eLife

Reviewed Preprint
v1 • November 7, 2025
Not revised

Reviewed Preprint
v2 • December 23, 2025
Revised by authors

For correspondence:
FlyGuy@unm.edu

Competing interests: No competing interests declared

Funding: See page 18

Reviewing editor: Michael E Zuber, SUNY Upstate Medical University, United States

Cell cycle-dependent cues regulate temporal patterning of the *Drosophila* central brain neural stem cells

Gonzalo N Morales Chaya[1,2], Mubarak Hussain Syed[1]

[1]Neural Diversity Lab, Department of Biology, University of New Mexico, Albuquerque, United States • [2]Institute of Neuroscience, Howard Hughes Medical Institute, University of Oregon, Eugene, United States

eLife Assessment

This manuscript reports **important** findings indicating that cell cycle progression and cytokinesis both contribute to the transition from early to late neural stem cell fates. Loss-of-function experimental evidence **convincingly** shows that disrupting the cell cycle or cytokinesis can alter cell fate. This work sets the stage for future investigations into the underlying mechanisms linking the cell cycle to the expression of temporal factors controlling cell fate.

https://doi.org/10.7554/eLife.108259.2.sa4

Abstract

During nervous system development, diverse types of neurons and glia are sequentially generated by self-renewing neural stem cells (NSCs). Temporal changes in gene expression within NSCs are thought to regulate neural diversity; however, the mechanisms regulating the timing of these temporal gene transitions remain poorly understood. Drosophila type 2 NSCs, like human outer radial glia, divide to self-renew and generate intermediate neural progenitors, amplifying and diversifying the population of neurons innervating the central complex, a brain region crucial for sensorimotor coordination. Type 2 NSCs express over a dozen genes temporally, broadly classified as early and late-expressed genes. A conserved gene, seven-up, mediates early to late gene expression by activating ecdysone receptor (EcR) expression. However, the timing of EcR expression and, consequently, the transition from early to late gene expression remains unknown. This study investigates whether intrinsic mechanisms of cell cycle progression and cytokinesis are required to induce the NSC early-late transition. By generating mutant clones that arrest the NSC cell cycle or block cytokinesis, we show that both processes are necessary for the early-to-late transition. When NSCs are cell cycle or cytokinesis arrested, the early gene Imp fails to be downregulated and persists in the old NSCs, while the late factors EcR and Syncrip fail to be expressed. Furthermore, we demonstrate that the early factor Seven-up is insufficient to drive the transition, despite its normal expression in cell cycle- or cytokinesis-inhibited NSCs. These results suggest that both cell-intrinsic (cell cycle/cytokinesis) and extrinsic (hormone) cues are required for the early-late NSC gene expression transition.

Introduction

From insects to humans, multipotent neural stem cells (NSCs) generate diverse neurons and glia, which mediate proper nervous system function. Both spatial and temporal patterning programs regulate the diversity of neurons generated during nervous system development (Doe, 2017; El-Danaf et al., 2023; Enriquez et al., 2015; Erclik et al., 2017; Hamid et al., 2023; Kohwi and Doe, 2013; Malin and Desplan, 2021; Oberst et al., 2019; Sen, 2023; Skeath and Thor, 2003; Wani et al., 2023). While much is known about the regulation of spatial identity, the mechanisms underlying temporal identity are not entirely understood. Elucidating the processes

through which NSCs undergo temporal patterning, enabling them to produce diverse neurons and glial cells in the nervous system, is critical for understanding brain development and disease. Studies on the Drosophila nervous system have identified temporal identity genes that express temporally in NSCs and diversify neural cell types (Bayraktar and Doe, 2013; Eldred et al., 2018; Isshiki et al., 2001; Li et al., 2013; Maurange et al., 2008; Syed et al., 2017; Yang et al., 2015; Chaya et al., 2025). Sim-ilar temporal identity programs were later shown to regulate vertebrate retinal and cortical cell type specification (Alsiö et al., 2013; Elliott et al., 2008; Javed et al., 2023; Kohwi and Doe, 2013; Koo et al., 2023; Pebworth et al., 2021; Telley et al., 2019). In Drosophila embryonic NSCs, these gene transitions were thought to be intrinsically regulated; however, our recent findings in larval central brain NSCs identified the role of extrinsic steroid growth hormone ecdysone in the early-to-late gene transition (Syed et al., 2017). Recently, thyroid hormone signaling has been identified as regulating the early-to-late born cone cell type in human retinal organoid cultures (Eldred et al., 2018). Over the past few years, various temporally expressed genes have been identified in both invertebrates and vertebrates; however, the factors that determine the precise timing of these gene transitions are poorly understood.

Based on division patterns, the Drosophila NSCs can be classified as type 0, type 1, or type 2 NSCs. Type 0 NSCs generate a single post-mitotic neuron with each division and have only been observed in embryonic stages (Arefin et al., 2020; Baumgardt et al., 2014). Type 1 NSCs generate a ganglion mother cell (GMC) with each division; each GMC makes two post-mitotic neurons (Doe, 2017; Doe and Goodman, 1985; Rossi and Desplan, 2017). While type 1 NSCs comprise the majority of NSCs in the brain, type 2 NSCs—though limited to just 16—generate more complex and diverse progeny by exhibiting a more complex division pattern, producing approximately 5,000 neurons of at least about 160 distinct cell types (Epiney et al., 2025). Type 2 NSCs divide to self-renew and generate Intermediate Neural Progenitors (INPs); each INP undergoes a series of 4-6 self-renewing divisions to produce 4-6 GMCs, which each generate two neurons (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Wang et al., 2014). Recent temporal RNA-sequencing experiments have revealed that type 2 NSCs undergo a gene expression cascade involving transcription factors (TFs) and RNA-binding proteins (RBPs). In early larvae 0-60h after larval hatching (ALH), the NSCs express TFs, Seven-up (Svp), Castor (Cas), Chinmo, and RBPs, IGF-II mRNA-binding protein (Imp), and Lin-28. In late larvae (60-120h ALH), the NSCs express TFs, Ecdysone receptor (EcR), Ecdysone-induced protein 93F (E93), Broad and RBP, Syncrip (Syp) (Ren et al., 2017; Syed et al., 2017). The early-expressed conserved transcription factor Svp regulates the expression of EcR, thereby rendering NSCs competent to respond to the extrinsic hormonal signal, which triggers the early-late gene expression switch (Syed et al., 2017). While the role of the extrinsic factor Ecdysone in triggering the early-late transition is understood, nothing is known about how intrinsic factors drive this transition and what regulates the timing of these events.

In vertebrate cortical development, the cell cycle plays a key role in regulating a neural progenitor's ability to respond to extrinsic cues. Transplanted progenitors in a late-stage host exhibit different responses to these cues depending on their cell cycle stage at the time of transplantation (McConnell and Kaznowski, 1991; Ohnuma and Harris, 2003). In Drosophila, several studies have demonstrated a relationship between the cell cycle and temporal gene expression programs in NSCs. Interestingly, this relationship differs markedly between embryonic and larval stages. In embryonic NSCs, the classic temporal cascade—Hunchback > Kruppel > Pdm1/2 > Castor—is largely cell-intrinsic and can proceed in isolated, G2-arrested cells (Grosskortenhaus et al., 2005). While the transition from Hunchback to Kruppel requires cytokinesis, subsequent transitions (Kruppel to Pdm1/2 to Castor) occur even when cell cycle progression and cytokinesis are blocked.

In contrast, larval NSCs exhibit temporal transitions from early factors (Imp/Chinmo) to late factors (Syp/Broad) that depend on the cell's metabolic state and progression through the G1/S phase of the cell cycle (van den Ameele and Brand, 2019). Notably, at the end of larval neurogenesis, late temporal programs schedule the end of neurogenesis, and NSCs undergo cell

cycle exit or apoptosis. These late-stage transitions are governed by the Svp-mediated induction of the EcR and its downstream effector E93 (Pahl et al., 2019; Homem et al., 2014; Maurange et al., 2008; Syed et al., 2017). However, whether Svp and EcR expression—and thus the timing of early-to-late transitions—are dependent on cell cycle progression remains unknown. Furthermore, it is not yet clear whether similar temporal and cell cycle interactions occur in type 1 and type 2 NSCs. Moreover, it is unclear whether the timing of NSC gene expression is driven by organismal cues (e.g., hormones or signaling pathways), internal cues (e.g., a cell cycle counting clock), or both. Here, we utilize larval central brain NSCs to investigate whether cell cycle and cytokinesis regulate the timing of the temporal gene expression program.

Results

Cell cycle and cytokinesis are both required for the early-late temporal factor switch in type 2 NSCs

Our previous work revealed that in type 2 NSCs, the transition from early to late temporal factors occurs around 55h ALH (Syed et al., 2017). To better understand the mechanisms regulating the precise timing of these gene expression shifts, we next focused on the roles of the cell cycle and cytokinesis. While the cell cycle has been shown to influence temporal patterning in some systems, it appears to be dispensable in others, suggesting context-dependent roles in regulating these transitions (van den Ameele and Brand, 2019; Grosskortenhaus et al., 2005; Syed et al., 2017; Ray and Li, 2022). To investigate whether the temporal progression of type 2 NSCs requires cell cycle progression, we blocked the NSC cell cycle or cytokinesis in all type 2 NSCs using the driver Wor-GAL4, Ase-GAL80, starting at 0h ALH (the experimental model is shown in (Figure 1). We inhibited the cell cycle in all type 2 NSCs by knocking down Cyclin-dependent kinase 1 (Cdk1), a cdc2 kinase that forms heterodimers with Cyclin A and Cyclin B and is required for the execution of mitosis. Larvae were collected at 0 h ALH, and temporal factor expression was assayed at 72 h ALH. The Cdk1RNAi effectively blocked the cell cycle, as we could not observe any GFP-labeled progeny near the NSCs, and the NSC cell volume was larger than that of the control, which has also been attributed to normal cell-cycle progression (Hartenstein et al., 1987)(Figure S1). While all control type 2 NSC lineages showed normal temporal factor expression progression, Cdk1RNAi type 2 NSCs exhibited prolonged expression of the early factor Imp into later stages of development, and failed to upregulate the late factors Syp and EcR (Figure S2), confirming that cell cycle is essential for the early-to-late gene transition.

To further ensure the cell-specificity of this phenotype, better quantify and allow NSCs to develop in an otherwise wild-type brain, we used the flip-out approach that induced random single cell clones of type 2 NSCs. We induced random clones by giving a heat shock at 0h ALH using the genotype hsflp, 10xUAS-mCD8GFP; Act>FRT-stop-FRT>-GAL4. Both control RNAi (UAS-mCherryRNAi) and experimental (UAS-Cdk1RNAi) clones were labeled by GFP, and type 2 hits were confirmed by lack of the type 1 Asense marker (Figure S1 E-G). After heat shock, we assayed the clones in 72h ALH larvae. In the control type 2 NSCs, as anticipated at 72h ALH, minimal or no expression of the early factor Imp was observed, alongside high expression of late factors EcR and Syp (Figure 2A-2C; quantified in Figure 2J). In contrast, the experimental type 2 NSCs, where the cell cycle was blocked, exhibited persistent expression of the early factor Imp and no expression of late factors EcR and Syp at 72h ALH (Figure 2D-2F; quantified in Figure 2J), thus confirming that cell cycle progression is indispensable for the early-late switch in temporal factor gene expression.

