Annu. Rev. Cell Dev. Biol. 2004. 20:619–47
doi: 10.1146/annurev.cellbio.19.111301.115142
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First published online as a Review in Advance on June 28, 2004

SPECIFICATION OF TEMPORAL IDENTITY IN THE DEVELOPING NERVOUS SYSTEM

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Key Words neuroblast, birth-order, competence, Hunchback

■ **Abstract** The nervous system of higher organisms exhibits extraordinary cellular diversity owing to complex spatial and temporal patterning mechanisms. The role of spatial patterning in generating neuronal diversity is well known; here we discuss how neural progenitors change over time to contribute to cell diversity within the central nervous system (CNS). We focus on five model systems: the vertebrate retina, cortex, hindbrain, spinal cord, and *Drosophila* neuroblasts. For each, we address the following questions: Do multipotent progenitors generate different neuronal cell types in an invariant order? Do changes in progenitor-intrinsic factors or progenitor-extrinsic signals regulate temporal identity (i.e., the sequence of neuronal cell types produced)? What is the mechanism that regulates temporal identity transitions; i.e., what triggers the switch from one temporal identity to the next? By applying the same criteria to analyze each model system, we try to highlight common themes, point out unique attributes of each system, and identify directions for future research.

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INTRODUCTION

The central nervous system (CNS) is unique in its complexity of cell types, highly precise cell interactions, and exquisite sensitivity to environmental inputs: For example, some neurons accept several hundred thousand inputs from other neurons (Butler & Hodos 1996); other neurons can change their neurotransmitter release in response to a single photon of light (Schneeweis & Schnapf 1995); and many neurons distinguish their unique synaptic targets among millions of available targets. Even as you read this review, the 10^{11} neurons and 10^{12} glial cells in your brain work in unison to detect the words on the page, relay them to the brain, process the words, put the words in syntax, put the syntax in context, and (hopefully) store the relevant information for recall later. The capability and flexibility of the nervous system is astonishing, yet it develops from relatively small pools of progenitor cells. How these progenitor cells change over time to generate neuronal diversity is the topic of this review.

We define a progenitor cell as a proliferative cell that has the ability to generate two or more different cell types (multipotent), whereas precursor cells are defined as proliferative but restricted to generating a single cell type. We define temporal identity as a cell fate that is specified on the basis of a temporal cue, one that is either intrinsic or extrinsic to the cell. Just as a cell can have a unique spatial fate that is predicted by its spatial position, so too can a cell have a temporal fate that is accurately predicted by the time at which it is born. In some cases temporal identity is tightly linked to cell-type identity (mammalian cortex, spinal cord, hindbrain), but it is important to note that temporal identity can be completely independent of the actual cell type produced. For example, temporal identity in *Caenorhabditis elegans* is coordinately regulated by the heterochronic pathway, but how each cell in the worm interprets its temporal identity depends on its unique spatial or lineal identity (Thummel 2001). Similarly, different *Drosophila* neuroblasts use the same temporal cues to generate different neuronal cell types (see below). Viewed more generally, spatial heterogeneity in neural progenitors may lead each to respond differently to the same intrinsic or extrinsic temporal identity cues and

thus produce different cell types. The key point is that temporal identity is another "axis of information"—generated intrinsically or extrinsically—that a progenitor can use to generate cell type diversity.

There are two conceptually different ways for specifying temporal identity, which are useful to consider in their extreme form (Figure 1), with the realization that most cells will use each mechanism to different degrees, as discussed below. (*a*) Intrinsic regulation of temporal identity (Figure 1; top row). Here the progenitor cell initially acquires a unique spatial identity on the basis of its anterior-posterior and dorsoventral position within the CNS; it then becomes independent of spatial patterning cues and initiates an invariant cell lineage. Progenitors using this mechanism would specify temporal identity strictly based on progeny birth-order (since they are unaffected by environmental changes). As discussed below, *Drosophila* neuroblasts and vertebrate retinal progenitors may use this type of mechanism to specify temporal identity. (*b*) Extrinsic regulation of temporal identity (Figure 1; bottom row). Here the progenitor also acquires a unique spatial identity on the basis of its anterior-posterior and dorsoventral position within the CNS; it then responds to changes in spatial patterning cues over time, which leads to the generation of different progeny over time. In this case, temporal factors modulate the palette of spatial cues the progenitor is exposed to over time. As discussed below, vertebrate cortical, hindbrain, and spinal cord progenitors may use this type of mechanism to specify temporal identity. We note that when extrinsic cues are highly stereotyped, the sequence of cell types produced can be nearly invariant, and thus simple cell lineage studies are not sufficient to distinguish between these two models; only in vitro isolation and transplantation experiments or molecular genetic analysis of candidate factors can provide evidence for one or the other model.

In this review, we discuss the specification of temporal identity in different regions of the CNS and in several organisms: *Drosophila* embryonic and larval neuroblasts, and the vertebrate retina, cerebral cortex, hindbrain, and spinal cord. Owing to space limitations, we do not discuss closely related areas that have been reviewed recently, including the timing of oligodendrocyte differentiation (Durand & Raff 2000), the role of Delta-Notch signaling in progenitor lineages (Marquardt 2003, Marquardt & Gruss 2002), and the role of cell cycle control and asymmetric cell divisions in neural patterning (Ohnuma & Harris 2003, Zhong 2003).

TEMPORAL IDENTITY IN THE *DROSOPHILA* CNS

Multipotent Progenitors and Invariant Sequence of Progeny?

The embryonic *Drosophila* CNS develops from progenitor cells called neuroblasts. Each neuroblast forms at a specific time and position by delaminating into the embryo from the ventral neuroectoderm, and each expresses a unique combination of molecular markers. In each half-segment, a total of 30 neuroblasts delaminate in a reproducible pattern called the neuroblast map, where each neuroblast is assigned

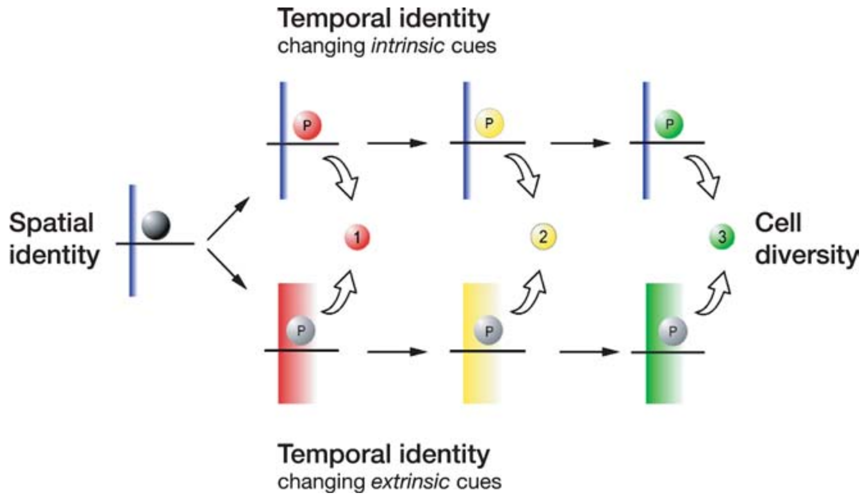


Figure 1 Models for specifying temporal identity. Progenitors (P) generate temporally distinct progeny (*smaller numbered circles*) over time. Hypothetical extrinsic spatial patterning cues are colored. **(***Top***)** Intrinsic temporal identity factors. Progenitors acquire spatial identity (*left panel*), and then are able to generate the proper sequence of cell types in the absence of extrinsic cues (e.g., in vitro). This model requires an intrinsic program that can specify temporal identity but does not rule out extrinsic influences (e.g., feedback inhibition from within a clone). Different progenitors can generate different cell types owing to their initial spatial heterogeneity, which may explain why cell type and birthdate are not correlated in some systems (e.g., neuroblasts and retina). **(***Bottom***)** Extrinsic temporal identity factors. Progenitors acquire an initial spatial identity (*left panel*), and then temporal changes in the spatial patterning cues result in the ordered production of different cell types. This model requires a mechanism for temporal regulation of extrinsic cues; it predicts (*a*) that sequential cell-type production is unlikely to occur without changes in extrinsic cues (e.g., single-cell cultures) and (*b*) that there will be a direct link between spatial patterning cues and cell-type specification. Cells undergoing terminal division are likely to be exposed to the same spatial patterning cue environment, which may explain why cell type and birthdate are well correlated in some systems (e.g., cortex, spinal cord, and retina).

