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Temporal control of neuronal diversity: common regulatory principles in insects and vertebrates?

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It is well established in species as diverse as insects and mammals that different neuronal and glial subtypes are born at distinct times during central nervous system development. In *Drosophila*, there is now compelling evidence that individual multipotent neuroblasts express a sequence of progenitor transcription factors which, in turn, regulates the postmitotic transcription factors that specify neuronal/glial temporal identities. Here, we examine the hypothesis that the regulatory principles underlying this mode of temporal specification are shared between insects and mammals, even if some of the factors themselves are not. We also propose a general model for birth-order-dependent neural specification and suggest some experiments to test its validity.

Introduction

The vast range of different neuronal subtypes in the central nervous system (CNS) was spectacularly revealed as early as the nineteenth century by Santiago Ramón y Cajal and Camillo Golgi's exquisite microscopy studies (Ramón y Cajal, 1989). How, then, is the remarkable diversity of different neurons and glia generated from a seemingly uniform pool of neural progenitors in the early embryo? Solving this question is not only a central challenge in neurobiology, but is also essential for developing safe and efficient stem-cell and regenerative brain therapies. Impressive progress has already been made in understanding one important source of neuronal and glial diversity – the spatial patterning cues that regulate the properties of progenitors and their neuronal/glial progeny (reviewed by Jessell, 2000; Skeath and Thor, 2003). Spatial patterning, however, is only part of the story, and we focus here on the mechanisms of temporal patterning. The importance of temporal specification during neurogenesis has been recognised ever since it was first clearly demonstrated that different types of neurons are born in a stereotypical order in the developing mammalian cerebral cortex (Berry et al., 1964). Subsequent investigations have revealed the existence of a regulatory link between birth order and neuronal/glial identity in many different regions of the mammalian CNS, as well as in the insect CNS, suggesting that it might well be a universal feature of all complex nervous systems (reviewed by Donovan and Dyer, 2005; Kessaris et al., 2001; Livesey and Cepko, 2001; Pearson and Doe, 2004; Yu and Lee, 2007).

Over the last decade, elegant studies in the developing CNS of the *Drosophila* embryo have identified several components of a temporal specification system (reviewed by Egger et al., 2008; Pearson and Doe, 2004). These correspond to a handful of transcription factors that are expressed in chronological sequence by individual multipotent progenitors, instructing them to generate different neuronal/glial subtypes at different stages of development. As in *Drosophila*, it is becoming clear that some regions of the vertebrate CNS contain multipotent neural progenitors that can sequentially generate two or more distinct cell identities (see Glossary, Box 1) (Qian et al., 2000; Shen et al., 2006). As yet, however, there is only limited evidence that the factors involved in insect neuronal temporal specification play conserved roles in vertebrates. We now review studies of *Drosophila* neurogenesis from many laboratories, and use these to set out a model for temporal neural specification, providing definitions for each of the components involved. Although many of the temporal factors themselves might not be functionally conserved in vertebrates, evolutionary comparisons lead us to hypothesise that there is a common underlying regulatory framework. We also outline some experiments that might test how similar the insect and vertebrate mechanisms of temporal neural specification are.

A mechanism linking birth order to neuronal fate in *Drosophila*

The basic building blocks of the *Drosophila* CNS are stem-cell-like multipotent progenitors called neuroblasts (reviewed by Doe, 2008). Each neuroblast divides many times in an asymmetric manner, renewing itself and budding off a smaller intermediate progenitor called a ganglion mother cell (GMC). In turn, GMCs usually divide only once to generate two postmitotic daughter cells that can be neurons or glia. However, recent studies show that a small number of specialised neuroblasts can generate modified GMCs that divide multiple times, acting as transit-amplifying cells that are somewhat analogous to vertebrate intermediate progenitors (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). Systematic lineage-labelling experiments have defined precisely which embryonic neurons are produced by each one of the 30 or so distinct types of neuroblasts in the *Drosophila* CNS (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997). These and other studies have revealed that a given neuroblast generates its repertoire of postmitotic progeny in a stereotypical sequence.

At the heart of the molecular machine that links birth order to neuronal fate lies a series of progenitor temporal transcription factors (progenitor TTFs, see Glossary in Box 1). These are expressed in a characteristic developmental sequence, the temporal series, within individual progenitors. Thus far, the expression of four progenitor TTFs, in the order Hunchback (Hb) → Kruppel (Kr) → Pdm → Castor (Cas), has been described (Isshiki et al., 2001; Kambadur et al., 1998) (Fig. 1). Loss- and gain-of-function studies have elegantly demonstrated that the same series of progenitor TTFs are necessary and sufficient to specify the temporal identities of neurons in several different neuroblast lineages (Grosskortenhaus et al., 2005; Grosskortenhaus et al., 2006; Isshiki et al., 2001; Pearson and Doe, 2003). Although progenitor TTFs are known to be present in neurons, as well as in neuroblasts and GMCs, they appear to be primarily required in progenitors, as their postmitotic expression alone is not sufficient to confer temporal identity (Pearson and Doe, 2003). Although each progenitor TTF is linked to a specific

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neuronal/glial cell identity within a given neuroblast lineage, between lineages the same factor can specify a different postmitotic cell identity. Presumably, this is because the overall cell identity of any neuron or glial cell is defined by a combination of its temporal identity, specified by progenitor TTFs, and its spatial identity, which varies between neuroblast lineages (reviewed by Bhat, 1999; Brody and Odenwald, 2002).

The competence of a progenitor to respond to a given TTF can change during the course of development. For example, experimental misexpression of a progenitor TTF at different times within the same progenitor does not always promote the same temporal identity in neurons (Cleary and Doe, 2006; Pearson and Doe, 2003). In addition, some progenitors appear to express a second endogenous burst of the same TTF, as has been observed for Kr and Cas in neuroblasts at late embryonic stages and for Cas (and also Seven up) during larval stages (Fig. 1C) (Cleary and Doe, 2006; Maurange et al., 2008). In principle, such redeployments within the same progenitor, together with changes in progenitor competence, allow the generation of more neuronal/glial temporal identities than there are progenitor TTFs. It is not yet clear how progenitors alter their competence states, but one potential mechanism involves transient progenitor TTFs that trigger much longer-lasting changes in the expression of progenitor competence factors (see Glossary, Box 1). Thus, although Cas is only expressed transiently in neuroblasts, it permanently switches off the Sox protein Dichaete and, concomitantly, triggers sustained expression of another transcription factor, Grainyhead. In turn, Grainyhead regulates several characteristic properties of late neuroblasts, including their cell-cycle speed and competence to undergo final cell-cycle withdrawal or apoptosis (Cenci and Gould, 2005; Maurange et al., 2008). Gain-of-function studies have also implicated Hb in the temporal regulation of competence states (Cleary and Doe, 2006; Pearson and Doe, 2003).

