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Neuronal specification in space and time

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Abstract

To understand how neurons assemble to form functional circuits it is necessary to obtain a detailed knowledge of their diversity and to define the developmental specification programs that give rise to this diversity. Invertebrates and vertebrates appear to share common developmental principles of neuronal specification in which cascades of transcription factors temporally pattern progenitors, while spatial cues modify the outcomes of this temporal patterning. Here, we highlight these conserved mechanisms and describe how distinct neural structures/animals use them in different ways. We present the questions that remain for a better understanding of neuronal specification. Single-cell RNA profiling approaches will potentially shed light onto these questions, allowing the characterization of neuronal diversity in adult brains, but also the investigation of the developmental trajectories leading to the generation and maintenance of this diversity.

One-sentence summary:

Principles of neuronal specification are shared between invertebrates and vertebrates and their contribution will be further elucidated using scRNAseq during development.

INTRODUCTION

Nervous system development requires many cell types generated in the proper order, number and location. This cellular diversity is generated from a small pool of progenitors initially defined by their spatial location and by sequential expression of temporal factors. Regulatory principles and some of the molecular players are conserved from insects to mammals. Here we highlight these similarities and discuss promising research avenues that use single-cell transcriptomics during development to understand neuronal specification.

Defining cell types one cell at a time

Although neurons have been classified since the beginning of modern neuroscience on the basis of morphology, function, electrophysiological properties or molecular markers (1), these criteria underestimate the diversity of neurons. Even in structures as well understood as the mammalian retina, morphology identifies 13 subtypes of bipolar cells but misses two additional types identifiable by transcriptional criteria (2). Although single-cell RNA sequencing (scRNAseq) allows unbiased identification of mature neurons, further understanding of nervous system function and disease requires knowledge not only of factors that define neuronal identity, but also of molecules driving neuronal specification, migration, and connectivity during development.

Modes of progenitor divisions and generation of neuronal units

Neural progenitors use two axes of information, spatial and temporal, to generate cellular diversity of the Central Nervous System (CNS) (3). The nervous system originates from simple neuroepithelial sheets where cells first proliferate by symmetric divisions and later become fate-restricted progenitors. We will focus on two examples of these progenitors, the apical radial glia (aRG) in the vertebrate cortex and the neuroblasts (NBs) in *Drosophila*.

aRG have long cellular processes that span from apical to basal sides of the developing cortex. aRG can give rise to neurons either through direct neurogenesis, in which the aRG divides asymmetrically to self-renew and generate a neuron, or indirect neurogenesis in which the aRG gives rise to intermediate progenitor populations, such as intermediate basal progenitors (bIPs), which usually divide once to produce neurons, and basal or outer RG (oRG), which have increased proliferative potential and can self-renew, amplifying the neuronal output of these lineages (4) (Figure 1A). These two types of intermediate progenitors appeared during mammalian evolution and have different proliferative capacities depending on the species. bIPs, which are not present in birds and reptiles (although zebrafish have non-apical progenitors), establish a new germinal niche, the subventricular zone (SVZ) that contributes neurons to all cortical layers. The SVZ appears to promote cortical expansion and the generation of new neuronal types in placental mammals, like callosal projection neurons, which connect different areas of the cortex and correlate with increased associative capacities (5). In mice, oRGs are present but far less abundant than in primates where they compartmentalize the SVZ by generating the outer SVZ. Thus an increase in oRG may have allowed an expanded cortex (4). Single-cell transcriptomics studies of human aRG and oRG show that oRGs preferentially express genes involved in growth factor signaling, cell migration and self-renewal, suggesting an increased stemness of oRGs as a mechanism underlying neocortex expansion in primates (6). Clonal analysis show the extensive proliferative capacity of human oRGs, which produce clones of neuronal and glial cells at mid-neurogenesis that are 1–2 orders of magnitude larger than mouse aRG clones (6, 7). Gene expression differences underlying distinct cortical progenitor behavior from rodents to humans have been investigated and recently reviewed (8).