an address according to its row and column number (e.g., 1–1, 4–2, etc.). The neuroblast map is symmetrical across the ventral midline and is serially repeated in each segment, with minor segmental differences. Neuroblasts divide asymmetrically to bud off a series of smaller daughter cells into the embryo, called ganglion mother cells (GMCs), which are named after their birth-order (e.g., GMC-1, GMC-2, etc.). GMCs have a limited proliferation potential, dividing just once to make two postmitotic neurons or glia. DiI labeling of single neuroblasts shows that every neuroblast makes a unique and reproducible clone of progeny, and that most

neuroblasts are multipotent (Bossing et al. 1996, Schmid et al. 1999, Schmidt et al. 1997). For example, neuroblast (NB) 1-1 (NB1-1) makes motor neurons, interneurons, and glia; NB7-1 makes motor neurons and interneurons; and NB5-6 makes interneurons and glia. Recent work on the early lineages of NB6-4, NB7-3, and NB7-1 has shown that each generates progeny in an invariant order (Higashijima et al. 1996, Novotny et al. 2002, Pearson & Doe 2003). NB7-3 has the shortest lineage (three GMCs) and has been completely defined (Novotny et al. 2002). NB7-1 has the longest lineage (~20 GMCs) and the exact lineage of the first 5 GMCs has been defined: It sequentially generates GMC-1 through GMC-5, which make the U1–U5 motor neurons and their sibling neurons, respectively (Pearson & Doe 2003), and then continues on to make about 15 GMCs that all produce interneurons (Bossing et al. 1996, Schmid et al. 1999, Schmidt et al. 1997). Taking the DiI lineage and birth-order data together, it appears that most neuroblasts are multipotent and many or all will generate their diverse progeny in an invariant sequence.

Recent work has shown that larval neuroblasts are also multipotent and generate distinctive progeny in an invariant order. For example, the AD brain neuroblasts generate olfactory projection interneurons, each of which projects its dendrites to a specific olfactory glomerulus (Jefferis et al. 2001, 2004). Early-born interneurons always project their dendrites to a specific glomerulus in the antennal lobe of the brain, while progressively later-born interneurons project to distinct glomeruli (Jefferis et al. 2001). Although there appears to be a precise link between interneuron birth-order and neuronal identity (assayed by characteristic dendritic projection patterns and transduction of a specific olfactory response), to date nothing is known about the molecular or cellular basis of temporal identity in the AD brain neuroblast.

Intrinsic Versus Extrinsic Regulation of Temporal Identity

What determines temporal identity in neuroblast lineages? Is each GMC born with an intrinsic temporal identity based on its birth-order (e.g., GMC-1, GMC-2, GMC-3, etc.)? Or are GMCs born equivalent and learn their temporal identity from the changing environment they are born into? There are several reasons to favor an intrinsic mechanism for timing and specifying temporal identity within neuroblast lineages. First, neuroblasts do not go through their lineages synchronously. Early-forming neuroblasts that are generating GMC-4 and GMC-5 are interspersed with late-forming neuroblasts that are generating GMC-1. Clearly a single environmental signal cannot provide temporal information to a population of asynchronous neuroblasts. Second, in vitro culture of *Drosophila* neuroblasts is more consistent with an intrinsic mechanism. Neuroblasts cultured in isolation undergo normal asymmetric division to make a series of GMCs (Broadus & Doe 1997) and, ultimately, clones that contain the correct number of a particular cell type. In one experiment, neuroblast clones were observed to have two serotonergic neurons (Huff et al. 1989), the proper number for the NB7-3 lineage (Novotny et al. 2002). In another experiment, clones were observed with non-overlapping populations of

neurons expressing Hunchback, Pdm, and Castor transcription factors (Brody & Odenwald 2000), similar to many in vivo lineages (Isshiki et al. 2001).

However, time-lapse analysis has not been done to ensure that the different types of neurons are generated in the normal order in the in vitro culture experiments; nor has any neuroblast been uniquely identified in culture to make sure it undergoes its specific lineage. In the future, it will be important to use genetic markers, such as neuroblast-specific green fluorescent protein (GFP) transgenes, to identify a neuroblast in vitro, and then use time-lapse analysis and cell type-specific markers to confirm that it makes the correct progeny in the proper order. In addition, these culture experiments do not rule out the possibility of feedback signaling from an early-born GMC to the neuroblast. Finally, even if neuroblast lineages are normal in vitro, it does not mean that extrinsic cues do not exist or may not be able to override or entrain an intrinsic program. This can be tested by heterochronic transplants of late lineage neuroblasts back into early hosts (or vice versa) to see if a different temporal environment is dominant over the intrinsic program of the neuroblast. In summary, in vitro and in vivo data support a model of temporal identity that is largely cell intrinsic (Figure 1, top row).

Factors Conferring Temporal Identity: Hunchback and Krüppel

The first candidate genes for regulating temporal identity throughout the embryonic CNS came when Kambadur et al. (1998) showed that three transcription factors are expressed in mutually exclusive layers in the late embryonic CNS (Kambadur et al. 1998). They found that Hunchback (Hb), a zinc finger transcription factor, was detected in deep layer neurons; Pdm1/Pdm2 (henceforth Pdm), both POU domain transcription factors, were detected in middle layer neurons; and Castor (Cas), previously called Ming (Cui & Doe 1992), a zinc finger transcription factor, was expressed in superficial layer neurons. This description was intriguing because DiI lineage analysis showed that neurons from early-born GMCs populate the deepest layer of the CNS, whereas later-born neurons are located more superficially (Bossing et al. 1996, Schmid et al. 1999, Schmidt et al. 1997), raising the possibility that Hb, Pdm, and Cas expression may correlate with neuronal birth-order and perhaps play a role in specifying temporal identity.

Isshiki et al. (2001) extended this descriptive analysis to include another zinc finger transcription factor, Krüppel (Kr), which is expressed at low levels in the Hb layer and in a distinct layer between Hb and Pdm (as well as at low levels in the Hb layer). They also precisely defined the timing of Hb, Kr, Pdm, and Cas expression in three different neuroblast lineages (Isshiki et al. 2001), finding that each protein is transiently detected in the neuroblast in the sequence Hb → Kr → Pdm → Cas (with low levels of Kr present during the Hb expression period). GMCs are born intermittently as the neuroblast transits through Hb → Kr → Pdm → Cas expression, with an average of one GMC per gene expression window, and the GMC and its neuronal progeny will maintain the gene expression profile present

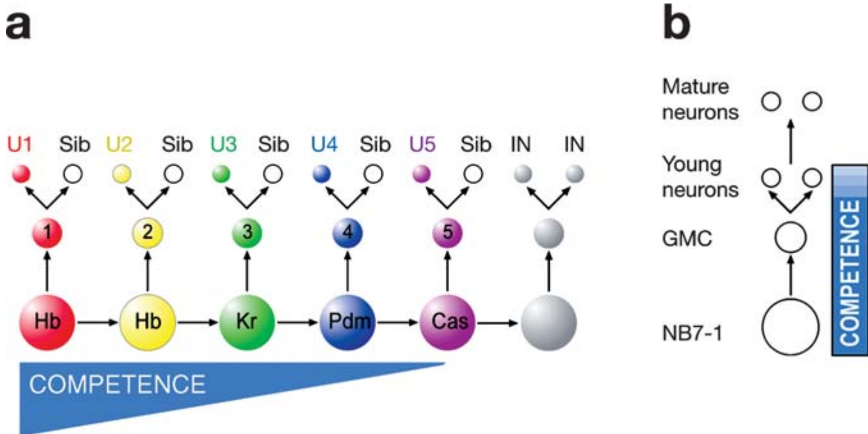


Figure 2 Hb → Kr → Pdm → Cas expression and the competence to respond to Hb in the *Drosophila* NB7-1 lineage. (*a*) A model neuroblast lineage is shown (NB7-1). Hb, Kr, Pdm, and Cas expression is indicated by color, with transient neuroblast expression maintained by the GMCs and neurons born during each window of gene expression. In this lineage, two GMCs are born during the window of Hb expression, although most lineages (e.g., NB7-3) produce only one GMC during the Hb expression window. Moreover, some GMCs can be born during a short window of gene overlap (a Pdm$^+$Cas$^+$ GMC is born during a brief window of Pdm$^+$Cas$^+$ overlap in NB2-4), but this does not occur in the NB7-1 lineage. Most importantly, a few neuroblasts do not express all of these genes: NB7-3 terminates its cell lineage after generating three GMCs and never expresses Cas, whereas NBs 2-1, 3-3, 5-1, 6-1, and perhaps 5-5 express Cas throughout their lineages (these neuroblasts typically make short clones of similar interneurons). Under the lineage is a representation of how NB7-1 shows progressive loss of competence to generate early-born neurons in response to Hb over time (Pearson & Doe 2003). (*b*) The competence to generate early-born neurons in response to Hb is lost by the time a neuron becomes postmitotic. Modified from Pearson & Doe (2003).

in the neuroblast at the time the GMC was born (Figure 2*A*). This is a remarkable pattern that raises many interesting questions: Does Hb → Kr → Pdm → Cas expression correlate with the production of distinct cell types? What is the function of each of these genes in specifying GMC temporal identity? How does the neuroblast time the Hb → Kr → Pdm → Cas sequence? How is gene expression maintained in GMC/neuronal progeny but not in the neuroblast? The first three of these questions are discussed below; we know nothing about the fourth.