The failure to switch from early to late factors could arise from blocking the cell-cycle progression (as part of a gene expression “clock” or “timer”) or from preventing cytokinesis, resulting in a daughter cell that may deliver feedback signals or partition transcriptional regulators out of the NSC. To discern between these possibilities, we inhibited cytokinesis, halting NSC progeny production while leaving its cell-cycle unaffected by expressing pavarotti RNAi (UAS-pavRNAi), a well-established cytokinesis inhibitor whose knockdown produces large, multinucleated cells (Gross-kortenhaus et al., 2005; Adams et al., 1998). Similar to our previous observations

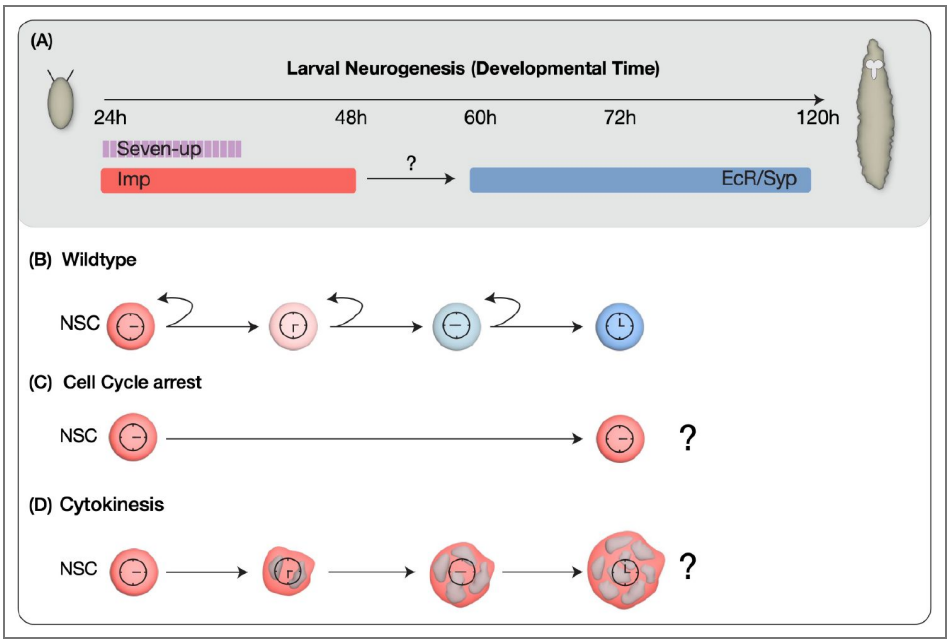

Figure 1. Models for early-to-late temporal progression transition of type 2 NSCs.

(A) Depicts timing of NSC temporal factor expression in type 2 NSCs during Drosophila larval development (B) Wild type NSCs undergo normal early to late temporal factor progression (C) Cell cycle progression as a regulator to allow timely expression of factors in NSCs (D) Cytokinesis dependent regulation of temporal gene expression.

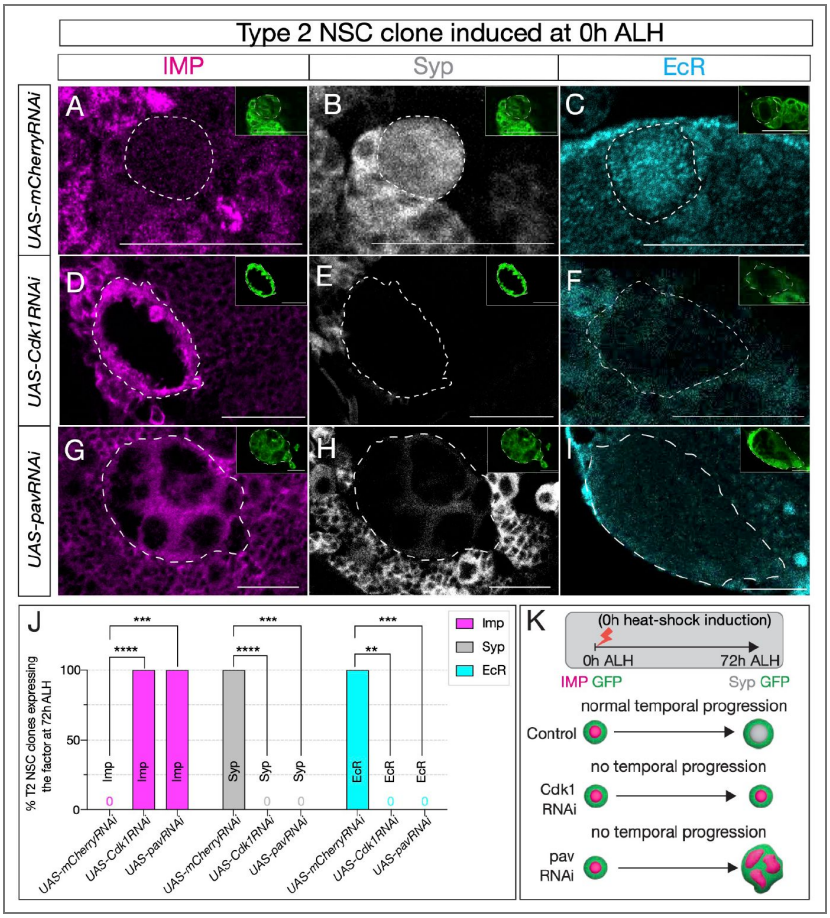

Figure 2. Cell cycle and cytokinesis are required for temporal gene expression progression in type 2 NSCs.

(A-C) Type 2 NSC control mCherryRNAi clones (circled) at 72h ALH show normal temporal gene expression progression. Early factor Imp is off, and late factors Syp and EcR are on at 72h ALH (D-F) Cdk1RNAi type 2 NSC clone fail to down-regulate early factor Imp, and late factors Syp and EcR are not turned on. (G-I) Similarly, cytokinesis-blocked type 2 NSC clones using pavRNAi fail to down-regulate Imp and upregulate Syp and EcR. (J) Quantification of early (Imp) and late (EcR, Syp) temporal factor expression in mCherryRNAi, Cdk1RNAi, and pavRNAi type 2 NSCs ($n \geq 4$ clones per genotype). Statistical analysis by Fisher's exact test: Imp: 0/5 vs 11/11 clones in Cdk1RNAi ($p = 0.0002$), 0/5 vs 8/8 in pavRNAi ($p = 0.0008$); EcR: 6/6 vs 0/4 in Cdk1RNAi ($p = 0.0048$), 6/6 vs 0/7 in pavRNAi ($p = 0.0006$); Syp: 5/5 vs 0/11 in Cdk1RNAi ($p = 0.0002$), 5/5 vs 0/8 in pavRNAi ($p = 0.0008$). Only one clone was found per animal. (K) Representation of experimental layout for inducing clones at 0h ALH and analysis at 72h ALH. Type 2 NSCs are identified as Asense-negative large cells expressing UAS-mcd8::GFP (green insets). Scale bars represent 20um.

(Figure S2), control type 2 NSCs showed no Imp expression and normal EcR/Syp expression (Figure 2A-2C; quantified in 2J), while the experimental pavRNAi type 2 NSCs resulted in large multi-nucleated NSCs (Figure S1 C-C') that maintained Imp expression and lacked EcR/Syp expression (Figure 2 G-2I; quantified in 2J). These results were consistent with blocking cytokinesis simultaneously in all type 2 NSCs (Figure S2). We conclude from these experiments that both, cell-cycle progression and cytokinesis are essential for the early-late gene expression switch in type 2 NSCs (Figure 2K).

The switching factor Svp is normally expressed in cell-cycle arrested NSCs

A conserved nuclear hormone receptor, Svp, mediates the early-to-late gene switch by regulating EcR expression (Dillon et al., 2024, Maurange et al., 2008, Syed et al., 2017). The svp mutant type 2 NSCs stay in their early gene expression module and fail to exit the cell cycle at the end of larval life. The mutant type 2 NSCs fail to downregulate the expression of early factors Chinmo and Imp and show no expression of the late factors EcR, Broad, Syncrip, and E93. While Svp shows its peak expression at 18-24h ALH, EcR expression starts at around 55h ALH (Dillon et al., 2024, Ren et al. 2017, Syed et al., 2017). Although svp is required for EcR expression, what regulates the precise timing of EcR expression around 55h ALH is unknown. The lack of EcR expression in cell cycle-arrested Type 2 NSCs could be due to either defective Svp expression or the lack of a cell cycle counter or both.

To test whether cell-cycle-arrested NSCs fail to express Svp and thus produce a phenotype similar to the svp mutant, we assayed Svp expression in type 2 cell-cycle-arrested NSCs. Since Svp expression is transient in these NSCs, we used a sv-pLacZ line, similar to our previous work (Syed et al., 2017), to achieve more stable protein expression. Upon counting LacZ-positive type 2 NSCs at 48h ALH, nearly all control type 2 NSCs showed svpLacZ expression (Figure 3A), and cell-cycle-arrested animals displayed similar expression levels with no statistically significant difference from controls (Figure 3B, quantified in 3C). We conclude that the timing of Svp expression is normal in cell-cycle-arrested NSCs.

Svp and cell cycle progression are independently required for the early-late temporal factor switch

Since Svp expression was normal in cell-cycle arrested type 2 NSCs, we wondered whether the cell cycle acts as a timer for the precise EcR expression following Svp expression. To address this question, we generated type 2 NSC clones expressing either Cdk1RNAi or pavRNAi at 42h ALH (see methods) well after the Svp expression window (18-24h ALH). We assayed for expression of NSC temporal markers at 72h ALH. If the transition fails to occur, it will confirm that Svp primes EcR expression, but the intrinsic cell cycle clock determines the precise timing. Conversely, if the transition occurs normally, cell cycle/cytokinesis plays a role in early gene expression (before 42h), but not in later, post-Svp expression. Compared to control type 2 NSC clones that showed normal Imp downregulation and EcR/Syp expression at 72h ALH (Figure 4A-4C; quantified in 4J), Cdk1 and pav mutant type 2 NSC clones had persistent Imp expression and no EcR/Syp expression (Figure 4D,4I; quantified in 4J), confirming that cell cycle progression post-Svp expression is critical for precise timing of EcR and early to late gene transition (Figure 3K).

Cell cycle and cytokinesis are required for the early-late temporal factor switch in type 1 NSCs

So far, we have focused on type 2 NSCs, which are relatively few; however, the majority of larval NSCs are type 1 NSCs, which express the same early and late temporal factors as type 2 NSCs (Syed et al., 2017). The timing of EcR expression and the transition from early to late gene expression also matches the type 2 NSC pattern (Syed et al., 2017). Thus, we sought to determine if cell cycle and cytokinesis also regulate temporal gene transitions in type 1 NSCs.

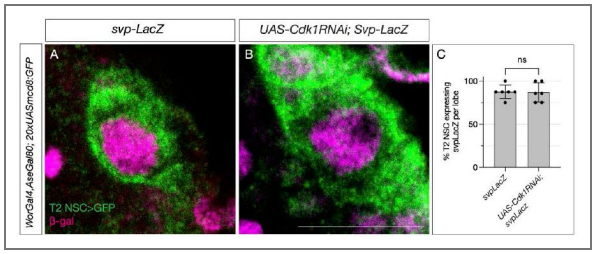

Figure 3. Switching factor Svp is expressed normally in cell-cycle-arrested type 2 NSCs.

(A) Control type 2 NSCs marked in green show Svp-LacZ expression at 48h ALH. (B) Cell-cycle-arrested (Cdk1RNAi) type 2 NSCs express Svp-LacZ similar to the control (C) Quantification of LacZ expression in control and cell cycle blocked type 2 NSCs, n=6 for each genotype. Unpaired t-test, p-value = 0.9168. Scale bars represent 10um.