The switching factors (see Glossary, Box 1) that are required for the transitions between progenitor TTFs appear to be primarily cell-intrinsic because neuroblasts are still able to undergo temporal transitions when isolated in vitro (Brody and Odenwald, 2000; Grosskortenhaus et al., 2005). Seven up (Svp), an orphan nuclear receptor, is a switching factor that regulates the transition from a Hb^+ state to a Hb^- Kr^+ identity by repressing the transcription of *hb* (Kanai et al., 2005; Mettler et al., 2006). Hence, Hb expression is prolonged in neuroblasts that lack Svp and, correspondingly, neurons with an early temporal identity are overproduced at the expense of those with later identities (Fig. 1A). In principle, switching through the temporal series could also be facilitated by cross-regulation between the progenitor TTFs themselves (Grosskortenhaus et al., 2006; Isshiki et al., 2001; Kambadur et al., 1998; Kanai et al., 2005). The two main network motifs involved are negative feedback and a negative (termed incoherent) type of feedforward loop (Alon, 2007). Together, these form a cross-regulatory unit, repeated at least twice during Hb → Cas progression, that could facilitate the exclusive expression of one, and only one, progenitor TTF at any given time (Fig. 1B). In general, however, such cross-regulation does not appear to be essential because loss of activity of Hb, Kr or Pdm merely leads to one temporal identity being skipped, rather than to all subsequent TTF switching being blocked. Nevertheless, for Cas, loss of activity does remove crucial negative feedback, leading to persistent Pdm expression and to a blockade of further temporal series progression (Grosskortenhaus et al., 2006). Hence, in addition to its role as a progenitor TTF, Cas also fulfils the definition of a switching factor.

What regulates the activity of switching factors with time and thus the frequency of temporal transitions? This, as yet, unknown mechanism, which might be described as a temporal series timer (see Glossary, Box 1), is predicted to be necessary for specifying the numbers of each neuronal/glial subtype that a neuroblast generates. One relevant observation here is that inactivating the cell-cycle components that regulate cytokinesis or the G2–M transition prevents the downregulation of Hb, which normally accompanies the transition to Kr expression, thus holding neuroblasts in a persistently 'young' state (Grosskortenhaus et al., 2005). Intriguingly, however, none of the progenitor TTF transitions from Kr → Pdm → Cas requires cell-cycle progression (Grosskortenhaus et al., 2005). Additional insights into the timer mechanism are likely to come from stop-restart experiments. For example, reintroducing a Cas burst into mutant lineages at a later-than-normal stage would show whether or not temporal specification is restored from the point at which it was originally blocked. This strategy might resolve whether switching factors are also components of the core timer mechanism. Yet more clues are likely to come from the identification of the upstream factors that temporally regulate Svp and Cas.

Box 1. A glossary of terms

Cell identity. Sometimes called cell fate, this is defined by the gene expression profile of a cell, which, in turn, specifies its morphology and functions. The overall identity of a neuronal or glial cell results from a combination of its temporal identity, which is conferred by postmitotic TTFs, and its spatial identity, which is imparted by anteroposterior and dorsoventral patterning genes.

Progenitor temporal transcription factors. Progenitor TTFs are transiently expressed and are required in neural progenitors to confer temporal identity in postmitotic daughter cells. They are sequentially expressed in a temporal series and can cross-regulate one another. Some progenitor TTFs are also expressed in neurons/glia, but their postmitotic expression is insufficient to confer temporal identity. For *Drosophila* neuroblasts, the four known progenitor TTFs are Hb, Kr, Pdm and Cas. For vertebrate progenitors, Fezf2, Sox9, Foxa2 and Phox2b are likely candidates.

Postmitotic temporal transcription factors. Postmitotic TTFs are expressed and required in temporal subsets of postmitotic neurons/glia for their temporal identity. Postmitotic TTF regulation by progenitor TTFs provides a way of passing temporal information from progenitors to neurons/glia, although the transmission mechanisms remain unclear. Postmitotic TTFs in *Drosophila* neurons include Chinmo and Collier, and in vertebrate cortical neurons Sox5, Ctip2 and Satb2.

Switching factors. These are required to switch between successive progenitor TTFs. Implicit here is that switching factors directly or indirectly regulate progenitor TTFs. In *Drosophila*, known switching factors are Svp and Cas (Cas also functions as a progenitor TTF). In vertebrates, the Svp orthologues Coup-TFI and Coup-TFII are required for switching from neurogenesis to gliogenesis. In addition, Hoxb1 can inhibit VM → 5HT switching in rhombomere 4. For the *Drosophila* Hb → Kr transition, components of the cell cycle also act as switching factors.

Temporal series timer. The hypothetical mechanism that regulates the activity of switching factors with time and thus the frequency of progenitor TTF transitions (see text and Fig. 4). It is unclear whether or not this mechanism counts units of time.

Progenitor competence factors. These influence the response of a progenitor to intrinsic or extrinsic cues. Neural progenitors undergo discrete transitions between different competence windows such that they can respond differently to the same progenitor TTF at two different developmental time points. Progenitor TTFs (and probably other factors) can establish competence windows by triggering long-lasting changes in the expression of progenitor competence factors. Dichaete and Grainyhead are examples of progenitor competence factors in *Drosophila* neuroblasts.