Does Hb → Kr → Pdm → Cas expression correlate with the production of distinct cell types? The answer to this key question is clearly no: Hb$^+$ first-born GMCs can produce interneurons, motor neurons, or glia, depending on the spatial identity of the neuroblast. Similarly, Kr$^+$, Pdm$^+$, and Cas$^+$ GMCs can generate motor neurons or interneurons and, in some cases, glia, depending on the neuroblast lineage. Thus Hb/Kr/Pdm/Cas expression is correlated with temporal identity

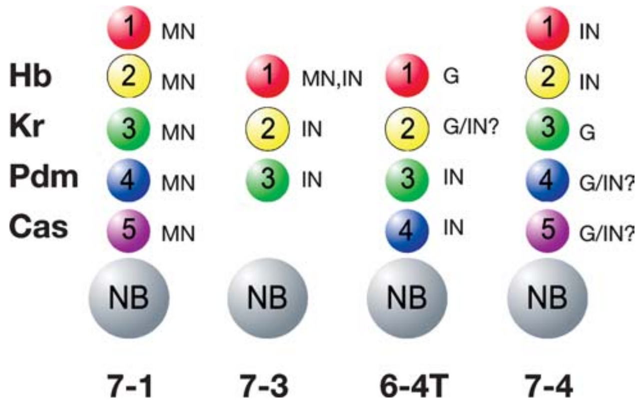


Figure 3 Birth-order versus cell type in *Drosophila* neuroblast lineages. In four well-characterized lineages, Hb is required in the first-born cells for their temporal identity, yet the first-born cell types can be motor neurons (MN), interneurons (IN), or glia (G). Thus, the unique spatial identity of each neuroblast, combined with the unique temporal identity of the progeny, results in different neural cell types in each lineage. This is in contrast to all first-born progeny being the same cell type (e.g., in the vertebrate cortex, hindbrain, and spinal cord). Modified from Isshiki et al. (2001).

(birth-order) and not cell type identity (Figure 3). Do these genes have a function in specifying temporal identity (birth-order) in multiple lineages? Here the answer is yes for at least Hb and Kr. Experiments in two different neuroblast lineages, NB7-1 and NB7-3, show that Hb and Kr are necessary and sufficient for specifying temporal identity, irrespective of the cell type involved (Isshiki et al. 2001). Using a combination of a *hb* null mutation plus a *hb* transgene that rescues the early segmentation function of Hb, Isshiki et al. (2001) showed that loss of Hb in the CNS resulted in death or cell fate transformation of the early-born GMCs in both lineages, without affecting later-born cells in the lineages. Conversely, neuroblasts forced to continually express Hb (using a variety of *Gal4* transgenes driving *UAS-hb* expression) resulted in continuous production of first-born GMCs—even past the normal number of GMCs made by that lineage—at the expense of later-born cells. Similarly, loss of *Kr* CNS expression resulted in the absence of Kr^+ cell types from the CNS, whereas forced *Kr* expression in neuroblasts generated ectopic second-born cell fates at the expense of all later-born fates (interestingly, the first-born Hb^+ cell fates are not affected by *Kr* misexpression). In conclusion, Hb and Kr are potent regulators of early temporal identity; the first regulators of temporal identity identified in the *Drosophila* or vertebrate CNS.

Regulation of Neuroblast Competence

The identification of Hb as an intrinsic determinant of early temporal identity allows it to be used to probe neuroblast competence. This is conceptually similar to using changing extrinsic cues to probe progenitor competence (either in vitro or

in heterochronic transplants). In this case, we asked when, if ever, NB7-1 loses competence to make early-born cell fates in response to Hb. We observed that during a brief window following the normal down-regulation of Hb (when the first couple of Hb-negative GMCs are being generated), the neuroblast remains competent to respond to a pulse of Hb by making extra early-born neurons. However, as the Hb pulse is given progressively later, the neuroblast gradually loses competence to respond (Pearson & Doe 2003). These data show that NB7-1 is progressively restricted in its ability to respond to Hb (Figure 2*A*); this is superficially similar to the progressive restriction proposed for cortical and retinal progenitors (see below). We were also interested in determining when competence to respond to Hb is lost during neuronal differentiation. Normally, Hb and Kr are expressed transiently in neuroblasts, but maintained in the GMCs and in their postmitotic neuronal progeny. Are all of these cell types equally competent to respond to Hb? We find that when Hb is misexpressed at high levels in postmitotic neurons, they are unable to alter their temporal identity based on a lack of change in multiple molecular markers (Pearson & Doe 2003). In contrast, when mitotic neuroblasts and GMCs are given a pulse of Hb that persists until just after GMC cell division, all the neurons assume a first-born fate, despite the lack of persistent Hb in the mature postmitotic neurons (Pearson & Doe 2003). Thus, competence to respond to Hb is lost in postmitotic neurons (Figure 2*B*). These results raise two interesting questions for future investigation. First is loss of competence in neurons due to cell cycle exit? This can be tested by manipulating the timing of cell cycle exit and Hb expression: For example, would driving an extra round of cell division in neurons allow them to respond to Hb? Second, how does transient Hb expression in neuroblasts and GMCs lead to permanent early temporal identity in neurons that contain no Hb protein? Hb may initiate a transcriptional cascade or may induce permanent chromatin remodeling. The latter model is appealing because Hb, and its vertebrate homolog Ikaros, are proposed to act with dMi-2 and Polycomb group proteins to establish stable chromatin domains during *Drosophila* segmentation (Kehle et al. 1998) and vertebrate hematopoiesis (Georgopoulos 2002, O'Neill et al. 2000).

Regulating Temporal Identity Transitions

It is important to precisely regulate the transitions from Hb to Kr to Pdm to Cas in neuroblasts: extended Hb or Kr expression leads to an overproduction of early-born cell types at the expense of later-born cell types. How does the neuroblast switch from Hb to Kr to Pdm to Cas? When neuroblasts are blocked in G2 (thus preventing both DNA replication and cytokinesis), they fail to down-regulate Hb (Isshiki et al. 2001); however, blocking neuroblast cytokinesis but not DNA replication leads to polyploid neuroblasts that also fail to down-regulate Hb transcription (R. Grosskortenhaus & C.Q. Doe, unpublished data). This is consistent with a model in which the new-born GMC signals back to the neuroblast to advance its gene expression profile (feedback signals), or with a model in which Hb transcriptional activators are asymmetrically partitioned into the GMC during neuroblast mitosis. In summary, cytokinesis but not DNA replication appears to be critical for

triggering the first step in the neuroblast gene expression cascade—down-regulation of Hb. The molecular nature of the signal is currently unknown, and it is also unknown whether the same mechanism regulates all subsequent steps in the gene expression pathway (Kr-Pdm-Cas).