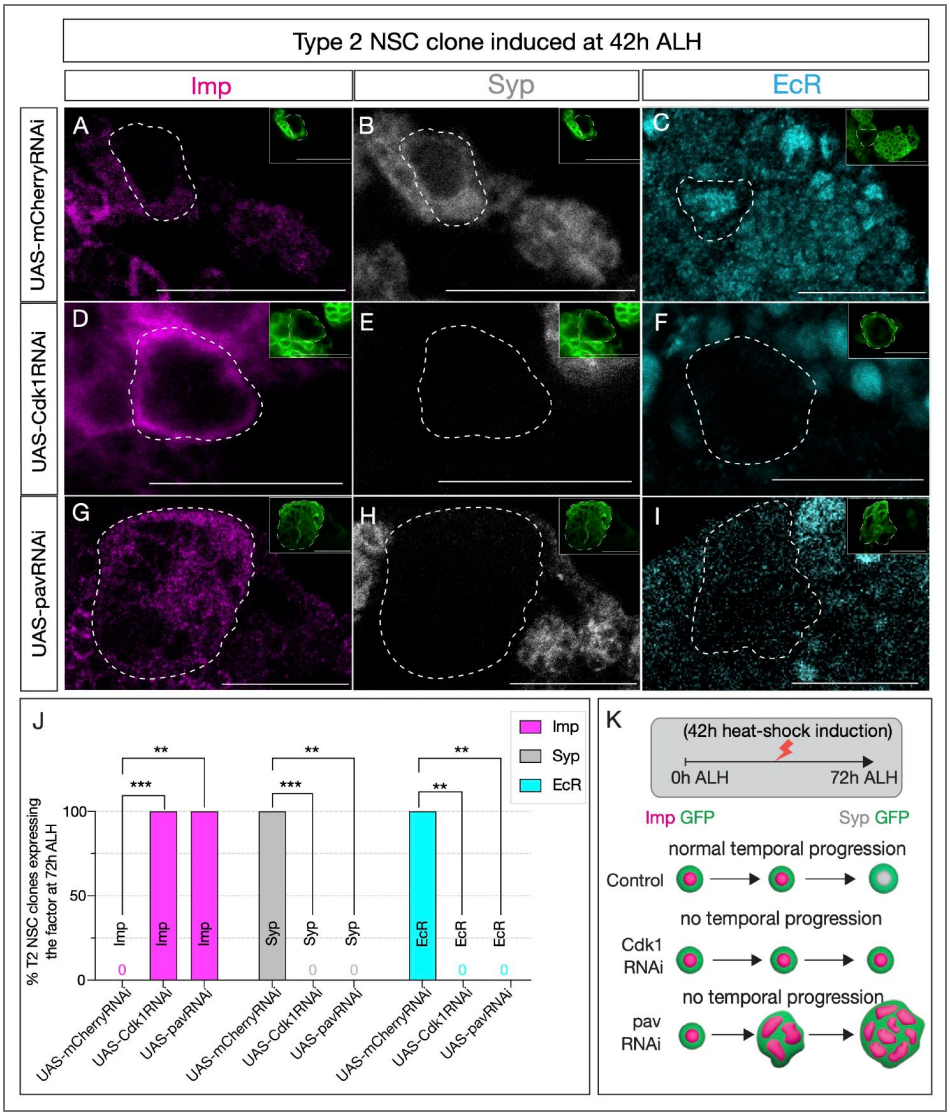

Figure 4. Early Svp expression is not sufficient to drive temporal factor progression in type 2 NSCs.

(A-C)(A-C) Type 2 NSC control mCherryRNAi clones (circled) induced at 42h ALH and stained at 72h ALH show normal progression of temporal factors. Early factor Imp is off, and late factors Syp and EcR are on. (D-F) Cdk1RNAi clones show consistent failure to down-regulate early factor Imp and activate Syp and EcR (G-I) Likewise, pavRNAi clones fail to express EcR and Syp and consistently express early factor Imp. (J) Quantification of early (Imp) and late (EcR, Syp) temporal factor expression in control (UAS-mCherryRNAi), UAS-Cdk1RNAi, and UAS-pavRNAi type 2 NSCs (n ≥ 4 clones per geno-type). Statistical analysis by Fisher's exact test: Imp: Cdk1RNAi vs control p = 0.0002, pavRNAi vs control p = 0.0079; EcR: Cdk1RNAi vs control p = 0.0079, pavRNAi vs control p = 0.0079; Syp: Cdk1RNAi vs control p = 0.0002, pavRNAi vs control p = 0.0079. Only one clone was found per animal. (K) Representation of experimental layout. Clones are identified as Asense negative large cells expressing UAS-mc-d8::GFP (green insets). Scale bars represent 20um.

Similar to our previous experiments, we generated clones using the genotype hsFLP, UAS-mCD8GFP; Act>FRT-stop-FRT>GAL4 / UAS-Cd-k1RNAi or UAS-mCherryRNAi and assayed GFP and Asense positive type 1 NSCs (Figure S1. E'-G'). Control RNAi induced at 0h and assayed at 72h showed normal down-regulation of Imp and expression of the late factors Syp (Figure 5A-C and 5K-M; quantified in 5J and 5T). In contrast, Cdk1 or pavRNAi type 1 NSC clones induced at 0h and assayed at 72h maintained Imp expression and lacked EcR/Syp expression (Figure 5D-I; quantified in 5J).

Similarly, Cdk1 or pavRNAi induced at 42h -- after the Svp expression window -- and assayed at 72h also maintained Imp expression and lacked EcR/Syp expression (Figure 5N-S; quantified in 5T). This suggests that most central brain NSCs possess a cell-cycle and cytokinesis-dependent, cell-intrinsic mechanism that regulates the Imp/ Syp expression transition. Our studies indicate that all central brain NSCs require cell cycle and cytokinesis, together with the switching factor Svp expression, to drive the early-late temporal factor expression transition.

Discussion

Temporal patterning plays a crucial role in specifying the diverse cell types of the nervous system. While many temporally expressed genes have been identified in both Drosophila and vertebrate neural progenitors (Javed et al., 2023; Koo et al., 2023; Pebworth et al., 2021; Syed et al., 2017; Telley et al., 2019; Walsh and Doe, 2017), what governs the timing of these gene expression transitions is not clearly understood. Is there an intrinsic timer within NSCs that counts and regulates the precise timing of temporal gene transitions? Is such a mechanism linked to cell-cycle progression and cytokinesis? How are NSC intrinsic and extrinsic environmental cues coupled? This study investigated the cell-intrinsic mechanisms regulating the early-to-late temporal gene expression transitions. Using Drosophila larval central brain NSCs, we identified that both cell cycle and cytokinesis-dependent cell-intrinsic timers regulate the progression of gene expression. In cytokinesis-blocked NSCs, the transition from early Imp expression to late EcR and Syp expression was impeded in both type 1 and type 2 NSCs. Upon temporal cell cycle block at 42h ALH, we showed that normal cell cycle progression is still essential for EcR expression despite the Svp expression being normal in the NSCs. These findings provide insights into the possible mechanisms regulating the temporal gene transition timing and also reveal the complex interplay of the cell cycle, cell-intrinsic genes, and cell-extrinsic hormonal cues in determining the precise timing of neuron type production within neural progenitors.

The possible role of cell cycle and cytokinesis in regulating temporal patterning and neuronal identity has been studied in vertebrates and invertebrates (Doe, 2008; Grosskortenhaus et al., 2005; Hagey et al., 2020; Kawaguchi, 2019; van den Ameele J, Brand AH. 2019). In vertebrates, the G2/M phase is required for the sequential generation of layer-specific cortical neurons. Knockdown of G2/M regulators has been shown to prevent radial glial cells from maintaining a "young," multi-potent state, leading to premature differentiation and a bias toward the production of early-born neurons (Hagey et al., 2020; Kawaguchi, 2019). In the case of Drosophila embryonic NSCs, which sequentially express Hunchback, Kruppel, Pdm1, and Castor, cell cycle is dispensable for these transitions. The initial Hunchback to Kruppel expression requires cytokinesis, while the Kruppel to Pdm1 to Castor transition occurs normally in G2 arrested NSCs (Grosskortenhaus et al., 2005). In contrast, our studies on larval NSCs show that both cell cycle and cytokinesis are essential for the early Imp/Chinmo to late EcR/Syncrip gene transition. The temporal gene progressions of embryonic NSCs occur normally in isolated NSCs, suggesting that these gene transitions are regulated intrinsically. In contrast, larval NSCs express EcR and require extrinsic growth hormone, ecdysone, for the transition from early to late gene expression. Our findings suggest that NSCs with more complex division patterns and dividing in situations where the growth and division patterns need to be coordinated require both cell-intrinsic and extrinsic cues. Studies on larval optic lobe NSCs thus far suggest that their temporal gene expression cascade is regulated cell-intrinsically, akin to embryonic NSCs (Konstantinides et al., 2022; Li et al., 2013). However, it remains unclear whether these temporal transitions depend on progression through the cell cycle/cytokinesis or both. This raises the intriguing

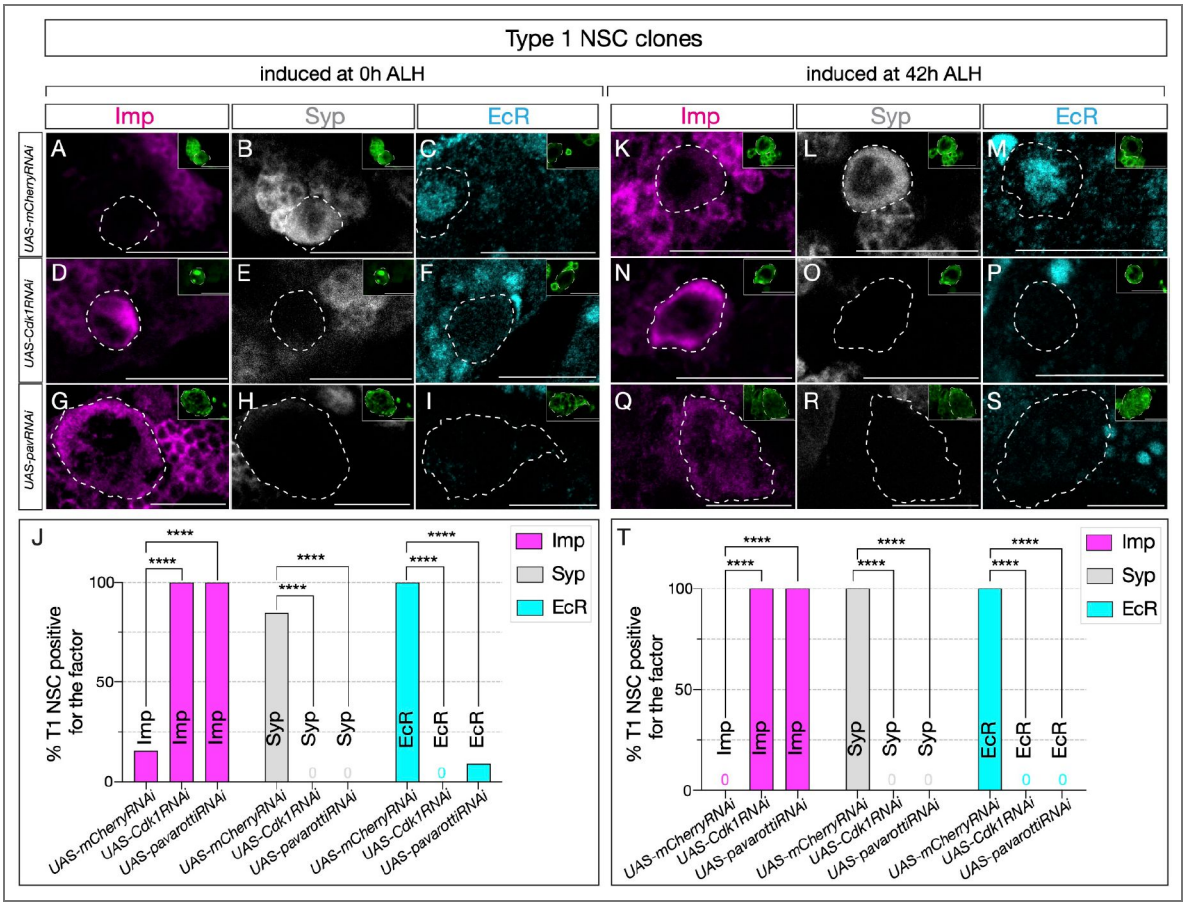

Figure 5. Cell cycle and cytokinesis are required for temporal progression in type 1 NSCs.

