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Control of neural stem cell self-renewal and differentiation in Drosophila

Kyung Hwa Kang[1*] and Heinrich Reichert[2]

[1]KAIST Institute of BioCentury, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, South Korea.

[2]Biozentrum, University of Basel, Klingelbergstrasse 50/70, CH-4056, Switzerland.

*Correspondence to: Kyung Hwa Kang <kyunghwa.kang@unibas.ch>

Abstract

The neural stem cells of *Drosophila,* called neuroblasts, have the ability to self-renew and at the same time produce many different types of neurons and glial cells. In the central brain and ventral ganglia, neuroblasts are specified and delaminate from the neuroectoderm during embryonic development under the control of proneural and neurogenic genes. In contrast, in the optic lobes, neuroepithelial cells are transformed into neuroblasts postembryonically by a spatial wave of proneural gene expression. Central brain and ventral nerve cord neuroblasts manifest a short embryonic proliferation period followed by a stage of quiescence and then undergo a prolonged postembryonic proliferation period during which most of the differentiated neurons of the adult CNS are generated. While most neuroblasts belong to a type I class that produces neuronal lineages through non self-renewing ganglion mother cells, a small subset of type II neuroblasts generates exceptionally large neuronal lineages through self-renewing intermediate progenitor cells that have transit amplifying function. All neuroblasts in the CNS generate their neural progeny through an asymmetric cell division mode in which the interplay of apical complex and basal complex molecules in the mitotically active progenitor results in the segregation of cell fate determinants into the smaller, more differentiated daughter cell. Defects in this molecular control of asymmetric cell division in neuroblasts can result in brain tumor formation. Proliferating neuroblast lineages in the developing CNS utilize transcription factor cascades as a generic mechanism for temporal patterning and birth order-dependent determination of differential neural cell fate. This contributes to the generation of a remarkable diversity of cell types in the developing CNS from a surprisingly small set of neural stem cell-like precursors.

Keywords: Neuroblast, Asymmetric cell division, Proliferation, Tumor, Temporal series

Introduction

In humans, as in all other higher animals, the central nervous system manifests the highest level of structural and functional complexity of any organ system. The huge diversity of neural cell types that characterize the complex circuits of the nervous system is produced by neural stem cells. During normal development, neural stem cells produce defined sets of neural progeny composed of specific cell types that interconnect to form functional circuitry. Understanding the molecular mechanisms that underlie this process and give rise to the astonishing number and diversity of precisely defined cell types in the nervous system is one of the most challenging problems in biology. In recent years, important contributions to the understanding of the molecular mechanisms involved in neural stem cell biology have been made in several vertebrate and invertebrate neurogenetic model systems, including the fruit fly *Drosophila* (Homem and Knoblich, 2012).

In *Drosophila,* the neural stem cells, called neuroblasts, are similar to vertebrate neural stem cells in their ability to self-renew and to produce many different types of neurons and glial cells. The *Drosophila* central nervous system (CNS), which can be divided into the central brain and optic lobe in the head and the ventral nerve cord (VNC) in the trunk region, consists of thousands of diverse neuronal cells, which are arranged in complicated neural circuits. All of these neuronal cells are generated by a remarkably restricted set of neuroblasts through precisely controlled proliferation and differentiation processes during development. In the last decade, significant progress has been made in understanding the generic developmental mechanisms that operate in these neuroblasts during their normal proliferation. Moreover, some insight into the molecular events by which deregulated neuroblast proliferation can lead to the formation of brain tumors has also been obtained.

In this review, we consider some of the recent insighs into the mechanisms by which these neuroblasts give rise to diverse neural lineages in CNS development. We first describe the generic series of events that result in the formation, proliferation and termination of neuroblasts in the CNS. We then examine the diversity of neuroblast types with a special focus on the role of transit amplifying neuroblast lineages in brain development. Subsequently we describe a central feature of all neuroblasts namely their ability to self-renew and generate differentiated daughter cells through asymmetric cell divisions, and we also assess how deregulation of this division mode can lead to tumorigenesis. Finally, we review the role of temporal patterning in neuroblasts for the orderly generation of different neural cell types during developmental progression.

The life history of a *Drosophila* neuroblast

The basic proliferative elements involved in building the *Drosophila* CNS are the stem cell-like multipotent neural progenitors referred to as neuroblasts. In the VNC and central brain, neuroblasts first arise by delamination from the neuroectoderm during embryonic development (Fig. 1A). In the embryonic neuroectoderm, groups of cells are singled out as proneural clusters through the expression of genes of the *achaete-scute* complex and *daughterless*. In these clusters, neuroblasts become specified by Notch-dependent lateral inhibition from neighboring non-neuroblast cells, in a process in which proneural gene activity is restricted to only the presumptive neuroblast, but not in its neighbors (Artavanis-Tsakonas and Simpson, 1991; Campos-Ortega, 1993; Hartenstein and Wodarz, 2013; Skeath and Thor, 2003). Additionally, members of the *Sox* transcription factor family have also been reported to be involved in the formation of neuroblasts in a Notch-independent manner (Buescher et al., 2002; Overton et al., 2002). Following their specification, the

neuroblasts of the VNC and central brain delaminate from the neuroectoderm, enlarge, and begin to proliferate during the short period of late embryogenesis to produce a small set of neurons that make up the simple larval CNS. These embryonically generated neurons are referred to as primary neurons and each neuroblast generates 10-20 primary neurons during embryonic development (Larsen et al., 2009; Lovick et al., 2013).