In *Drosophila,* there are different types of NB division: Type 0 NBs asymmetrically divide multiple times, and, like for aRG direct neurogenesis, each time they self-renew they generate a single neuron. Type I NBs are the most abundant and also undergo multiple asymmetric divisions to self-renew but they produce a ganglion mother cell (GMC) that divides once more to generate either two neurons or glial cells. Type II NBs have expanded lineages: they divide to self-renew and produce multiple intermediate neural progenitors (INPs) that themselves divide asymmetrically to produce 4–6 GMCs (3). Hence, type II NBs produce lineages with larger number of neurons in ways that resemble the indirect neurogenesis in mammals (Figure 1B).

Temporal patterning of progenitors

Most of our understanding of cortical neurogenesis comes from studies of excitatory projection neurons, which comprise about 70–80% of the neuronal population in the mammalian cortex. GABAergic inhibitory interneurons (representing ~20–30%), migrate

into the cortex from their birthplaces, mainly in ganglionic eminences (Figure 3B) (9). The mature mammalian cortex comprises 6 neuronal layers that are generated in an inside-out manner: early born neurons occupy deep layers (DL: VI-V), closer to the neural progenitors, while later born neurons progressively occupy upper layers (UL: IVIII-II), after migrating along the processes of aRG through earlier born neurons (Figure 2A). Projection cortical neurons are thus organized in columns that are parallel to aRG processes and represent the functional units of each cortical area (9). A similar columnar structure is shared by the medulla neuropil in the fly optic lobe, in which each of the 800 columns generated by approximately the same number of NBs represents a functional processing unit (each column is composed of about 80 neuron types), allowing the retinotopic integration of visual information relayed from the 800 unit eyes (10). *Drosophila* medulla neurons are displaced away from the parent NBs as newly born neurons are generated, creating an early-to-late birth order-dependent axis in the opposite manner to the mammalian cortex (Figure 2B).

The generation of neural diversity in *Drosophila* depends on temporal and spatial patterning of neural progenitors. The first example of temporal patterning was described in embryonic NBs of the *Drosophila* ventral nerve cord. As the NBs age, they sequentially express a series of temporal transcription factors (tTFs) (Hunchback (Hb) → Krüppel → Pdm → Castor → Grainy head), generating specific neuronal progeny in each of the different temporal identity windows defined by these factors (3). Expression of these tTFs in different lineages leads to different neuronal outcomes. For example, Hb expression specifies the U1–U2 motor neuron identity in the NB7–1 lineage but specifies Rp1-Rp4 motor neuron identity in the NB3–1 lineage (3), indicating that spatial positioning additionally patterns these NBs. Other fly lineages have since been shown to express a series of tTFs, whose identity varies from structure to structure (3). The medulla NBs in the fly optic lobe sequentially express a series of six tTFs that are different from ventral nerve cord tTFs (11, 12) (Figure 2B). Expression of consecutive tTFs overlaps, generating additional temporal identity windows and expanding the diversity of neuronal progeny that can be produced. In addition, subtemporal genes act downstream of tTFs and further subdivide temporal windows (3). Each NB asymmetric division generates a GMC, which after its final division produces two different neurons, one of which activates the Notch pathway ($Notch^{ON}$) while the other does not ($Notch^{OFF}$), thus doubling the diversity of neural progeny (11). A different series of tTFs has also been found for the INP lineages that are orthogonal to the temporal series in type II central brain NBs (13) (Figure 1B), further expanding the array of neuronal types generated from a single type II NB.

Two prevailing models posit how mammalian cortical layers are generated. In the first (Figure 2A), a single multipotent progenitor has the competence to generate neurons for all the cortical layers. This progenitor undergoes asymmetric cell divisions and progressively becomes fate-restricted, first producing deep layers then switching to producing more superficial layers. Supporting this model, transplantation experiments and retroviral lineage tracing showed that early stage progenitors either transplanted into an old cortex or labeled early in corticogenesis can generate neurons for all the cortical layers, whereas old-stage progenitors either transplanted into a young cortex or labeled late in corticogenesis can only generate neurons residing in superficial layers (14). This model is consistent with the notion

that progenitors express a series of intrinsic temporal factors that induce with time the different neurons of successive layers. The second model of initially fate-restricted progenitors posits the existence of independent progenitors that each generates one of the different neuronal populations (15)(16).

