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seven-up Controls Switching of Transcription Factors that Specify Temporal Identities of *Drosophila* Neuroblasts

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Summary

Drosophila **neuronal stem cell neuroblasts (NB) constantly change character upon division, to produce a different type of progeny at the next division. Transcription factors Hunchback (HB), Krüppel (KR), Pdm (PDM), etc. are expressed sequentially in each NB and act as determinants of birth-order identity. How a NB switches its expression profile from one transcription factor to the next is poorly understood. We show that the HB-to-KR switch is directed by the nuclear receptor Seven-up (SVP). SVP expression is confined to a temporally restricted subsection within the NB's lineage. Loss of SVP function causes an increase in the number of HB-positive cells within several NB lineages, whereas misexpression of** ***svp*** **leads to the loss of these early-born neurons. Lineage analysis provides evidence that** ***svp*** **is required to switch off HB at the proper time. Thus,** ***svp*** **modifies the self-renewal stem cell program to allow chronological change of cell fates, thereby generating neuronal diversity.**

Introduction

Multipotent precursor cells often generate cellular diversity of their progeny according to a constant temporal program. For example, in vertebrate cortical development, the construction of the well-defined "inside-out" pattern of the layer structure correlates with the birth order of each neuron from its progenitor (McConnell, 1995). In the insect central nervous system, temporal information plays an important role in the generation of neuronal diversity (Skeath and Thor, 2003). Neurons are born from the sequential division of the neural stem cells called neuroblasts (NB). A NB divides asymmetrically to regenerate itself, while producing a differentiating daughter cell called a ganglion mother cell (GMC), which then divides symmetrically to produce two postmitotic neurons. Ablation experiments in grasshopper demonstrated that the fate of each GMC is determined by the birth order from the parental NB (Doe and Goodman, 1985). Thus, a NB must constantly change its properties to generate different progeny upon each division.

A likely mechanism that regulates cell determination during cell lineage development is a genetic switch involving transcription factors that control temporal identity in response to lineage progression. In *Drosophila*, several factors required for temporal cell fate determination are identified. NBs change their character by sequentially expressing transcription factors such as HB, KR, PDM, and Castor (CAS) (Cui and Doe, 1992; Isshiki et al., 2001; Kambadur et al., 1998; Mellerick et al., 1992; Novotny et al., 2002), and HB and KR are necessary and sufficient for specifying early-born cell fates (Isshiki et al., 2001; Novotny et al., 2002). The expression profiles of these transcription factors are maintained in the progeny from NBs and thus can serve as a birth-date marker of differentiated neurons (Isshiki et al., 2001). Genetic manipulations that disrupt the expression profile of these transcription factors cause the reduction of neuronal cell identities within the lineage (Brody and Odenwald, 2000; Isshiki et al., 2001; Kambadur et al., 1998; Novotny et al., 2002). Therefore, in order to generate proper neuronal diversity, it is essential that NBs switch their expression pattern of HB, KR, etc. within the NB lineage. Although regulatory interactions exist among these transcription factors, inactivating these transcription factors does not prevent the appearance of factors specifying the later part of the lineage (Brody and Odenwald, 2000; Isshiki et al., 2001; Kambadur et al., 1998). This suggests that factors other than HB, KR, etc. are involved in specifying the timing of the switch and turning on the late lineage program (Isshiki et al., 2001).

A good candidate which provides temporal information for NB switching is SVP, a transcription factor belonging to the COUP-TF subgroup of the nuclear receptor superfamily. Many members of this family, including SVP, are "orphan receptors" with no known ligand and are expected to act in a ligand-independent fashion (Wang et al., 2003). The *svp* gene was originally identified as a genetic switch between two neuronal subtypes in the *Drosophila* compound eye through its specific expression in a photoreceptor subtype (Mlodzik et al., 1990). In the embryonic CNS, *svp* is also expressed in a restricted pattern during neurogenesis. An analysis using an enhancer trap strain of *svp* revealed that, although most of the NBs will turn on *svp*, its expression within the NB lineage is limited to a subsection of each NB lineage; for example, NB7-1, which forms at late stage 8, starts *svp* expression only at stage 9, and NB3-1

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does not express *svp* until long after its formation at early stage 10 (Doe, 1992). This temporally restricted expression of *svp* in the developmental history of NBs raises the possibility that SVP could provide temporal information within the NB lineage and may be involved in the switching the expression of HB, KR, and so on.