In the central brain and in the thoracic ganglia, most embryonic neuroblasts enter quiescence in the late embryonic stage (Egger et al., 2008; Younossi-Hartenstein et al., 1996). Exceptions are the four neuroblasts that generate the intrinsic neurons of the mushroom body, along with a fifth brain neuroblast, which do not undergo quiescence, and divide continuously throughout all larval stages to generate exceptionally large lineages of neurons in adult CNS. Neuroblast entry into quiescence is mediated by intrinsically acting Hox genes as well as by temporal identity factors (Tsuji et al., 2008). Following quiescence, most of the remaining neuroblasts enlarge and restart cell division in the late first instar or early second instar of the larva. Re-entrance of the neuroblasts into the cell cycle is triggered by extrinsic signals, including nutritional or hormonal signals such as ecdysone (Colombani et al., 2012; Randhawa and Cohen, 2005). Interestingly, the fat body and a glial cell niche mediate this process. In presence of nutrition, an unknown secreted molecule from the fat body triggers release of the *Drosophila* insulin like protein (Dilp) from glial cells. Through the insulin receptor (InR), Dilp activates the PI3K/AKT-Target of Rapamaycin (TOR) signaling pathway in neuroblasts and this, in turn, induces the neuroblasts to exit quiescence, increase volume, and re-enter the cell cycle. (Chell and Brand, 2010; Shim et al., 2013; Sousa-Nunes et al., 2011). In contrast to the neuroblasts that undergo quiescence and reactivation, in the abdominal ganglia many of the embryonic neuroblasts are eliminated at late embryogenesis through programmed cell death

The majority of the neurons that make up the adult central brain and VNC, termed secondary or adult-specific neurons, are generated by neuroblasts postembryonically during a prolonged period of intense proliferative activity which typically lasts from the end of the first laval instar until late larval/early pupal stages (Ito and Hotta, 1992; Prokop and Technau, 1991; Truman and Bate, 1988). Thus, the development of VNC and central brain is accomplished in two distinct periods of neurogenesis, a brief first period in embryonic stages and an extensive second period in larval stages. In the central brain, approximately 90% of the neurons present in the adult brain are produced postembryonically by a stereotyped array of 100 embryonically derived neuroblast pairs (Technau et al., 2006; Urbach and Technau, 2004).

While these neuroblasts, which can be further divided into type I and type II neuroblasts (see below), arise from the neuroectoderm of the early embryo, the neuroblasts of the optic lobe (OL) are generated from the neuroepithelial cells of the optic anlagen in larval stages (Fig. 1B). During early larval development, the embryonic optic placode generated by invagination of the OL primordium in early embryonic stage, expands dramatically in size through symmetric cell divisions and becomes segregated into two separate epithelia termed inner proliferation center (IPC) and outer proliferation center (OPC). At the medial edge of the OPC, the neuroepithelial cells of the neuroectoderm are sequentially converted into neuroblasts of the medulla, which represents the largest neuropile of the OL (Egger et al., 2007). The dynamic transition of neuroectodermal cells to neuroblasts is triggered by a synchronized medial to lateral wave of expression of the proneural gene *lethal of Scute (l'sc)*, which is more refined by integration of Notch signaling. (Egger et al., 2010, 2011). This neuroepithelium-to-neuroblast transition by the proneural wave is negatively regulated by JAK/STAT signaling and positively

regulated by Fat-Hippo signaling (Reddy et al., 2010; Yasugi and Mizuno, 2008; Yasugi et al., 2008).

Tight regulation of the precise time at which neuroblasts stop their proliferative divisions is critical for achieving the correct balance of early versus late-born neuronal fate and for the determining the final number of neurons in the mature CNS. In the VNC and central brain, termination of neuroblast proliferation occurs either through apoptosis or by terminal differentiation (Reichert, 2011). Since neuroblasts end their proliferative periods at different times in different regions of the developing CNS, the molecular mechanisms for terminating proliferation are varied for distinct neuroblasts. For example, a pulse of Hox protein expression leads to elimination of specific embryonic and postembryonic neuroblasts in the abdominal ganglia of the VNC, and the activation of pro-apoptotic genes, such as *reaper, grim, and hid* is involved in this process (Bello et al., 2003; Peterson et al., 2002). Hox gene expression in these neuroblasts is suppressed until the appropriate time by the *Polycomb group (PcG)* genes (Bello et al., 2007). In contrast, the mushroom body neuroblasts of the central brain, which do not undergo quiescence and continue proliferating until end of the pupal stage, are prevented from premature cell cycle exit by mechanisms that involve Tailless (Tll) transcription factor and the leucine-zipper protein Bunched (Kurusu et al., 2009; Siegrist et al., 2010). In the central brain and thoracic ganglia, most neuroblasts disappear due to terminal differentiation, which involves step-wise changes of the neuroblast's cellular properties, including shrinkage of cell size, attenuation of the cell cycle, and expression of homeodomain transcription factor Prospero (Pros), to terminate their proliferation. Pros promotes terminal differentiation of neuroblasts by inducing genes required for the cell cycle exit and the terminal differentiation (Maurange et al., 2008). In many cases, the timing of cell cycle exit of neuroblasts is controlled by the expression of a series of transcription factors (temporal transcription factor series; see below), which is also

important for generating different cell types in a given neuroblast lineage (Almeida and Bray, 2005; Cenci and Gould, 2005; Maurange et al., 2008).

Diversity of neuroblast types in the CNS

With few exceptions, almost all neuroblasts in the CNS generate their postmitotic neural progeny through secondary progenitors, that can be either non self-renewing or self-renewing. The so-called type I neuroblasts generate non self-renewing secondary progenitors, referred to as ganglion mother cells (GMCs). Each stem cell-like division of the parent type I neuroblast (which self-renews) gives rise to one GMC which in turn divides only once to produce two postmitotic daughter cells, either neurons or glial cells (Fig. 2A). Due to the asymmetric segregation of the Notch signaling inhibitor Numb during this terminal GMC division, one of its daughter cells has active Notch signaling ("Notch-On") while the other daughter has inhibited Notch signaling ("Notch-Off"). This difference translates into lineage-specific differences in the cellular and molecular properties of the two daughters such as axonal targeting, dendritic innervation or survival. Since each type I neuroblast gives rise to numerous GMCs during its period of proliferative activity, its lineage of neural progeny comprises two "hemilineages", one of which is Notch-On while the other is Notch-Off (Karcavich and Doe, 2005; Karcavich, 2005). This generic binary mechanism of asymmetric Notch signaling operating in all neuroblast lineages is an important factor in generating the remarkable neural diversity in the CNS and notably in the central brain and OL of *Drosophila* (Kumar et al., 2009; Li et al., 2013; Lin et al., 2010; Truman et al., 2010).