The behavior of the overall progenitor population in the studies above does not explain how a single progenitor can generate all neuronal subtypes. MADM (Mosaic Analysis with Double Markers) lineage tracing experiments labeling single progenitors in mice support the multipotent progenitor model for the generation of cortical projection neurons: aRG generate defined clones averaging 8–9 neurons during the neurogenic phase that span all layers of the cortex when induced early (E10 to E13) and shift to the production of only superficial layer neurons when induced in later time points (E15) (7) (Figure 2A). Although these results support the multipotent progenitor model, and imply the existence of temporal patterning in the cortex, this type of progenitor could coexist with other fate-restricted progenitors that remain to be identified.

Intrinsic temporal factors identified in vertebrate neurogenesis are often homologous to those found in *Drosophila*. Ikaros (the ortholog of the early tTF Hb in the fly ventral nerve cord) is expressed early in cortical and retinal progenitors and specifies early born neuronal fates in both tissues, while Casz1 (the vertebrate ortholog of the late fly tTF Castor) is expressed in older retinal progenitors, specifying late born fates (17, 18) (Figures 2A and C). Induction of the FoxG1 TF (ortholog of *Drosophila* Slp1) induces DL neurogenesis through derepression of the TF Fezf2 (19). Brn1/2 are involved in the transition from early to mid-neurogenesis in the mouse cortex as UL neurons fail to be generated in *Brn-1/Brn-2* double mutants (20), while their mis-expression produces later born neurons. Similar to the role of Seven-up as a switching factor from early to late neuron generation in *Drosophila* (3), knockdown of its CoupTFI/II ortholog in the developing mouse forebrain prolongs generation of early born neurons at the expense of late born neurons (21). Therefore, tTFs generate neuronal diversity in both insects and vertebrates. However, in most cases, it remains unknown whether these factors act in vertebrate progenitors or in neurons, and whether they are organized in temporal series, as has been shown in flies NBs.

The timing of fate switches in neural progenitors is essential for the generation of the proper number and identity of neuronal subtypes. In several cases, feedforward mechanisms whereby an early tTF activates the expression of the next tTF in the series have been identified, while negative feedbacks allow later tTFs to repress earlier tTFs (11) (Figure 2B). Extrinsic factors can also be involved in transitions: the hormone ecdysone regulates the transition from early to late tTFs in *Drosophila* central brain type II NBs (22). The mechanisms by which vertebrate tTFs regulate each other and how these tTFs control gene expression to define cell fates are largely unknown (18).

Temporal patterning generates neuronal diversity and also allows the production of different neuronal types in an invariant order, which likely facilitates assembly of neural circuits. In *Drosophila*, deterministic lineages generate hard-wired circuits in an ordered and stereotypic manner (23–25). In the mammalian brain, lineage relationships between cortical neurons instruct both laminar organization and also functional relationships: excitatory neurons

preferentially form chemical synapses between lineage-related neurons rather than with non-sibling neurons (26). The formation of electrical synapses also occurs preferentially between lineage-related inhibitory interneurons (27). These examples highlight the instructive role of developmental time in the sequential generation and assembly of cortical circuitry, which is further refined in vertebrates by neuronal activity and stochastic processes. For instance, retinal progenitor cells (RPC) generate the seven distinct retinal cell classes in overlapping temporal sequences following the multipotent progenitor model (28) (Figure 2C). However, clones derived from single RPC are variable in size and composition, suggesting that stochastic factors also control lineage progression. Time-lapse imaging of single labeled RPC suggests that RPC are equipotent but have certain probabilities of dividing or differentiating, such that each cell type is biased but not deterministically specified in a restricted temporal window (29). At the end, integration of stochastic and deterministic mechanisms at the population level allows the mouse retina to reach a defined size and cellular composition (28).