Conclusions and Future Directions

Drosophila neuroblasts have become a model system for investigating temporal identity. We know that individual progenitors can reproducibly generate multiple cell types, that most progenitors sequentially express Hb → Kr → Pdm → Cas transcription factors, that Hb and Kr are potent intrinsic regulators of early temporal identity, and that transient Hb in mitotic progenitors can lead to stable early temporal identity in mature neurons. We know that one neuroblast (NB7-1) is progressively restricted in its competence to respond to Hb and that postmitotic neurons have also lost competence to respond to Hb. Taken together, these data strongly suggest that *Drosophila* neuroblasts are molecularly heterogeneous, multipotent progenitors that use intrinsic cues to specify temporal identity; it remains possible, however, that extrinsic feedback cues occur within the lineage to drive the successive transitions in temporal identity. Despite recent progress, what we do not know is also considerable. Some neuroblasts (NB1-1 and NB4-2) show little or no competence to respond to Hb (Isshiki et al. 2001); why are these neuroblasts so different from the others examined in detail (NB7-1 and NB7-3)? What are the functions of Pdm and Cas in temporal identity? What regulates temporal identity in larval neuroblast lineages? What are the functions of Hb, Kr, Pdm, and Cas orthologs in the vertebrate CNS? In addition, we know very little about what molecular mechanism controls the neuroblast gene expression clock—cytokinesis is required for Hb downregulation but is this mechanism used for Kr, Pdm, and Cas downregulation as well? What is the exact cellular and molecular mechanism? These are only the most obvious questions, and these plus many more remain to be answered over the next few years.

It is interesting to speculate that altering the timing of Hb, Kr, Pdm, or Cas gene expression in neuroblasts would be an effective way to modulate the neuroblast lineage diversity or length during evolution. Primitive insects have the same number of neuroblasts per segment as newer species, yet they make many fewer neurons (Truman & Ball 1998). Thus it appears that natural selection has tinkered more with neuroblast lineage length than with neuroblast numbers. It will be interesting to see if more primitive species have the same Hb → Kr → Pdm → Cas expression profile and, if so, which portions of the lineages are truncated compared with *Drosophila* neuroblast lineages.

TEMPORAL IDENTITY IN THE VERTEBRATE RETINA

The retina begins as an extension of the anterior neural plate, where it separates to form the optic vesicle. The optic vesicle invaginates to form the bilayered optic cup, which is made up of an outer layer (pigmented epithelium) and an inner layer

(neural retina). Retinal progenitors divide to produce the seven major cell types of the eye: ganglion cells, horizontal cells, bipolar cells, amacrine cells, cone photoreceptors, rod photoreceptors, and Müller glia. These cells are organized into the outer nuclear layer (ONL, containing rods and cones), the inner nuclear layer (INL, containing horizontal, bipolar, amacrine, and Müller cells), and the ganglion cell layer (GCL) (Young 1985). In this review, we focus on experiments directly related to how temporal identity is established in the retina. Other recent reviews summarize how bHLH genes and Notch signaling regulate cell diversity in the retina (Cepko 1999, Harris 1997, Marquardt 2003, Marquardt & Gruss 2002, Perron & Harris 2000a) and how cell cycle control and asymmetric cell division can alter cell type ratios (Dyer & Cepko 2001; Ohnuma & Harris 2003; Ohnuma et al. 2001, 2002; Zhong 2003).

Multipotent Progenitors and Invariant Sequence of Progeny?

Early studies in rodents, chick, *Xenopus*, or fish using pulses of BrdU or H^3-thymidine showed that the birthdate of each cell type occurs in a stereotyped, yet highly overlapping order: ganglion cells, horizontal cells, cones, and amacrines always differentiate first; whereas bipolars, rods, and Müller glia always differentiate last (Cepko et al. 1996, Chang & Harris 1998, Hu & Easter 1999, Young 1985). However, birthdating studies cannot distinguish whether single progenitors (with temporally distinct birthdates) make only one cell type or whether the progenitors are multipotent. To obtain this information, single-progenitor retroviral lineage studies were performed (Fekete et al. 1994, Holt et al. 1988, Moody et al. 2000, Turner & Cepko 1987, Turner et al. 1990, Wetts & Fraser 1988), and all reached the same major conclusions. (*a*) Many progenitor cells are multipotent, and some can even produce all seven cell types of the retina. (*b*) Clones induced earlier are large and contain both early- and late-born cell types, whereas clones induced later are smaller and contain only late-born cell types. (*c*) Sibling cells often have different cell fates (from two-cell clone analysis). (*d*) Progenitor clones vary wildly in size and cell type composition; for example, a single clone may contain 33 rods and no other cell type (Turner et al. 1990), whereas another clone of six cells may contain four different cell types (Wetts & Fraser 1988).

Taken together, the birthdating and lineage studies prove that the retina is generated by multipotent progenitors that have a bias toward producing some cell types first and other cell types later. These data allow us to rule out models where the retina is generated by seven different cell type-restricted precursor pools or models in which progenitors make any cell type in any order (because late clones never contain early cell types). Yet we do not know whether different cell types are always born in the same order in all progenitor lineages or whether progenitors are molecularly heterogeneous and consequently have unique lineages; these questions cannot be resolved without developing methods for following individual progenitor lineages over time. In contrast, the relative role of intrinsic and extrinsic cues in regulating the sequential cell type production has been an active area of research (see below).

Intrinsic Versus Extrinsic Regulation of Temporal Identity

Heterochronic transplants, heterochronic cocultures, and single-cell in vitro culture experiments have been done to tease out the relative contribution of intrinsic and extrinsic cues. Several experiments have been done to test for extrinsic regulation of the late-born rod cell fate.

When young embryonic retinal cells are mixed with an excess of older postnatal retinal cells, the young donor cells wait and express a rod marker at the donor-specific time (Belliveau & Cepko 1999, Morrow et al. 1998, Rapaport et al. 2001). Young retinal tissue plugs grafted into older retinal environments also showed donor-specific timing of rod differentiation, even at the borders of the plug, where donor and host cells touch (Rapaport et al. 2001). Moreover, co-culturing postnatal progenitors with an excess of embryonic retinal cells also does not alter the timing of rod differentiation in the late cells, although rod cell numbers are reduced (Belliveau et al. 2000). This suggests that rod cell differentiation is regulated by a cell-intrinsic timer. Interestingly, the intrinsic programming of rod cell fate may be lost following prolonged culture of retinal cells in vitro; old progenitor cells that normally never make early-born ganglion cells in vivo or in late-early co-cultures acquire the potential to make ganglion cells following five days of in vitro culture (James et al. 2003). How this culture system reprograms old progenitor cells so they can revert to making an early-born cell type is an open question.

Despite an abundance of experiments that show autonomous or intrinsic development of late-born rods, there is also evidence for extrinsic regulation of late-born rod cell fate. Late retinal cells co-cultured with early cells make five times fewer rods (although timing of their differentiation is normal) and a concomitant increase in bipolar cells; this effect can even be observed in post-mitotic presumptive rods (Ezzeddine et al. 1997). This may be the result of defects in sibling cell specification, because rods and bipolars are both late-born cell types that are often siblings in two-cell clones (Belliveau et al. 2000). In any case, the signal appears to be mediated, in part, through the CNTF/LIF cytokine pathway, although it is unknown what early retinal cell type is generating this cue (Belliveau et al. 2000, Neophytou et al. 1997).

Early-born retinal cell types are also sensitive to extrinsic cues. Young retinal cells co-cultured with older cells lose the ability to generate early-born amacrine cells (Belliveau & Cepko 1999) and ganglion cells (Waid & McLoon 1995, 1998). In each case, the inhibitory signal comes from differentiated amacrine or ganglion cells, respectively, providing evidence for a cell type-specific feedback signal (Belliveau & Cepko 1999, Waid & McLoon 1998). The ganglion cell feedback signal is mediated by Sonic hedgehog (Shh), produced by the mature ganglion cells, and acts on cells at or prior to their terminal cell division (Zhang & Yang 2001).

The amacrine feedback signal also acts on cells at or prior to their final division (Belliveau & Cepko 1999); the identity of the signal is unknown. In co-culture experiments where amacrine/ganglion cell numbers are reduced, there are no obvious compensatory increases in any other cell type. This suggests that the feedback

signals are not providing an instructive cue to distinguish alternate cell fates. It is more likely that these feedback signals trigger a temporal identity transition to make later-born cell types (see below) or to limit amacrine/ganglion cell proliferation or survival.