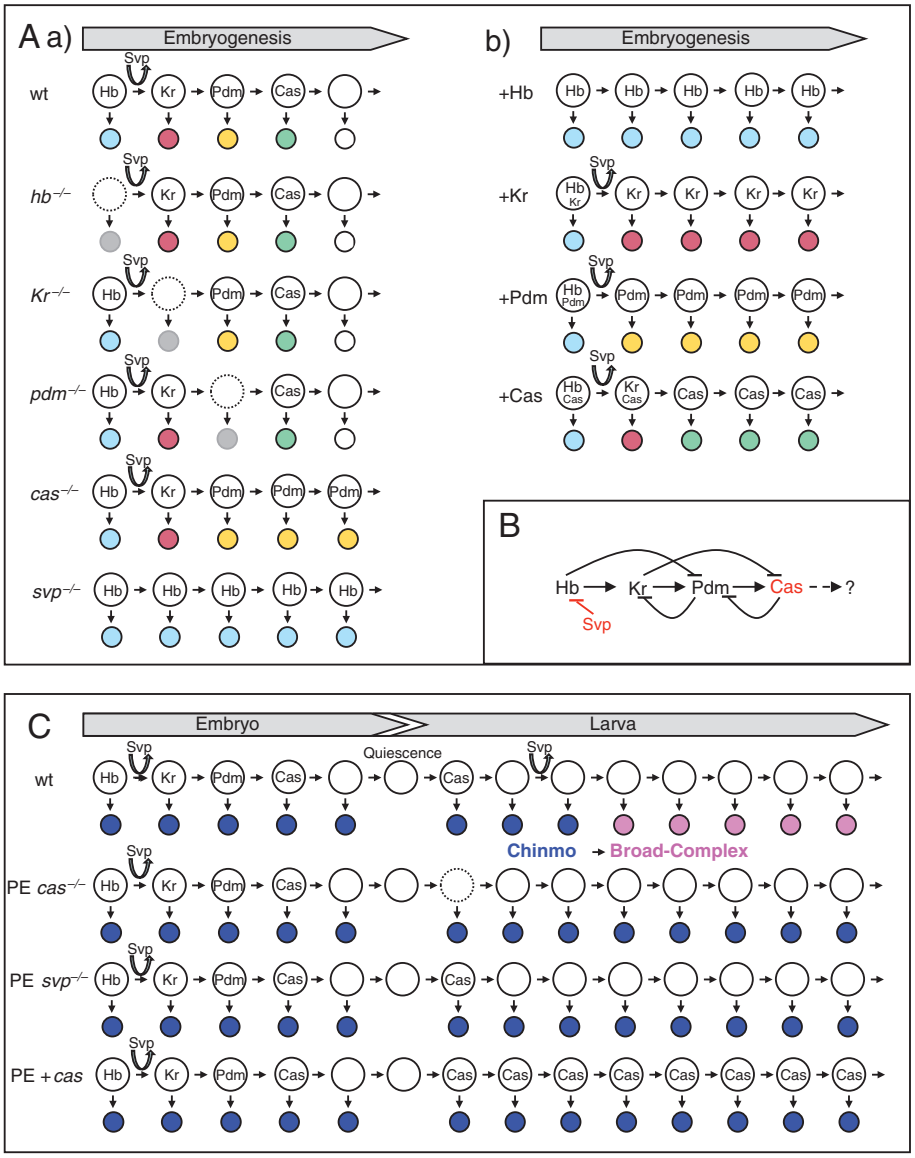

Fig. 1. Progenitor temporal transcription factors (TTFs) in *Drosophila*. Wild-type (wt) *Drosophila* neuroblasts (large circles) express four progenitor TTFs, at different times during embryogenesis, in the following sequence: Hunchback (Hb) → Kruppel (Kr) → Pdm → Castor (Cas). (**A**) Each progenitor TTF is associated with postmitotic progeny (small circles) of a different temporal identity (blue, red, yellow or green). (a) Loss-of-function of a single progenitor TTF leads either to the skipping of one temporal identity (shown in grey for *hb*$^{-/-}$, *Kr*$^{-/-}$ or *pdm*$^{-/-}$) or to stalled temporal series progression, associated with supernumerary early temporal identities that are Pdm-dependent (*cas*$^{-/-}$) or Hb-dependent (*svp*$^{-/-}$). (b) Continuous misexpression of any of the four progenitor TTFs leads to supernumerary progeny with the corresponding temporal identity. (**B**) Known negative cross-regulatory interactions between progenitor TTFs and the switching factor Svp. Cas is not only a progenitor TTF but, like Svp, also a switching factor (red). Note that other known progenitor TTFs (black), such as Hb, do not fulfil this definition because although misexpression blocks progenitor TTF progression, loss-of-function does not (Grosskortenhaus et al., 2005; Isshiki et al., 2001). (**C**) Progenitor TTFs are also expressed during postembryonic (larval and pupal) stages. Most, if not all, neuroblasts first generate Chinmo^{+} (blue) neurons during embryonic and early larval stages. They then switch to producing Broad-Complex^{+} (pink) neurons during late larval and pupal stages. Neuroblasts fail to undergo the Chinmo^{+} → Br-C^{+} switch if the postembryonic progression of progenitor TTFs is blocked by the removal of the postembryonic (PE) pulse of Cas (PE *cas*$^{-/-}$) or of Svp (PE *svp*$^{-/-}$), or by misexpressing Cas (PE + *cas*).

The temporal identity of neurons is not only influenced by progenitor TTFs but also by postmitotic TTFs (see Glossary, Box 1). In the mushroom body (MB), an anterior region of the *Drosophila* CNS that is associated with learning and memory, each neuroblast sequentially generates five distinct subtypes of interneurons (Lee et al., 1999). Chinmo is a putative transcriptional repressor that is expressed in immature postmitotic progeny, with different levels defining each of the first three temporal identities of MB neurons (Yu and Lee, 2007; Zhu et al., 2006). Genetic manipulations of postmitotic TTFs, such as Chinmo, in neurons can lead to transformations in temporal identity that are just as striking as those resulting from progenitor TTF manipulations in neuroblasts (Fig. 2A). This raises the important question of whether the two types of TTF act independently of one another or whether progenitor TTFs might regulate postmitotic TTFs. The latter scenario would provide the beginnings of a possible mechanism for transmitting temporal information from progenitors to their postmitotic daughter cells. A recent study suggests that, at least for Chinmo, this is highly likely to be the case (Maurange et al., 2008). Chinmo is strongly expressed in the early-born neurons generated by most, if not all, neuroblasts in the *Drosophila* CNS and not just those in the MB. Neurons produced during embryonic and early larval stages express Chinmo, whereas a related transcription factor, Broad Complex (Br-C; Broad – FlyBase), is expressed in neurons generated at late larval and pupal stages (Fig. 1C). The finding that bursts of Cas and Svp in larval neuroblasts are required for the transition from Chinmo^{+} to Br-C^{+} neurons provides evidence that progenitor TTFs can regulate postmitotic TTFs, although a function for Br-C in the temporal identity of neurons has yet to be demonstrated. Another possible way of transmitting temporal information would be for a neuron/glial cell to inherit a postmitotic TTF from its progenitor. This possibility is suggested by a study of the transcription factor Collier (Col; Knot – FlyBase) in one neuroblast lineage (called 5-6) in the *Drosophila* embryo (Baumgardt et al., 2007). Although Col acts as a postmitotic TTF to specify the peptidergic identity of the late-born Tvb neuron, it is also expressed in the late-stage neuroblast and in the late-born GMC from which Tvb is derived. The transmission of temporal information from neuroblast to GMC to neuron might also involve bridging mechanisms other than the direct inheritance of transcription factor expression. For example, in the