Spatial patterning of neural progenitors

Intrinsic temporal patterning of neural progenitors, although a conserved strategy, cannot explain the diversity of neurons generated. Neural progenitors are additionally patterned by other mechanism based on their spatial location. Temporal and spatial patterning intersect to generate neural diversity in the *Drosophila* optic lobe. The neuroepithelium that generates optic lobe NBs is regionalized into different compartments by the restricted expression of spatial homeodomain TFs such as Vsx, Optix, and Rx as well as by the signaling molecules Decapentaplegic (Dpp), Wingless (Wg) and Hedgehog (Hh), generating eight NB domains along the dorso-ventral axis (Figure 3A) (30). The NBs generated in each domain (except the Wg domain) transit through the same series of tTFs as they age but the combinatorial input of spatial and temporal factors allows the generation of an increased diversity of neuronal types whereby spatial information modifies the type of neurons produced by tTFs. Unicolumnar neurons (in a 1:1 ratio with medulla columns), generated from all spatial compartments, appear to ignore spatial information, whereas multi-columnar neurons (which are fewer in number because they arborize in more than one column) are made from fewer NBs in restricted spatial compartments (30).

In the vertebrate neural tube, progenitors are patterned by spatial cues in the form of Hox gene expression along the rostro-caudal axis in response to morphogenetic gradients including fibroblast growth factors (FGFs) and retinoic acid, while Sonic Hedgehog (Shh), Bone Morphogenetic Proteins (BMPs) and Wnt pattern the dorsoventral axis (Figure 3B). Opposing signaling gradients confer positional information to neural progenitors and instruct expression of distinct TFs, which respond differentially to signaling inputs. Moreover, cross-repression between TFs expressed in neighboring progenitor domains generates sharp gene expression boundaries (31). Each progenitor compartment then generates different neuronal types. Temporal patterning also appears to be involved in spinal cord neurogenesis, as early-born motor- and inter-neurons differ from those produced later, although clonal relationships remain to be determined (32) (33) (Figure 3B).

Additional factors controlling neuronal specification

Both spatial and tTFs act in progenitors to specify neuronal progenies. In postmitotic neurons, additional TFs termed terminal selectors act individually or in combination to activate expression of terminal differentiation genes that define neuronal attributes such as neurotransmitter expression, neurite morphology and electrophysiological properties (34). Spatial and temporal factors acting combinatorially regulate the expression of terminal selectors (35)(36). In addition, extrinsic mechanisms are involved in generation of cortical diversity; they include TGFβ signaling, as well as thalamic input. Feedback cues from previously generated DL neurons influence the production of UL cortical neurons or promote the switch from neurogenesis to gliogenesis (14). These extrinsic mechanisms provide an additional level of regulation and plasticity to corticogenesis, which would not be possible by relying only on intrinsic temporal and spatial factors.

Shifting to single-cell approaches during development

While much of what is known about spatial and temporal patterning of neurogenesis derives from molecular genetic techniques, much will be learned using sc-RNAseq technologies. sc-RNAseq has amplified our perception of neural diversity, redefining the concept of cell type. It has allowed validation of previously proposed cell types and uncovered new ones (37). However, the relationship of transcriptomic cell types to *bona fide* cell types has often not been established. Accurate cell-type classification will require correlations between molecular and morphological, physiological, and connectomic characteristics. The transcriptional signature of a neuron should be predictive of its function. Indeed, a transcriptomic study of neuronal diversity in the fly optic lobe identified TFs explaining much of the neuronal diversity in this brain region (38), where the same neurotransmitter identity can be assigned by distinct TFs combinations in different neuronal types, suggesting phenotypic convergence and supporting principles described in *C. elegans* (34).

Unresolved however is how the adult transcriptional profile arises from the establishment of neuronal identity during development when most gene expression changes occur, often coincident with circuit assembly (39, 40). Since sc-RNAseq yields snapshots of expression data whereas neuronal specification through development is a continuum of different states, computational methods such as trajectory inference algorithms are necessary to temporally order these states and to allow the identification of branching points during developmental trajectories (41). CRISPR mutation-based high-throughput lineage tracing approaches (41) although still technically challenging, are beginning to allow the reconstruction of lineage trees. Methods for reconstructing the positional information of a given cell in the tissue being subjected to transcriptomic analysis (spatial transcriptomics) are also being implemented (41). Parallel improvements in computational methods applied to sc-RNAseq studies are necessary to interpret the increasing complexity of data generated using these approaches. Additionally, functional validation is essential to test hypotheses emerging from sc-RNAseq studies. scRNAseq have allowed researchers to begin a comprehensive characterization of cell types in the CNS, to discover new cell types, to identify new markers that enable their manipulation, to trace developmental cellular decisions and to uncover disease-related genes (reviewed in (37)). Integration of transcriptomic, epigenomic and

proteomic analyses at the single-cell level is within reach and will reveal the cellular diversity and development of systems as complex as the human brain.