Here, we demonstrate that *svp* has an essential function for proper switching of transcription factors within the NB and the generation of neuronal diversity in *Drosophila* CNS development. Loss of function of *svp* increased the number of cells of the early-born cell type within several NB lineages and decreased the number of the late-born cell types. Lineage analysis provided evidence that, in *svp* mutant embryo, NBs fail to terminate HB expression with proper timing, causing duplication of progeny with early-born identity. These results suggest that SVP is a fundamental regulator for NB switching and provide important insights about how cellular diversity is acquired during development.

Results

SVP Is Expressed Transiently within Several NB Lineages

Previous studies on *svp* expression in the CNS were done using an enhancer trap strain (Doe, 1992) and had limitations in the temporal resolution owing to the stability of the β-galactosidase protein. We thus generated an antibody that recognizes SVP protein and used it to examine the SVP expression profile in the embryo. In addition to the CNS, SVP was expressed in several tissues, such as dorsal vessel, oenocyte, and fat body (Supplemental Figure S1A), as previously reported, using an enhancer trap strain or in situ hybridization (Doe, 1992; Elstob et al., 2001; Hoshizaki et al., 1994; Kerber et al., 1998; Lo and Frasch, 2001; Mlodzik et al., 1990; Sen et al., 2003; Urbach et al., 2003; Urbach and Technau, 2003). Expression of SVP in these tissues was stable and could be observed until stage 16, the latest stage examined. In contrast, the expression profile in the CNS was extremely dynamic. For example, at stage 11, SVP was expressed in NB2-4 but not in NB7-3, which was just after its formation (Figures 1A–1D). After NB7-3 had divided, SVP was expressed in NB7-3 and the GMC that it had generated but was no longer detectable in NB2-4 (Figures 1E–1H). Thus, the expression of SVP is confined to temporally restricted subsections of the NB lineage. While SVP was expressed in many NB and GMCs, only a small number of neurons were SVP positive (data not shown). This indicates that, unlike HB and KR, the expression profile of SVP in the NBs is not maintained in their neuronal progeny.

seven-up Mutants Overproduce Early-Born Neurons

To study the defect in the *svp* mutant CNS, we used markers that are expressed in a small subset of CNS neurons derived from identified NBs. The *Lim-3A-TauMyc* reporter is expressed in three RP motoneurons that are generated from NB3-1 (Schmid et al., 1999; Thor et al., 1999). While normal embryos contain three Lim-3A-positive cells in each hemisegment, the *svp* mutant embryo had 3–7 reporter-positive cells per hemisegment (average = 4.3, n = 126) (Figures 1I–1L). All of the *Lim-3A-TauMyc*-positive cells in the *svp* mutant still sent their axons toward the periphery, suggesting that they possessed their correct identity as motoneurons. We also examined another NB lineage, NB7-1, which generates five U neurons that can be marked by the expression of Even-skipped (EVE) (Bossing et al., 1996; Broadus et al., 1995). In the *svp* mutant, the average number of EVE-positive U neurons was increased to 8.4 (n = 378) (Figures 1M–1P; Supplemental Table S1). Thus, *svp* mutation causes an increase in the number of specific neurons produced in multiple NB lineages.

To analyze how the loss of *svp* function alters the number of specific neuronal progeny, we chose NB7-1 because the birth order of the U neurons is known, and U neurons can be identified individually by their gene expression patterns (Isshiki et al., 2001). U1 and U2, the first two U neurons born from NB7-1, express both HB and KR. U2 also expresses ZFH-2 and thus can be distinguished from U1. U3 is double positive for KR and ZFH-2, while U4 and U5 are singly positive for ZFH-2 and CAS, respectively, and also express Runt (Figure 2A). The *svp* mutant hemisegment contained 6.7 EVE-positive U neurons that coexpressed HB and KR (n = 120), whereas only two, U1 and U2, were double positive in normal embryos (Figure 2B; Supplemental Table S1). Furthermore, in the *svp* mutant, there were 6.2 neurons (n = 132) that were HB positive and ZFH-2 negative, i.e., the gene expression pattern characteristic of the first-born U1 neuron. We thus conclude that the *svp* mutant overproduces U1, the neuron possessing the character of the early-born identity.

In contrast, the number of late-born U neurons such as U4 and U5 was dramatically reduced in the *svp* mutant CNS. The *svp* mutant hemisegment contained only 1.1 (n = 48) EVE-positive U neurons that expressed Runt, as opposed to the normal number of 2 (n = 24), and only 0.5 (n = 24) CAS-positive U neurons instead of 1 (Figure 2B; Supplemental Table S1). These results suggest that, in the *svp* mutant, there is an expansion of the early phase of the NB7-1 lineage, accompanied by a truncation of the lineage.

