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Proliferation control in neural stem and progenitor cells

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Abstract

Neural circuit function can be drastically affected by variations in the number of cells that are produced during development or by a reduction in adult cell number due to disease. Unlike many other organs, the brain is unable to compensate for such changes by increasing cell numbers or altering the size of the cells. For this reason, unique cell cycle and cell growth control mechanisms operate in the developing and adult brain. In *Drosophila melanogaster* and mammalian neural stem and progenitor cells these mechanisms are intricately coordinated with the developmental age and the nutritional, metabolic and hormonal state of the animal. Defects in neural stem cell proliferation that result in the generation of incorrect cell numbers or defects in neural stem cell differentiation can cause microcephaly or megalencephaly.

Introduction

Cell proliferation can be defined as an increase in total cell number that is achieved through cell growth and division. At the tissue level, proliferation rates are influenced by the size of the initial progenitor pool, the total number of progenitor divisions, the frequency with which progenitors divide and the fraction of proliferative and non-proliferative daughter cells that they generate.

In many organs, changes in cell number can be compensated by alterations in cell size. For example, in the fruit fly, *Drosophila melanogaster*, a decrease in total cell number in the wing disc is compensated by an increase in cell size this way ensuring total organ size is not altered [1]. In the brain, however, the number of neurons produced during development is critical for circuit formation [2] and cell size cannot be varied without affecting function. Thus, the regulation of cell division is particularly important in the CNS.

Neurons in the brain originate from a relatively small number of neural stem and progenitor cells (NSPCs). Neural stem cells (NSCs) can be defined as cells that can generate all the cell types in the brain, whereas neural progenitors (NPs) have more restricted potential. During development, NSCs initially expand through symmetric self-renewing divisions. Later, they undergo asymmetric neurogenic divisions in which one of the daughter cells remains a proliferating progenitor and the other daughter cell undergoes differentiation after one or multiple rounds of transit amplifying divisions. Over time, NSPCs change their competence [3,4]. This allows them to produce the various neuron types [5] that are essential for

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circuit formation and also contributes to timely cell cycle exit. Once NSCs have completed their set of neurogenic divisions, they typically undergo apoptosis or terminal division, or enter senescence and decrease proliferation. For this reason, the number of NSCs is low in post-embryonic animals, with only a few stem cells remaining in the adult brain [6].

The number of neurons in the brain is therefore determined by the proliferative potential of NSPCs, their lineages, the number of symmetric and asymmetric divisions that they undergo and their cell-cycle frequency. In this review, we discuss each of these types of proliferation control in NSPC lineages. We focus on *D. melanogaster*, in which several fundamental mechanisms of neural proliferation control have recently been identified, and the mouse, in which those mechanisms are remarkably conserved. In addition, we describe how the proliferative capacity of NSPCs changes over time and how this is regulated and coordinated in the developing brain to control neurogenesis. Finally, we consider the consequences of aberrant proliferation in the brain.

NSPC lineages and types of division

The number of neurons generated during neurogenesis is not only determined by the initial number of NSPCs and the duration of their proliferative period but also by their lineage. NSPCs can both self-renew and generate more differentiated daughter cells with reduced proliferative capacity. Various types of transit amplifying cells that can increase the number of neurons formed as a result of each NSC division also exist in different neural progenitor lineages.

Drosophila neural stem cell lineages

NSCs in the developing *Drosophila* brain are called neuroblasts (NBs). NBs undergo multiple rounds of asymmetric cell division to generate one larger and one smaller daughter cell [7]. During each division, NBs distribute the fate determinants Numb, Prospero and Brat to the basal cell cortex and atypical protein kinase C (aPKC) to the apical cell cortex [8]. After mitosis, each daughter cell therefore inherits a different set of determinants (Figure 1A). As a result, one of the daughter cells remains a NB and continues proliferating, while the other becomes more committed to differentiation.

Based on their lineages, two different types of NBs can be distinguished (Figure 1B). Type I NBs divide asymmetrically to self-renew and generate a ganglion mother cell (GMC) that, in turn, divides symmetrically to produce two neurons or glial cells (Figure 1C). Type II NBs also self-renew but, unlike type I NBs, they generate a so-called intermediate neural progenitor (INP, Figure 1C). INPs initially go through a 4 to 6 hour long maturation stage, during which they do not divide but sequentially initiate expression of the transcription factors Asense (Ase) and Deadpan (Dpn). After this initial stage, they undergo 3-5 additional rounds of asymmetric division, generating another INP and a GMC that divides terminally into two neurons or glia cells. The presence of INPs dramatically increases the number of neurons produced by type II NBs. This provides an important example of how differences in NSC lineages can affect the number of neurons produced. Type II NBs are further characterized by the absence of expression of Ase and the presence of the transcription factors Pointed and Buttonhead (Btd; the homologue of mammalian Sp8). The cooperation

of Btd and Pointed [9] is both required and sufficient for specifying type II NB fate [10,10-12]. Notch signaling is another important regulator of NB types and a reduction of Notch levels leads to a complete loss of type II NBs [13]. Type I NBs are less sensitive: reduced Notch levels only lead to a reduction in the number of type I NBs in the central brain [14] do not affect the number in the ventral nerve chord [15].

Lineage progression in type II NBs is regulated by two transcriptional networks. The first network regulates NB self-renewal and is composed of three redundant transcription factors, HLHmγ, Deadpan (Dpn) and Klumpfuss (Klu) 12,16,17. The network is controlled by the Notch signaling pathway, which directly promotes expression of HLHmγ and Dpn to promote NB growth and proliferation [17]. Notch also regulates regrowth of the daughter NB after division [18,19]. In the more differentiated daughter cells, this self-renewal network is turned off by Numb and Brat. Numb inhibits Notch signaling [20,21] and Brat acts as a translational inhibitor [22,23]. When these determinants are missing or Dpn, HLHmγ or Klu are overexpressed, INPs transform into NB-like cells, causing uncontrolled overproliferation and the formation of a brain tumor [16-18].

In INPs, the transcriptional network described above is also active; however, self-renewal is restricted by a second network that both limits the number of INP divisions and provides temporal identity (see below). This network is induced by the chromatin remodeling SWI/SNF complex and the transcription factor Earmuff (Erm; the *Drosophila* homolog of the mammalian Zn-finger transcription factors Fez and Fezl; [3,24,25]). Erm is specifically expressed in INPs where it is thought to [3] restrict the proliferative potential and stabilize cell fate by activating Pros expression and repressing Notch signaling [24]. Erm acts through the SWI/SNF complex, which is present both in INPs and in type II NBs. In type II NBs, however, SWI/SNF is inactive because Erm is not expressed and SWI/SNF target loci are occupied by the components of the NB self-renewal network [3]. In INPs, SWI/SNF initiates a cascade of temporal transcription factors (see below) that prevents INPs from reverting back into type II NBs [3,25,26]. Thus, the sequential activity of both networks ensures lineage directionality in type II NB lineages.