Factors Conferring Temporal Identity

Three criteria need to be addressed for any factor to be considered a positive regulator of temporal identity: (*a*) expression at the time temporal identity is specified, (*b*) mutants show an absence of a specific temporal identity, and (*c*) misexpression produces additional cells of a particular temporal identity. For genes that act to inhibit a specific temporal identity (e.g., repress competence to make early temporal identity) the opposite criteria would apply: (*a*) exclusion from progenitors generating a specific temporal identity, (*b*) mutants show ectopic temporal identity, and (*c*) misexpression suppresses the temporal identity. There is no gene that meets either set of criteria in the retina. One intriguing candidate, however, is Pax6, which is expressed widely in progenitors and then in early-born amacrines, ganglion cells, and horizontal cells (Belecky-Adams et al. 1997, Livesey & Cepko 2001). When *pax6* is removed from progenitors after they form, the progenitors appear to lose the ability to generate anything but amacrine cells (Marquardt et al. 2001). These data suggest that Pax6 may be required to maintain progenitor multipotency or for the transition from amacrine to later-born cell type production. Other candidate temporal identity genes show expression in progenitors and one mature retinal cell type (e.g., Prox-1, Hes-1, Chx-10) or have a mutant phenotype lacking a single mature cell type (e.g., Brn3a), but in each case the gene is not temporally regulated in progenitors and has not been tested for its misexpression phenotype (Livesey & Cepko 2001).

Regulating Temporal Identity Transitions

Simple extrinsic or intrinsic models are insufficient to account for the observed temporal identity transitions in the retina. A global extrinsic cue does not fit with the overlap in cell type birthdates, unless there is also variation in cell type–specific proliferative expansion. However, feedback signals clearly regulate the transition to producing later-born cell types (by limiting production of the early-born cell types), and the relevant signaling pathways are beginning to be identified (see above). Similarly, a purely intrinsic clock does not account for the failure of early progenitors to make early cell types when placed in an older retinal environment; yet there is some evidence for a cell-intrinsic clock of rod cell differentiation (Belliveau & Cepko 1999, Morrow et al. 1998, Rapaport et al. 2001). In fact, a combination of global cues and intrinsically different progenitors provides the best model to explain the timing of temporal identity transitions (Cepko 1999, Harris 1997, Marquardt 2003, Marquardt & Gruss 2002, Perron & Harris 2000). For example, in the early retina only some progenitors might be competent to respond to a global ganglion cell–promoting signal, whereas in the late retina only some

progenitors might remain competent to respond to a rod/bipolar signal. This model highlights the importance of understanding the nature of the signaling systems as well as the nature of cellular competence.

Conclusions and Future Directions

Despite a great deal of recent progress, there are still many possible models for how specification of temporal identity occurs in the retina:

1. Heterogeneous progenitors; fixed birth-order (Figure 1, top): There may be multiple progenitor pools that each have a distinctive fixed lineage, summing up to the appropriate distribution of cell types in the retina (the only constraint would be that the group of early fates would always precede the group of late fates). For example, one progenitor might generate only ganglion cells → horizontals → amacrines; another might generate only amacrines → horizontals; yet another might generate only ganglion cells → rods → bipolars → Müller glia. This model presupposes a great deal of molecular heterogeneity in the progenitor population and is supported by the observation that a subset of progenitors expressing the extracellular VC1.1 epitope will preferentially generate amacrine and horizontal cells (Alexiades & Cepko 1997). This may be the first insight into the molecular and developmental complexity of the progenitor population. Importantly, in this model, temporal identity would not have to be linked to cell type identity. Instead, unique progenitor type plus birth-order (temporal) identity could equal cell type identity. For example, progenitor type A could make amacrines first, whereas progenitor type B could make ganglion cells first, even if the same temporal identity factor is used to specify first-born identity. Although this seems like a complex model, it is precisely how cell type identity appears to be specified in the relatively simple *Drosophila* CNS (Figure 3).
2. Equivalent progenitors; variable lineages: Each progenitor could respond to extrinsic cues that determine cell type identity, again summing up to the appropriate distribution of cell types in the retina. This model requires spatial or temporal heterogeneity in the extrinsic cues to account for the overlap in cell type birthdates.

Of course, a combination of these models is also possible, and has in fact been proposed: Changing extrinsic signals, combined with intrinsic differences in progenitor competence to respond, leads to the observed sequence of retinal cell types (Cepko 1999, Harris 1997). In the future, it will be essential to describe more precisely the lineage of individual progenitors in vivo or in vitro. This could be determined by in vivo time-lapse lineage analysis, coupled with vital or postmortem cell type-specific markers. The technology to do these studies has recently been developed (Das et al. 2003). It will also be important to define the extrinsic cues that are known to affect progenitor clone composition and to determine whether these cues affect cell fate choice, cell survival, or proliferative expansion of either

progenitors themselves or cell type-restricted precursors. Conversely, it is vital to investigate the intrinsic differences between progenitors. Recent molecular profiling studies should shed light on this question (Blackshaw et al. 2003, Livesey et al. 2004), but with the mature retina containing $>$400,000 cells/mm^2 and expressing an estimated 25,000 different transcripts (Sharon et al. 2002), mapping progenitor gene profiles could take some time.

TEMPORAL IDENTITY IN THE VERTEBRATE CEREBRAL CORTEX

The mammalian cerebral cortex develops from the pseudostratified epithelium of the anterior neural tube. Mitotic progenitors are located in the ventricular zone and give rise to the neurons and glia of the mature cerebral cortex. The mature cortex is a laminar structure, arranged in layers 1, 2/3, 4, 5, 6 with layer 1 the most superficial and layer 6 the deepest. Each layer contains neurons that have a lamina-specific projection pattern; typically, deep layer neurons project out of the cortex, whereas superficial layers contain neurons that form cortico-cortical projections.

Multipotent Progenitors and Invariant Sequence of Progeny?

Birthdating studies using H^3-thymidine show that the mammalian cerebral cortex follows an inside-out mode of histogenesis (Berry & Rogers 1965, Berry et al. 1964, McConnell 1988). The first-born neurons populate deep layer 6, next-born migrate to layer 5, and so on until the last-born neurons settle in superficial layer 2/3. The only exception to the inside-out timing of neurogenesis are the neurons of layer 1 and the subplate, which are born before all other layers (Luskin & Shatz 1985a,b). Retroviral lineage studies show that single progenitors are multipotent and can generate progeny in more than one layer (Reid et al. 1997, Walsh & Reid 1995). Because specific layers are generated on specific days, and because progenitors are multipotent, it appears that progenitors generate neurons of each layer sequentially. In addition, similar to many regions of the CNS, cortical progenitors generate glial cells after neurogenesis is complete. Thus birth-order (temporal identity) and laminar cell type identity are tightly linked; this is different from *Drosophila* neuroblasts and retinal progenitors, where cell type identity is not linked to temporal identity. What remains unclear, however, is how often a single progenitor contributes to all the layers, if some progenitors are limited to generating only deep or superficial layers, and to what degree progenitor heterogeneity leads to distinctive cell lineages within the cortex (either within a well-defined spatial area such as the visual cortex or between different cortical areas).

Intrinsic Versus Extrinsic Regulation of Temporal Identity

The timing of laminar birth-order is relatively tight, with each layer predominantly generated in a single interval with little overlap. This is in contrast to the fly CNS,

where there is significant overlap in the production of each temporal identity because of the different starting points for each lineage; it is also different from the overlapping birthdates for each cell type in the retina (see above). The tight window of laminar birthdates raises the possibility of a global timing cue that instructs progenitors to generate each laminar fate in synchrony.

To determine the relative contribution of intrinsic/extrinsic cues in specifying laminar (temporal) identity, McConnell & Kaznowski (1991) performed a series of elegant heterochronic transplant experiments. When early donor progenitors (making layer 5/6 neurons) were isolated, labeled with H^3-thymidine, and transplanted into an older host (making layer 2/3 neurons), they could alter their laminar identity from donor (layer 5/6) to host (layer 2/3). About 50% of the labeled neurons went to the host-appropriate layer 2/3, and the other 50% stayed at the normal 5/6 fate (McConnell & Kaznowski 1991). Why is it that only some of the progenitors are competent to respond to the host cues? Cells transplanted immediately after labeling, before most had gone through their terminal cell division, were competent to respond to host cues and migrate to host-appropriate layers. In contrast, cells transplanted after 4 h of labeling, when the majority had become postmitotic, produced neurons that migrated to the donor-appropriate layers. In addition, these authors reanalyzed their early → late transplants (McConnell 1988), looking for lightly labeled versus darkly labeled H^3-thymidine cells. They found that 98% of lightly labeled cells (presumed to have undergone an additional division in the host environment) were indeed in the host-appropriate layer 2/3. In total, these data show that commitment to a specific laminar identity occurs around the final G_2/M phase of the cell cycle. This remains one of the best descriptions of how cell cycle and cell specification are coordinated. These experiments reveal two important points: (*a*) Early cortical progenitors are not intrinsically restricted to generating a single early-born laminar fate; they have the potential to develop into any later-born laminar fate if provided the appropriate cues; and (*b*) the use of naïve early progenitors as probes reveals the presence of extrinsic cues at each stage of laminar development; these temporally different cues can direct young donor progenitors into different laminar fates.