(A-I). Clones were induced at 0h ALH, and for (K-S), clones were induced at 42h ALH. (A-C) Typ. (A-I). Clones were induced at 0h ALH, and for (K-S), clones were induced at 42h ALH. (A-C) Type 1 NSC control RNAi clones show normal expression of temporal factor progression. (D-F) Cell cycle blocked type 1 NSC clones using Cdk1RNAi show failure to up-regulate EcR and Syp, and consistent expression of early factor Imp. (G-I) pavRNAi type I NSC clones fail to downregulate early factor Imp, and late factors Syp and EcR fail to express. (K-M) Control type 1 NSC clones induced at 42h ALH show normal expression of early and late factors, while Cdk1RNAi clones (N-P) and pavRNAi clones (Q-S) fail to downregulate Imp and fail to activate late factors Syp and EcR. (J, T) Quantification of early and late factor expression of 0h and 42h ALH–induced clones. (J) In clones induced at 0h ALH, Imp was expressed in 10/10 Cdk1RNAi clones (2/13 control, p = 0.000067) and 12/12 pavRNAi clones (2/13 control, p = 0.000020). EcR was not detected in Cdk1RNAi (0/11) or pavRNAi clones (0/11), while it was expressed in 12/12 control clones (p = 0.000001 for both). Syp was absent in Cdk1RNAi (0/10) and pavRNAi clones (0/12), while present in 11/13 control clones (p = 0.000067 and p = 0.000020, respectively). (T) In clones induced at 42h ALH, Imp expression persisted in 10/10 Cdk1RNAi (0/10 control, p = 0.000011) and 13/13 pavRNAi clones (0/10 control, p = 0.000001). EcR expression was not detected in Cdk1RNAi (0/8) or pavRNAi clones (0/13), compared to 10/10 control (p = 0.000023 and p = 0.000001). Syp expression was not detected in Cdk1RNAi (0/10) or pavRNAi clones (0/13), but was regular in 10/10 control (p = 0.000011 and p = 0.000001). Only one clone was found per animal. For each genotype n≥8. Type 1 clones are identified as Asense-positive, large cells expressing mcd8::GFP (green insets). Scale bars represent 20um..

question of whether optic lobe NSCs follow an embryonic-like model, in which temporal patterning occurs independently of cell division, or resemble larval central brain NSCs, where temporal progression is tightly coupled to the cell cycle and cytokinesis Through the cell cycle, neural progenitors generate neurons populating distinct layers in the vertebrate cortex (Borrell and Calegari, 2014; Hagey et al., 2020; Ohtsuka and Kageyama, 2019; Salomoni and Calegari, 2010); however, the relationship between cell cycle and temporal patterning has remained elusive. Given that the cell cycle is essential for temporal gene transitions, it could be one potential way of timing the generation of distinct progeny with precise temporal control. While we were able to demonstrate the cell-intrinsic roles of cell cycle in timing temporal gene transitions, given the fact that NSC resides in a glial niche and in close proximity to its lineages (Doe, 2008), and the cell-cycle blocked lineages have few or not progeny, we cannot not rule out the possibility that type 2 NSCs with cell cycle arrest failed to undergo normal temporal progression indirectly due to a lack of feedback signaling from their progeny. Additionally, we note that in some large multinucleated pavRNAi clones, a weak EcR signal can sometimes be observed near the cell membrane or within cytoplasmic regions (Fig. 2I). However, the nuclear compartments of these polynucleated cells—where EcR is normally localized and active—show no detectable EcR staining. The origin of this membrane-proximal EcR signal remains unclear; it may reflect non-specific antibody binding, altered EcR trafficking or degradation in cytokinesis-defective cells, or imaging artefacts arising from the large and irregular morphology of these clones. Importantly, the absence of nuclear EcR in the scored NSCs supports our conclusion that both cell cycle progression and cytokinesis are required for the acquisition of nuclear EcR expression and for the early-to-late temporal transition.

We acknowledge that we cannot formally exclude gene-specific, cell-cycle–independent roles of Cdk1 or Pav. However, several lines of evidence favor the interpretation that disruption of cell-cycle progression or cytokinesis underlies the phenotype. First, Cdk1 and Pav are canonical regulators of mitosis and cytokinesis, respectively; second, the same qualitative temporal-block phenotype (persistence of early markers and failure to induce late markers) is produced by orthogonal manipulations that prolong G1/S or slow proliferation, including G1/S inhibitors and mitochondrial OxPhos inhibition (which increases G1/S occupancy), reported by others (van den Ameele and Brand, 2019). Thus, although we cannot rule out additional, gene-specific contributions of Cdk1 or Pav, the concordant effects of multiple, mechanistically distinct cell-cycle perturbations make altered cell-cycle dynamics the most parsimonious explanation. Definitive understanding of the molecular mechanism (for example, whether mitosis-dependent chromatin remodeling, partitioning of factors during cytokinesis, or cell-cycle–regulated signaling triggers late-factor induction) will require further experiments.

Our results suggest that only part of the temporal gene cascade depends on cell-cycle progression. Depletion of Cdk1 disrupts the induction of late temporal factors (Syp, EcR) while leaving the onset of early switching factor Svp intact, indicating that the early expression of the temporal transition switching program can proceed independently of cell-cycle progression. This distinction raises specific mechanistic possibilities: for example, mitosis- or cytokinesis-coupled processes such as transcriptional reactivation, cell-cycle–dependent post-translational modification of regulators, or daughter-cell–mediated signaling/partitioning could be required for late-factor induction but not for early svp activation. Defining the precise mechanism is beyond the scope of this study; however, our findings provide a testable framework for future experiments to determine how cell-cycle–regulated changes in nuclear or cytoplasmic state enable the transition from early Imp/Chinmo to late Syp/EcR expression.

During cortical development, both cell cycle and extrinsic factors govern neuronal fate, and it was proposed that neuronal progenitors undergo cyclic changes in their ability to respond to extrinsic cues (McConnell and Kaznowski, 1991; Okamoto et al., 2016). Similarly, in Drosophila, Delta expression in the neighboring GMC and glia regulate Notch signaling, which is required for terminating neurogenesis in the central brain (Sood et al., 2024). Further studies are needed to elucidate the mechanism by which the cell cycle influences EcR expression and to confirm the role of progeny within the lineage in regulating temporal gene expression transitions in NSCs.

Materials and methods

Immunohistochemistry

Larval brains were dissected in PBS and mounted on poly-D-lysine-coated coverslips. Samples were fixed for 23 minutes in 4% PFA in PBST, and then washed in PBST for 3×20 minutes. Following, the samples were blocked with 2% normal donkey serum and 2% normal goat serum (Jackson ImmunoResearch Laboratories, Inc.) in PBST for 40 minutes at room temperature. Samples were then incubated in a primary antibody mix diluted in PBST overnight or for 1-2 days at 4°C. Primary antibodies were removed, and samples were thoroughly washed with PBST. Samples were then incubated in secondary antibodies for 2 hours at room temperature. Secondary antibodies were removed, and samples were washed in PBST. Samples were dehydrated with an ethanol series of 30%, 50%, 75%, and 100% ethanol, then incubated in xylene (Fisher Chemical X5-1) for 2×10 minutes. Samples were mounted onto slides with DPX (Sigma-Aldrich 06552) and cured for 3-4 days, then stored at 4°C until imaged.

Image acquisition and analysis

Fluorescent images were acquired on both Zeiss LSM 710 and 800. Scale bars were given for all stacks within maximum intensity projection images. Brightness and contrast were adjusted in the figure images individually for better visualization across the entire image in control and experimental samples. Statistics and bar graphs were computed using Prism 10.

Figure Preparation

Confocal images were prepared using FIJI or Imaris 10.0.0. Figures and schematics were assembled using Adobe Illustrator (2025).

Generation of clones

To generate RNAi clones, hsFLP;10xUASmc-d8::GFP; ActFRTstopFRTGAL4 flies were crossed to the RNAi lines for the gene of interest or control UAS-mCherryRNAi line. Embryos were collected over a period of 4 hr. After hatching, larvae were collected and then heat shocked in a 37°C water bath for 8 minutes and reared at 25°C until the desired time point. Temporal factor expression (Imp, Syp, EcR) was scored as positive or negative in individual neuroblasts. Because pavR-NAi clones are frequently large and often multinucleated, reliable nuclear segmentation and per-nucleus intensity quantification were not feasible. To ensure reproducibility across samples, we normalized fluorescence signal intensity for each neuroblast to the mean intensity of neighboring wild type neuroblasts imaged in the same field. A neuroblast was considered positive when its normalized intensity in the nucleus was at least 2× the local background. This scoring criterion was applied uniformly to all genotypes and time points. Only one clone was quantified per animal unless otherwise indicated.

Supplementary figures and tables

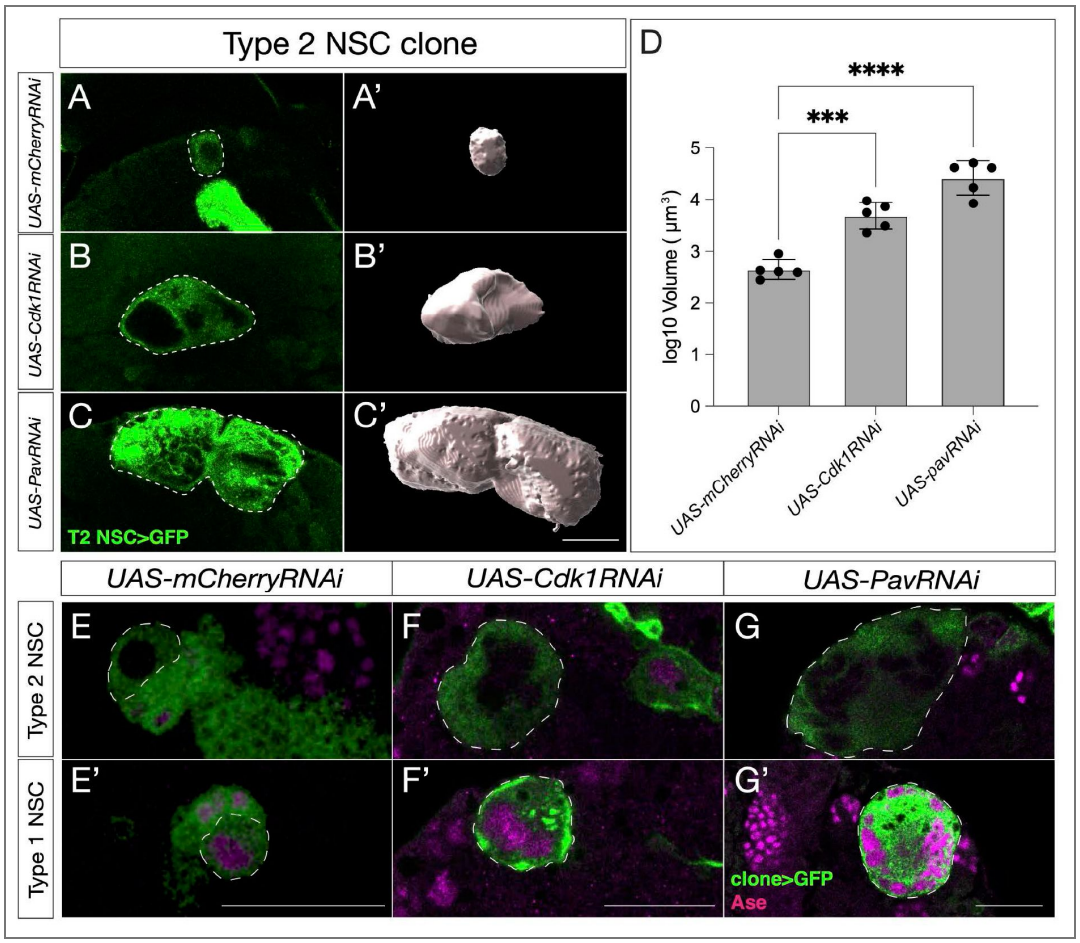

Figure S1. pav and Cdk1RNAi type 2 NSC clones are larger in volume. Confocal scans with their respective Imaris reconstruction are shown on the right for mCherryRNAi (A-A'), Cdk1RNAi (B-B'), and pavRNAi (C-C') clones. (D) Quantification of log10-transformed clone volumes (µm^3); n = 5 clones per condition, plotted as mean ± SEM. Overall treatment effect by Welch's ANOVA, $p < 0.0001$. Post-hoc Dunnett's T3 vs. mCherryRNAi: Cdk1RNAi, $p = 0.0003$; pavRNAi, $p < 0.0001$. only one clone was quantified per animal. Type 2 (E–G) and type 1 (E'–G') clones show negative and positive nuclear Asense (Ase) staining, respectively, in (E, E') mCherryRNAi, (F, F') Cdk1RNAi, and (G, G') pavRNAi. Scale bar represents 20um.