Since neither SVP protein nor *svp* mRNA was detectable in U neurons themselves (data not shown), *svp* likely acts in precursor cells that generate U neurons, rather than in postmitotic U neurons. For example, an increase in the number of U1 neurons may be due to an overproliferation of GMC-1, which normally produces a single U1 neuron, or due to cell fate changes of GMCs that normally produce late-born U neurons toward the GMC-1 fate. To address whether *svp* expression within the NB7-1 lineage correlates with its requirement, we used an enhancer trap strain of *svp* that expresses β-galactosidase. Because β-galactosidase is stable for many hours within a cell, neuronal progeny from a NB that expresses the *svp-lacZ* reporter is expected to inherit and retain the β-galactosidase protein. Among the five EVE-positive U neurons, β-galactosidase was expressed strongly in U2 through U5 but was undetectable in U1 (Figure 2D). This suggests that SVP is not yet expressed when the NB divides to produce GMC-1 that generates U1, and thus the overproduction of U1 neurons in the *svp* mutant is not due to the abnormal beha-

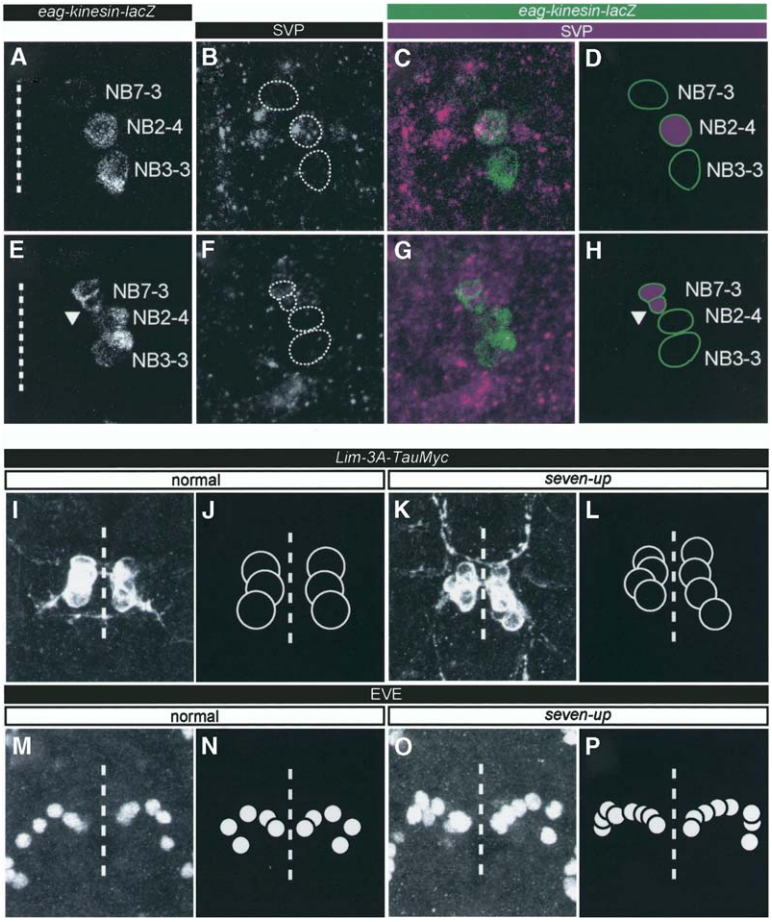

Figure 1. Dynamic Expression of SVP Is Required for Proper CNS Development

(A–H) SVP (B, C, F, and G; magenta) expression in the NB layler, shown with *eagle-kinesin-lacZ* (A, C, E, and G; green), used as a marker for three NB lineages shown in dotted circles in (B) and (F). NB7-3 has not divided at early stage 11 (A–D), but has given rise to a GMC-1 (arrowhead) at late stage 11 (E–H). SVP expression is dynamic; between these two time points NB7-3 turned on SVP expression, whereas NB2-4 turned it off. Other NBs also express SVP, which contributes to staining outside the region outside dotted circles.

(I–L) RP neurons generated from NB3-1 labeled by *Lim-3A-tauMyc* reporter gene in normal (I and J) and $svp^{e22/e300}$embryo (K and L).