Mammalian neural progenitor cells

In the mammalian cortex, neuroepithelial progenitors initially expand through symmetric divisions prior to neurogenesis (Figure 1D). After the onset of neurogenesis, however, they gradually start expressing glial markers [27,28] and are therefore called radial glial (RG) cells. RG cells form the apical most layer of the developing cortex, the ventricular zone (VZ). They extend apical and basal processes to the ventricular surface and the basement membrane. RG cells divide to generate one self-renewing daughter cell and one cell that becomes a post-mitotic neuron or a basally localized intermediate progenitor cell (IPC). IPCs are located between the VZ and the cortical plate, in the subventricular zone (SVZ) (Figure 1D). Unlike RG cells, they typically divide only once to generate a pair of cortical neurons [29,30]. Another progenitor type are the so-called short neural precursors which reside in the VZ [31] but lack a long basal process and give rise to neurons instead of IPCs [32]. In many mammals, including ferrets and primates (but typically not in rodents), the number of neurons is amplified by an additional type of progenitor, the outer radial glial cells (oRG

cell, also known as a basal RG cell, Box 1). oRG cells arise from asymmetric divisions of RG cells but continue to divide asymmetrically, generating multiple IPCs and eventually large numbers of neurons [33-35]. Recently, several new types of basal neural progenitors have been described in the outer subventricular zone of the macaque, underlining the lineage complexity of the developing brain [36]. Despite the very different size and morphology of the mammalian and fly brains, the lineages of their progenitors are remarkably similar: rodent neurogenesis is similar to the divisions of type I NBs, whereas the more complex lineages of the primate dorsal cortex resemble the type II NB lineage.

Asymmetric division in the mammalian brain

At the peak of neurogenesis, RG cells in the cortex undergo almost exclusively asymmetric divisions. This was convincingly demonstrated by a recent clonal analysis in which the daughter cells of an RG cell were differentially labeled, allowing their fates to be tracked [4]. How the two daughters of an RG cell adopt different identities is much less clear. It was initially assumed that – as in *D. melanogaster* - asymmetric inheritance of apical or basolateral determinants regulates cell fate [37,38]. Indeed, a fraction of the asymmetrically dividing RG cells have a mitotic spindle that is slightly oblique, which would predict unequal inheritance of the very narrow apical plasma membrane domain [39]. However, several observations are inconsistent with this simple model. First, the fraction of non-planar divisions is too low to explain the high rate of asymmetric cell division during peak neurogenesis [39-41]. Second, the rate of asymmetric cell division is unaffected by randomization of spindle orientation through the removal of G-protein-signalling modulator 2 (GPSM2, also known as LGN) [42-44]. Furthermore, reducing the number of non-planar mitotic spindles in mice by mutating mammalian Inscuteable gene (mInsc), which controls spindle orientation does not convert asymmetric into symmetric divisions [41]. Orientation of the mitotic spindle is also regulated by a specialized type of astral microtubules that attach spindle to the apical and basal part of the cell cortex and stabilize planar mitotic spindes [45]. In contrast to the data from the mInsc mutant mice [41], spindle verticalization by means of interference with the basal astral microtubules promoted asymmetric cell divisions [45]. The inheritance of apical plasma membrane is not tightly coupled to daughter cell fate [43]. Apical process is inherited equally between the two daughter cells[46], however at least one component of the apical domain, Par3, has been described to segregate asymmetrically and regulate cell fate decisions [47]. Daughter cell fate correlates better with inheritance of the basal process [43,46] but so far, a basal determinant in the cortex remains to be identified.

An interesting role in regulating asymmetric cell fate has been proposed for the centrosome. Both in *D. melanogaster* and in mouse, the centrioles inherited by the two daughter cells of each NSC differ in maturity and protein content. In *D. melanogaster* the ‘daughter’ centriole is inherited by the NB, whereas the older centriole is inherited by the differentiating daughter cell [48,49], although this asymmetry does not seem to influence cell fate [49]. In the mouse cortex, the older ‘mother’ centriole is consistently retained by the RG cell whereas the younger centriole is inherited by the differentiating daughter cell (Figure 2A; [50]). Preventing RG cells from inheriting the old mature mother centriole causes premature depletion of progenitors from the VZ [50]. The primary cilium, an organelle implicated in many signaling pathways [51], is associated with the mother centriole and is inherited by the

daughter cell that retains stem cell character [52]. As a consequence, the apical daughter cell extends a new cilium earlier than the basal daughter cell (and in the latter case the cilium emerges from the basolateral rather than the apical surface) [52]. Experiments that equalized the two centrioles have suggested that this asymmetry might contribute to fate specification [50]. Though, it must act redundantly with other factors, as cortical neurogenesis is unaffected by the absence of centrioles when p53, a tumor suppressor that inhibits cell cycle in response to DNA damage, is also mutated [53].

In addition to those intrinsic pathways, the fate of the RG daughter cells is influenced by intercellular communication. As in *D. melanogaster*, the Notch pathway controls the balance between self-renewal and neuronal differentiation in the mouse cortex. Numb, the main regulator of Notch signalling in *D. melanogaster* NBs, is present in RG cells, but its role in regulating Notch signaling in the mammalian brain is not yet fully resolved (reviewed in [54]). Notch activity in the mammalian brain is regulated by interactions between RG cells and their differentiated daughter cells. During neurogenesis, these differentiating cells (mainly the IPCs) are the source of Notch ligands that activate Notch receptors in their neighboring RG cells [55-58] (Figure 2B, D). The main targets of Notch signaling in the mammalian CNS are the Hes (primarily Hes1 and Hes 5) and Hey family genes, which inhibit the expression of proteins required for neuronal differentiation [54]. Thus, high Notch activity maintains the undifferentiated state of NSCs by repressing the expression of proneural factors [59], whereas low Notch signaling leads to neuronal differentiation [60].

How Notch is activated and maintained in RGs at earlier stages is less clear. An elegant model proposes that there are oscillations in Notch signaling in neural progenitors. Telencephalon NSCs express Hes1 in an oscillatory pattern (Figure 2C, E) [61] and this induces oscillations in expression of proneural factor Neurogenin 2 and Notch ligand Delta-like 1. Reducing Notch signaling disrupts these oscillations (Figure 2E) and promotes sustained expression of the proneural genes, which in turn induces differentiation [61]. In addition, oscillatory expression of Hes1 also seems to be important for the proliferation of NPCs, since cells with sustained high expression of Hes1 show reduced proliferation[61,62]. This model could complement the lateral-inhibition model, which proposes that the differentiating cell with higher expression of the Notch ligands, promotes self-renewal of the neighboring cell, and explain how Notch signaling regulates cell fate during the early stages of cortical development.

The fates of RG daughter cells are also regulated by cell cycle length. Generally, RGs undergoing symmetric self-renewing divisions have a longer cell cycle than progenitors undergoing asymmetric cell divisions (Figure 2F; [63,64]). Increasing cell cycle length triggers premature neurogenesis [65] whereas reducing G1 phase length inhibits neurogenesis [66] and promotes IPC generation [67]. This correlates well with the observation that length of the G1 phase increases with the progression of neurogenesis and increased number of asymmetric divisions [68]. It has been proposed that S-phases in cells undergoing symmetric self-renewing divisions are longer because more extensive DNA repair is required to meet the higher requirements for replication fidelity in these cells [64]. Alternatively, cell cycle length could affect cell fate by modulating the oscillation patterns of Notch targets and proneural genes.

Starting and stopping proliferation

Final neuron numbers in the brain are also determined by the time at which neural progenitors start and stop dividing. Both initiation and termination of NSC proliferation must be coordinated with the animal's developmental stage and different types of neurons must be generated at specific times. The mechanisms initiating and terminating cell division, as well as a transcriptional clock conferring temporal identity to NBs, have recently been identified in *D. melanogaster* and shown to be remarkably conserved in vertebrates [69].

Initiation

In *Drosophila*, embryonic NBs form by delaminating from the neuroectoderm. At the end of embryogenesis, many NBs undergo apoptosis but a fraction become quiescent and re-enter the cell cycle during early larval stages. Most adult neurons are generated during this second wave of neurogenesis (Figure 3A).