CONCLUSIONS

In both invertebrates and vertebrates, conserved mechanisms of temporal and spatial patterning allow neural progenitors to generate an astonishing diversity of cell types in the CNS. As the brain evolved with greater complexity, new progenitor types were generated. Extrinsic influences and developmental plasticity acquired increased roles in cellular patterning, allowing integration of increased numbers and diversity of cells.

Single-cell analyses have allowed identification of cell type specific markers and drivers, which will be invaluable for testing hypotheses regarding the development of neuronal diversity. Insight into temporal and spatial factors that specify neuronal diversity will be crucial for producing the desired neuronal types for cell replacement therapy. Finally, comparison across species of neural diversity and development will contribute to our understanding of the evolution of the brain.

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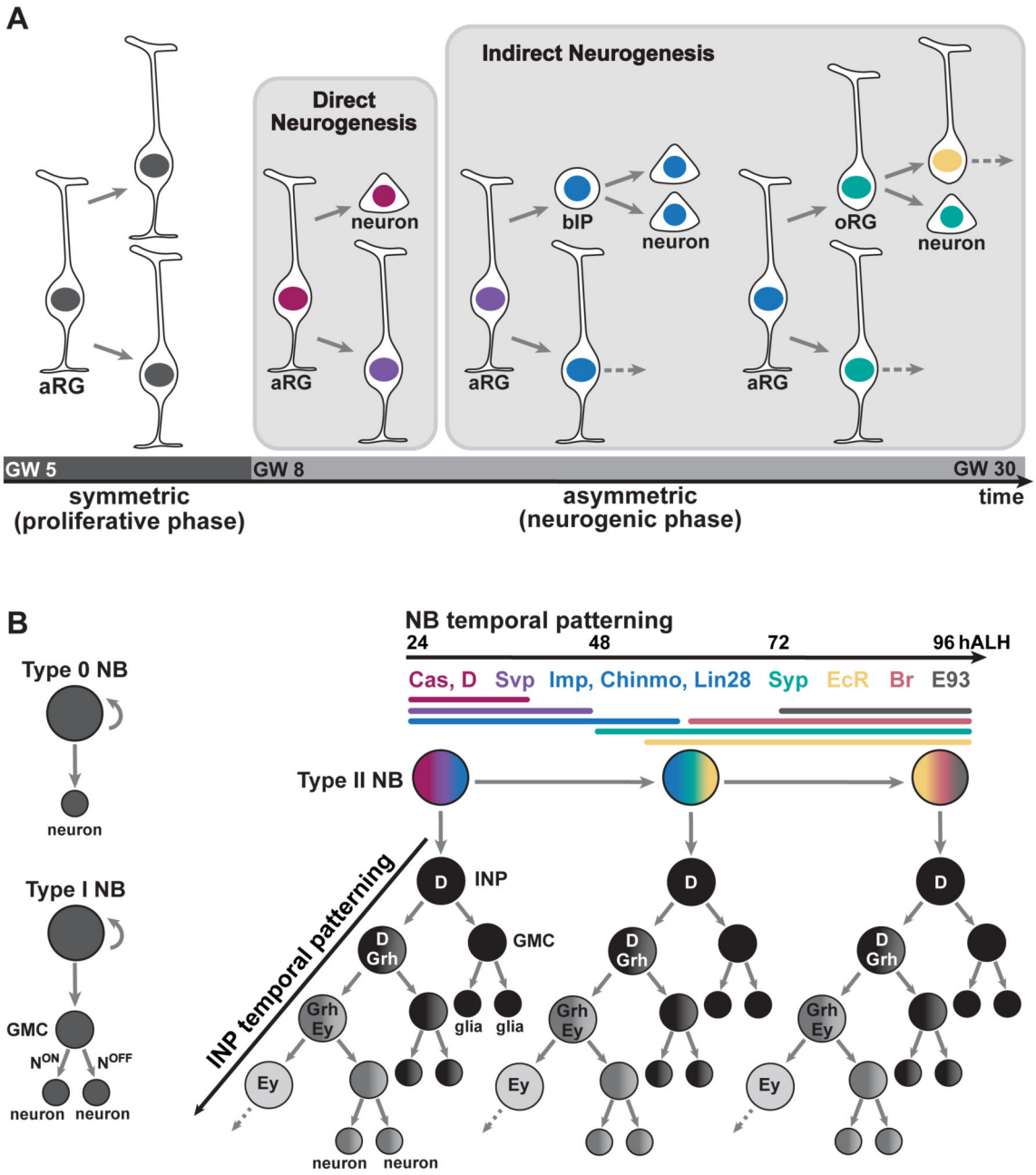