All of the neuroblasts in the VNC and most of the neuroblasts in the central brain belong to the type I class. Although their characterization is still incomplete, the

neuroblasts that generate the medulla neurons of the optic lobe also appear to belong to the type I class (Fig. 2C). In contrast, 8 neuroblasts located in the central brain hemispheres belong to a different class referred to as type II (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). These type II neuroblasts can be distinguished from type I neuroblasts by the absence of expression of the proneural transcription factor Asense and the cell fate determinant Pros (Bello et al., 2008; Boone and Doe, 2008). Type II neuroblasts generate their lineages of neural progeny through transit amplifying self-renewing secondary progenitors called intermediate neural progenitors (INPs). Each INP undergoes a limited series of proliferative divisions, in each of which it self-renews and generates a GMC which divides once more to produce two postmitotic neural cells (Fig. 2B). Since each type II neuroblast generates numerous INPs and each INP generates several GMCs, a marked amplification of proliferation ensues, and lineages that are 4-5 fold larger than any type I lineages are produced. These remarkably large type II neuroblast lineages comprise up to 500 neural cells and, hence, make a substantial contribution to the complex circuitry of the central brain (Bello et al., 2008; Reichert, 2011). For example, type II neuroblasts generate numerous neural cells, neurons and glia, that contribute to an extensive midline neuropile structure, the central complex of the Drosophila central brain (Izergina et al., 2009; Viktorin et al., 2011). Moreover, and more strikingly, they also contribute to the optic lobe by generating glial cells, which migrate out of the central brain and differentiate into lobula giant glial cells (Viktorin et al., 2013). Interestingly, the pronounced amplification of proliferation achieved in type II neuroblast lineages is balanced by extensive programmed cell death in these lineages, and this likely helps to generate the precise number of differentiated neuron needed in corresponding brain circuitry (Jiang and Reichert, 2012).

Recently, considerable insight into the mechanisms that control proliferation and lineage progression in type II neuroblast lineages, and notably in their INP

sublineages, has been obtained. Immediately following their generation, INPs are in an immature state characterized cellularly by mitotic inactivity and arrest in the G2 phase and molecularly by the absence of expression of Asense and the bHLH-O transcription factor Deadpan (Bowman et al., 2008). During the following 4-5 h of cell cycle arrest, INPs mature and acquire the restricted developmental potential necessary for several ensuing asymmetric cell divisions. During each of these cell divisions the mature Asense- and Deadpan-positive INPs self-renew and generate a GMC which gives rise to two neuronal or glia cells (Bayraktar et al., 2010). During the initial asymmetric division of the type II neuroblast, the cell fate determinants Brain tumor (Brat) and Numb are segregated into the INP daughter where they play an essential role to establishing INP potential (Bowman et al., 2008). Numb specifies INP identity by antagonizing the Notch pathway. Brat, on the other hand, contributes to the identity of INPs by blocking their potential dedifferentiation into neuroblast-like progenitors, and this process is likely to be mediated by suppressing the action of the self-renewal factor Klumpfuss through attenuation of β-catenin/Armadillo activity (Berger et al., 2012; Komori et al., 2014; Xiao et al., 2012). Additional restriction of INP dedifferentiation potential is mediated by dFezf/Earmuff (Erm), which is expressed in mature INPs and prevents their dedifferentiation by activating Prospero to limit proliferation as well as by antagonizing Notch signaling (Weng et al., 2010; Weng and Lee, 2011). Mutation in any one of genes that encode these INP specifying molecules including *brat*, *numb* or *erm* results in the failure of neural differentiation and overgrowth of Type II neuroblasts or INPs (see below) (Bowman et al., 2008; Weng et al., 2010). Recently, several new genes involved in proliferation and differentiation of type I and type II neuroblast have been identified by genome-wide transgenic RNAi screening (Neumuller et al., 2011). Further investigation of these new candidate genes is likely to result in additional information concerning the mechanisms that control neurogenesis in different neuroblast types.

Neuroblasts proliferate in a stem cell mode

A defining feature of stem cells is their ability to self-renew and at the same time generate daughter cells, that are committed to further differentiation, in one and the same cell cycle. This feature is usually linked to the ability of stem cells to undergo asymmetric cell divisions. All of the neural stem cell-like neuroblasts in the developing CNS of *Drosophila*, be they type I, type II, or OL neuroblasts, divide in an asymmetric stem cell mode (Benito-Sipos et al., 2011; Brody and Odenwald, 2000; Egger et al., 2008; Isshiki et al., 2001; Kambadur et al., 1998; Karlsson et al., 2010; Reichert, 2011; Touma et al., 2012; Tran and Doe, 2008). Indeed, many of the basic cellular processes and molecular mechanisms that operate in asymmetric stem cell division have been elucidated in the *Drosophila* neuroblast models (Januschke and Gonzalez, 2008; Knoblich, 2008; Schaefer and Knoblich, 2001; Wu et al., 2008; Zhong and Chia, 2008). While type I and type II neuroblasts differ in some aspects of their asymmetric cell division modes, a fundamental property of the asymmetric divisions manifested by these neuroblasts is the unequal segregation of proteins that assign cell polarity and cell fate to the two asymmetric daughter cells, the self-renewing neuroblast and the more differentiated daughter cell (GMC or INP) (Doe, 2008; Homem and Knoblich, 2012; Knoblich, 2008; Neumuller and Knoblich, 2009). This unequal segregation of molecular determinants involves two major molecular complexes that act in the neuroblast during the cell cycle (Fig. 3).

A so-called apical complex is essential for determining the axis of polarity and the orientation of the mitotic spindle in the neuroblast. This apical complex consists of the Par3/Par6/aPKC subcomplex and the Pins/Gαi/Mud subcomplex, both of which are localized in the apical region of the neuroblast and are linked via the Inscuteable protein. The Pins/Gαi/Mud protein complex is required for proper spindle orientation.

Mud binds directly to astral microtubules so that Pins/Gαi/Mud-Insc-par3/Par6/aPKC can exert a pulling force on the spindle of the dividing neuroblast (Izumi et al., 2006; Kraut and Campos-Ortega, 1996; Kraut et al., 1996; Siller et al., 2006; Speicher et al., 2008). The Par3/Par6/aPKC complex is involved in setting up and maintaining the apical-basal axis of polarity in the neuroblast. This complex is also responsible for the basal localization of cell fate determinants through sequential phosphorylation events that occur in the apical region of the neuroblast (Betschinger et al., 2003; Knoblich, 2008; Wirtz-Peitz et al., 2008; Yamanaka et al., 2006). For example, the mitotically active kinase Aurora A (Aur-A) phosphorylates Par6 resulting in activation of aPKC which then phosphorylates specific cell fate determinants located in the apical region of the neuroblast's cell cortex resulting in their release from the cortex apically and, hence, in their basal accumulation (Fig. 3B).