Cell cycle re-entry of larval NBs is triggered by larval feeding, which leads to increased levels of circulating amino acids (Figure 3B). Amino acids are sensed by the fat body, where the amino acid transporter Slimfast (Slif) detects their presence and activates the TOR pathway. Upon TOR activation, the fat body generates a hormonal signal, whose molecular identity is unknown [70]. In the glial cells that form the larval blood-brain barrier [71] this hormone stimulates PI3K/TOR signaling and induces release of insulin like peptides (Ilps). These Ilps are sensed by quiescent NBs and induce cell cycle re-entry through the insulin signaling pathway [72,73]. Combined activation of the Insulin and TOR pathways stimulates protein biosynthesis and inhibits the transcription factor Foxo to stimulate growth and NB division.

A slightly different mechanism is used by the optic lobe (OL) NBs, which only arise during larval stages [74]. In early larval stages OL neuroepithelial (NE) cells divide symmetrically. After this initial stage of expansion, a wave of proneural gene expression converts NE cells to asymmetrically dividing NBs. Larval feeding also activates the TOR/InR network to initiate proliferation of NE cells. Dietary nutrients also sustain mitotic activity during NE expansion and, later, promote the NE to NB conversion [2].

In contrast to *Drosophila* NBs, mammalian NE cells are not quiescent prior to the onset of neurogenesis. Nevertheless, Insulin signaling also regulates mammalian brain development [75]. The cerebrospinal fluid (CSF) is an important source of Insulin-like growth factors (IGF), such as IGF-2, which stimulates RG cell proliferation in an age dependent manner [76]. Deletion of either IGF-2 or Igf1R receptor decreased progenitor proliferation at late neurogenesis and led to smaller brains. Similarly, overexpression of insulin-like growth factor-I (IGF-I) in the CNS stimulates neural cell proliferation during embryogenesis and inhibits their apoptosis [77].

Temporal control of cell cycle exit in fly

NSCs stop proliferating at a predetermined developmental stage. In *D. melanogaster*, this is regulated by the transcriptional cascade that confers NSC temporal identity. This cascade allows progenitor cells to generate different types of neurons over time and its terminal stage

triggers their disappearance by either apoptosis or differentiation. The establishment and regulation of this cascade has recently been extensively reviewed [69] and we will therefore only discuss it briefly here.

Most embryonic NBs sequentially express Hunchback (Hb), Seven up (Svp), Kruppel (Kr), Pou domain transcription factor 1 and 2 (Pdm1/2) and Castor (Cas) [78-80]. Expression of these transcription factors is inherited by the GMCs and determines the type of neuron generated by these cells [79-82].

Postembryonic NBs re-express Cas and Svp [83,84] which provide these larval NBs with temporal information and determine the fate of the neurons they generate and the time of NB cell cycle exit. Various models have been proposed for how this is achieved. In all NBs, cell cycle exit is preceded by a reduction in cell size [83,85]. For this to occur, the steroid hormone Ecdysone induces a change in glucose metabolism that depletes the building blocks for lipid and protein biosynthesis and reduces cell growth (discussed below) [85]. Ultimately, Pros accumulates in the nucleus of NBs, where it is thought to induce terminal differentiation [83].

Other types of NBs are terminated by different means. In the mushroom bodies (MB), NBs survive much longer. Prior to their elimination at approx. 96 hours of pupal development, a decrease in insulin/PI3K signaling induces nuclear localization of Foxo, which reduces growth and proliferation in these NBs. The resulting very small MB NBs are eliminated by caspase-dependent cell death [86]. Apoptosis also terminates NBs in the abdominal parts of the ventral nerve cord (VNC; Figure 1B). Here, a transient pulse of the Hox gene *abdominal A* (*AbdA*) induces expression of pro-apoptotic genes [87,88]. The expression of AbdA in NBs is also controlled by Notch signaling, which is activated by Delta-ligands expressed by their neighboring cells [89]. Correct temporal identity is important for this event as interrupting the temporal series of transcription factor expression prevents AbdA expression and NB death [88].

How the temporal transcription factors connect to the various events terminating NBs is an important open question. One potential link is the Hedgehog (Hh) pathway, which is a target of Cas. Hh signaling progressively increases in NBs until it is sufficient to reduce the expression of Grainyhead (Grh), a transcription factor necessary to maintain proliferation of post-embryonic NBs [15,84,90]. How the increase in Hh signaling is achieved is currently unclear.

Two other temporal transcription factor series regulate proliferation in other parts of the *Drosophila* brain. In type II NB lineages, INPs successively express Dichaete, Grainyhead, and Eyeless [5,91]. Besides conferring temporal identity, this transcriptional cascade ensures cell cycle exit after all fates have been generated. The cascade is started by SWI/SNF, which initiates the expression of Dichaete. SWI/SNF also induces Hamlet, a putative histone methyltransferase that promotes the transition from early to late stages and ultimately the termination of INP divisions [26]. Hamlet is the common *Drosophila* homolog of Prdm3 and Prdm16, two factors that regulate various fate transitions in mammalian development [92] by initiating histone H3K9 methylation and heterochromatin formation, a possible mechanism by which Hamlet could regulate INP clock gene expression [93].

Another sequential transcription factor cascade is used in OL medulla NBs, which sequentially express Homothorax, Eyeless, Sloppy paired, Dichaete and Tailless as they age [94,95]. Expression of these transcription factors is inherited by the daughter cells generated during each of the expression periods so that a series of INPs with distinct gene expression patterns is generated [95]. The diversity is further increased by different levels of Notch signaling in these INPs, allowing for an enormous number of distinct neurons to be generated [95].

Thus, instead of undergoing true self-renewal some types of *Drosophila* neural precursor cells pass through distinct transcriptional states. Completion of this transcriptional program terminates their proliferative capacity thus ensuring the generation of a complete set of neuronal types.

Temporal control of neurogenesis in mammals

Sequential generation of distinct neuronal subtypes is also crucial for the developing mammalian cortex. A series of transplantation experiments revealed that neural progenitors become progressively restricted, so that the progenitors generating upper layer neurons cannot revert back and give rise to the deep layer neurons [96,97]. Similarly, mouse neural progenitors derived from embryonic stem (ES) cells in culture are able to sequentially generate neurons expressing markers for individual cortical layers [98-100] supporting the model that layer identities are determined by intrinsic pathways. Recent results support another model, in which distinct progenitor populations exist for different cortical layers [101]. It was thought that neurons in the outer layers of the cortex are specifically generated by a subpopulation of RG expressing the transcription factor Cux2. Later results, however, could not confirm this hypothesis [102]. In particular, an elegant clonal analysis in which RG daughter cells were labeled with distinct colors demonstrated that neurons from one RG cell can populate all layers of the cortex [4] and provided strong support for a model in which individual progenitors can generate neurons of multiple cortical layers.

Consideration of sequence homology suggests that several transcription factors have a conserved role in conferring temporal identity to neurons – although those relationships are sometimes complex. *Pax6* is the vertebrate homolog of the *D. melanogaster* clock gene *eyeless* and is expressed by cortical radial glia [103]. Cux-2 is the vertebrate homologue of cut, an important regulator of neurogenesis in *D. melanogaster* [104]. Chicken ovalbumin upstream promoter-transcription factor I and II (COUP-TFI and COUP-TFII) are required for the temporal specification of NSPCs. COUP-TFI and II are the vertebrate homologues of *D. melanogaster*'s temporal transcription factor Svp. Knockdown of COUP-TFI and II in the developing cortex leads to production of the extra early-born neurons at the expense of late-born neurons expressing Brn2 [105]. Interestingly Brn1 and Brn2 are POU domain transcription factors, homologues of *D. melanogaster*'s Pdm1/2 that are required for the generation of late-born neurons. Other *D. melanogaster* temporal transcription factors, such as Hb (mouse Ikaros/Znfn1a1) and Cas (mouse Casz1), have also been shown to play a role in conferring temporal fate to cortical and retinal progenitor cells, respectively [106,107]. These data suggest that these factors might have a cell-autonomous role in the production of fate-committed neuronal precursors that is conserved between mice and *Drosophila*.