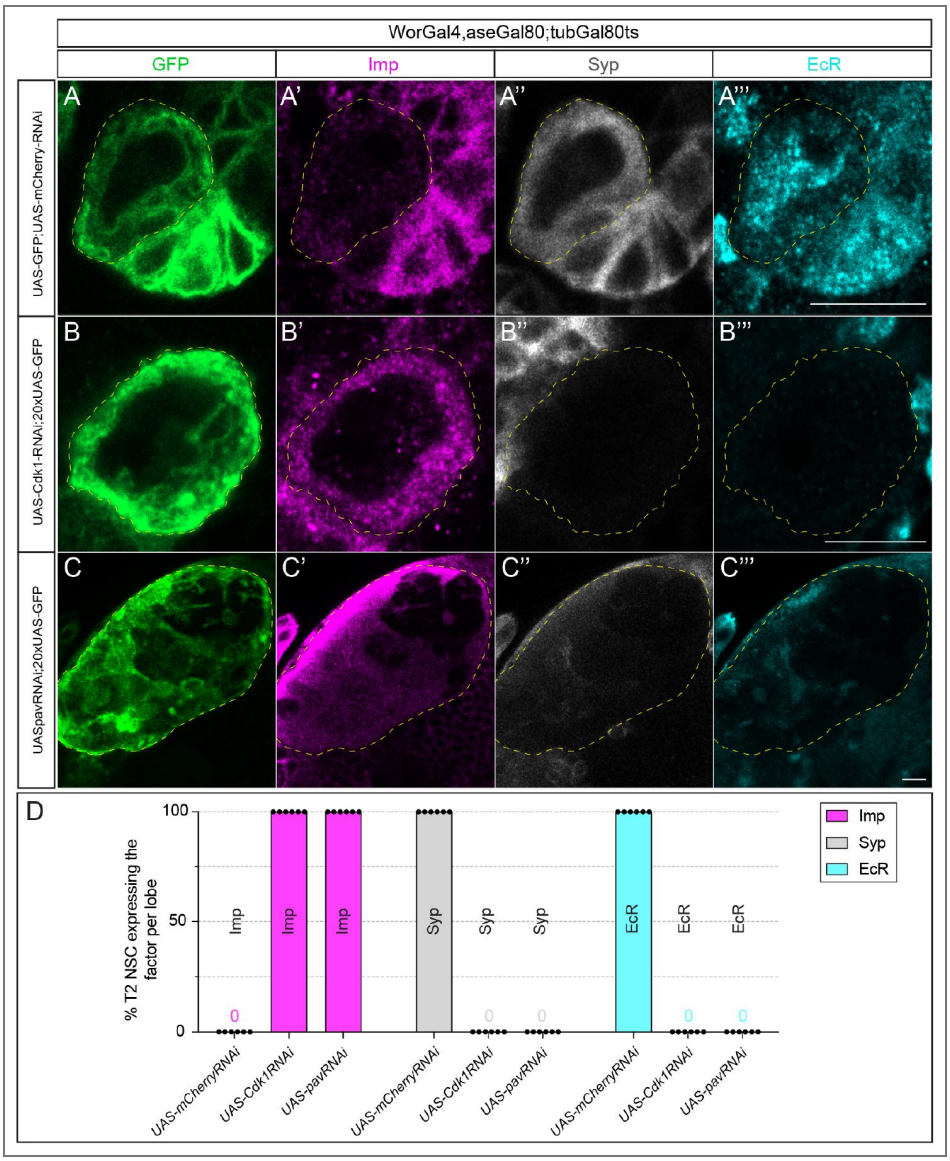

Figure S2. Cell-cycle and cytokinesis inhibit the early-to-late transition of temporal factors in type 2 NSCs.

(A-A''') Type 2 NSC control UAS-mCherryRNAi (circled) at 72h ALH show normal temporal gene expression progression. Early factor Imp is off, and late factors Syp and EcR are on at 72h ALH (B-B''''). Cdk1RNAi type 2 NSCs fail to downregulate early factor Imp, and late factors Syp and EcR are not turned on. (C-C''') Similarly, cytokinesis-blocked type 2 NSCs using pavRNAi fail to downregulate Imp and upregulate Syp and EcR. (D) Quantifications of early and late temporal factor expression in control, cell cycle, and cytokinesis blocked type 2 NSCs; n=6 for each genotype. Type 2 NSCs are identified as large cells expressing UAS-mcd8::GFP. Scale bars represent 10um.

Antibodies	Source	Identifier
Chicken anti-GFP (1:å1500)	Aves Labs	Cat# AB_2314866; RRID_AB_2314866
Rabbit anti-Asense (1:1000)	Chien-Yu Lee	N/A
Rat anti-IMP (1:200)	Claude Desplan	N/A
Rabbit anti b -GAL (1:1000)	MP Biomedicals	Cat# 559762
Mouse anti-EcR-B1 (1:2000)	Carl Thummel	N/A
Guinea pig anti-Syncrip (1:2000)	Ilan Davis	N/A
Chemicals		
16% Paraformaldehyde	Electron Microscopy Sciences	Cat# 15710
Triton X-100	Sigma-Aldrich	Cat# T8787
Apple Juice	S. Martinelli & Co	N/A
Schneider's Insect medium	Sigma-Aldrich	Cat# S0146
Agar	Sigma-Aldrich	Cat# A1296
DPX mounting medium	Sigma-Aldrich	Cat# 06522
Sucrose	Research products International	Cat# 57-50-1
Xylene	Fisher Scientific	Cat# 1330-20-7, 100-41-4

Resource Table.

Strains		
hsFlp,UAS-mCD8GFP; Act-FRTstopFRT-GAL4	This study	NA
UAS-pavRNAi	BDSC	BDSC_43963
UAS-Cdk1RNAi	BDSC	BDSC_36117
UAS-mCherryRNAi	BDSC	BDSC_35785
Wor-GAL4, Ase-GAL80; 20xUASmcd8GFP	Syed Lab	NA
Wor-GAL4, Ase-GAL80; tub-GAL80ts	Syed Lab	NA
UAS-mcd8GFP (II)	BDSC	5137
20xUAS-mcd8GFP (III)	BDSC	32194
svp-LacZ	BDSC	BDSC_26669
Software and Algorthms		
Imagej	Fiji	Version: 2.9.0/1.53t
Adobe Illustrator (version 24.005.20307)	Adobe Systems	
GraphPad Prism 9	GraphPad Software	https://www.graphpad.com/
Imaris v9.9 and above	Oxford Instruments	RRID: SCR_007370; https://www.oxinst.com/news/imaris-launches-version-9.9-with-machine-learning-and-open-source-connections

Resource Table. (continued)

Table 1. **Fly genotype with associated figures.**

Experimental line	Main	Supplementary
hsFlp,UAS-GFP;Act-FRTstopFRT-GAL4 crossed to UAS-mCherryRNAi	Fig. 2A, 2B, 2C	Fig. S1A
hsFlp,UAS-GFP; Act-FRTstopFRT-GAL4 crossed to UAS-Cdk1RNAi	Fig. 2D, 2E, 2F,	Fig. S1B
hsFlp,UAS-GFP;act-FRTstopFRT-GAL4 crossed to UAS-pavRNAi	Fig. 2G, 2H, 2I	Fig. S1C
Wor-GAL4, Ase-GAL80;tub-GAL80ts crossed to UAS-mcd8::GFP;UAS-mCherryRNAi		Fig. S2A-A’’’
Wor-GAL4, Ase-GAL80;tub-GAL80ts crossed to UAS-mcd8::GFP;UAS-Cdk1RNAi		Fig. S2B-B’’’
Wor-GAL4, Ase-GAL80;tub-GAL80ts crossed to UAS-mcd8::GFP;UAS-pavRNAi		Fig. S2C-C’’’
Wor-GAL4,AseGAL80; 20xUASmcd8:GFP crossed to Svp-LacZ	Fig. 3A	
WorGAL4,AseGAL80; 20xUASmcd8:GFP crossed to UAS-Cdk1-RNAi;Svp-LacZ	Fig. 3B	
hsFlp,UASmCD8GFP; Act-FRTstopFRT-GAL4 crossed to UAS-mCherryRNAi	Fig. 4A, 4B, 4C	
hsFlp,UASmCD8GFP; Act-FRTstopFRT-GAL4 crossed to UAS-Cdk1RNAi	Fig. 4D, 4E, 4F	
hsFlp,UASGFP; Act-FRTstopFRT-GAL4 crossed to UAS-pavRNAi	Fig. 4G, 4H, 4I	
hsFlp,UASmCD8GFP; Act-FRTstopFRT-GAL4 crossed to UAS-mCherryRNAi	Fig. 5A, 5B, 5C, 5K, 5L, 5M	
hsFlp,UASmCD8 GFP; Act-FRTstopFRT-GAL4 crossed to UAS-Cdk1RNAi	Fig. 5D, 5E, 5F, 5N, 5O, 5P	
hsFlp,UASGFP; Act-FRTstopFRT-GAL4 crossed to UAS-pavRNAi	Fig. 5G, 5H, 5I, 5Q, 5R, 5S	

Data availability

All data generated in this study are included in the manuscript

Acknowledgements

We thank Adil Wani, Asif Bakshi, Sen-Lin Lai, and Chris Doe for providing feedback on the manuscript. Thanks to Claude Desplan for sharing antibodies generously. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. We thank UNM CETI Biology Cell Biology Core for providing a confocal microscope facility. The research was supported by the Sloan Research Fellowship FG-2023-20617, 2023 BBRF Young Investigator Grant, McKnight Scholars Award, National Science Foundation CAREER Award IOS-2047020, Air Force Office of Scientific Research FA9550-24-1-0214 and NINDS R01NS136555 to MHS

Additional information

Author contributions

Conceptualization: G.M.C and M.H.S.; methodology: G.M.C.; visualization: G.M.C. and M.H.S.; writing-original draft: G.M.C.; editing: M.H.S.; funding acquisition: M.H.S. and supervision: M.H.S.