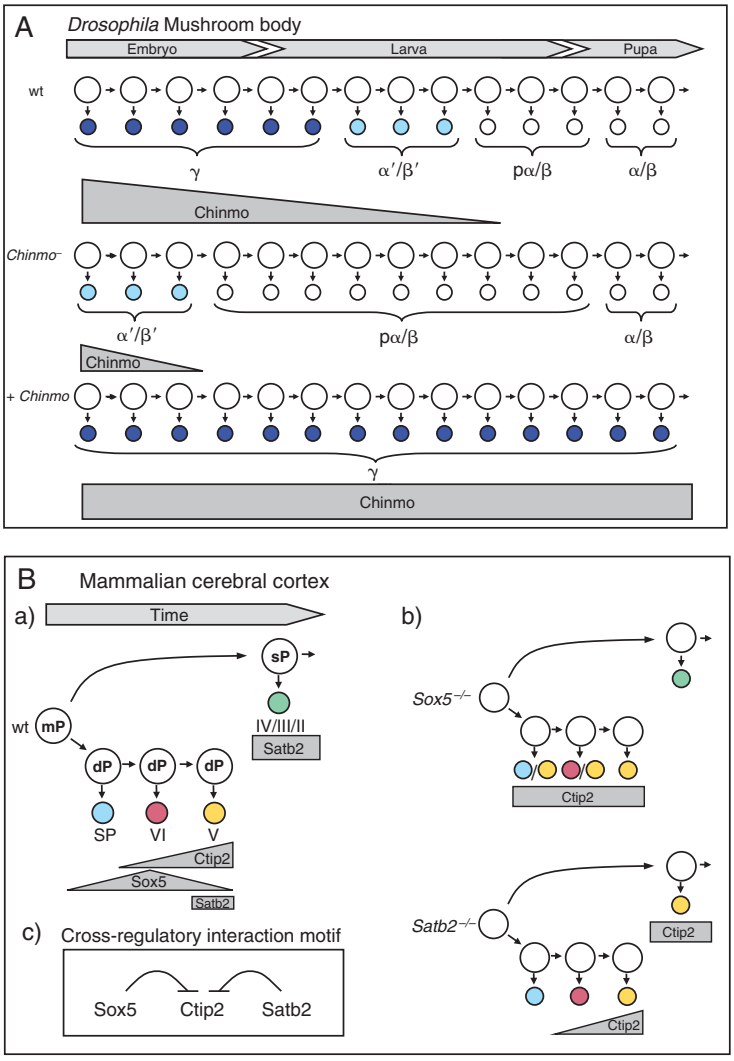

Fig. 2. Postmitotic TTFs in *Drosophila* and mammals. (**A**) In *Drosophila*, wild-type (wt) mushroom body (MB) neuroblasts (large circles) generate MB neurons (small circles). Early-born MB neurons express high levels of the transcription factor Chinmo (dark blue), whereas later-born MB neurons express either low levels (light blue) or none (white). Postmitotic levels of Chinmo specify the different temporal identities of γ, α′/β′, pα/β or α/β neurons. A decrease in Chinmo expression (*Chinmo*⁻) leads to fewer γ neurons and to the precocious generation of supernumerary pα/β neurons. Chinmo does not appear to specify the temporal identity of α/β neurons. If high levels of Chinmo are maintained in all postmitotic neurons (+ *Chinmo*), the early temporal identity (γ) is continuously generated at the expense of all later temporal identities (α′/β′, pα/β and α/β). (**B**) (a) In the mouse cerebral cortex, multipotent progenitors (mP) generate two distinct pools of progenitors: deep-layer progenitors (dP) and superficial-layer progenitors (sP). In turn, dP and sP sequentially generate the different neuronal subtypes (coloured circles) that are associated with deep (SP/VI/V) and superficial (IV/III/II) cortical layers, respectively. Postmitotic projection neurons of the different layers express different combinations of Sox5 (SRY-box 5), Ctip2 (Coup-TF-interacting protein 2) and Satb2 (special AT-rich sequence binding protein 2). Sox5 is normally expressed at different levels in the neurons of each of the layers SP, VI and V. (b) *Sox5* inactivation leads to a reduction in the sub-plate (SP) neuronal layer. SP neurons appear to be replaced by ectopic Ctip2⁺ neurons (yellow), characteristic of layer V. For clarity, ectopic Ctip2⁺ neurons located in superficial layers IV/III/II have been omitted as their origin is unclear. Inactivation of *Satb2*, which is predominantly expressed by layer IV/III/II neurons, leads to these late-born neurons acquiring an earlier Ctip2⁺ identity. (c) Sox5 and Satb2 repress *Ctip2*, and biochemical studies suggest that the Satb2 repression is direct (Alcamo et al., 2008; Britanova et al., 2008). As Ctip2 and Satb2 are transiently coexpressed by some layer V neurons (Alcamo et al., 2008), it might be that a stable layer-specific cell identity is only acquired sometime after neurons become postmitotic.

embryonic 4-2 lineage, the transcription factor Klumpfuss (Klu) acts within the second-born GMC, distinguishing its postmitotic progeny from those of the first-born GMC (Yang et al., 1997). Klu might therefore represent a bridging factor that mediates the transmission of temporal information from neuroblast to neuron. Further experiments are required to address whether progenitor TTFs in neuroblasts are required for the expression of Klu in GMCs and whether bridging factors acting in GMCs are widespread in other lineages. Taken together, the data from *Drosophila* studies are consistent with a model for temporal neural specification that relies on transiently expressed progenitor TTFs regulating the temporal identity of postmitotic cells and, in some cases, also the competence of progenitors. Transient progenitor TTFs might alter progenitor competence and other progenitor properties in a long-term manner via the sustained expression of target genes. In addition, they might transmit temporal-identity information from progenitor to postmitotic cell via the regulation of postmitotic TTFs.