At the end of neurogenesis in the developing mouse neocortex, most RG cells undergo symmetric neurogenic division and disappear. A fraction of RG, however, will remain and produce glia [4,29,108,109]. This neurogenic to gliogenic switch is orchestrated by cell-intrinsic epigenetic changes. These allow RG to respond to gliogenic cues and to stimulation of the JAK/STAT pathway by IL6 family cytokines secreted by differentiated cells. During the neurogenic phase of cortical development, promoters of astrocyte-specific genes are silenced by DNA methylation and thus unresponsive to the gliogenic stimuli [110].

The Notch pathway also plays a role in acquisition of gliogenic competence [111]. Notch signaling induces expression of Nuclear factor I-A (Nfia) which in turn promotes expression of the astrocyte specific genes [112]. In addition, polycomb group complex (PcG) limits the neurogenic potential of the NPCs by repressing proneural transcription factor Neurogenin 1 [113]. Newborn neurons secrete IL6 family cytokine Cardiotrophin 1 which activates JAK/STAT signaling in RG [114]. Activated STATs can in turn act on demethylated astrocyte specific genes to trigger astrocyte differentiation.

Thus, both in *Drosophila* and in mammals NSPCs undergo identity changes over time. The transcriptional cascade controlling temporal specification of neuronal subtypes and termination of neurogenesis in mammals is not precisely understood yet, but homology data from *Drosophila* could provide further insight into these mechanisms.

Proliferation control and metabolism

Recent experiments have revealed exciting connections between neural progenitor proliferation and metabolism. Insulin signaling is responsible for initiating NB proliferation in *Drosophila* larvae (see above). During starvation, the kinase Alk maintains Insulin pathway activity even when Insulin levels are low (Box 2). In addition, metabolic changes induced by hormonal signaling initiate the termination of NB proliferation.

Central brain NBs regrow to their original size after each division [115,116]. During the pupal stages, just before terminal differentiation, however, they reduce their growth and become progressively smaller (Figure 3A,C) [85]. The key trigger for this change in growth is Ecdysone, a steroid hormone released in pulses throughout development (Figure 3C). Ecdysone secretion is tightly linked to nutritional state so that the next stage of metamorphosis is only entered under favorable food conditions [117-119]. In NBs, Ecdysone acts together with the multisubunit Mediator complex to change the expression of metabolic enzymes (Figure 3C; Figure 4A; [85]). Together, those changes up-regulate oxidative Phosphorylation (OxPhos) which induces NB shrinkage and cell-cycle exit. Reducing OxPhos prevents NB shrinkage, extends NB life-span and increases the number of neurons produced [85]. Inhibiting amino acid and lipid oxidation by reducing electron transfer flavoprotein beta subunit (ETF) levels has the same effect [85,120]. Interestingly there are no differences in cellular ATP levels between growing and shrinking NBs, suggesting that it is the supply of biosynthesis intermediates rather than energy that is crucial for NB regrowth after mitosis [85]. Thus cell intrinsic metabolic changes can directly influence cell fate and proliferation during development.

Metabolism is also an important regulator of adult mouse NSPCs (Figure 4B). Proliferating NSPCs contain high levels of Fasn, an enzyme that catalyzes the production of palmitate from malonyl-CoA and Acetyl-CoA. Palmitate is used as a substrate for the synthesis of new fatty acids. Reduction in Fasn levels impairs *de novo* lipogenesis and leads to reduced proliferation of mouse NSPCs [121]. By contrast, slowly proliferating NSPCs highly express Spot14, an inhibitor of malonyl-CoA synthesis and *de novo* lipogenesis [121]. Thus, the levels of *de novo* lipogenesis directly correlate with NSPC proliferation. Newly synthetized lipids in NSPCs are predominantly integrated at membranes, suggesting that proliferating stem cells require a large amount of lipids for membrane bio-synthesis.

The metabolic states of both mammalian and *Drosophila* proliferative stem cells are thus highly anabolic (prioritize bio-synthesis) suggesting that the levels of biosynthesis are crucial not only to regulate cell proliferation but also to determine cell fate. Together these studies show that regulation of metabolism is an evolutionary conserved mechanism to regulate stem cell fate, quiescence and proliferation during development and in adults.

Defects in proliferation control

Defects in stem cell or progenitor proliferation can lead to neurodevelopmental disorders. In this section we discuss how increased or decreased proliferation of NSCs can impact on neurogenesis.

2.1. Microcephaly

Microcephaly is a brain developmental disorder leading to a severe reduction in brain size. The disease is known as autosomal recessive primary microcephaly (MCPH) when the head circumference is less than −2 standard deviations away from the mean without severe effects on brain structure. The mechanisms that lead to microcephaly are still unclear, but there several models to explain origin have been proposed.

MCPH proteins (Table 1) include those implicated in centrosome biogenesis (CENPJ / CPAP [53,122], PLK4 [123]), centrosome maturation (Cdk5Rap2 [124,125]) and spindle regulation (Aspm [126]). Defects in the genes encoding these proteins are thought to affect neurogenesis by causing centrosome defects that can lead to mis-positioned spindles. This would primarily affect the early symmetric divisions responsible for expansion of neuroepithelial cells before neurogenesis. However, LGN knockout mice, which have misoriented spindles very early in neural development (embryonic day 10) do not show defects in neuronal production [43]. It is therefore interesting to consider whether other potential functions of the MCPH proteins might also contribute to brain size reduction.

The involvement of centrosomes and spindles in correctly positioning RG cells [53] as well as other functions of MCPH proteins in DNA segregation provide alternative explanations for the disease. Defects in centrosomes or in the mitotic spindle could lead to delayed mitosis, defects in DNA segregation and aneuploidy leading to RG and IPC cell death. In addition, some MCPH genes, such as Aspm also affect neuronal migration or localization of myosin [127], possibly through Wnt regulation [128]. Defects in neuronal migration could lead to

decreased neuronal layer thickness and provide an alternative explanation for the reduced brain size.

Other MCPH genes have more complex roles. Loss of Wdr62 in neural progenitors results in mitotic delay, cell death and consequently smaller brains in mice [129]. MCPH2/Wdr62 is important for both centrosome maturation and mitotic spindle assembly and stability [129]. The protein interacts with and activates the kinase Aurora A, which could explain its dual function [129]. MCPH1/Microcephalin is similarly complex. It couples mitosis to the centrosome cycle and controls spindle positioning and chromosome segregation indirectly [130]. MCPH1 deficiency causes defects in DNA-damage repair, DNA condensation, centrosome assembly/maturation, cell-cycle progression, mitotic spindles, and in chromatin remodeling. These defects lead to a premature switch from symmetric to asymmetric divisions in progenitors and can also cause cell death and reduce neuronal cell number.

Interestingly the function of several MCPH genes is evolutionary conserved and the study of their orthologues in *D. melanogaster* has revealed that these genes also have important roles during fly brain development. Mutations in *asp*, *Drosophila*'s orthologue of *Aspm*, also lead to a smaller brain while causing no obvious defect in body size [127]. In *asp* mutants a significant percentage of brain optic lobe neuroepithelial cells have defects in chromosome segregation, which leads to aneuploidy and cell death [127]. Asterless (Asl) is the *Drosophila* orthologue of MCPH4 and is also involved in centrosome regulation. It is a centriolar protein required for pericentriolar material recruitment and centrosome maturation [131,132]. Although Asl cellular function is conserved, *asl* mutations do not cause obvious defects in *Drosophila* brains [133].