(M–P) U neurons generated from NB7-1 labeled by EVE expression gene in normal (M and N) and $svp^{e22/e300}$ embryo (O and P). The number of specific neurons labeled are increased in *svp* mutant. (D), (H), (J), (L), (N), and (P) are tracings of (C), (G), (I), (K), (M), and (O), respectively. Dotted line indicates the midline.

vior of the precursor cells of the U1 neuron. It is more likely that the absence of *svp* function altered the fate of *svp*-positive precursor cells that normally generate U2–U5 neurons, resulting in the overproduction of U1.

SVP Is Required for the Generation of the GMC-2 Fate in the NB7-3 Lineage

To address whether the *svp* mutant affects birth-order identity in other NB lineages, we examined the NB7-3 lineage, in which all cells generated from the NB can be labeled with lineage marker Eagle (Lundell and Hirsh, 1998) or *eagle-kinesin-lacZ* (Higashijima et al., 1996). In abdominal segments, the NB7-3 lineage is composed of four neurons: EW1, which expresses HB and KR; GW, which expresses HB, KR, and ZFH-1; EW2, which expresses KR and ZFH-2; and EW3, which expresses only ZFH-2 (Isshiki et al., 2001) (Figure 3A). The first GMC from this lineage generates EW1 and GW, the second GMC (GMC-2) gives rise to EW2 and a cell that undergoes cell death, and GMC-3 produces EW3 (Isshiki et al., 2001; Lundell and Hirsh, 1998; Novotny et al., 2002). In the *svp* mutant, the total number of neurons generated from NB7-3 was 4.2 (n = 182), only slightly increased from the normal number of 4. Among these cells, 3 cells expressed HB instead of the normal number of 2, and 3.7 instead of 3 were positive for KR (Figure 3B; Supplemental Table S2). All mutant lineages contained three cells that were double positive for HB and KR, a property shared by two neurons that are generated from GMC-1. While one of these cells (GW) is expected to express ZFH-1, there were none in the *svp* mutant NB7-3 lineage that expressed this marker. Nonetheless, one of the HB, KR double-positive cells exhibited an axonal morphology characteristic of GW (Figure 3B). In contrast, the average numbers of cells that expressed late-born marker ZFH-2 were decreased from 2 to 1.4 (n = 88) (Supplemental Table S2). Thus, just as in the NB7-1 lineage, *svp* mutant NB7-3 overproduced neurons with the early-born fate, with concomitant decrease in neurons with the late-born fate (Figure 3F).

To study the temporal pattern of SVP expression in the NB7-3 lineage, we examined the expression of *svp* mRNA and protein in conjunction with the lineage marker *eagle-kinesin-lacZ* (Figure 3D). Accumulation of *svp* mRNA started in NB7-3 when it had yet to produce a GMC, and *svp* mRNA was not detectable after the second NB division (data not shown). After the first division, SVP protein was detected in both NB and the first GMC. After the second division, SVP protein was dramatically downregulated and was undetectable either in the NB or its progeny within the NB7-3 lineage. These