Fig.1: Modes of division of neural progenitors. A. Neurogenesis in the primate cortex. aRG undergo symmetric cell divisions to expand their pool (proliferative phase), and then transit to a neurogenic phase where they generate neurons directly or indirectly through intermediate progenitors such as bIPs and oRGs. Changes in TF expression in progenitors over time are shown as changes in cell color. aRG: apical radial glia. bIP: basal intermediate progenitor. oRG: outer radial glia. GW: gestational week. **B. Different modes of NB division in *Drosophila*.** Type 0 NBs self-renew and generate a single neuron at each division. Type I NBs self-renew and produce GMCs that divide once to produce one Notch^{ON} and one Notch^{OFF} neuron. Type II NBs have an increased neuronal output by

generating INPs, which themselves asymmetrically divide multiple times to produce GMCs. Both NBs and INPs sequentially express series of tTFs as they age (examples of these and additional temporal factors in NBs are shown). Cas: Castor. D: Dichaete. Svp: Seven-up. Imp: IGF-II mRNA-binding protein. Chinmo: Chronologically inappropriate morphogenesis. Syp: Syncrip. EcR: ecdysone receptor. Br: Broad. E93: Eip93. Grh: Grainy head. Ey: Eyeless. hALH: hours after larval hatching. Modified after Doe *et al.*, 2017 (3).