Funding

Funder	Grant reference number	Author
Alfred P. Sloan Foundation (APSF)	FG-2023-20617	Mubarak Hussain Syed
McKnight Foundation (McKnight)	McKnight Scholars Award	Mubarak Hussain Syed
DOD | AF | AMC | AFRL | Air Force Office of Scientific Research (AFOSR)	FA9550-24-1-0214	Mubarak Hussain Syed
National Science Foundation (NSF)	IOS-2047020	Mubarak Hussain Syed
HHS | NIH | National Institute of Neurological Disorders and Stroke (NINDS)	R01NS136555	Mubarak Hussain Syed

Author ORCID iDs

Gonzalo N Morales Chaya: https://orcid.org/0009-0005-4263-1323
Mubarak Hussain Syed: https://orcid.org/0000-0003-2424-175X

References

Adams RR, Tavares AAM, Salzberg A, Bellen HJ, Glover DM (1998) pavarotti encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. *Genes Dev* **12**:1483-1494 https://doi.org/10.1101/gad.12.10.1483

Alsiö JM, Tarchini B, Cayouette M, Livesey FJ (2013) Ikaros promotes early-born neuronal fates in the cerebral cortex. *Proc Natl Acad Sci U S A* **110**:E716-725 https://doi.org/10.1073/pnas.1215707110

Arefin B, Bahrampour S, Monedero Cobeta I, Curt JR, Stratmann J, Yaghmaeian Salmani B, Baumgardt M, Benito-Sipos J, Thor S. (2020) *Development of the Drosophila melanogaster embryonic CNSPatterning and Cell Type Specification in the Developing CNS and PNS* Elsevier. pp. 617-642 https://doi.org/10.1016/B978-0-12-814405-3.00025-4

Baumgardt M, Karlsson D, Salmani BY, Bivik C, MacDonald RB, Gunnar E, Thor S. (2014) Global Programmed Switch in Neural Daughter Cell Proliferation Mode Triggered by a Temporal Gene Cascade. *Dev Cell* **30**:192-208 https://doi.org/10.1016/j.devcel.2014.06.021

Bayraktar OA, Doe CQ (2013) Combinatorial temporal patterning in progenitors expands neural diversity. *Nature* **498**:449-455 https://doi.org/10.1038/nature12266

Bello BC, Izergina N, Caussinus E, Reichert H. (2008) Amplification of neural stem cell proliferation by intermediate progenitor cells in Drosophila brain development. *Neural Develop* **3**:5 https://doi.org/10.1186/1749-8104-3-5

Boone JQ, Doe CQ (2008) Identification of Drosophila type II neuroblast lineages containing transit amplifying ganglion mother cells. *Dev Neurobiol* **68**:1185-1195 https://doi.org/10.1002/dneu.20648

Borrell V, Calegari F. (2014) Mechanisms of brain evolution: Regulation of neural progenitor cell diversity and cell cycle length. *Neurosci Res* **86**:14-24 https://doi.org/10.1016/j.nes.2014.04.004

Bowman SK, Rolland V, Betschinger J, Kinsey KA, Emery G, Knoblich JA (2008) The Tumor Suppressors Brat and Numb Regulate Transit-Amplifying Neuroblast Lineages in Drosophila. *Dev Cell* **14**:535-546 https://doi.org/10.1016/j.devcel.2008.03.004

Chaya GNM, Hamid A, Wani AR, Gutierrez A, Syed MH (2025) Developmental Genetic and Molecular Analysis of Drosophila Central Complex Lineages. *Cold Spring Harb Protoc* **2025**:pdb.top108429 https://doi.org/10.1101/pdb.top108429

Dillon NR, Manning L, Hirono K, Doe CQ (2024) Seven-up acts in neuroblasts to specify adult central complex neuron identity and initiate neuroblast decommissioning. *Development* **151**:dev202504 https://doi.org/10.1242/dev.202504

Doe CQ, Goodman CS (1985) Early events in insect neurogenesis. *Dev Biol* **111**:206-219 https://doi.org/10.1016/0012-1606(85)90446-4

Doe CQ (2008) Neural stem cells: balancing self-renewal with differentiation. *Development* **135**:1575-1587 https://doi.org/10.1242/dev.014977

Doe CQ (2017) Temporal Patterning in the Drosophila CNS. *Annu Rev Cell Dev Biol* **33**:219-240 https://doi.org/10.1146/annurev-cellbio-111315-125210

El-Danaf RN, Rajesh R, Desplan C. (2023) Temporal regulation of neural diversity in Drosophila and vertebrates. *Semin Cell Dev Biol* **142**:13-22 https://doi.org/10.1016/j.semcdb.2022.05.011

Eldred KC, Hadyniak SE, Hussey KA, Brenerman B, Zhang P-W, Chamling X, Sluch VM, Welsbie DS, Hattar S, Taylor J, *et al.* (2018) Thyroid hormone signaling specifies cone subtypes in human retinal organoids. *Science* **362**:eaau6348 https://doi.org/10.1126/science.aau6348

Elliott J, Jolicoeur C, Ramamurthy V, Cayouette M. (2008) aros Confers Early Temporal Competence to Mouse Retinal Progenitor Cells. *Neuron* **60**:26-39 https://doi.org/10.1016/j.neuron.2008.08.008

Enriquez J, Rio LQ, Blazeski R, Bellemin S, Godement P, Mason C, Mann RS (2018) Differing Strategies Despite Shared Lineages of Motor Neurons and Glia to Achieve Robust Development of an Adult Neuropil in Drosophila. *Neuron* **97**:538-554.e5 https://doi.org/10.1016/j.neuron.2018.01.007

Enriquez J, Venkatasubramanian L, Baek M, Peterson M, Aghayeva U, Mann RS (2015) Specification of Individual Adult Motor Neuron Morphologies by Combinatorial Transcription Factor Codes. *Neuron* **86**:955-970 https://doi.org/10.1016/j.neuron.2015.04.011

Epiney D, Chaya GNM, Dillon NR, Lai S-L, Doe CQ (2025) Transcriptional complexity in the insect central complex: single nuclei RNA-sequencing of adult brain neurons derived from type 2 neuroblasts. *eLife* **14**:RP105896 https://doi.org/10.7554/eLife.105896.2

Erclik T, Li X, Courgeon M, Bertet C, Chen Z, Baumert R, Ng J, Koo C, Arain U, Behnia R, *et al.* (2017) Integration of temporal and spatial patterning generates neural diversity. *Nature* **541**:365-370 https://doi.org/10.1038/nature20794

Grosskortenhaus R, Pearson BJ, Marusich A, Doe CQ (2005) Regulation of Temporal Identity Transitions in Drosophila Neuroblasts. *Dev Cell* **8**:193-202 https://doi.org/10.1016/j.devcel.2004.11.019

Hagey DW, Topcic D, Kee N, Reynaud F, Bergsland M, Perlmann T, Muhr J. (2020) CYCLIN-B1/2 and -D1 act in opposition to coordinate cortical progenitor self-renewal and lineage commitment. *Nat Commun* **11**:2898 https://doi.org/10.1038/s41467-020-16597-8

Hamid A, Gutierrez A, Munroe J, Syed MH (2023) The Drivers of Diversity: Integrated genetic and hormonal cues regulate neural diversity. *Semin Cell Dev Biol* **142**:23-35 https://doi.org/10.1016/j.semcdb.2022.07.007

Hartenstein V, Rudloff E, Campos-Ortega JA. (1987) The pattern of proliferation of the neuroblasts in the wild-type embryo of Drosophila melanogaster. *Rouxs Arch Dev Biol* **196**:473-485 https://doi.org/10.1007/BF00399871

Homem CCF, Steinmann V, Burkard TR, Jais A, Esterbauer H, Knoblich JA (2014) Ecdysone and Mediator Change Energy Metabolism to Terminate Proliferation in Drosophila Neural Stem Cells. *Cell* **158**:874-888 https://doi.org/10.1016/j.cell.2014.06.024

Isshiki T, Pearson B, Holbrook S, Doe CQ (2001) Drosophila Neuroblasts Sequentially Express Transcription Factors which Specify the Temporal Identity of Their Neuronal Progeny. *Cell* **106**:511-521 https://doi.org/10.1016/S0092-8674(01)004

Javed A, Santos-França PL, Mattar P, Cui A, Kassem F, Cayouette M. (2023) Ikaros family proteins redundantly regulate temporal patterning in the developing mouse retina. *Development* **150**:dev200436 https://doi.org/10.1242/dev.200436

Kawaguchi A. (2019) Temporal patterning of neocortical progenitor cells: How do they know the right time?. *Neurosci Res* **138**:3-11 https://doi.org/10.1016/j.neures.2018.09.004

Kohwi M, Doe CQ (2013) Temporal fate specification and neural progenitor competence during development. *Nat Rev Neurosci* **14**:823-838 https://doi.org/10.1038/nrn3618

Konstantinides N, Holguera I, Rossi AM, Escobar A, Dudragne L, Chen Y-C, Tran TN, Martínez Jaimes AM, Özel MN, Simon F, *et al.* (2022) A complete temporal transcription factor series in the fly visual system. *Nature* **604**:316-322 https://doi.org/10.1038/s41586-022-04564-w

Koo B, Lee K-H, Ming G, Yoon K-J, Song H. (2023) Setting the clock of neural progenitor cells during mammalian corticogenesis. *Semin Cell Dev Biol* **142**:43-53 https://doi.org/10.1016/j.semcdb.2022.05.013

Li X, Erclik T, Bertet C, Chen Z, Voutev R, Venkatesh S, Morante J, Celik A, Desplan C. (2013) Temporal patterning of Drosophila medulla neuroblasts controls neural fates. *Nature* **498**:456-462 https://doi.org/10.1038/nature12319

Malin J, Desplan C. (2021) Neural specification, targeting, and circuit formation during visual system assembly. *Proc Natl Acad Sci* **118**:e2101823118 https://doi.org/10.1073/pnas.2101823118

Maurange C, Cheng L, Gould AP (2008) Temporal Transcription Factors and Their Targets Schedule the End of Neural Proliferation in Drosophila. *Cell* **133**:891-902 https://doi.org/10.1016/j.cell.2008.03.034

McConnell SK, Kaznowski CE (1991) Cell Cycle Dependence of Laminar Determination in Developing Neocortex. *Science* **254**:282-285 https://doi.org/10.1126/science.1925583

Oberst P, Agirman G, Jabaudon D. (2019) Principles of progenitor temporal patterning in the developing invertebrate and vertebrate nervous system. *Curr Opin Neurobiol* **56**:185-193 https://doi.org/10.1016/j.conb.2019.03.004

Ohnuma S, Harris WA (2003) Neurogenesis and the Cell Cycle. *Neuron* **40**:199-208 https://doi.org/10.1016/S0896-6273(03)00632-9

Ohtsuka T, Kageyama R. (2019) Regulation of temporal properties of neural stem cells and transition timing of neurogenesis and gliogenesis during mammalian neocortical development. *Semin Cell Dev Biol* **95**:4-11 https://doi.org/10.1016/j.semcdb.2019.01.007

Okamoto M, Miyata T, Konno D, Ueda HR, Kasukawa T, Hashimoto M, Matsuzaki F, Kawaguchi A. (2016) Cell-cycle-independent transitions in temporal identity of mammalian neural progenitor cells. *Nat Commun* **7**:11349 https://doi.org/10.1038/ncomms11349

Pebworth M-P, Ross J, Andrews M, Bhaduri A, Kriegstein AR (2021) Human intermediate progenitor diversity during cortical development. *Proc Natl Acad Sci* **118**:e2019415118 https://doi.org/10.1073/pnas.2019415118

Pahl MC, Doyle SE, Siegrist SE (2019) E93 Integrates Neuroblast Intrinsic State with Developmental Time to Terminate MB Neurogenesis via Autophagy. *Curr Biol* **29**:750-762.e3 https://doi.org/10.1016/j.cub.2019.01.039

Ray A, Li X. (2022) A Notch-dependent transcriptional mechanism controls expression of temporal patterning factors in Drosophila medulla. *eLife* **11**:e75879 https://doi.org/10.7554/eLife.75879

Ren Q, Awasaki T, Wang Y-C, Huang Y-F, Lee T. (2018) Lineage-guided Notch-dependent gliogenesis by Drosophila multi-potent progenitors. *Development* dev.160127 https://doi.org/10.1242/dev.160127

Ren Q, Yang C-P, Liu Z, Sugino K, Mok K, He Y, Ito M, Nern A, Otsuna H, Lee T. (2017) Stem Cell-Intrinsic, Seven-up-Triggered Temporal Factor Gradients Diversify Intermediate Neural Progenitors. *Curr Biol* **27**:1303-1313 https://doi.org/10.1016/j.cub.2017.03.047