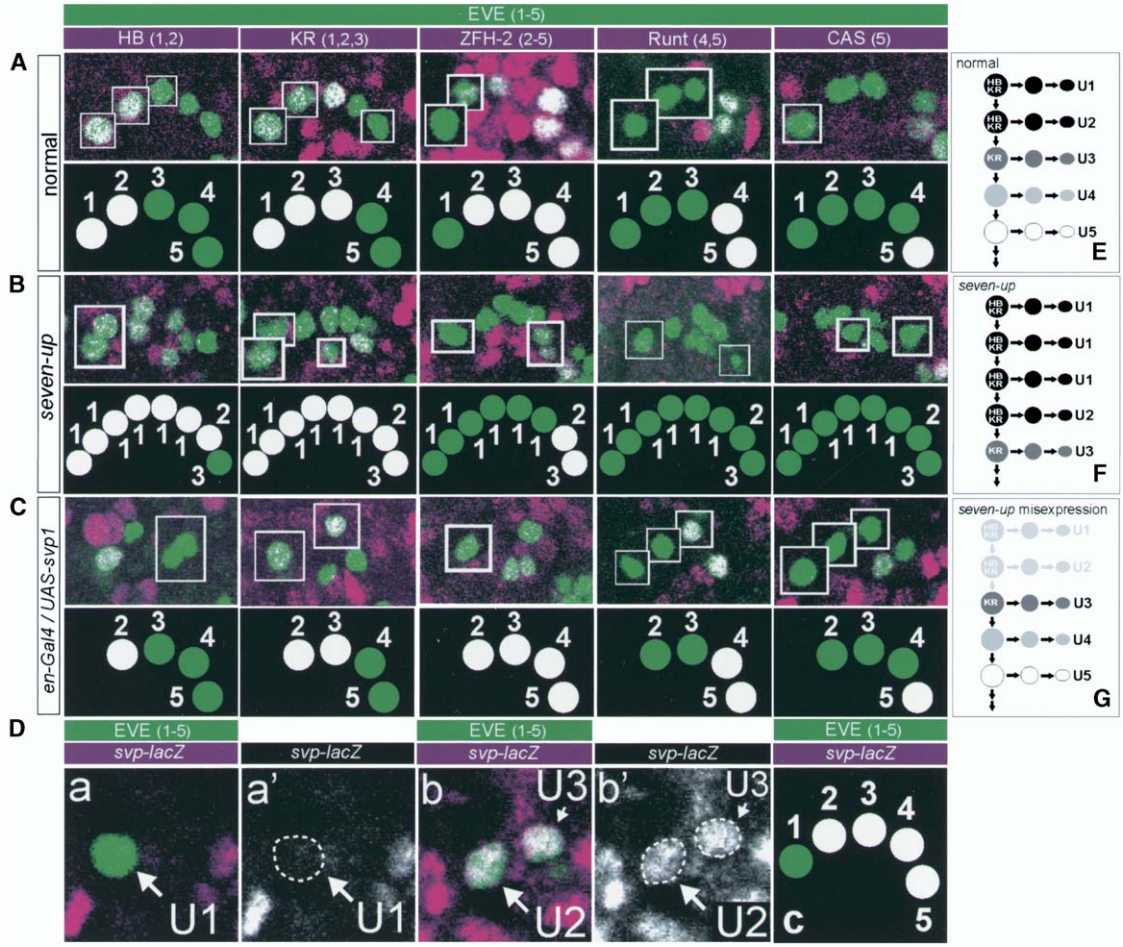

Figure 2. Expression and Function of *svp* in the NB7-1 Lineage

EVE-positive U neurons (U1–U5) (green) generated from NB7-1. White box indicates that this portion was spliced in from a different focal plane. The cell type and birth order of U neurons were identified using several neural markers (magenta). HB: U1 (1) and U2 (2), KR: U1, U2, and U3 (3), ZFH-2: U2–U5 (5), Runt: U4 (4) and (5), CAS: U5 (5). (A) normal embryo, (B) *svp*$^{e22/e300}$ embryo, and (C) *engrailed-Gal4/UAS-svp 1*. (D) *svp-lacZ* enhancer trap expression in the NB7-1 lineage. Of five EVE (green)-positive U neurons, U1 did not express β-galactosidase (a, a′; magenta), indicating that NB7-1 starts *svp* expression after generating the first GMC that produces U1. (E–G) Proposed NB7-1 lineages, inferred from gene expression patterns of the progeny. All images are from stage 15 embryos.

results indicate that the expression of SVP protein in the NB7-3 lineage is restricted to a subsection of the lineage corresponding to the stage when the NB switches from the HB-positive state to the KR-positive state.

Misexpression of *svp* Causes Loss of Early-Born Neurons

Since *svp* has a critical role in limiting the number of neurons with the early-born fate, we next addressed whether *svp* plays an instructive function in suppressing early-born neurons. Forced expression of *svp* in NB7-3 and NB7-1 lineages was achieved by the UAS/GAL4 method using *engrailed-Gal4*. Because this driver is expressed in the posterior part of each segment from late stage 8, this manipulation results in precocious expression of SVP in these NB lineages, starting at the time of NB formation. In the NB7-3 lineage, HB-positive cells were missing in 40% of hemisegments (n = 60), indicating that the generation of early-born neurons is compromised by the precocious expression of *svp* (Figure 3C). In 16.1% (n = 180) of hemisegments, all the cells that were generated from NB7-3 were missing. This could be a secondary effect of disrupting the early phase of the NB lineage, or misexpression of *svp* in the neuroectoderm may have affected the delamination or the formation of the NB.