Temporal specification in the vertebrate CNS

Three lines of argument suggest that qualitatively different temporal specification mechanisms could operate in the CNS of vertebrates and *Drosophila*. First, although all of the known progenitor TTFs in *Drosophila* have vertebrate orthologues, thus far there is no evidence that a Hb → Kr → Pdm → Cas neural progenitor sequence is conserved. Second, there are compelling data that extrinsic signals have an input into establishing the birth order of neurons and glia in vertebrates (Cepko, 1999; Desai and McConnell, 2000; McConnell and Kaznowski, 1991; Miller and Gauthier, 2007; Sockanathan and Jessell, 1998; Yun et al., 2002), but, as yet, this has not been demonstrated in *Drosophila*. A third and even more fundamental issue is that the cellular basis of the observed birth-order sequence of neuronal/glial subtypes remains unclear in many regions of the vertebrate CNS. In principle, the repertoire of postmitotic cells could be generated in full by a single multipotent progenitor (as in *Drosophila*) or piecemeal by multiple unipotent progenitors, each dividing at a different time to produce a distinct neuronal/glial subtype. Resolving which of these two extremes is the case, or whether the reality lies somewhere in between, will require extensive cell-lineage analysis. At present, a comprehensive region-by-region analysis would be technically challenging in vivo but, in future, new clonal analysis methods based on Brainbow and mosaic analysis with double markers might help (Livet et al., 2007; Zong et al., 2005).

We now review three examples of temporal specification in the vertebrate CNS and discuss the extent to which they might fit into the regulatory framework of *Drosophila* temporal specification. This

discussion about possible shared mechanisms between species will remain more of a hypothesis than a review until the three caveats above, particularly the vertebrate cell-lineage issue, are resolved. Although we provide examples from various regions of the developing vertebrate CNS, including the hindbrain, spinal cord and telencephalon, the retina is not included and has been reviewed elsewhere (Cepko, 1999; Cepko et al., 1996; Livesey and Cepko, 2001; Marquardt and Gruss, 2002).

The switch from visceral motor to serotonergic neurons

Progenitors in the ventral hindbrain of the chick and mouse first generate visceral motor (VM) and then serotonergic (5HT) neurons (Fig. 3A). They express the transcription factor paired-like homeobox 2b (Phox2b) early, during VM neurogenesis, whereas they express forkhead box A2 (Foxa2) later, during 5HT neurogenesis (Jacob et al., 2007; Pattyn et al., 2000). Interestingly, in the absence of Foxa2, the generation of VM neurons is prolonged and there is a corresponding block in 5HT neuronal production (Jacob et al., 2007). Conversely, a targeted deletion of *Phox2b* in mice leads to the precocious generation of 5HT neurons and a lack of VM neurons (Pattyn et al., 2003). Therefore, by analogy to *Drosophila*, Phox2b (Pattyn et al., 2003) and Foxa2 appear to act as progenitor TTFs. The cross-repressive circuit between these factors contains a negative-feedback loop (likely to be indirect) from Foxa2 to Phox2b that is reminiscent of that between Cas and Pdm. Foxa2 is thus required to prevent the continued expression of the preceding progenitor TTF and so, like Cas, might be both a progenitor TTF and a switching factor. Interestingly, in one segment of the hindbrain (rhombomere 4), the Phox2b → Foxa2 transition is normally suppressed, such that VM production is prolonged and 5HT neurons are absent. This is because the resident Hox protein in rhombomere 4, Hoxb1, maintains progenitor expression of Phox2b for longer than in other regions (Pattyn et al., 2003; Samad et al., 2004). Hoxb1 expression in progenitors, in turn, depends upon the combined activities of NK6 homeobox protein (Nkx6) and another Hox protein, Hoxb2 (Pattyn et al., 2003). As all three transcription factors are required to prevent ectopic 5HT neurogenesis in rhombomere 4, they can be thought of as components that 'freeze' a temporal transition, an effect opposite to the promotion of progenitor TTF switching by Svp. The true extent of parallels with the *Drosophila* model will only become clear in this system once it is known whether or not a common progenitor generates VM, 5HT and perhaps other types of neurons. Another unresolved question is whether ventral progenitors change competence after the VM → 5HT switch. Experiments assessing the progenitor response to Phox2b misexpression, specifically at late stages, should help to clarify this issue.

The switch from neurons to glia

Vertebrate neurons are generated before glia in vivo and this sequence can be recapitulated in vitro (Qian et al., 2000). Lineage-tracing studies and clonal analysis in culture have demonstrated that, as in *Drosophila*, there are common progenitors in vertebrates for neurons and glia (Leber et al., 1990; Qian et al., 2000; Walsh and Reid, 1995). The neuron → glia switch is known to involve a complex interplay between environmental cues and intrinsic factors in the cerebral cortex, and this might well be the case in other regions of the CNS (Guillemot, 2007; Miller and Gauthier, 2007; Rowitch, 2004). Two types of ventral spinal cord progenitor are known to switch from neurogenesis → gliogenesis (Fig. 3B). Those expressing the basic helix-loop-helix (bHLH) transcription factor, stem cell leukaemia (Scl; Tal1 – Mouse Genome Informatics), first generate V2 interneurons followed by astrocytes, whereas those expressing another bHLH factor, oligodendrocyte transcription factor 2 (Olig2), sequentially generate motoneurons and then oligodendrocytes (Lu et al., 2002; Muroyama et al., 2005; Orentas et al., 1999; Zhou and Anderson, 2002). For both the Olig2 and Scl progenitor types, the late onset of expression of SRY-box-containing gene 9 (*Sox9*), which encodes a high-mobility-group (HMG)-domain transcription factor, correlates with the timing of neuron → glial switching, and its loss blocks gliogenesis with a concomitant increase in V2 interneurons and motoneurons (Stolt et al., 2003). Thus, as for *Drosophila* progenitor TTFs, Sox9 can specify different cell identities in different lineages, in this case two distinct glial subtypes.