Rossi AM, Desplan C. (2017) Asymmetric Notch Amplification to Secure Stem Cell Identity. *Dev Cell* **40**:513-514 https://doi.org/10.1016/j.devcel.2017.03.010

Salomoni P, Calegari F. (2010) Cell cycle control of mammalian neural stem cells: putting a speed limit on G1. *Trends Cell Biol* **20**:233-243 https://doi.org/10.1016/j.tcb.2010.01.006

Sen SQ (2023) Generating neural diversity through spatial and temporal patterning. *Semin Cell Dev Biol* **142**:54-66 https://doi.org/10.1016/j.semcdb.2022.06.002

Skeath JB, Thor S. (2003) Genetic control of Drosophila nerve cord development. *Curr Opin Neurobiol* **13**:8-15 https://doi.org/10.1016/S09594388(03)00007-2

Sood C, Nahid MA, Branham KR, Pahl M, Doyle SE, Siegrist SE (2024) Delta-dependent Notch activation closes the early neuroblast temporal program to promote lineage progression and neurogenesis termination in Drosophila. *eLife* **12**:RP88565 https://doi.org/10.7554/eLife.88565.3

Syed MH, Mark B, Doe CQ (2017) Steroid hormone induction of temporal gene expression in Drosophila brain neuroblasts generates neuronal and glial diversity. *eLife* **6**:e26287 https://doi.org/10.7554/eLife.26287

Telley L, Agirman G, Prados J, Amberg N, Fièvre S, Oberst P, Bartolini G, Vitali I, Cadilhac C, Hippenmeyer S, *et al.* (2019) Temporal patterning of apical progenitors and their daughter neurons in the developing neocortex. *Science* **364**:eaav2522 https://doi.org/10.1126/science.aav2522

van den Ameele J, Brand AH (2019) Neural stem cell temporal patterning and brain tumour growth rely on oxidative phosphorylation. *eLife* **8**:e47887 https://doi.org/10.7554/eLife.47887

Walsh KT, Doe CQ (2017) Drosophila embryonic type II neuroblasts: origin, temporal patterning, and contribution to the adult central complex. *Development* dev.157826 https://doi.org/10.1242/dev.157826

Wang Y-C, Yang JS, Johnston R, Ren Q, Lee Y-J, Luan H, Brody T, Odenwald WF, Lee T. (2014) Drosophila intermediate neural progenitors produce lineage-dependent related series of diverse neurons. *Development* **141**:253-258 https://doi.org/10.1242/dev.103069

Wani AR, Chowdhury B, Luong J, Chaya GM, Patel K, Isaacman-Beck J, Shafer O, Kayser MS, Syed MH (2023) Stem cell-specific ecdysone signaling regulates the development and function of a Drosophila sleep homeostat. *bioRxiv* https://doi.org/10.1101/2023.09.29.560022

Yang C-P, Fu C-C, Sugino K, Liu Z, Ren Q, Liu L-Y, Yao X, Lee LP, Lee T. (2015) Transcriptomes of lineage-specific Drosophila neuroblasts profiled via genetic targeting and robotic sorting. *Development* dev.129163 https://doi.org/10.1242/dev.129163

Peer reviews

Reviewer #1 (Public review):

Summary:

Drosophila larval type II neuroblasts generate diverse types of neurons by sequentially expressing different temporal identity genes during development. Previous studies have shown that transition from early temporal identity genes (such as Chinmo and Imp) to late temporal identity genes (such as Syp and Broad) depends on the activation of the expression of EcR by Seven-up (Svp) and progression through the G1/S transition of the cell cycle. In this study, Chaya and Syed examined if the expression of Syp and EcR is regulated by cell cycle and cytokinesis by knocking down CDK1 or Pav, respectively, throughout development or at specific developmental stages. They find that knocking down CDK1 or Pav either in all type II neuroblasts throughout the development or in single type neuroblast clones after larval hatching consistently leads to failure to activate late temporal identity genes Syp and EcR. To determine whether the failure of the activation of Syp and EcR is due to impaired Svp expression, they also examined Svp expression using a Svp-lacZ reporter line. They find that Svp is expressed normally in CDK1 RNAi neuroblasts. Further, knocking down CDK1 or Pav after Svp activation still leads to loss of Syp and EcR expression. Finally, they also extended their analysis to type I neuroblasts. They find that knocking down CDK1 or Pav, either at 0 hours or at 42 hours after larval hatching, also results in loss of Syp and EcR expression in type I neuroblasts. Based on these findings, the authors conclude that cycle and cytokinesis are required for the transition from early to late late temporal identity genes in both types of neuroblasts. These findings add mechanistic details to our understanding of the temporal patterning of Drosophila larval neuroblasts.

Strengths:

The data presented in the paper are solid and largely support their conclusion. Images are of high quality. The manuscript is well-written and clear.

Weaknesses:

The authors have addressed all the weaknesses in this revision.

https://doi.org/10.7554/eLife.108259.2.sa3

Reviewer #2 (Public review):

Summary:

Neural stem cells produce a wide variety of neurons during development. The regulatory mechanisms of neural diversity are based on the spatial and temporal patterning of neural stem cells. Although the molecular basis of spatial patterning is well-understood, the temporal patterning mechanism remains unclear. In this manuscript, the authors focused on the roles of cell cycle progression and cytokinesis in temporal patterning and found that both are involved in this process.

Strengths:

They conducted RNAi-mediated disruption on cell cycle progression and cytokinesis. As they expected, both disruptions affected temporal patterning in NSCs.

Weaknesses:

Although the authors showed clear results, they needed to provide additional data to support their conclusion sufficiently.

For example, they can examine the effects of cell cycle acceleration on the temporal patterning.

https://doi.org/10.7554/eLife.108259.2.sa2

Reviewer #3 (Public review):

Summary:

The manuscript by Chaya and Syed focuses on understanding the link between cell cycle and temporal patterning in central brain type II neural stem cells (NSCs). To investigate this, the authors perturb the progression of the cell cycle by delaying the entry into M phase and preventing cytokinesis. Their results convincingly show that temporal factor expression requires progression of the cell cycle in both Type 1 and Type 2 NSCs in the Drosophila central brain. Overall, this study establishes an important link between the two timing mechanisms of neurogenesis.

Strengths:

The authors provide solid experimental evidence for the coupling of cell cycle and temporal factor progression in Type 2 NSCs. The quantified phenotype shows an all-or-none effect of cell cycle block on the emergence of subsequent temporal factors in the NSCs, strongly suggesting that both nuclear division and cytokinesis are required for temporal progression. The authors also extend this phenotype to Type 1 NSCs in the central brain, providing a generalizable characterization of the relationship between cell cycle and temporal patterning.

Weaknesses:

One major weakness of the study is that the authors do not explore the mechanistic relationship between cell cycle and temporal factor expression. Although their results are quite convincing, they do not provide an explanation as to why Cdk1 depletion affects Syp and EcR expression but not the onset of svp. This result suggests that at least a part of the temporal cascade in NSCs is cell-cycle independent which isn't addressed or sufficiently discussed.

https://doi.org/10.7554/eLife.108259.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *Drosophila larval type II neuroblasts generate diverse types of neurons by sequentially expressing different temporal identity genes during development. Previous studies have shown that the transition from early temporal identity genes (such as Chinmo and Imp) to late temporal identity genes (such as Syp and Broad) depends on the activation of the expression of EcR by Seven-up (Svp) and progression through the G1/S transition of the cell cycle. In this study, Chaya and Syed examined whether the expression of Syp and EcR*

> *is regulated by cell cycle and cytokinesis by knocking down CDK1 or Pav, respectively, throughout development or at specific developmental stages. They find that knocking down CDK1 or Pav either in all type II neuroblasts throughout development or in single-type neuroblast clones after larval hatching consistently leads to failure to activate late temporal identity genes Syp and EcR. To determine whether the failure of the activation of Syp and EcR is due to impaired Svp expression, they also examined Svp expression using a Svp-lacZ reporter line. They find that Svp is expressed normally in CDK1 RNAi neuroblasts. Further, knocking down CDK1 or Pav after Svp activation still leads to loss of Syp and EcR expression. Finally, they also extended their analysis to type I neuroblasts. They find that knocking down CDK1 or Pav, either at 0 hours or at 42 hours after larval hatching, also results in loss of Syp and EcR expression in type I neuroblasts. Based on these findings, the authors conclude that cycle and cytokinesis are required for the transition from early to late temporal identity genes in both types of neuroblasts. These findings add mechanistic details to our understanding of the temporal patterning of Drosophila larval neuroblasts.*
>
> *Strengths:*
>
> *The data presented in the paper are solid and largely support their conclusion. Images are of high quality. The manuscript is well-written and clear.*

We appreciate the reviewer's detailed summary and recognition of the study's strengths.

> *Weaknesses:*
>
> *The quantifications of the expression of temporal identity genes and the interpretation of some of the data could be more rigorous.*
>
> *(1) Expression of temporal identity genes may not be just positive or negative. Therefore, it would be more rigorous to quantify the expression of Imp, Syp, and EcR based on the staining intensity rather than simply counting the number of neuroblasts that are positive for these genes, which can be very subjective. Or the authors should define clearly what qualifies as "positive" (e.g., a staining intensity at least 2x background).*

We thank the reviewer for this helpful suggestion. In the new version, we have now clarified how positive expression was defined and added more details of our quantification strategy to the Methods section (page 11, lines 380-388; lines 426-434 in tracked changes file). Fluorescence intensity for each neuroblast was normalized to the mean intensity of neighboring wild-type neuroblasts imaged in the same field. A neuroblast was considered positive for a given factor when its normalized nuclear intensity was at least 2× the local background. This scoring criterion was applied uniformly across all genotypes and time points. All quantifications were performed on the raw LSM files in Fiji prior to assembling the figure panels.

> *(2) The finding that inhibiting cytokinesis without affecting nuclear divisions by knocking down Pav leads to the loss of expression of Syp and EcR does not support their conclusion that nuclear division is also essential for the early-late gene expression switch in type II NSCs (at the bottom of the left column on page 5). No experiments were done to specifically block the nuclear division in this study specifically. This conclusion should be revised.*

We blocked both cell cycle progression and cytokinesis, and both these manipulations affected temporal gene transitions, suggesting that both cell cycle and cytokinesis are essential. To our knowledge, no mechanism/tool exists that selectively blocks nuclear division while leaving cell cycle progression intact. We have added more clarification on page 4, line 123 onwards (lines 126 onwards in tracked changes file).

> *(3) Knocking down CDK1 in single random neuroblast clones does not make the CDK1 knockdown neuroblast develop in the same environment (except still in the same brain) as wild-type neuroblast lineages. It does not help address the concern whether "type 2 NSCS with cell cycle arrest failed to undergo normal temporal progression is indirectly due to a lack of feedback signaling from their progeny", as discussed (from the bottom of the right column on page 9 to the top of the left column on page 10). The CDK1 knockdown neuroblasts do not divide to produce progeny and thus do not receive a feedback signal from their progeny as wild-type neuroblasts do. Therefore, it cannot be ruled out that the loss of Syp and EcR expression in CDK1 knockdown neuroblasts is due to the lack of the feedback signal from their progeny. This part of the discussion needs to be clarification.*

Thanks to the reviewer for raising this critical point. We agree and have added more clarification of our interpretations and limitations to our studies in the revised text on page 8, line 278-282 (lines 296-300 in tracked changes file)

> *(4) In Figure 2I, there is a clear EcR staining signal in the clone, which contradicts the quantification data in Figure 2J that EcR is absent in Pav RNAi neuroblasts. The authors should verify that the image and quantification data are consistent and correct.*

When cytokinesis is blocked using pav-RNAi, the neuroblasts become extremely large and multinucleated. In some large pav RNAi clones, we observed a weak EcR signal near the cell membrane. However, more importantly, none of the nuclear compartments showed detectable EcR staining, where EcR is typically localized. We selected a representative nuclear image for the figure panel. To clarify this observation, we have now added an explanatory note to the discussion section on page 8, lines 283-291 (lines 301-309 in tracked changes file).