Why is the brain particularly sensitive to those mutations? The prevailing hypothesis is that these mutations are specific for neuronal cells, or that neural progenitor cells may have specific requirements for centrosomes for the coordination of symmetric and asymmetric cell divisions [134]. However, the ubiquitous expression of many MCPH genes suggests an alternative possibility: although most developing tissues can correct reductions in proliferation and cell number through latter compensation or repair mechanisms the intricate lineage control mechanisms in the brain exclude this repair. For example, once RG cells have switched to gliogenesis, they are no longer able to generate cortical projection neurons [135], and therefore they can no longer compensate for insufficient number of neurons produced earlier in embryonic development.

Mouse models of MCPH do not recapitulate all aspects of the disease, and in several cases, brain size reduction is less severe when compared to humans [126,136]. The recent establishment of a culture system for at least one form of MCPH [137] might help to bridge this gap and tell us whether the same cellular defect underlies all forms of MCPH or whether different cellular defects lead to the same overall disease phenotype.

2.2. Megalencephaly

Megalencephaly is a growth development disorder characterized by an increased brain size, with an average weight 2.5 standard deviations from the mean of the general population.

Mutations in the phosphatase and tensin homologue deleted on chromosome ten (Pten) tumor suppressor gene cause an increase in mouse brain sizes similar to human megalencephaly [138]. Brain enlargement in *Pten* mutants results from increased cell proliferation and decreased cell death of NSPCs in the VZ [138]. Indeed, mutations in components of the PI3K/PTEN-AKT-mTOR pathway have been identified in human patients with megalencephaly and hemi megalencephaly [139-142].

A recent report showed that mutations leading to the stabilization of cyclin D2 (CCDN2) cause megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome [143]. Interestingly, cells from patients with mutations in PI3K-AKT pathway also showed stabilized levels of CCND2, suggesting that CCND2 could be a relevant downstream target of the PI3K-AKT pathway [143].

Outlook

Recent years have seen tremendous progress in our understanding of the control of proliferation in the developing nervous system. Work in *D. melanogaster* has established surprising and unexpected connections between proliferation control and metabolism. We know now that both initiation of NSC proliferation and its termination are controlled by key metabolic changes. These metabolic processes were previously, and somewhat disrespectfully, called “housekeeping” and thought to be uniform in all cells without influence on cell fate. Other basic housekeeping genes, such as the Mediator complex or the SWI/SNF complex, were also found to regulate very specific NSC lineage decisions, possibly explaining their involvement in very specific diseases, such as microcephaly and mental retardation [144,145].

It will be exciting to determine the extent to which those functions are conserved in mammals. Compared to *D. melanogaster*, proliferation control in the mammalian brain is more complex, with multiple genes acting in a partially redundant fashion to control similar processes. A big revolution in the field has comes from the ability to manipulate human tissue to understand human brain development. The discovery of human and primate specific lineages and cell types have been one of the major breakthroughs in this area [33,35,146] and will lead to insights into why our brain is so more complex than that of animals. The next few years are predicted to provide key insights into this question, aided by key recently-developed technologies. Among those are the ability to follow human brain development in real time in human slice cultures [33], the ability to edit the human genome using CRISPR/Cas9 [147], and the ability to generate brain tissue from pluripotent stem cells [98,100,137,148]. Together, these technological developments have the potential to revolutionize our understanding of human brain development, and address one of the most exciting questions in biology: what are the proliferation control mechanisms that enable human brains to generate so many more neurons than those of other animals and how do these changes lead to the enormous cognitive abilities we only see in humans?

Glossary Terms

Senescence

The phenomenon by which cells cease to proliferate, usually associated with aging.

Terminal division

Cell division in which both daughter cells undergo terminal differentiation.

Transit amplifying cells

A progenitor cell population with restricted potential and limited proliferative potential.

Neural progenitor lineages

Neuronal lineages that originate from a neural stem cell.

Gyrencephaly

a morphological feature of the brain in which an expansion of the cortical surface leads to the formation of characteristic folds.

Fat body

an organ in *Drosophila melanogaster* that combines the functions of mammalian fat tissue and liver.

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Box 1. Outer Radial Glia

Primate brains have a significantly higher number of outer radial glia (oRG) cells compared to mouse brains. In primate brains oRG cells form an additional layer called the outer subventricular zone (OSVZ) [149] that is thought to be the major source of neurons. oRG cells are multipotent progenitor cells that arise from radial glia (RG) cells and continue to self-renew while sequentially generating multiple IPCs (Figure 1D**;** [33,146,150]). The precise origin and cell biological features of oRG cells are not clear. oRGs have a long basal process but lack an apical process and thus are not connected to the ventricular surface. They do not express apical polarity proteins, such as Par3 [146], and might therefore simply be RG cells that have lost the apical plasma membrane domain. Alternatively, they might arise from specific types of RG divisions [150]. In support of the latter possibility, reorienting the division plane in mouse RG cells can lead to the formation of cells with oRG characteristics [43,46,151] and an expansion of cortical neuron numbers [41]. Human, but not mouse, RG cells express PDGF and high levels of PDGF receptor beta (PDGFRβ) and PDGF signaling is required for human progenitor proliferation [152]. Ectopic expression of PDGF in mouse RG cells is sufficient to increase their numbers and results in the formation of RG cells outside the ventricular zone, suggesting that PDGF signaling might be a major determinant of oRG formation [152]. Mechanisms controlling formation of gyri are still elusive, however thalamic innervations seem to modulate fold size [34]. Recently, two groups reported induction of folding in normally smooth mouse brains by manipulating levels of nuclear protein Trnp1 and Rho GTPase activating protein ARHGAP11B [153,154].

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Box 2. Influence of nutritional status

An animal's nutritional status is an important regulator of its development, determining the rate and duration of its growth period and thus its size [155]. However, the brain is usually spared from the effects of nutritional deprivation. Nutritional restriction during human intrauterine growth, for example, results in small babies that have proportionally large heads [156]. Interestingly, this brain-sparing phenomenon is conserved in *Drosophila melanogaster*.

During *D. melanogaster* brain development there are two developmental phases that are sensitive to dietary nutrients. In early larval stages nutrients are required to reactivate neuroblast (NB) proliferation after the quiescent period that occurs during the embryo-to-larva transition [70,72,73] and in the optic lobe (OL) they are required to trigger the initial expansion of neuroepithelial cells and their conversion to NBs [2]. However, during later stages of larval development, the brain is no longer affected by poor nutrition (whereas other fly organs grow at a reduced rate in such circumstances) [157].

In the fly ventral nerve cord this brain sparing is mediated by anaplastic lymphoma kinase (Alk), a receptor tyrosine kinase [158]. Alk is activated by its ligand Jelly belly, which is constitutively expressed by glial cells in a nutrient-independent manner [157]. Alk directly induces phosphorylation of the effector targets of the nutrient-regulated TOR and Insulin pathways, effectively bypassing these pathways. Thus, in older brains, Alk uncouples the control of NB proliferation from the animal's nutritional cues. It remains unclear how the Alk pathway is differentially regulated in young *vs.* older larval stages and the mechanisms that spare late larval OL NBs are still unknown. Sustained nutritional challenge throughout larval development leads to reduced number of OL neuroepithelial progenitors and consequently reduced numbers of medulla NBs [2]. Interestingly this reduced pool of NBs still forms all the correct neuron types, albeit at a reduced number, showing that NBs prioritize neuronal variety over neuronal number [2].