In contrast, older progenitors appear to have lost the potential to form early-born laminar fates. Franz & McConnell (1996) labeled old progenitors (normally making layers 2/3) and transplanted them into younger animals where the host progenitors were making layer 5. They found that 90% of the labeled cells migrated to the donor-appropriate layer of 2/3, despite the fact that the donor cells had to undergo a extended period of migration as they waited for layer 2/3 to form (Frantz & McConnell 1996). The authors scored light and heavy H^3-thymidine labeled cells and saw no significant difference between the two populations, suggesting that even lightly labeled late progenitors that went through a round of cell division in the early environment still failed to respond to early cues (Frantz & McConnell 1996). It has been previously shown that older progenitors divide fewer times than young progenitors (Walsh & Reid 1995). Consistent with this, the authors also observed that the late progenitors produce fewer divisions in the younger environment, as

they normally would, further supporting the idea that old progenitors are unaffected in the early environment (Frantz & McConnell 1996). Thus late progenitors are intrinsically limited in the cell fates they can produce.

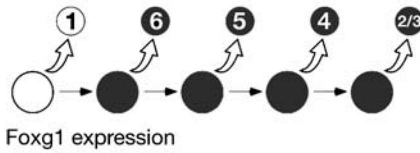
What is the identity of the extrinsic signals that can reprogram early progenitors to make later-born laminar fates? When early progenitors are labeled, cultured for several hours at low density, and then transplanted into old host brains, they make host-appropriate cell fates (Bohner et al. 1997). However, if early progenitors were labeled, cultured at low density, but pelleted before transplant (restoring cell contact), the ability of the progenitors to make early fates was restored (60% in layers 5/6). Thus simple cell-cell contact or short range signals are needed for early progenitors to maintain their capacity to produce early fates. The experiments have a substantial in vitro step, however, which seems to alter properties of the progenitors: They produce about 40% layer 1 neurons (Bohner et al. 1997), which is not seen if the progenitors are not cultured (McConnell 1988, McConnell & Kaznowski 1991). In addition, the molecules behind the cell-cell contact or short range signals that maintain the progenitor's competence to generate early-born cell fates have not been found.

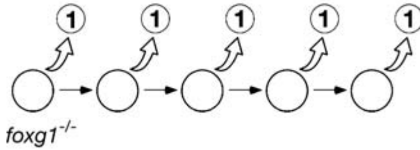
Although it is clear that different extrinsic cues are present during cortical histogenesis (see above), this does not mean that cortical progenitors are completely naïve. Older progenitors undergo progressive restriction (Desai & McConnell 2000). In addition, in vitro time-lapse lineage analysis of individual cortical progenitors shows that they can generate Reelin$^+$ neurons prior to ER81$^+$ neurons (Shen & Temple 2001). Because Reelin is an early-born layer 1 marker and ER81 is a middle-born layer 5 marker, this experiment suggests that cortical progenitors have the intrinsic potential to make the appropriate sequence of laminar (temporal) identities. In addition, cell lineage analysis of isolated cortical progenitors shows they typically produce neurons before glia (Qian et al. 1998, 2000), suggesting that the temporal identity switch between neurons and glia in the cortex is mediated by an intrinsic or short-range extrinsic signal.

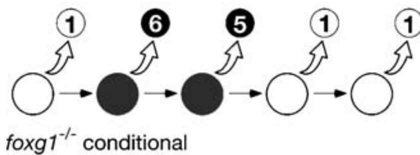
Factors Conferring Temporal Identity

The best candidate for a temporal identity factor is the winged-helix transcriptional repressor, Foxg1. Expression of *foxg1* begins in progenitors after the production of layer 1 neurons and persists in the post-mitotic neurons of layers 2–6 (Hanashima et al. 2004). In *foxg1* mutants, cortical progenitors continually reiterate layer 1 neurons at the expense of later-born cell types (Hanashima et al. 2004). Moreover, if *foxg1* is removed from progenitors after the generation of layer 5 neurons (middle-aged), re-initiation of layer 1 neuron production is observed (Hanashima et al. 2004) (summarized in Figure 4). These results suggest that *foxg1* normally has an active role in suppressing early temporal identities in late progenitors and that late progenitors may maintain a cryptic competence to generate early-born neurons that has never been observed in transplantation experiments. Whereas the exploration of *foxg1* is still in its infancy, the initial

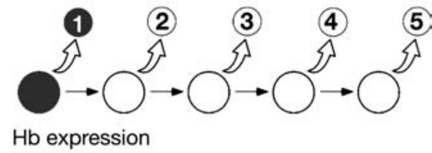
Foxg1 *represses* early-born temporal identity

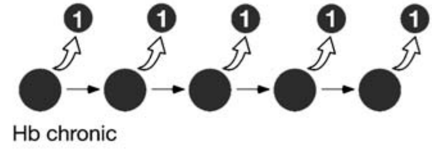






Hb *promotes* early-born temporal identity





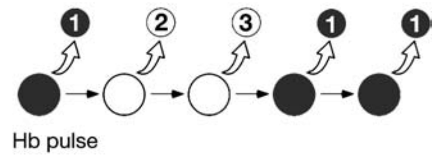


Figure 4 Intrinsic regulation of early-born temporal identity: repression by Foxg1 and activation by Hunchback. Foxg1 transcription factor represses early-born temporal identity in the mammalian cortex, whereas Hunchback (Hb) promotes early-born temporal identity in the *Drosophila* CNS. *foxg1* or Hb expression, black shading; progenitors, large circles; progeny, small circles. (*Top row*) Wild-type expression of *foxg1* and Hb. Murine *foxg1* is expressed in progenitors only after early-born neurons are generated and is maintained in all later-born progeny. *Drosophila* Hb is expressed in young neuroblasts and maintained in early-born progeny. (*Middle row*) *foxg1* actively represses first-born temporal identity, so in *foxg1* mutants, only first-born fates are produced. Similarly, only first-born temporal identities are produced when *Drosophila* neuroblasts are forced to continually express Hb because Hb actively promotes first-born identity. (*Bottom row*) Progenitor competence can be probed by conditional removal or mis-expression of *foxg1* and Hb, respectively. If *foxg1* is removed from older progenitors, the progenitors are competent to go back and make first-born cells again. Similarly, if an older *Drosophila* neuroblast is forced to mis-express Hb, it is competent to respond and remake first-born cells as well. This figure highlights the fact that even though temporal identities can be actively repressed (*foxg1*) or actively promoted (Hb), the outcome is the same: unique temporal specification of progeny.

results strongly suggest it has a role in negatively regulating early temporal identity. Determining the *foxg1* misexpression phenotype will be interesting, as well as investigating how *foxg1* expression is temporally regulated. It is fruitful to compare murine Foxg1 and *Drosophila* Hb temporal identity factors. Whereas Foxg1 is an intrinsic factor that represses early-born temporal identity, Hunchback is an intrinsic factor that promotes early-born temporal identity. In both cases, old

progenitors can be made to resume producing early-born temporal fates, either by the removal of Foxg1 or by the misexpression of Hb (Figure 4). This illustrates the potential importance of both positive and negative regulation of temporal identity and provides two molecular entry points (Foxg1 and Hb) for investigating progenitor competence.

In addition to *foxg1*, a few transcription factors are expressed in specific layers of neurons in the cortex, which make them candidates for specifying temporal identity. The POU-domain transcription factor Oct6, a homolog of the fly *pdm* genes, is detected strongly in layer 5 and weakly in layers 2/3 (Frantz et al. 1994a). However, *oct6* is not expressed in progenitors, so it is more likely to play a role in differentiation or function of layer 5 neurons, not in their temporal identity. A better candidate is the Otx1 homeodomain transcription factor, which is detected at high levels in the cytoplasm of early progenitors and maintained in the nucleus of mature layer 5/6 neurons (Frantz et al. 1994b). The mouse *otx1* knockout was first reported to have a loss of cortical lamination (Acampora et al. 1996), but a more recent analysis using molecular markers shows no change in the fate or migration of layer 5/6 neurons, but rather defects in axon targeting (Weimann et al. 1999). In the future, identification of new genes that are transiently expressed in progenitors and maintained in their post-mitotic neurons, such as *foxg1* and *otx1* in mouse or *hb* and *Kr* in *Drosophila* neuroblasts, would be excellent candidates for regulating temporal identity. If intrinsic determinants of early temporal identities are found, it will be interesting to misexpress these factors in older progenitors to see if an intrinsic challenge can reprogram the progenitors to a younger state (which has not been observed by merely placing the older progenitor in a younger environment). This type of experiment would give insight into the relative importance of changing extrinsic cues versus changes in intrinsic competence to respond.