Three major cell fate determinants, Numb, Brat, and Pros, and two adaptor proteins, Miranda (Mira) and Partner-of-Numb (Pon) make up the so-called basal complex in the proliferating neuroblast. During asymmetric cell division of the neuroblast, these basally localized proteins are segregated into the smaller daughter cell, where they act in promoting differentiation and suppressing proliferation. Numb is a membrane bound Notch inhibitor containing a phosphoserine-binding (PTB) domain. Numb participates in specifying GMC fate by promoting endocytosis of Notch, thus maintaining Notch at a lower level in GMC than that of neuroblast (Bowman et al., 2008; Rhyu et al., 1994; Spana and Doe, 1996; Spana et al., 1995; Uemura et al., 1989; Wang et al., 2007; Zhong et al., 1996). Pros is involved in specifying neuronal and glial cell types in the developing nervous system, and during asymmetric cell division of the neuroblast, Pros is segregated together with Mira into GMC. Upon completion of cell division, Mira is degraded and Pros is released from the cortex and enters into nucleus, where it specifies GMC identity by promoting the expression of GMC-specific genes and repressing the expression of neuroblast-specific genes

(Atwood and Prehoda, 2009; Choksi et al., 2006; Ikeshima-Kataoka et al., 1997; Li and Vaessin, 2000; Shen et al., 1997). Thus, Prospero negatively regulates the expression of cell cycle genes such as *cyclin A*, *cyclin E*, and *string*, a *Drosophila* homolog of Ccdc25, and positively regulates the expression of *dacapo*, a cyclin-dependent kinase inhibitor. Pros also activates many genes involved in terminal differentiation of neurons such as *fasciclin II* and *netrin B* (Choksi et al., 2006). Brat, an NHL containing translation regulator, is thought to regulate ribosomal protein biosynthesis and to inhibit the transcription factor Myc at the posttranscriptional level. Like with Pros, Brat is exclusively segregated with Mira into the GMC during mitosis and contributes to GMC specification by decreasing protein synthesis (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006c).

As in other stem cells lineages, maintaining the precise balance between self-renewal and differentiation in asymmetrically dividing neuroblast lineages is essential to ensure normal development of the CNS as well as to prevent accumulation of aberrant neural stem cell-like progenitors. Indeed, recent studies using *Drosophila* neuroblasts have shown that defects in the key molecular mechanisms involved in asymmetric cell division control can result in loss of differentiated cells and uncontrolled overgrowth of neuroblast-like cells leading to brain tumor formation (Bello et al., 2007; Caussinus and Gonzalez, 2005; Chang et al., 2012; Knoblich, 2008) (Fig. 4). Notably, mutations in genes that result in defects in function or asymmetric localization of cell fate determinants such as mutations in Pros, Numb, Brat or in their adaptors Mira and Pon result in massive tumorous overproliferation in the brain due to the production of supernumerary self-renewing daughters at the expense of differentiated cells (Bello et al., 2006; Betschinger et al., 2006; Choksi et al., 2006; Wang et al., 2006a). Neural tumors also result from mutation of other genes involved in asymmetric cell division such as *discs large* (*dlg*), *lethal giant larva* (*lgl*), and *scribble* (*scrib*) or the genes encoding the Aur-A and Polo kinases

(Beaucher et al., 2007; Lee et al., 2006a; Ohshiro et al., 2000; Peng et al., 2000; Reichert, 2011; Wang et al., 2007; Wang et al., 2006b). All of the resulting neural tumor cells undergo massive overgrowth upon transplantation into wild-type hosts, kill the host within weeks, and become immortalized and can be serially transplanted into successive hosts over years (Beaucher et al., 2007; Caussinus and Gonzalez, 2005). These transplanted cells can also exhibit metastatic behavior, migrating away from the site of the primary tumor, passing through several cell layers, and establishing secondary colonies. As might be expected, type II neuroblasts are more susceptible to tumorigenesis, since their lineages comprise two cell types with self-renewing capability, namely neuroblasts and INPs.

Temporal patterning of neuroblast proliferation

The ensemble of neuroblasts in the *Drosophila* CNS can give rise to an astounding diversity of neural cell types. While the molecular mechanisms that make this possible are incompletely understood, the requirement of both positional and temporal information in proliferating neuroblasts for the generation of different neural cell types in its lineal progeny has been firmly established. Positional information is provided to each neuroblast of the central brain and VNC by the early embryonic expression of anteroposterior and dorsoventral patterning genes (Bossing et al., 1996; Broadus and Doe, 1995; Doe, 1992; Doe and Technau, 1993; Schmidt et al., 1997; Urbach and Technau, 2003). These two sets of developmental control genes, which include the Hox genes, the gap genes, the segment polarity genes and the columnar genes, establish a Cartesian grid-like molecular coordinate system in the neuroectoderm, from which the neuroblasts derive. As a result, each neuroblast acquires a specific combination of developmental control genes, which contribute to the specific identity of the neuroblast. As shown by an enormous body of genetic

evidence, this “combinatorial code” of transcription factors can directly influence the neural cell types that a given neuroblast generates (Skeath, 1999; Skeath and Thor, 2003; Technau et al., 2006; Urbach and Technau, 2004).

In addition to positional information, temporal information is also required in neuroblasts, notably for the generation of different cell types in its lineage of progeny at different times during the proliferation process. The time at which a given progeny is produced and exits the cell cycle is referred to as its birth date, and different progeny are generated by the parent neuroblast in a fixed birth order. The basic molecular mechanism that links birth order to neuronal fate involves a stereotyped temporal series of transcription factors expressed in the parent neuroblast. This temporal transcription factor series was first identified in the proliferating embryonic neuroblasts of the VNC (Fig. 5A), where a serial cascade of transient expression of the five transcription factors Hunchback (Hb), Krüppel (Kr), Pdm, Castor (Cas), and Grainyhead (Grh) takes place (Baumgardt et al., 2009; Benito-Sipos et al., 2010; Brody and Odenwald, 2000; Grosskortenhaus et al., 2005; Grosskortenhaus et al., 2006; Isshiki et al., 2001; Kambadur et al., 1998; Novotny et al., 2002; Pearson and Doe, 2003). The temporal transition of transcription factors is facilitated by cross-regulation among these transcription factors, which usually involves both positive feedforward regulation and negative feedback regulation (Baumgardt et al., 2009; Nakajima et al., 2010) However, this cross-regulation is not always required, and is sometimes even sufficient, since loss of one of the transcription factors Hb, Kr, or Pdm does not result in a blockage of the temporal series but only in the skipping of one temporal identity (Brody and Odenwald, 2000; Grosskortenhaus et al., 2006; Isshiki et al., 2001; Maurange et al., 2008; Tran and Doe, 2008). The specific molecular signals that control the switch in expression from one transcription factor to the next are still unclear.