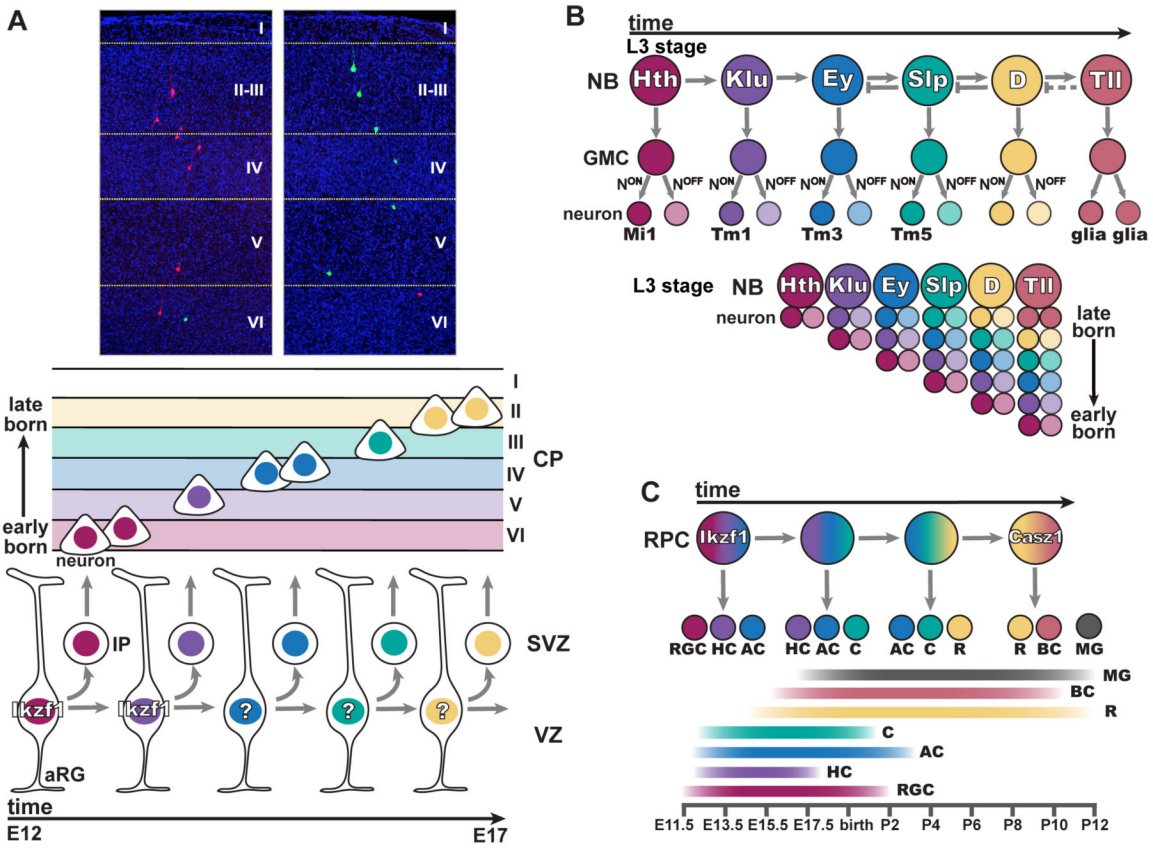

Fig.2: Temporal patterning of progenitors. A. Multipotent progenitor model of neurogenesis in the mammalian cortex. Upper panel:

Asymmetric MADM clone showing the progeny of a single aRG that span each layer (II–VI) of the mouse cortex (Picture from S. Hippenmeyer (7)). **Bottom panel:** A common progenitor generates neurons for the different cortical layers (VI through II) sequentially in an inside-out fashion. Ikaros (Ikzf1) is an example of a TF specifying deep layer neuronal identity. Changes in TF expression in progenitors over time are shown with color changes. aRG: apical radial glia. IP: intermediate progenitor. VZ: ventricular zone. SVZ: subventricular zone. CP: cortical plate. E: embryonic day. **B. Temporal patterning in *Drosophila* optic lobe NBs. Upper panel:** Sequential expression of tTFs in *Drosophila* type I optic lobe NBs, specifying distinct neuronal (e.g. Mi1, Tm1, Tm3, Tm5) and glial identities in each temporal window. Cross-regulatory interactions between tTFs are shown. **Bottom panel:** Newly born neurons displace older siblings away from the parent NB, generating a birth order-dependent layered neuronal arrangement in the medulla cortex in third instar larvae (L3). **C. Vertebrate retina.** RPCs sequentially generate the seven retinal cell types in overlapping waves. The TF Ikzf1 specifies early-born fates while Casz1 specifies late born fates. RPC: retinal progenitor cell. RGC: retinal ganglion cell. HC: horizontal cell. AC: amacrine cell. C: cone. R: rod. BP: bipolar cell. MG: Müller glia. E: embryonic day. P: postnatal day.