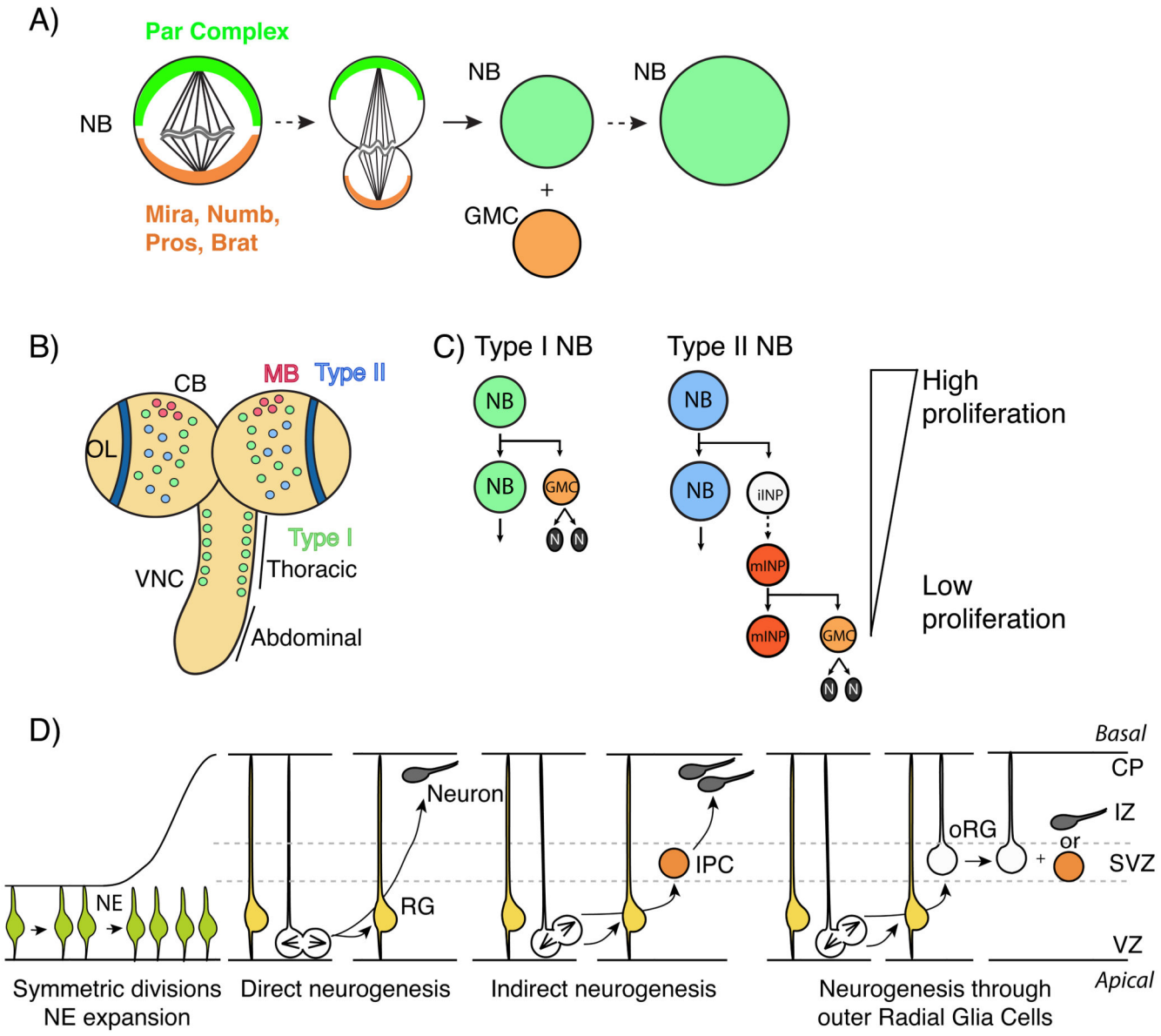

Figure 1. *Drosophila melanogaster* and mouse neural stem cell lineages

(A) *D. melanogaster* neuroblasts (NBs) divide asymmetrically to self-renew and to generate a more differentiated daughter cell. The Par complex (green) localizes to the apical cortex of NBs, and directs the cell fate determinants Mira, Numb, Pros and Brat (orange) to the basal cell cortex. The apical Par complex orients the mitotic spindle with respect to the established apical-basal axis. The NB divides asymmetrically and segregates the basal cell fate determinants into the ganglion mother cell (GMC), in which they promote differentiation. (B) A 3rd instar *D. melanogaster* larval brain. The larval brain can be divided into the central brain (CB), optic lobe (OL) and ventral nerve cord (VNC). Several types of NBs (including type I, type II and mushroom body (MB) NBs) can be found in the CB and in the thoracic and abdominal regions of the VNC. (C) Lineage organization of type I and type II NBs. Type I NBs divide to self-renew and to generate a GMC, which divides once more to form

two neurons (N). Type II NBs divide to self-renew and to generate an immature intermediate progenitor (iINP). INPs undergo through a period of maturation (to form a mature INP (mINP)) with no cell division, after which they undergo several rounds of division to self-renew and generate GMCs. Each GMC divides symmetrically to form two neurons or glia. (D) Development of the mouse neocortex. Before the onset of neurogenesis neuroepithelial cells (NE, dark blue line in panel B) divide symmetrically to expand their number. When neurogenesis begins NE transform into radial glia (RG) cells that can divide to self-renew and generate a neuron (direct neurogenesis) or divide to self-renew and generate an intermediate progenitor cell (IPC) that can then divide to generate neurons (indirect neurogenesis). RG cells can also divide to generate outer radial glia (oRG) cells that can themselves divide to self-renew and generate IPC or neurons. CP-cortical plate; IZ-intermediate zone; N- neuron; SVZ-sub-ventricular zone; VZ-ventricular zone.