Each of the transcription factors in this temporal series is expressed in the proliferating neuroblast during a specific time window, and the GMC that is generated by the neuroblast during that time window inherits the expression of that transcription factor. In consequence, the neurons that derive from the GMC inherit and maintain the expression of the same transcription factor, which is both required and sufficient for their birth order-dependent neuronal specification (Homem and Knoblich, 2012; Li et al., 2014). While the positional information acquired by each neuroblast in a neurogenic array is distinct, the temporal information manifest in proliferating neuroblasts has a more generic character. Many of the neuroblasts in the embryonic VNC manifest the same temporal series of Hb, Kr, Pdm, Cas, and Grh expression. However, since different neuroblasts generate different lineal cell types, this temporal series does not control neural cell type per se. Rather it specifies birth order-dependent neural identity, which together with positional identity provided by spatial combinations of transcription factor expression (and with hemilineage-specific Notch signaling) is translated into the specific neural cell types produced in a neuroblast lineage.

Temporal specification is not limited to embryogenesis but also occurs during postembryonic neurogenesis. In VNC neuroblasts two transcription factors, Cas and Sevenup (Svp), act in a postembryonic temporal series; Cas expression in late embryonic neuroblasts is maintained in postembryonic neuroblasts after exit from quiescence and is followed by a wave of Svp expression (Maurange et al., 2008; Zhu et al., 2006). Other members of the postembryonic temporal series must also exist, however, they have not yet been identified. A more complete characterization of a postembryonic temporal series has been carried out in OL development where a different temporal series of transcriptional factors has been identified (Fig 5B, change from 4). In the OL neuroblasts of the developing medulla, a temporal transcription factor series composed of Homothorax (Hth), Klumpfuss (Klu), Eyeless (Ey), Sloppy-

paird (Slp), Dichaete (D), and tailless (Tll) is expressed (Li et al., 2013; Suzuki et al., 2013). Moreover, crossregulatory interactions are required between some, but not all, of these transcription factors. Mutational inactivation or overexpression of individual members of this temporal series in OL neuroblasts affects birth order-dependent expression of different neuronal markers in the neural cells that are generated by these progenitors implying that the temporal transcription factors control OL neuronal fate. An interesting concatenation of two different temporal transcription factor series is seen during postembryonic development in type II neuroblast lineages (Bayraktar and Doe, 2013). The type II neuroblasts themselves serially express the transcription factors D/Cas and Svp, and more temporal transcription factors are likely to exist as well in these neuroblasts. In addition, each INP daughter cell generated by a type II neuroblast also expresses its own series of temporal transcription factors, namely D, Grh and Ey, in the sublineage of cells that it generates. Mutation or overexpression of the temporal transcription factors in INPs demonstrate the requirement of these factors in fate determination of the lineal neural progeny in INP sublineages, and also show that the sequential expression of these transcription factors is tightly controlled by cross-regulation mechanisms. This type of combinatorial temporal patterning composed by two different axis of temporal transcription factor cascades leads to a larger diversity of neurons and glial cells in complex neural lineages of type II neuroblasts.

Taken together, these findings indicate that virtually all neuroblast lineages in the developing CNS utilize transcription factor cascades as a generic mechanism for temporal patterning and determination of neural cell fate. The specific transcription factor combinations utilized in type I, type II, and OL neuroblasts differs. However, the functional role of the resulting temporal information, integrated together with positional information and binary Notch signaling, is a common one, namely the

generation of the remarkable diversity of cell types in the developing CNS from a surprisingly small set of neural stem cell-like precursors.

Conclusion

Drosophila neuroblasts have emerged as an excellent model for understanding the cellular molecular mechanisms involved in neural stem cell self-renewal and differentiation. The genetic basis for the generation of these neural stem cells from the neuroectoderm as well as many of the mechanisms that operate in these primary progenitors during their asymmetric proliferative cell divisions have been elucidated. Moreover, the processes that integrate amplification of proliferation with restricted lineage progression in transit amplifying intermediate progenitors are beginning to be understood. Finally, insight into the combinatorial molecular code that imparts positional and temporal information to neural stem cells as well as the role of these two types of information in specifying the diversity of differentiated neural cell types generated by individual neural stem cells is being obtained. Given the remarkable conservation of molecular mechanisms involved in nervous system development in *Drosophila* and vertebrates including mammals, the investigations of all of these features of neural stem cell biology in the fly model is likely to help in understanding the roles of neural stem cells in generating the highly complex human brain. From this perspective, the use of the *Drosophila* model for unraveling the mechanisms underlying neural stem cell derived brain tumors may also lead to important insight into the aberrant molecular mechanisms that cause brain tumors in human patients.

Abbreviations

aPKC: Atypical protein kinase C, Par3: Partitioning defect 3, Par6: Partitioning defect 6, CNS: Central nerve system, INP: Intermediate neural progenitor, Pon: Partner of Numb, Pins: Partner of Inscuteable, Mud: Mushroom body defect, Gαi: G protein α i subunit 65A

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Figure legends

Fig.1 Neurogenesis in the CNS of *Drosophila*

Drosophila neurogenesis occurs in two distinct periods, at embryonic and larval stage. **A**, Neuroblasts of the ventral nerve cord derive from the neuroectoderm (NE) by delamination. Proliferating neuroblasts self-renew and generate one ganglion mother cell (GMC) by asymmetric division. The GMC, in turn, divides once more to produce two postmitotic cells, neurons or glia cells. **B**. Neuroblasts in the postembryonic CNS. Schematic view of the *Drosophila* CNS in the third instar larva. Different types of neuroblasts are distributed in three anatomically different regions, the central brain (CB), optic lobe (OL), and ventral nerve cord (VNC). The central brain has three different types of neuroblasts, Type I, Type II, and Mushroom body (MB) neuroblast.