The available data strongly suggest that a bipotent Olig2$^+$ progenitor sequentially generates motoneurons and oligodendrocytes. Evidence for this comes from the chick spinal cord, where lineage tracing demonstrates that a common progenitor generates motoneurons and oligodendrocytes (Leber et al., 1990). It has also been shown that chick Olig2$^+$ progenitors express the bHLH transcription factors neurogenin 1 and 2 (Neurog1/2) during the neurogenic, but not the gliogenic, phase and that, in this context, Neurog1/2 function to inhibit precocious oligodendrocyte production (Zhou et al., 2001). Transplantation experiments indicate that the timing mechanism that schedules the neuron → glia switch utilises, at least in part, cell-intrinsic factors. Hence, young spinal cord Olig2$^+$ progenitors transplanted into young hosts generate both motoneurons and oligodendrocytes, whereas old progenitors transplanted into young hosts only generate oligodendrocytes (Mukouyama et al., 2006). Recently, two murine counterparts of *Drosophila* Svp, Coup-TFI and Coup-TFII (chicken ovalbumin upstream promoter-transcription factors I and II; also known as Nr2f1 and Nr2f2), have been shown to participate in the neuron → glia switch (Naka et al., 2008). Coup-TFI and Coup-TFII are transiently expressed in early neural progenitors from various regions of the CNS prior to the switch to gliogenesis, and knocking down the expression of both factors prolongs neurogenesis at the expense of gliogenesis (Fig. 3B). Naka et al. also conducted a stop-restart experiment showing that delayed rescue of the *Coup-TFI/II* knockdown initiates gliogenesis at a later time point than normal (Naka et al., 2008). Hence, Coup-TFI/II and Svp play evolutionarily related roles in temporal specification, probably functioning as cell-intrinsic switching factors. By analogy with *Drosophila* Svp, some of the downstream targets of the Coup-TFs in neural progenitors, which have yet to be identified, would be expected to correspond to progenitor TTFs.

Multiple temporal identities in the cerebral cortex

Birth order is linked to neuronal/glial identity throughout the vertebrate CNS but perhaps the most striking manifestation of this is found in the developing cerebral cortex, where different neuronal temporal identities are organised into six morphologically distinct layers. The cerebral cortex is therefore ideally suited to studying temporal neural specification, and impressive progress has recently been made in this system (reviewed by Leone et al., 2008; Molyneaux et al., 2007). The first postmitotic cells that appear in the developing cerebral cortex are Cajal-Retzius (CR) neurons, which occupy the most superficial layer, layer 1. CR neurons arise from specialised progenitors in restricted locations of the telencephalon (reviewed by Soriano and Del Rio, 2005). Neurons in the remaining strata, layers 2-6, are formed in an 'inside-out' manner, meaning that those in deeper layers are born before those that occupy more-superficial layers (Berry and Rogers, 1965; Berry et al., 1964). Retroviral lineage-tracing experiments in mammals show that young cortical progenitors generate neurons that are