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Neural stem cells produce a wide variety of neurons during development. The regulatory mechanisms of neural diversity are based on the spatial and temporal patterning of neural stem cells. Although the molecular basis of spatial patterning is well-understood, the temporal patterning mechanism remains unclear. In this manuscript, the authors focused on the roles of cell cycle progression and cytokinesis in temporal patterning and found that both are involved in this process.*
>
> *Strengths:*
>
> *They conducted RNAi-mediated disruption on cell cycle progression and cytokinesis. As they expected, both disruptions affected temporal patterning in NSCs.*

We appreciate the reviewer's positive assessment of our experimental results.

> *Weaknesses:*
>
> *Although the authors showed clear results, they needed to provide additional data to support their conclusion sufficiently.*
>
> *For example, they need to identify type II NSCs using molecular markers (Ase/Dpn).The authors are encouraged to provide a more detailed explanation of each experiment. The current version of the manuscript is difficult for non-expert readers to understand.*

Thanks for your feedback. We have now included a detailed description of how we identify type II NSCs in both wild-type and mutant clones. We have also added a representative Asense staining to clearly distinguish type 1 (Ase^{+}) from type 2 (Ase^{-}) NSCs see Figure S1. We

have also added a resources table explaining the genotypes associated with each figure, which was omitted due to an error in the previous version of the manuscript.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *The manuscript by Chaya and Syed focuses on understanding the link between cell cycle and temporal patterning in central brain type II neural stem cells (NSCs). To investigate this, the authors perturb the progression of the cell cycle by delaying the entry into M phase and preventing cytokinesis. Their results convincingly show that temporal factor expression requires progression of the cell cycle in both Type 1 and Type 2 NSCs in the Drosophila central brain. Overall, this study establishes an important link between the two timing mechanisms of neurogenesis.*
>
> *Strengths:*
>
> *The authors provide solid experimental evidence for the coupling of cell cycle and temporal factor progression in Type 2 NSCs. The quantified phenotype shows an all-ornone effect of cell cycle block on the emergence of subsequent temporal factors in the NSCs, strongly suggesting that both nuclear division and cytokinesis are required for temporal progression. The authors also extend this phenotype to Type 1 NSCs in the central brain, providing a generalizable characterization of the relationship between cell cycle and temporal patterning.*

We thank the reviewer for recognizing the robustness of our data linking the cell cycle to temporal progression.

> *Weaknesses:*
>
> *One major weakness of the study is that the authors do not explore the mechanistic relationship between the cell cycle and temporal factor expression. Although their results are quite convincing, they do not provide an explanation as to why Cdk1 depletion affects Syp and EcR expression but not the onset of svp. This result suggests that at least a part of the temporal cascade in NSCs is cell-cycle independent, which isn't addressed or sufficiently discussed.*

Thank you for bringing up this important point. We are equally interested in uncovering the mechanism by which the cell cycle regulates temporal gene transitions; however, such mechanistic exploration is beyond the scope of the present study. Interestingly, while the temporal switching factor Svp is expressed independently of the cell cycle, the subsequent temporal transitions are not. We have expanded our discussion on this intriguing finding (page 9, line 307-315; lines 345-355 in tracked changes file). Specifically, we propose that svp activation marks a cell-cycle–independent phase, whereas EcR/Syp induction likely depends on cell-cycle–coupled mechanisms, such as mitosis-dependent chromatin remodeling or daughter-cell feedback. Although further dissection of this mechanism lies beyond the current study, our findings establish a foundation for future work aimed at identifying how developmental timekeeping is molecularly coupled to cell-cycle progression.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) Figure 1 C and D, it would be better to put a question mark to indicate that these are hypotheses to be tested.*

We appreciate this suggestion and have added question marks in Figure 1C and 1D to clearly indicate that these panels represent hypotheses under investigation clearly.

> *(2) Figure 2A-I, Figure 4A-I, Figure 5A-I and K-S, in addition to enlarged views of single type II neuroblasts, it would be more convincing to include zoomed-out images of the entire larval brain or at least a portion of the brain to include neighboring wild-type type II neuroblasts as internal controls. Also, it would be ideal to show EcR staining from the same neuroblasts as IMP and Syp staining.*

We thank the reviewer for this valuable input. In our imaging setup, the number of available antibody channels was limited to four (anti-Ase, anti-GFP, anti-Syp, and antiImp). Adding EcR in the same sample was therefore not technically possible, we performed EcR staining separately.

> *(3) The authors cited "Syed et al., 2024" (in the middle of the right column on page 5), but this reference is missing in the "References" section and should be added.*

The missing citation has been added to the reference section.

> *(4) It would be better to include Ase staining in the relevant figure to indicate neuroblast identity as type I or type II.*

We agree and now include representative Ase staining for both type 1 and type 2 NSC clones in Figure S1, along with corresponding text updates that describe these markers.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *Major comments*
>
> *(1) The present conclusion relies on the results using Cdk1 RNAi and pav RNAi. It is still possible that Cdk1 and Pav are involved in the regulation of temporal patterning independent of the regulation of cell cycle or cytokinesis, respectively. To avoid this possibility, the authors need to inhibit cell cycle progression or cytokinesis in another alternative manner.*

We thank the reviewer for raising this important point. While we cannot completely exclude gene-specific, cell-cycle-independent roles for Cdk1 or Pav, we observe consistent phenotypes across several independent manipulations that slow or block the cell cycle. Also, earlier studies using orthogonal approaches that delay G1/S (Dacapo/Rbf) or impair mitochondrial OxPhos (which lengthens G1/S; van den Ameele & Brand, 2019) produce similar temporal delays. These concordant phenotypes strongly support the interpretation that altered cell-cycle progression—rather than specific roles of a single gene—is the primary cause of the defect. While we cannot exclude additional, gene-specific effects of Cdk1 or Pav, the concordant phenotypes across independent perturbations make the cell-cycle disruption model the most parsimonious interpretation. We have clarified this reasoning in the discussion section on pages 8-9, lines 293-305 (lines 311-343 in tracked changes file).

> *(2) To reach the present conclusion, the authors need to address the effects of acceleration of cell cycle progression or cytokinesis on temporal patterning.*

We thank the reviewer for this insightful suggestion. To our knowledge, there are currently no established genetic tools that can specifically accelerate cell-cycle progression in Drosophila neuroblasts. However, our results demonstrate that blocking the cell cycle impairs the transition from early to late temporal gene expression. These findings suggest that proper cell-cycle progression is essential for the transition from early to late temporal identity in neuroblasts.

> *Minor comments*
>
> *(3) P3L2 (right), ... we blocked the NSC cell cycle...*

> *How did they do it?*
>
> *Which fly lines were used?*
>
> *Why did they use the line?*

These details are now included in the Materials and Methods and the Resource Table (pages 11-13). We used Wor-Gal4, Ase-Gal80 to drive UAS-Cdk1RNAi and UASpavRNAi in type 2 NSCs

> *(4) P5L1(left), ... we used the flip-out approach...*
>
> *Why did they conduct it?*
>
> *Probably, the authors have reasons other than "to further ensure."*

We have clarified in the text on page 4, lines 137-139, that the flip-out approach was used to generate random single-cell clones, enabling quantitative analysis of type 2 NSCs within an otherwise wild-type brain.

> *(5) P5L8(left), ... type 2 hits were confirmed by lack of the type 1 Asense... The authors must examine Deadpan (Dpn) expression as well. Because there are a lot of Asense (Ase) negative cells in the brain (neurons, glial cell, and neuroepithelial cells).*
>
> *Type II NSCs can be identified as Dpn+/Ase- cells.*

We agree that Dpn is a helpful marker. However, we reliably distinguished type II NSCs by their lack of Ase and larger cell size relative to surrounding neurons and glia, which are smaller in size and located deeper within the clone. These differences, together with established lineage patterns, allow unambiguous identification of type 2 NSCs across all genotypes. We have now added representative type I and type 2 NSC clones to the supplemental figure S1 (E-G') with Asense stains to demonstrate how we differentiate type I from type II NSCs.

> *(6) P5L32(left), To do this, we induced...*
>
> *This sentence should be made more concise.*
>
> *Please rephrase it.*

The sentence has been rewritten for clarity and concision.

> *(7) P5L42(left), ...lack of EcR/Syp expression (Figure 2). However, EcR expression is still present (Figure 2I).*

In some large pavRNAi clones, a weak EcR signal can be observed near the cell membrane; however, none of the nuclear compartments—where EcR is typically localized—show detectable staining. We selected a representative nuclear image for the figure and addressed this observation on page 8, lines 283-291 (lines 301-309 in tracked changes file).

> *(8) P7L29(left), ...had persistent Imp expression...*
>
> *Imp expression is faint compared to that in Figure 2G.*
>
> *The differences between Figures 2G and 3G should be discussed.*

We thank the reviewer for this comment. We have added a note in the Methods section clarifying that brightness and contrast were adjusted per panel for optimal visualization; thus, apparent differences in signal intensity do not reflect biological variation. Fluorescence intensity for each neuroblast was normalized to the mean intensity of neighboring wild-type

neuroblasts imaged in the same field. A neuroblast was considered Imp-positive when its normalized nuclear intensity was at least 2× the local background. This scoring criterion was applied uniformly across all genotypes and time points. All quantifications were performed on the raw LSM files in Fiji prior to assembling the figure panels.

> *(9) P8 (Figure 5)*
>
> *The Imp expression is faint compared to that in Figure 5Q.*
>
> *The difference between Figure 5G and 5Q should be discussed further.*

As mentioned above, we have clarified our image processing approach in the Methods section to explain any differences in signal appearance between these figures.

> *(10) P10 Materials and Methods*
>
> *The authors did not mention the fly lines used. This is very important for the readers.*

We thank the reviewer for bringing this oversight to our attention. The Resource Table was inadvertently omitted from the initial submission. The complete list of fly lines and reagents used in this study is now provided in the updated Resource Table.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *Major points*
>
> *(1) The authors mention that the heat-shock induction at 42ALH is well after svp temporal window and therefore the cell cycle block independently affects Syp and EcR expression. However, Figure 3 shows svp-LacZ expression at 48ALH. If svp expression is indeed transient in Type 2 NSCs, then this must be validated using an immunostaining of the svp-LacZ line with svp antibody. This is crucial as the authors claim that cell cycle block doesn't affect does affect svp expression and is required independently.*

We thank the reviewer for bringing this important issue to our attention. As noted, Svp protein is expressed transiently and stochastically in type 2 NSCs (Syed et al., 2017), making direct antibody quantification challenging upon cell cycle block. Consistent with previous work (Syed et al., 2017), we used the svp-LacZ reporter line to visualize stabilized Svp expression, which reliably captures Svp expression in type 2 NSCs (Syed et al., 2017 *https://doi.org/10.7554/eLife.26287*, and Dhilon et al., 2024 *https://doi.org/10.1242/dev.202504*).

> *(2) The authors have successfully slowed down the cell cycle and showed that it affects temporal progression. However, a converse experiment where the cell cycle is sped up in NSCs would be an important test for the direct coupling of temporal factor expression and cell cycle, wherein the expectation would be the precocious expression of late temporal factors in faster cycle NSCs.*

We agree that such an experiment would be ideal. However, as noted above (Reviewer #2 comment 2), to our knowledge, no suitable tools currently exist to accelerate neuroblast cell-cycle progression without pleiotropic effects.

> *Minor point*
>
> *The authors must include Ray and Li (https://doi.org/10.7554/eLife.75879) in the references when describing that "...cell cycle has been shown to influence temporal patterning in some systems,...".*

We thank the reviewer for this helpful suggestion. The cited reference (Ray and Li, eLife, 2022) has now been included and appropriately referenced in the revised manuscript.

https://doi.org/10.7554/eLife.108259.2.sa0