Fig.2. Different types of neuroblasts and their proliferation modes

A, Type I neuroblasts(NB) divide asymmetrically to generate one neuroblast and one ganglion mother cell (GMC). The neuroblast self-renews and the GMC divides terminally into two neurons or glia. **B**, Type II neuroblasts, eight of which are present in each hemisphere of the larval brain divide asymmetrically to generate one self-renewing neuroblast and one immature intermediate neural precursor (INP) with transit amplifying function. The INP matures through expression of genes that inhibit dedifferentiation and promote lineage progression. Mature INPs produce one immature INP and one GMC through another asymmetric division. **C**, Optic Lobe neuroblasts are generated by transition from neuroepithelial cells (NE) to neuroblasts induced at the medial edge of the outer proliferation center by a proneural wave. They proliferate in the type I mode. A, apical ; B, basal.

Fig.3 Asymmetric cell division of neuroblasts

A,Through asymmetric cell division neuroblasts self-renew and simultaneously generate a more differentiated GMC. In the mitotically active neuroblast a Par3/Par6/aPKC protein complex localized asymmetrically at the apical cortex is linked to the Pins/ Gαi/Mud protein complex via the scaffolding protein Inscuteable. Cell fate determinants including Pros, Brat, and Numb are asymmetrically localized at basal cortex together with their adaptor proteins, Mira and Pon. During asymmetric cell division, these cell fate determinants are exclusively segregated into the GMC where they induce various differentiation events. **B**, The apical protein complexes mediate the basal localization of cell fate determinants through protein phosphorylation cascades. Aur-A phosphorylates Par6 to activate aPKC in the complex. aPKC phosphorylates Lgl, Numb, and Mira. Phosphorylated Mira carries Pros and Brat to basal cortex. Polo is also involved in asymmetric protein distribution by phosphorylating Numb and Pon. A, apical ; B, basal.

Fig.4 Defects in asymmetric cell division of neuroblasts cause tumorigenesis

Defects in the molecular machinery involved in asymmetric cell division, including mutations of cell fate determinant genes, *pros*, *brat* and *numb,* cause tumor cell like overgrowth. While the mutant neuroblasts often still divide asymmetrically, their secondary progenitor progeny (GMC in type I neuroblasts and INP/GMC in type II neuroblasts) do not generate differentiated neural cells but rather revert to neuroblast-like cells that continue to divide in an uncontrolled manner. **A**, Normal neuroblast proliferation leading to differentiated neural cells. **B**, Mutant neuroblast overproliferation leading to tumorigenesis

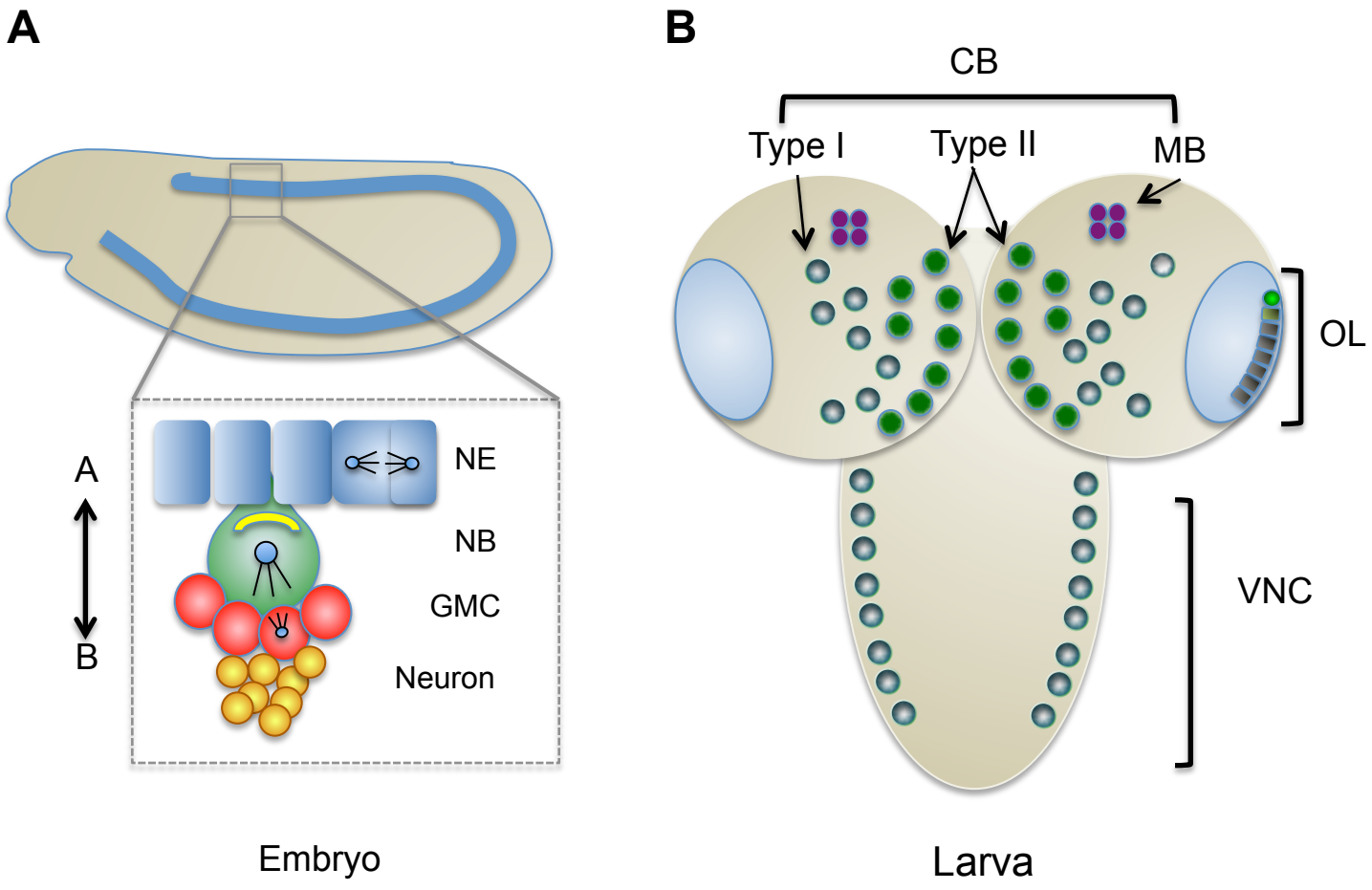
Fig.5 Temporal patterning of neuroblast proliferation