Regulating Temporal Identity Transitions

Little is known about the mechanism that directs a progenitor to switch from generating one laminar identity to the next. The progenitors do not appear to be counting cell divisions, because middle stage progenitors (that would normally contribute neurons to layer 4) can skip ahead to make later-born layer 2/3 neurons upon transplantation into an older host without undergoing extra rounds of division (Desai & McConnell 2000). These results are more consistent with a model in which extrinsic signals (e.g. feedback inhibition from earlier-born neurons) prevent progenitors from generating pre-existing neuronal fates. The molecular nature of these cues remain unknown.

Conclusions and Future Directions

The cortex is a unique and elegant system to study temporal identity because of the tight relationship between birthdate and laminar identity. In fact, only in this system do we know exactly when temporal identity is specified: prior to or during the terminal mitosis of the neuron. Models for the specification of temporal

identity in the cortex are similar to those for the retina, except that extrinsic or intrinsic (progenitor) heterogeneity is less likely to muddy the issue by resulting in large overlaps in cell type birthdates (Figure 1, bottom). The mammalian cortex seems ripe for a comprehensive genomic analysis similar to that being currently done in the retina (Livesey et al. 2004, Sharon et al. 2002). It would be useful to identify genes specifically expressed in each cortical layer as well as transiently in progenitors. This group of genes would be excellent candidates for regulating temporal identity.

TEMPORAL IDENTITY IN THE VERTEBRATE SPINAL CORD AND HINDBRAIN

In the previous sections we describe individual neural progenitors that appear to make four or more different cell types over time. Here we describe two potentially simpler systems, the ventral spinal cord and the ventral hindbrain, in which progenitors make two cell types in a reproducible temporal order. These systems may be more tractable and still provide insights into temporal patterning that can be applied to more complex systems.

Multipotent Progenitors and Invariant Sequence of Progeny?

Most regions of the brain and spinal cord generate neurons followed by glia (Kessaris et al. 2001). In the spinal cord, clones containing motor neurons and glia are common (Leber et al. 1990) and birthdating studies show that motor neurons are generated first, followed by glia (Altman & Bayer 1984, Soula et al. 2001). In the spinal cord, these conclusions are supported by recent studies following the expression of the basic helix-loop-helix proteins Olig2 and Neurogenin1 (Ngn1) and the homeodomain protein Nkx2.2 within the ventral pMN domain. Early progenitors in this domain are Olig2^{+} Ngn1^{+} Nkx2.2^{-} and give rise to Olig2^{+} Ngn1^{+} Nkx2.2^{-} motor neurons; then later, the same progenitor domain, and most likely the same progenitors, switches to an Olig2^{+} Ngn1^{-} Nkx2.2^{+} gene expression profile and generates Olig2^{+} Ngn1^{-} Nkx2.2^{+} oligodendrocytes (Mizuguchi et al. 2001, Novitch et al. 2001, Zhou et al. 2001). In the hindbrain, birthdating studies show that within the pMN domain motor neurons are born early and serotonergic interneurons are born later (Pattyn et al. 2003). Thus it is likely that many spinal cord and hindbrain progenitors, including those in the pMN domain, are multipotent and generate different cell types in an invariant sequence.

Factors Conferring Temporal Identity

In the spinal cord, the Olig2/Ngn1 combination is correlated with early-born motor neuron identity, whereas the Olig2/Nkx2.2 combination is correlated with late-born oligodendrocyte identity (Mizuguchi et al. 2001, Novitch et al. 2001, Zhou et al.

2001). Do these transcription factors regulate temporal identity, cell type identity, or neither? Overexpression of the Olig2/Ngn1 combination leads ectopic motor neurons, even in dorsal regions of the spinal cord that never make motor neurons; similarly, misexpression of the Olig2/Nkx2.2 combination results in ectopic oligodendrocytes (Mizuguchi et al. 2001, Novitch et al. 2001, Zhou et al. 2001). This suggests that these transcription factors are more tightly linked to specifying cell type than temporal identity. Mutant analysis is consistent with this interpretation. In *olig2* mutants, ventral progenitors are transformed to a more dorsal fate, yet they still undergo their normal temporal program of differentiation, generating V2 interneurons early and astrocytes late (Lu et al. 2002, Zhou & Anderson 2002). Taken together, mutant and misexpression experiments show that Olig2/Ngn1 and Olig2/Nkx2.2 have a primary role in cell type specification, rather than in specifying temporal identity. The intrinsic or extrinsic mechanism that provides temporal information to pMN progenitors remains unknown (see below).

In the chick hindbrain, young pMN progenitors first express the transcription factors Phox2b and Nkx2.2, but subsequently the ventral border of the Phox2b domain shifts dorsally, leaving the pMN progenitors expressing only Nkx2.2 (Pattyn et al. 2003). The timing of this transition correlates well with the progenitor's switch from making motor neurons to making serotonergic interneurons, including the observation that cells in rhombomere 4 never down-regulate *phox2b* and generate additional motor neurons at the expense of serotonergic interneurons. Interestingly, *hoxb1* mutants, *phox2b* mutants, and *nkx6.1/nkx6.2* double mutants all show premature down-regulation (or absence) of *phox2b* expression in rhombomere 4, and there is a concomitant switch to the precocious production of serotonergic interneurons. This suggests that Phox2b may be actively suppressing late-born temporal identity (serotonergic interneuron identity in this region of the CNS). Misexpression experiments, however, provide strong evidence that the primary function of Phox2b is to induce visceral motor neuron identity, rather than regulate temporal identity. Ectopic Phox2b in the dorsal spinal cord, where there are no visceral motor neurons (or even somatic motor neurons), results in the production of ectopic visceral motor neurons, as seen with limited marker analysis (Dubreuil et al. 2000). Thus it appears that Phox2b is necessary and sufficient to specify visceral motor neuron cell type. This pushes the specification of temporal identity back one step, to the mechanism timing *phox2b* expression in the hindbrain.

Regulating Temporal Identity Transitions

How does a ventral spinal cord progenitor coordinately switch from $Ngn1^{+}$ to $Nkx2.2^{+}$? This change in gene expression is due to a shift in the spatial boundaries of Ngn1 and Nkx2.2 expression domains (Mizuguchi et al. 2001, Novitch et al. 2001, Soula et al. 2001, Zhou et al. 2001), resulting in a temporal switch in the pattern of gene expression within ventral progenitors. It is unknown whether intrinsic or extrinsic cues drive this change in gene expression in ventral progenitors. One

attractive model is that a late increase in Sonic hedgehog (Shh) signaling from the ventral neural tube expands the expression domain of Nkx2.2, a known positive target of Shh signaling (Briscoe et al. 1999) and at the same time restricts the domain of Ngn1, thereby creating an $Olig2^{+}$ $Nxk2.2^{+}$ $Ngn1^{-}$ progenitor domain (Zhou et al. 2001). The importance of intrinsic and extrinsic cues could be tested by in vitro culture of ventral progenitors. If they undergo the expected change in gene expression under constant growth factor conditions, it would suggest an intrinsic model; if gene expression timing can be regulated by Shh levels, it would support an extrinsic model.

A related series of questions apply to the ventral hindbrain. Do intrinsic or extrinsic cues regulate the rhombomere-specific timing of *phox2b* expression? Hoxb1 is an intrinsic factor that blocks *phox2b* down-regulation in rhombomere 4; it could render progenitors incompetent to respond to an extrinsic signal or interfere with an intrinsic transcriptional program. Heterotopic transplants or in vitro progenitor culture could be used to distinguish these models.

Conclusions and Future Directions

Ventral spinal cord and hindbrain progenitors reveal an important and potentially widespread mechanism for specifying temporal identity: (*a*) changing the borders of spatially regulated patterns of gene expression over time leads to (*b*) single precursors changing transcription factor profiles over time, which results in (*c*) sequential production of different cell types. This model may be most relevant for systems in which extrinsic cues are known to shape the gene expression profiles within progenitor pools, such as the DV axis of the spinal cord and hindbrain (Figure 1, bottom). It is likely that highly dynamic spatial gene expression patterns characterize many progenitor domains (cortex, retina, hindbrain, and spinal cord), but what is unclear is how single progenitors translate these dynamic gene expression profiles into the sequential production of different cell types and whether progenitors undergo intrinsic and irreversible changes during this process.