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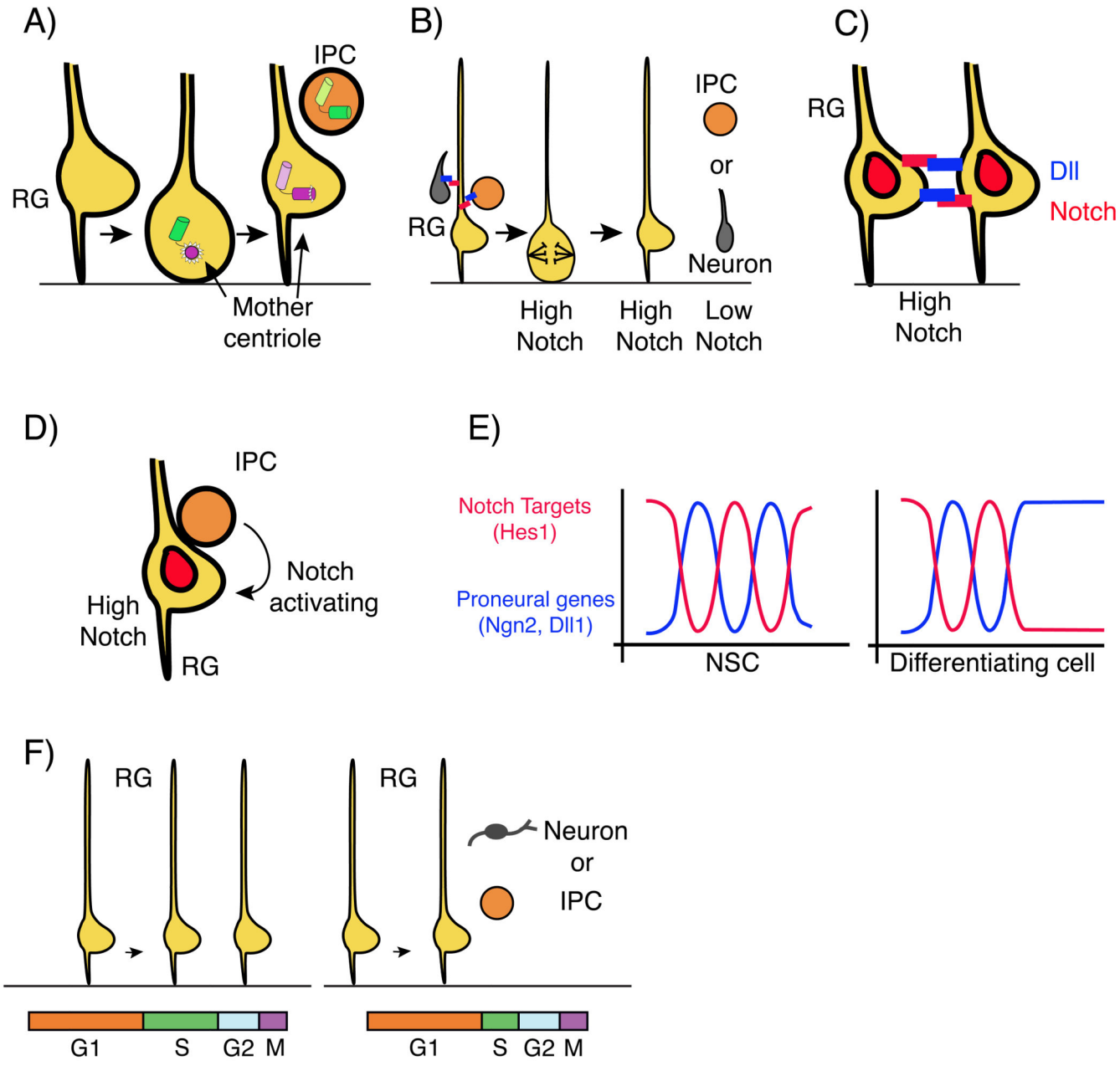

Figure 2. Asymmetric cell division in the mammalian neocortex

(A-C) Proposed models of radial glia (RG) cell asymmetric cell fate generation (A) Asymmetric inheritance of differently aged centrioles (the ‘mother’ centriole being older than the ‘daughter’ centriole) into the two daughter cells could determine their differential fate. (B) Different levels of Notch signaling between the RG and the differentiating intermediate progenitor cells (IPC) and neurons are an important regulator of cell fate. This may arise as a result of lateral inhibition, which proposes that the differentiating cell with higher expression of the Notch ligands, promotes self-renewal of the neighboring cell. (C) Notch signaling is also an important regulator of symmetric RG divisions. At early stage of neurogenesis RG cells express both Notch ligands and receptors, and this way activate Notch

signaling in neighbouring RG cells, promoting their RG cell fate. (D) During mid-neurogenesis, IPCs provide Notch ligands to RG to promote their self-renewal. (E) Notch signaling results in an oscillatory pattern of expression of Notch target Hes1 and proneural genes Ngn2 and Dll-1 in self-renewing cells. In differentiating neural cells, the oscillations cease and cells express low levels of Notch targets and high levels of proneural genes promoting differentiation. (F) Cell cycle length can influence neural cell fate. S phase is shorter in asymmetrically dividing RGs comparing to self-renewing cells.
Dll-Delta like; IPC-intermediate progenitor cell; NSC-neural stem cell.

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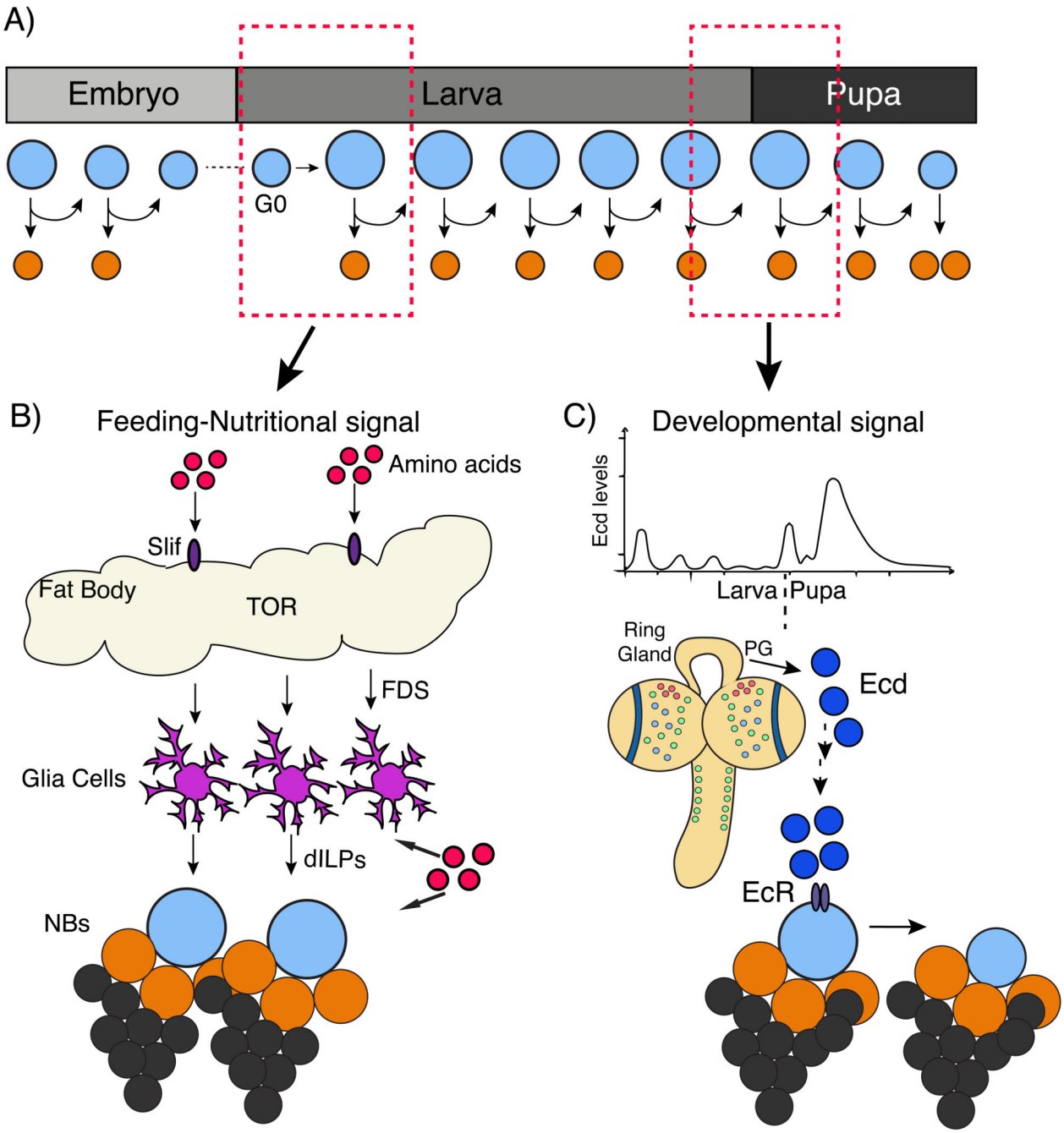

Figure 3. Different stages of *Drosophila* neurogenesis

(A) Different stages of *Drosophila* neurogenesis. NBs (blue) are generated during embryonic development; enter quiescence at the embryo to larva transition; re-enter the cell cycle in early larval stages; and cease proliferating during pupal stages. Embryonic and pupal NBs do not increase back to the size of their parent NB after each asymmetric cell division, whereas larval NBs do re-grow. (B) NB exit from quiescence in early larval stages is triggered by larval feeding. Amino acids are sensed by the receptor Slimfast (Slif) in the fat body. Slif activates the TOR pathway and the release of a fat body derived signal (FDS). The

FDS stimulates glial cells to secrete Insulin like peptides (dILPs) that activate Insulin signaling and PI3K/TOR signaling in NBs and consequently their proliferation. (C) The switch from growing larval NBs to shrinking pupal NBs is triggered by a pulse of Ecdysone (Ecd) at the larva to pupa transition. Ecd is produced and released by the prothoracic gland (PG), a component of the ring gland. The secreted Ecd binds to its receptor, EcR, in NBs where it induces this change in NB growth properties and, ultimately, NB disappearance.

