB neuroblasts from quiescence, although it is not yet clear which cell types produce these signalling molecules [49].

Increasing progenitor numbers via a symmetric division strategy

The most dramatic cell proliferation within the postembryonic CNS occurs within the OL. The embryonic OL primordium invaginates in the early embryo as a placode of 30–40 neuroepithelial cells. This expands dramatically during postembryonic stages by symmetric divisions, segregating into two separate epithelia known as the Inner Proliferation Centre (IPC) and the Outer Proliferation Centre (OPC). Most OL neurons are generated from these two centres although smaller numbers are also generated from the Glial Precursor Cell (GPC) areas [50]. The mechanism generating IPC neuroblasts has yet to be elucidated but, for the OPC, it is now clear that neurogenesis involves an early-larval phase of neuroepithelial expansion via symmetric divisions followed by a late-larval differentiative phase involving asymmetric neuroblast divisions [51••,52–53] (Figure 2). This two-step process has attracted much recent attention as it may have close parallels with neurogenesis in vertebrates (*see reviews in this issue by H. Sawa and Y. Gotoh*). OL neuroblasts begin to form from the medial edge of the OPC neuroepithelium by a wave of expression of the proneural protein Lethal of Scute that is negatively regulated by JAK/STAT signalling [51••,52,54••]. This neuroepithelial-to-neuroblast conversion continues for approximately four days until the neuroepithelium has been fully depleted.

Increasing progenitor numbers via an asymmetric division strategy

Recently, a second strategy was discovered for expanding progenitor numbers (and thus neuronal numbers), this time utilising a modified form of asymmetric division. In the dorso-posterior medial part of the postembryonic CB, eight primary neuroblasts (probably the pl and pm subgroups) divide asymmetrically to self-renew and bud off intermediate neural progenitors (INPs) that, unlike GMCs, can undergo multiple divisions [20••,55,56••,57••]. We now refer to these atypical primary progenitors as type-II neuroblasts to distinguish them from the more abundant type-I neuroblasts in the CB, Th and Ab (Table 1). Unlike their type-I counterparts, type-II neuroblasts do not express Pros or Asense (Ase) although both transcription factors become expressed by INPs. As each type-II neuroblast generates numerous INPs, their overall lineages are ~4-fold larger than those of other type-I neuroblasts, except for MB neuroblasts that also have large lineages as they do not undergo quiescence (Figure 2). The absence of Ase in type-II neuroblasts is functionally important because its overexpression inhibits INP formation. However, removing Ase activity from type-I neuroblasts does not convert them into INP-generating progenitors, indicating that the presence/absence of Ase is not sufficient to account for type-I/type-II differences [20••]. Lack of Pros expression appears to make type-II neuroblasts particularly susceptible to overgrowth as *brat* or *numb* brain tumours derive specifically from these and not other lineages and can be suppressed by overexpressing Pros [19,20••]. Further studies will be required to shed light on the origin of type-II neuroblasts and whether or not they are derived from a more typical Ase^{+} type-I neuroblast in the embryo. Either way, there are interesting parallels between type-II lineages and the transit-amplifying modes of progenitor expansion during vertebrate neurogenesis.

Mechanisms for terminating neuroblast divisions

The time at which neuroblasts finally and irreversibly stop dividing is crucial, not only for achieving the correct balance of early versus late-born neuronal/glial fates but also for determining the final size of the growing CNS. This neuroblast termination process occurs at very different times in different regions but is complete by the end of metamorphosis, such that there are no identifiable neuroblasts in the adult CNS [12]. We now discuss what is known about the molecular mechanisms regulating the timing and mechanism of neuroblast termination.

Some type-I neuroblasts terminate early in development, generating small lineages that contribute only a handful of neurons to the adult CNS. This is the case in the Ab region of the VNC, where type-I neuroblasts terminate in the late embryo or larva via a mechanism involving Hox-induced apoptosis [58–61]. By contrast, most type-I

neuroblasts in the CB and Th do not terminate until the pupal stage, after each one has generated around 100 progeny [10]. CB and Th neuroblasts then go through a series of changes including shrinkage, lengthening of the cell cycle, expression of nuclear Prospero and then cell-cycle exit via a differentiative division [35••]. In light of their distinct termination mechanisms, we propose subdividing type-I neuroblasts ending proliferation via *a*poptosis or a *d*ifferentiative division into type-IA and type-ID respectively (Table 1). MB neuroblasts generate 5-fold more neurons than most type-ID neuroblasts and do not express Pros nor require it for termination but, intriguingly, they do not appear to undergo apoptosis either [62]. These neuroblasts are also atypical in that they require the orphan nuclear receptor Tailless (Tll) and the leucine-zipper protein Bunched to maintain divisions at late stages [62,63]. Although more studies are needed, it seems that MB neuroblasts may utilise yet a third category of termination mechanism: Pros-independent cell-cycle exit.

Recent work indicates that temporal transcription factors and their target genes regulate the timing of type-I neuroblast termination in the postembryonic CB and VNC, regardless of whether this is executed via Hox-induced apoptosis or Pros-dependent cell-cycle exit [35••]. Loss of the temporal transcription factors Svp or Cas can immortalise neuroblasts, allowing them to divide during adulthood, whereas loss of one downstream target of Cas, the transcription factor Grainyhead, slows down the cell cycle and induces premature termination [35••,61]. Hence, temporal transcription factors and their targets allow type-IA and type-ID neuroblasts to undergo stepwise changes with developmental time that not only impact on progeny identity but can also affect cell-cycle speed, entry into quiescence, and, ultimately, time of termination. Further studies in type-II and OPC lineages should reveal whether reaching the end of the temporal transcription factor series is a global signal for neuroblast termination.

Conclusions and future directions

An important conclusion from recent studies is that the formation and mode of division of *Drosophila* neuroblasts are more diverse than previously thought. Symmetric expansion of neuroepithelial cells facilitates local increases in neuroblast number and the 'classic' asymmetric neuroblast lineage can be adapted to make more cells via INPs. In addition, some types of neuroblasts enter quiescence whereas others do not, some neuroblasts finally terminate their divisions via apoptosis yet others employ cell-cycle exit. Even the latter exit strategy appears to be further subdivided into Pros-dependent and independent mechanisms. It seems certain that over the next few years many more of the regulators and effectors of type-specific neuroblast properties will be identified. It will also be interesting to see how many of these properties, which are all dynamic, lie downstream of the temporal transcription factor series. Another area of current interest relates to the possibility that the *Drosophila* CNS might provide a useful model in which to study repair responses to injury. In this regard, it is tantalising that localised DNA synthesis by glia, and possibly other cells, has recently been detected in the adult CNS, both as a normal wild-type feature and in response to mechanical damage [64,65].

At least some of the regulatory principles underlying neurogenesis in *Drosophila* and mammals are conserved but there are sure to be significant differences too (recently reviewed by [37]). Although more research is needed before the true extent of the cellular and molecular similarities becomes clear, progress is so rapid that this is unlikely to remain obscure for long. A longer term challenge will be to learn enough about the regulation of temporal identity and proliferation during development to ensure that neural stem cells can be efficiently and safely manipulated in the clinic.

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