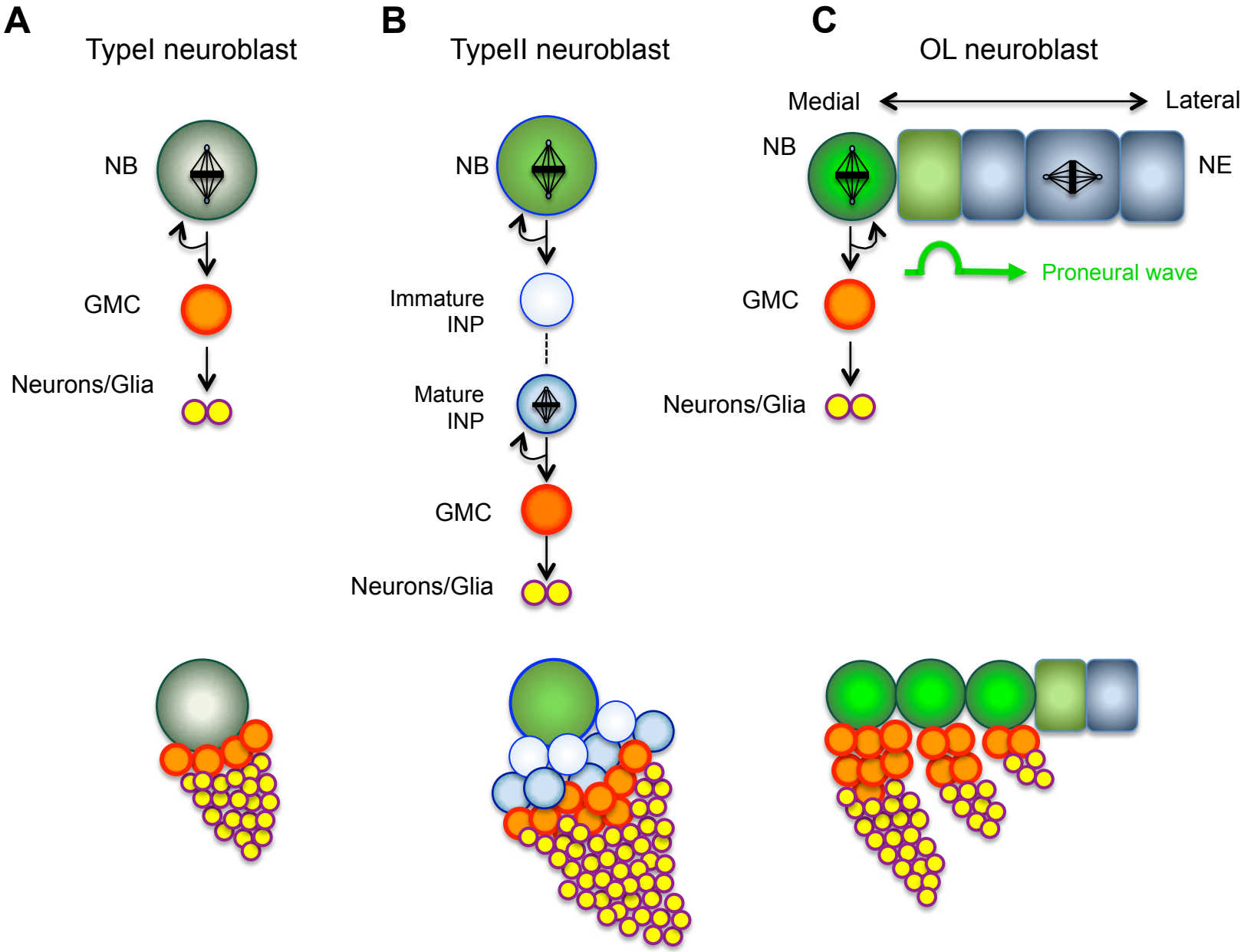
A, Embryonic neuroblasts in the VNC express a temporal series of the transcription factors, Hb, Kr, Pdm, Cas and Grh as they age. The temporal transcription factor expressed in the neuroblast is inherited by its GMC and specifies the identity of its

two neural cell progenies. During embryogenesis, a transient burst of Svp expression is required for the switch from Hb to Kr expression. Cas expression is maintained through quiescence and defines the temporal identity of the larval neuroblast until Svp is re-expressed. **B**, Serial expression of Hth, Klu, Ey, Slp, D, and Tll transcription factors in the medulla neuroblasts of the OL during postembryonic development.