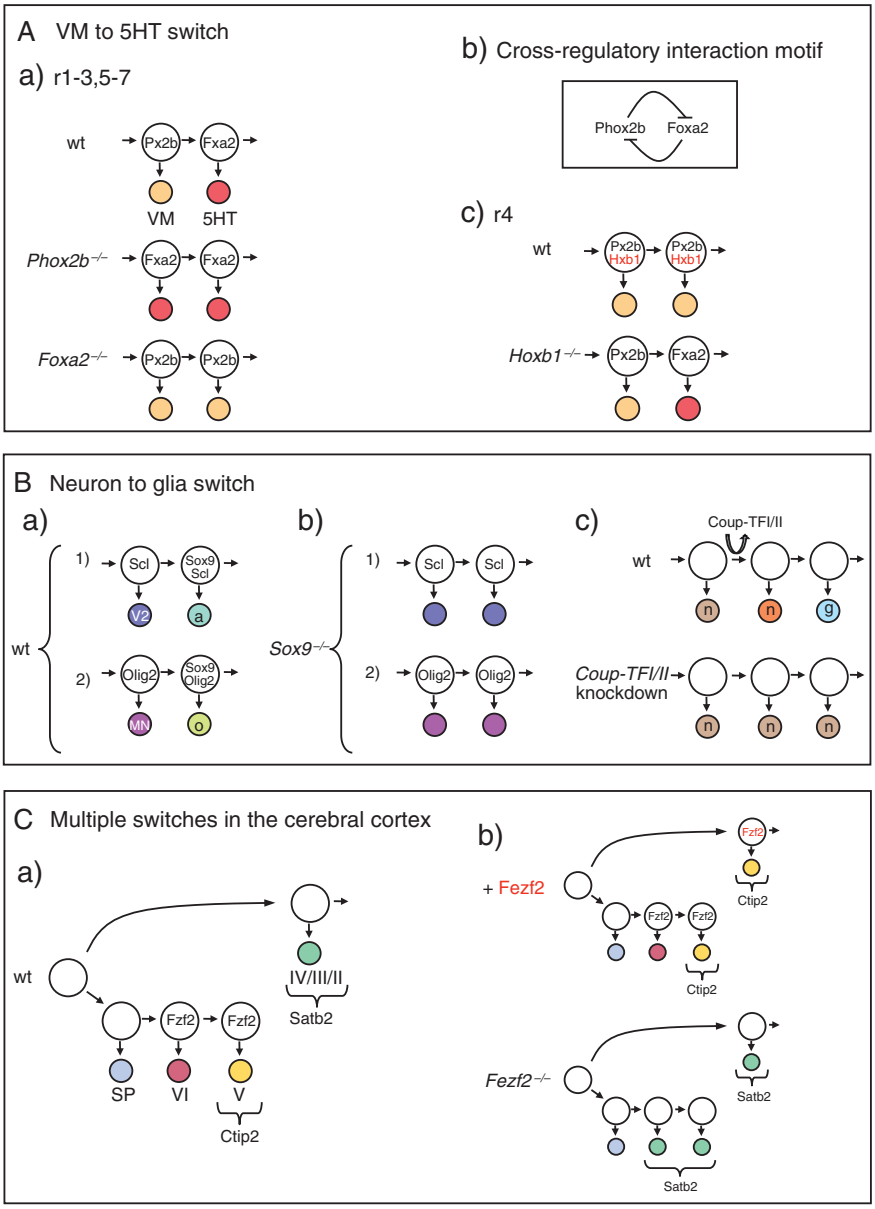

Fig. 3. Candidate progenitor TTFs in vertebrates. (**A**) (a) In most rhombomeres (r) of wild-type (wt) mouse and chick hindbrains, ventral progenitors (large circles) express Phox2b (Px2b) early and then Foxa2 (Fxa2) later, correspondingly generating VM (yellow) then 5HT (red) neurons. In *Phox2b*$^{-/-}$ mice, progenitors express Foxa2 and generate 5HT neurons precociously. In *Foxa2*$^{-/-}$ mice, VM generation is prolonged. (b) Loss- and gain-of-function experiments show that it is sequential cross-repression that promotes the switch between the VM and 5HT neuronal identities. (c) Hoxb1 (Hxb1) expression in r4 maintains Phox2b expression, thus preventing the switch to 5HT neurogenesis in this particular segment of the hindbrain. (**B**) (a) In the spinal cord, two types of progenitors (1 and 2) first generate neurons then glia. One progenitor type generates V2 interneurons (V2) and then astrocytes (a) and the other generates motoneurons (MN) and then oligodendrocytes (o). The lineage-specific factors Scl and Olig2, acting in combination with the temporal factor Sox9, influence whether an astrocytic or an oligodendrocytic cell identity is specified. (b) Loss of Sox9 activity in either progenitor type appears to prevent the neuronal-to-glial switch. (c) The orphan nuclear receptors Coup-TFI/II are transiently expressed in early neural progenitors and appear to act as switching factors as their knockdown prevents the switch from neurons (n) to glia (g). (**C**) (a) Cortical progenitors (large circles) can sequentially generate different neuronal subtypes (small coloured circles) during mouse embryogenesis that are each associated with different layers (SP, VI, V or IV/III/II). Fezf2 (Fzf2) is expressed in progenitors at the time they generate neurons that colonise layers VI and V. (b) Misexpression (red text) of Fezf2 during late stages of corticogenesis forces late progenitors to generate Ctip2^{+} neurons (yellow) typical of layer V. *Fezf2* inactivation results in an excess of Satb2^{+} neurons (green), typical of superficial layers IV/III/II, at the expense of layer VI and V neurons.

distributed across deep and superficial layers, whereas older progenitors only produce neurons in superficial layers (Luskin et al., 1988; Price and Thurlow, 1988; Rakic, 1988; Reid et al., 1995; Walsh and Cepko, 1988). These and other types of experiments (Desai and McConnell, 2000; Frantz and McConnell, 1996; McConnell and Kaznowski, 1991) indicate that there is a progressive restriction in the neuronal potential of progenitors with developmental time, as occurs with *Drosophila* neuroblasts. However, although multipotent progenitors in the ventricular zone of the cortex ultimately give rise to neurons in all layers, this occurs via an intermediate branching of the lineage that generates two separate pools of restricted progenitors, which are themselves specific for either deep- or superficial-layer neurons (reviewed by Molyneaux et al., 2007). As in *Drosophila*, it appears that the core mechanism for generating neuronal diversity from a multipotent progenitor relies largely on cell-intrinsic cues. Thus, cortical progenitors isolated in vitro can generate multiple neuronal subtypes in the same temporal order as they do in vivo (Shen et al., 2006). Remarkably, even mouse embryonic stem cells cultured under the correct conditions in vitro can generate neurons that express different cortical-layer markers in a sequence that recapitulates native corticogenesis (Gaspard et al., 2008).

One progenitor transcription factor implicated in temporal specification of the telencephalon is forkhead box G1 (Foxg1). The conditional inactivation of Foxg1 forces progenitors in the mouse cerebral cortex (pallium) that would not normally generate CR neurons to initiate the CR programme ectopically (Hanashima et al., 2007; Hanashima et al., 2004; Muzio and Mallamaci, 2005). In addition, stop-restart experiments in vitro show that transient knockdown of *Foxg1* in cultured mouse cortical progenitors leads to the persistent generation of CR neurons, followed by all the other layer-specific neuronal identities, apparently without intervening fate skipping (Shen et al., 2006). Taken together, these studies suggest that Foxg1 does not act as a switching factor for most cortical progenitors in vivo, rather it permanently suppresses the generation of neurons with a CR-like identity. However, Foxg1

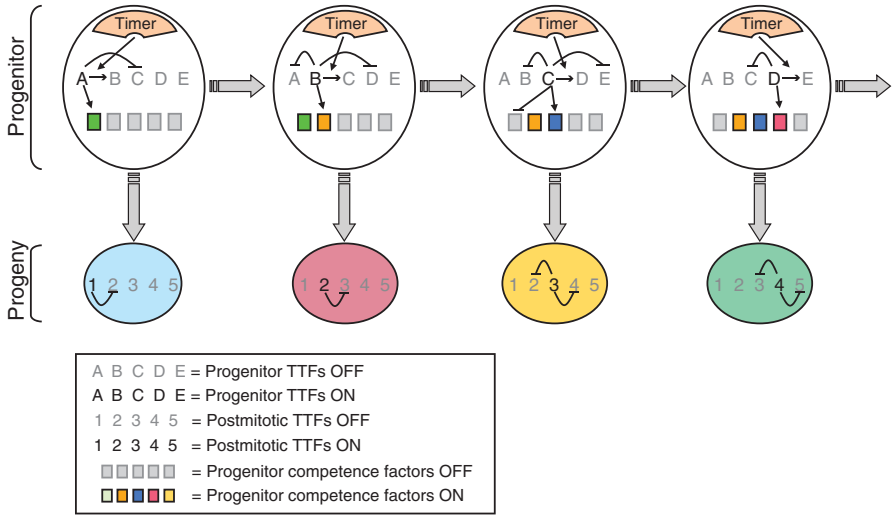

Fig. 4. Model for an asymmetrically dividing multipotent progenitor. A single multipotent progenitor (large oval) is shown at several different time points during development. The progenitor divides asymmetrically to 'self renew' and to generate a sequence of postmitotic progeny (small ovals), each with a different temporal identity (represented by the different colours). Within the progenitor, a temporal series timer (crescent) regulates the activity of switching factors with time and thus the frequency of the transitions (indicated by a sweeping arrow) between different progenitor TTFs (A → B → C → D → E). The core of the temporal series timer would be progenitor-intrinsic and could include both oscillatory and hourglass-like elements (reviewed by Pourquie, 1998; Rensing et al., 2001). Cross-regulatory repressions between some progenitor TTFs can promote these transitions (lines above letters indicate a selection of possible interactions), which may occur after one or many intervening cell cycles. Transient expression of progenitor TTFs can induce long-lasting changes in the expression pattern of a set of target genes – the progenitor competence factors. These, in turn, can modify several properties of the progenitors, including their ability to respond to later progenitor TTFs in the sequence. Progenitor TTFs also function, in combination with progenitor competence factors, to regulate the postmitotic TTFs (1, 2, 3, 4 and 5) that define the temporal identity of postmitotic progeny. Temporal identities can be stabilised by cross-regulatory interactions between the postmitotic TTFs (lines between numbers indicate a selection of possible repressions). Possible mechanisms for transmitting and transducing progenitor temporal information into the temporal identity of postmitotic daughter cells are discussed in the main text. For clarity, only one linear progenitor sequence (branch) is shown and intermediate progenitors are omitted. However, the main features of this general model also apply to progenitor lineages with more than one branch, such as those in the cerebral cortex and haematopoietic system.

might also act more like a switching factor in those spatially restricted progenitors that do normally generate a cohort of CR neurons.