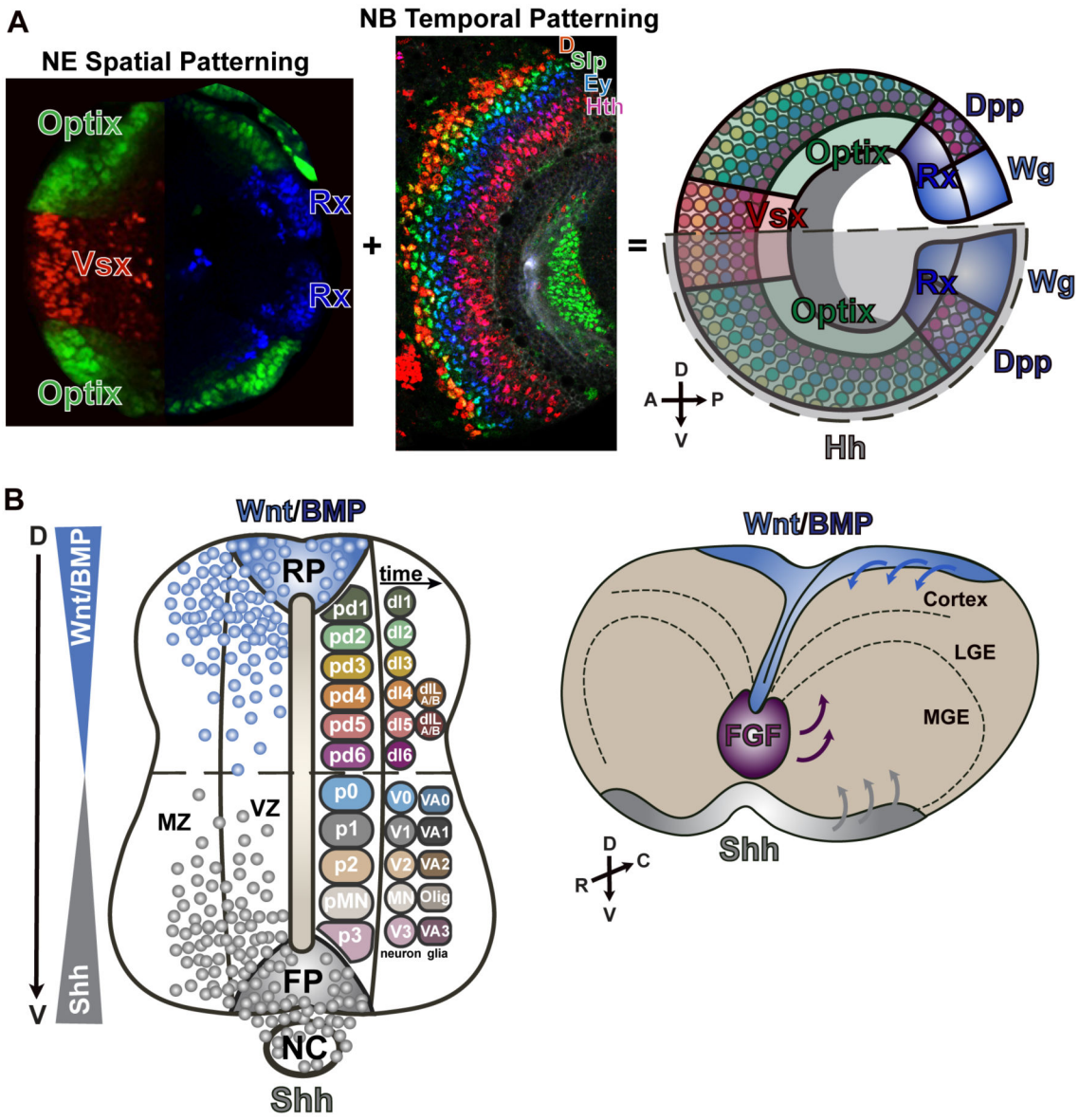

Fig. 3.: Spatial patterning of progenitors. A. Integration between spatial and temporal patterning in the *Drosophila* optic lobe. Left panel:
Regionalization of the *Drosophila* optic lobe neuroepithelium (NE) by the expression of the TFs Optix, Vsx and Rx. **Middle panel:** Sequential expression of the tTFs Hth-Ey-Slp-D in optic lobe NBs of different ages. **Right panel:** Schematic of spatial and temporal factors acting in optic lobe NBs. The neuroepithelium is additionally patterned by the signaling molecules Dpp and Wg and by Hh in the ventral part, creating eight compartments along the dorsoventral axis. **B. Morphogens spatially pattern the mammalian spinal cord and telencephalon. Left panel:** Dorso-ventral patterning of the neural tube by Wnt/BMP and Shh that regulate the expression of TFs in progenitors, which produce different types of neurons. Early born neurons are different from those produced at later stages. The progenitor domains and the neurons generated in each one of them are indicated. NC: notochord. FP: floor plate. RF: roof plate. VZ: ventricular zone. MZ: mantle zone. **Right panel:** Schematic

of the mouse embryonic telencephalon that is similarly patterned by the morphogens Wnt and BMPs from the dorsal hem and ventrally by the secretion of Shh. FGFs are secreted from a rostral signaling center (violet). GABAergic interneurons are generated from the lateral (LGE) and medial ganglionic eminences (MGE) in the ventral telencephalon and migrate tangentially to reach the cortex.