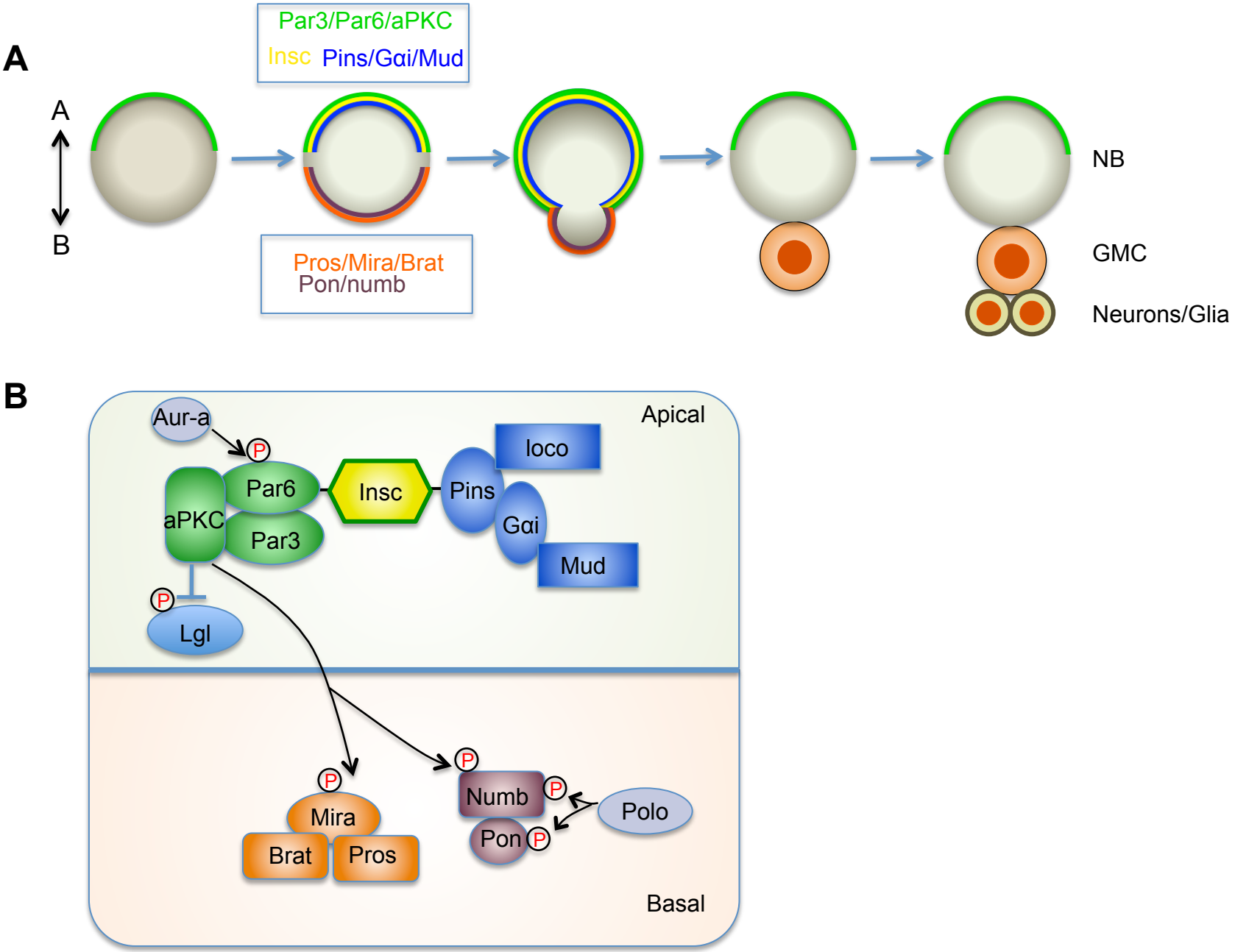
C, Combinatorial temporal patterning in type II neuroblast lineages. In addition to a temporal series expressed in the type II neuroblasts, a second different temporal series comprising D, Grh, and Ey is expressed in each INP. Thus two axes of temporal transcription factor cascades interact to generate a large diversity of neural cell types in these lineages.

Kang and Reichert Fig.1

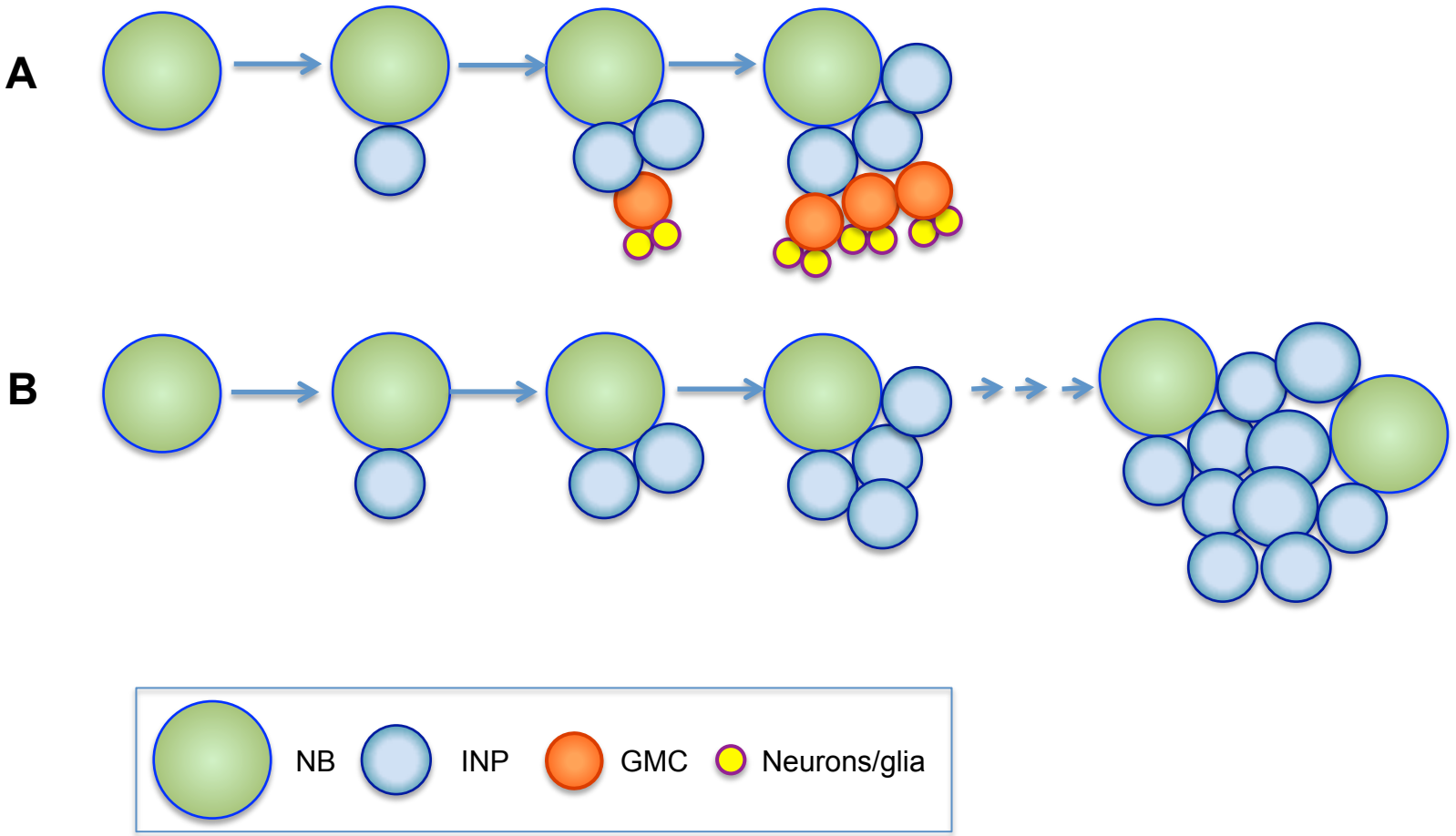



A
TypeI neuroblast
NB
GMC
Neurons/Glia
B
TypeII neuroblast
NB
Immature INP
Mature INP
GMC
Neurons/Glia
C
OL neuroblast
Medial
Lateral
NB
NE
Proneural wave
GMC
Neurons/Glia

Kang and Reichert Fig.3



Kang and Reichert Fig.4



Kang and Reichert Fig.5