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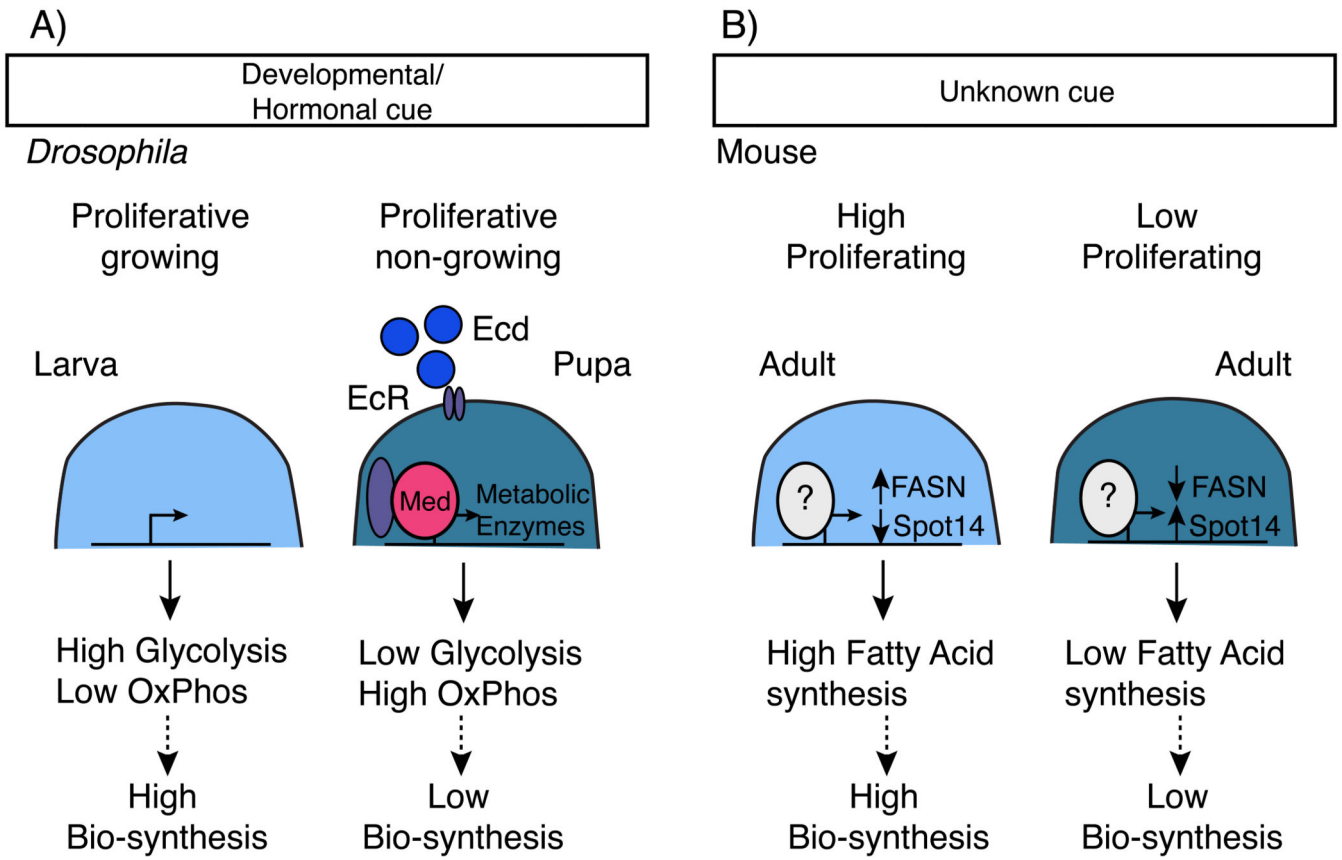

Figure 4. Metabolic regulation of neural stem cell fate and proliferation

(A) In *Drosophila melanogaster*, a pulse of the steroid hormone Ecdysone (Ecd) induces global transcriptional changes in metabolic genes causing a shift in the metabolic profile between larval and pupal neuroblasts (NBs). In pupal NBs, Ecd/EcR together with the Mediator complex change the transcription levels of several metabolism enzymes which results in an increase in Oxidative phosphorylation (OxPhos) and in a decrease in glycolysis. Larval NBs thus depend highly on glycolysis, whereas pupal NBs depend on OxPhos. Highly glycolytic metabolism is thought to promote bio-synthesis of macromolecules and therefore cell growth. By contrast, metabolism that is dependent on OxPhos can produce more energy at the expense of intermediate metabolites and bio-synthesis.

(B) The highly proliferative mouse adult neural stem cells (NSCs) have high expression of FASN and low expression of Spot14 enzymes, which leads to high levels of fatty acid synthesis. By contrast, low proliferating mouse adult NSCs have the opposite pattern of expression of FASN and Spot14, and have therefore low levels of fatty acid synthesis. High levels of fatty acids promote bio-synthesis of macromolecules required for NSCell growth and proliferation.

EcR-Ecdysone receptor; Med-Mediator.

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Table 1

Features and functions of MCPH genes

Human Disease locus	Gene symbol	Gene name	Mouse orthologue	Proposed function	*Drosophila* orthologue	References
MCPH1	BRIT1 Microcephalin	BRCT-repeat inhibitor of hTERTexpression	MCPH1	DNA repair; Coupling of the centrosome cycle with mitotic entry	MCPH1	130,159-162
MCPH2	WDR62	WD repeat domain 62	WDR62	Spindle pole protein	CG7337	163-165
MCPH3	CDK5RAP2	Cyclin-dependent kinase 5 regulatory associated protein 2	CDK5RAP2	PCM protein	Centrosomin (Cnn)	166
MCPH4	CEP152 (AKA MCPH9; SCKL5)	Centrosomal protein 152 kDa	CEP152	Centrosomal protein	Asterless (Asl)	167
MCPH5	ASPM	Abnormal spindle like microcephaly associated protein	ASPM	Spindle pole protein	Abnomrmal Spindle (Asp)	168
MCPH6	CENPJ or CPAP	Centromeric protein J	Sas-4	Centrosome biogenesis	Sas-4	166
MCPH7	STIL	SCL/TAL1 interrupting locus	STIL	Centrosome associated protein	Anastral spindle 2 (Ana2)	169,170
MCPH8	CEP135	Centrosomal protein 135	CEP135	Centriole biogenesis	CEP135/ Bld10	171,172
	Plk4	Polo-like Kinase 4	PLK4	Centrosome biogenesis	Sak kinase	123
	CEP63	Centrosomal protein 63	CEP63	Centriole biogenesis	*??*	173

MCPH- Autosomal recessive primary microcephaly; hTERT- human Telomerase reverse transcriptase;PCM- pericentriolar material; SAS-4- Spindle assembly abnormal 4 ortholog (C. elegans).