Ventral spinal cord and hindbrain progenitors represent one of the most tractable systems for investigating temporal patterning in the CNS, and many important questions can be answered with existing methodology. What is the respective contribution of intrinsic and extrinsic cues in regulating the temporal profiles of gene expression in progenitor domains? This can be addressed with in vitro culture experiments. Do progenitors change their competence as they divide, and do progeny lose competence as they differentiate? Perhaps the study of how Hoxb1 blocks progenitors from responding to the "down-regulate *phox2b* cue" will help illuminate how competence is regulated in progenitors and their progeny. What are the temporal factors that drive the observed changes in progenitor gene expression? The simplicity of the spinal cord/hindbrain system (small number of cell types, well-defined positions, extended temporal windows) should make it relatively easy to identify genes that are correlated with early or late temporal identity, whether they are transcription factors or signaling proteins.

DISCUSSION

In this review, we have focused on how progenitor cells change over time to generate cell diversity (Table 1). We propose that progenitor identity is initially specified by spatial cues; the progenitor is then exposed to intrinsic or extrinsic temporal cues and ultimately responds by generating specific cell types over time. We suggest that there are three levels of regulation used to create neuronal diversity: (*a*) spatial cues that change progenitors based on their position in the CNS, (*b*) temporal cues that change the progenitor over time, and (*c*) cell type identity genes that interpret the combination of spatial and temporal cues to initiate a particular differentiation program (e.g., visceral motor neuron, serotonergic interneuron, layer 5 cortical neuron).

TABLE 1

Question	System	Result	Experiment	Most relevant references
Progenitors multipotent?	Fly CNS	Yes	In vivo clones	(Schmid et al. 1999)
	Retina	Yes	In vivo clones	(Turner et al. 1990, Wetts & Fraser 1988)
	Cortex	Yes	In vivo clones	(Reid et al. 1997)
	Spinal cord	Yes	In vivo clones	(Leber et al. 1990)
Invariant birth-order?	Fly CNS	Yes	Gene expression, clones	(Isshiki et al. 2001)
	Retina	?		
	Cortex	Yes	Isochronic transplants	(Berry et al. 1964)
	Spinal cord	Yes	Gene expression	(Soula et al. 2001)
Birthdate = cell type?	Fly CNS	No	Clonal analysis	(Novotny et al. 2002, Pearson & Doe 2003)
	Retina	No	Clonal analysis	(Turner et al. 1990)
	Cortex	Yes	H^3-thymidine	(Berry et al. 1964)
	Spinal cord	Yes	Gene expression	(Soula et al. 2001)
Known temporal cues?	Fly CNS	Yes	Hb, Kr	(Isshiki et al. 2001)
	Retina	No		
	Cortex	Yes	Foxg1	(Hanashima et al. 2004)
	Spinal cord	No		
Competence: early → late switch?	Fly CNS	?		
	Retina	No	In vivo transplant	(Rapaport et al. 2001)
	Cortex	Yes	In vivo transplant	(McConnell 1988)
	Spinal cord	?		
Competence: late → early switch?	Fly CNS	Yes	Hb pulse	(Pearson & Doe 2003)
	Retina	No	In vivo transplant	(Rapaport et al. 2001)
		Yes	In vitro culture	(James et al. 2003)
	Cortex	No	In vivo transplant	(Frantz & McConnell 1996)
		Yes	Foxg1 loss	(Hanashima et al. 2004)
	Spinal cord	?		

We have chosen to discuss systems in which progenitors are known to be multipotent and have been shown (or are likely) to generate their distinctive progeny in an invariant order. In some systems (fly neuroblasts and vertebrate retina) it appears that intrinsic changes in the progenitor play a large role in determining temporal identity (Figure 1, top). In the retina, many aspects of late temporal identity appear intrinsically controlled, but there is strong evidence for feedback signals from early-born neurons to limit further production of early-born cell types. These feedback signals may be part of a mechanism for promoting early to late temporal identity transitions. In vitro culture of isolated retinal progenitors has been successful only for late progenitors (Cayouette & Raff 2003) and supports an intrinsic regulation model. *Drosophila* neuroblasts appear to go through a normal lineage when cultured in isolation in vitro, supporting an intrinsic model, but they have not been tested for their ability to respond to a heterochronic in vivo environment. A role for feedback signals from GMCs to the neuroblast is currently one attractive model for promoting temporal identity transitions. For both retina and neuroblasts, we hypothesize that progenitors may have a high degree of molecular heterogeneity that distinguishes progenitors from each other. As a result, the same temporal identity cues lead to the production of different cell types in each progenitor (Figure 1, top).

In the cortex, hindbrain, and spinal cord it is not clear yet whether intrinsic or extrinsic cues play the primary role in specifying temporal identity (because few culture experiments have been done). The most attractive model is that changing spatial cues specify temporal identity in the hindbrain and spinal cord. This is because several relevant cell type markers such as Nkx2.2, Olig2, Ngn1, and Phox2b are known to change expression domains along the DV axis over time and are known to be responsive to Shh signaling along the DV axis (Figure 1, bottom). It remains to be seen whether changes in Shh signaling or other signaling molecules are sufficient to explain the temporal profile of gene expression and cell type production occurring in the hindbrain and spinal cord. Indeed, there are currently no data to rule out the possibility of changing progenitor competence or the existence of feedback signals.

One commonality between most systems discussed is the loss of progenitor competence over time. It is dangerous to extrapolate too much between systems, however, because quite different experiments have been used to define competence. In *Drosophila* neuroblasts, competence has been probed by misexpression of an intrinsic factor for early temporal identity, whereas in other systems, competence has been defined by a response to heterochronic signals. Only recently has it been possible to compare both methods in the cerebral cortex. Old cortical progenitors that lack the competence to make layer 1 neurons in heterochronic transplants can produce them when the Foxg1 protein is conditionally removed, showing that Foxg1 is an intrinsic factor that inhibits early-born temporal identity. This suggests that intrinsic factors may be more potent at changing cellular competence and illustrates how little we know about how extrinsic and intrinsic factors regulate progenitor competence.

Investigation of progenitor cell competence is a difficult question, but will be important to address in all systems discussed in this review. In flies, older neuroblasts gradually lose competence to respond to Hb (Pearson & Doe 2003). What is the molecular basis for this limitation in competence? Is it because Hb cofactors are also temporally regulated? Alternatively, do Hb target loci become inaccessible over time (e.g., owing to changes in chromatin structure)? These questions are experimentally tractable in *Drosophila*, but it is too early to know whether they are relevant to the study of vertebrate progenitor competence.

Another important question of wide relevance is how temporal identity transitions are regulated. This could be mechanistically very different for each system. A neuroblast lineage-intrinsic mechanism appears to regulate temporal identity gene expression in *Drosophila* neuroblasts (R. Grosskortenhaus & C.Q. Doe, unpublished data). This may involve a feedback inhibition signal as proposed for the retina. In contrast, a very different mechanism is likely to regulate the temporal dynamics of Shh expression within the spinal cord.

Overall, this review has highlighted our progress on understanding how temporal identity is regulated during CNS development. There has been much progress in all systems: fly neuroblasts and vertebrate retina, cortex, hindbrain, and spinal cord. Despite this progress, the field is still in its infancy. Huge gaps in our knowledge of progenitor cell lineage remain (in vivo and in vitro); even in *Drosophila* we have only recently begun collecting birth-order lineage data (Pearson & Doe 2003). We know few intrinsic or extrinsic temporal identity factors, just Hb and Kr in *Drosophila* (Isshiki et al. 2001), and Foxg1 in the cortex (Hanashima et al. 2004). And we know little about how transitions are made between different temporal identities, although there is excellent evidence for feedback signals in the retina and cortex. In the future, improved methods of live time-lapse imaging will permit in vivo cell lineage studies (Das et al. 2003); new insights into chromatin remodeling may help understand the molecular nature of cellular competence, and new methods for prospective progenitor isolation and genomic profiling will greatly facilitate temporal identity gene discovery (Blackshaw et al. 2003, Livesey et al. 2004). It is an exciting time to be exploring the fourth dimension of CNS development, temporal identity.

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