The transcription factor, Fez family zinc-finger 2 (Fezf2, also known as Fezl), is expressed by early cortical progenitors (Fig. 3C). In *Fezf2*-null mice, there is a loss of deep-layer projection neurons, accompanied by an expansion of neurons that express superficial-layer (late-born) neuronal markers (Chen, B. et al., 2005; Chen et al., 2008; Chen, J. G. et al., 2005; Molyneaux et al., 2005). Conversely, misexpression of Fezf2 in late progenitors, which would normally generate superficial-layer neurons, leads to the ectopic generation of neurons that express molecular markers and axon projections characteristic of deep-layer neurons (Molyneaux et al., 2005). Fezf2 is thus a strong candidate to be a cortical progenitor TTF, providing deep-layer temporal identity to cortical neurons. If this is the case, then birthdating studies would be predicted to show that superficial-layer neurons are born precociously in *Fezf2* mutants. A further complication is that Fezf2 is not only expressed in early cortical progenitors, but also in their deep-layer neuronal progeny (Chen, J. G. et al., 2005; Molyneaux et al., 2005). Therefore, before its role can be clearly defined, experiments are needed to elucidate in which cells Fezf2 acts.

Several recent studies have shown that vertebrate layer-restricted transcription factors function in specifying temporal neuronal identities in the cortex, a role that is similar to that of the *Drosophila* postmitotic TTFs (reviewed by Fishell and Hanashima, 2008; Leone et al., 2008; Molyneaux et al., 2007). Three such factors, namely SRY-box 5 (Sox5), Coup-TF-interacting protein 2 (Ctip2; Bcl11b – Mouse Genome Informatics) and special AT-rich sequence binding protein 2 (Satb2), acting in a cell-autonomous manner, can account for the sequential generation of distinct subtypes of cortical pyramidal neurons (Alcamo et al., 2008; Britanova et al., 2008; Lai et al., 2008) (Fig. 2B). Sox5 and Ctip2 specify deep-layer pyramidal neurons that project subcortically, whereas Satb2 is a determinant of callosal neurons, which are mostly found in more-superficial layers. The absence of any one of these factors results in the loss of the corresponding cell population and in the ectopic expansion of cells typical of the adjacent layer. These observations, together with gain-of-function experiments, indicate that Sox5 and Satb2 repress *Ctip2* (Fig. 2B). In *Drosophila*, the importance of analogous cross-repressive interactions between the few postmitotic TTFs that have been functionally characterised thus far is less clear, although it is known that Chinmo and Br-C do not repress each other in postembryonic neurons (Maurange et al., 2008). It is also far from clear at present whether the cortical progenitor-to-neuron transmission of temporal information uses the same regulatory logic as *Drosophila* neuroblasts. Intriguingly, however, at least some parallels seem likely as it has been shown that the candidate progenitor TTF, Fezf2, activates a postmitotic TTF, Ctip2, and represses another, Satb2, either directly or via Ctip2 (Chen et al., 2008; Molyneaux et al., 2005). Furthermore, different levels of Sox5 in cortical neurons contribute to distinct deep-layer identities in a manner that is reminiscent of the graded action of Chinmo in *Drosophila* MB neurons (Lai et al., 2008).

Conclusions

The observation that neurons and glia are sequentially generated in the developing CNS of organisms as diverse as fruit flies and mice suggests the existence of a common set of underlying regulatory principles. The shared cellular framework for this common regulatory logic is a multipotent progenitor that is able to generate two or more distinct temporal identities in a stereotypical sequence. Within this context, we have outlined a general model for a multipotent progenitor (Fig. 4). This cell expresses a series of progenitor TTFs that, in turn, can regulate progenitor competence factors. The combination of progenitor TTFs and competence factors then specifies which postmitotic TTFs will be expressed in neuronal/glial progeny. If postmitotic TTFs are initially transcribed in progenitors, they can then be inherited by daughter cells, either by direct protein/mRNA perdurance or via the maintenance of a transcriptionally active status. Where postmitotic TTFs are first transcribed only in intermediate progenitors or in neurons/glia, more-indirect transmission mechanisms are required, such as those involving bridging factors. Little is known about the timing mechanism that controls the frequency of transitions between progenitor TTFs. However, the transitions themselves are known to require switching factors that participate in negative feedback and/or cross-repressive motifs that involve progenitor TTFs. Thus, transcription factor repression is likely to play a similar role in defining discrete cell fates during temporal patterning as it is known to in spatial patterning (Affolter and Basler, 2007; Briscoe and Ericson, 2001). In this regard, it is intriguing that the chronological sequence of known *Drosophila* progenitor TTFs in neuroblasts resembles the spatial order in which these are expressed during the earlier developmental process of blastoderm segmentation, and that similar cross-repressive interactions are utilised in both contexts (Isshiki et al., 2001).

Since the classic 'inside-out' studies of mammalian corticogenesis provided the initial impetus for exploring neural temporal specification, dramatic progress has been made in both insects and vertebrates. However, many important and interesting questions remain unresolved. What are the in vivo lineage relationships between vertebrate progenitors and their progeny? Which cellular contexts, other than a multipotent progenitor undergoing temporal transitions, can generate birth-order-dependent neural identities? Which molecular mechanisms transmit temporal information from progenitors to daughters? How is the temporal specification mechanism integrated with lineage-specific spatial patterning cues? What regulates the frequency of temporal transitions? How do local niches, feedback from progeny and other extrinsic influences regulate temporal specification? Finally, the temporal series is known to regulate the mitotic activity of progenitors in *Drosophila* (Maurange et al., 2008). Is this also the case in vertebrates? These are such fast-moving and exciting times that perhaps the only thing we can be sure of is that not everything in this hypothesis piece will turn out to be correct.

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