A similar effect of precocious SVP expression was observed in NB7-1 in the generation of U neurons: 33.3% of HB-positive cells were absent, whereas late-born neurons were only mildly affected (Figure 2C); the average numbers of Runt and CAS were 1.48 (n = 60) and 1.0 (n = 48), respectively. Because SVP is normally not expressed at the stage when NBs generate HB-positive progeny, these data suggest that SVP can block the production of HB-positive progeny when misexpressed precociously. Interestingly, when *svp* misexpression was achieved in all neurons using *elav-Gal4*, we did not observe any defect in the number of HB-positive cells either in the NB7-3 lineage or the NB7-1

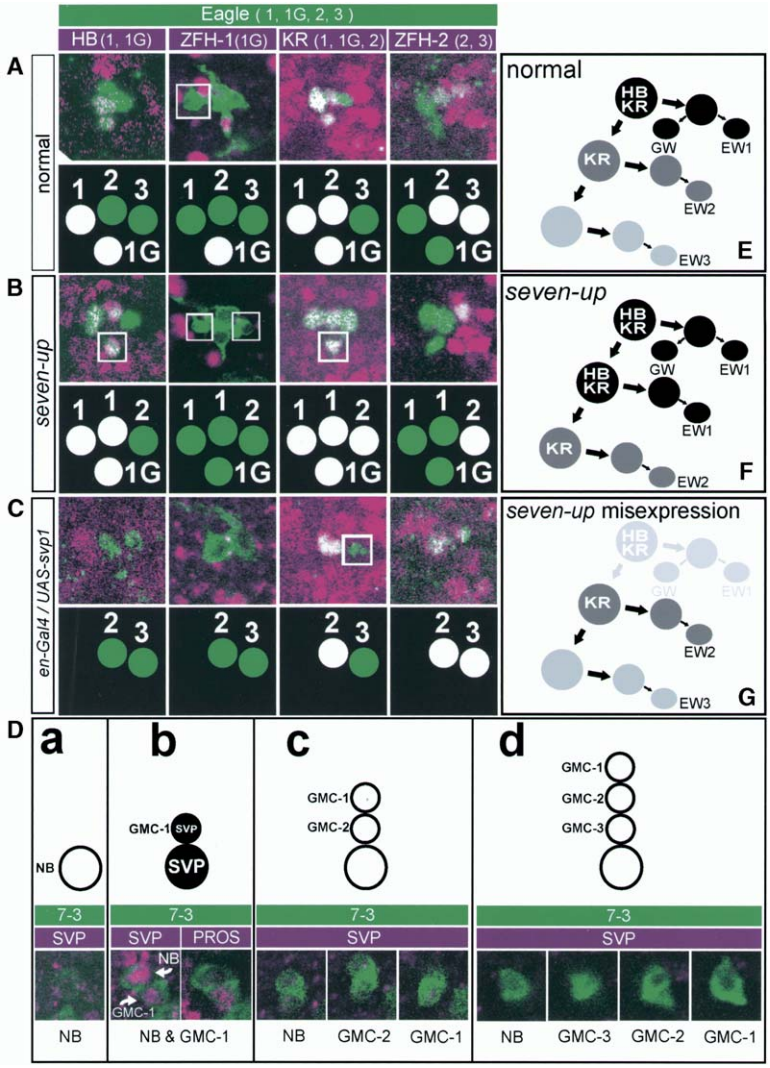

Figure 3. Expression and Function of *svp* in the NB7-3 Lineage

NB7-3 was marked with a lineage marker Eagle or *eagle-kinesin-lacZ* (green). Four neurons generated from NB7-3 can be distinguished with the combination of several transcription factors: HB, KR, ZFH-1, and ZFH-2 (magenta). HB: EW1 neuron (1) and GW neuron (1G); ZFH-1: GW neuron; KR: EW1, GW, and EW2 (2); ZFH-2: EW2 and EW3 (3). (A) normal, (B) *svp*$^{e22/e300}$, (C) *engrailed-Gal4/ UAS-svp1*. (D) Expression of SVP protein in the NB7-3 lineage. NB7-3 and its progeny were labeled with *eagle-kinesin-lacZ* reporter, and GMCs were identified owing to the expression of Prospero. NB7-3 does not express SVP protein at the time of its formation (a). When it generates the first progeny, SVP is expressed in both NB and GMC-1 (b). SVP is dramatically downregulated when NB7-3 generates its second progeny GMC-2 (c). At the stage when NB7-3 generates GMC-3, SVP is undetectable within this lineage.

(E–G) Proposed NB7-3 lineages, inferred from gene expression patterns of the progeny. Preparations shown in (A), (B), and (C) are stage 15; those in (D) are from stages 11 and 12.

lineage (data not shown). This suggests that SVP can only act in precursor cells that generate postmitotic neurons.

SVP Is Required for the HB-to-KR Switch in the NB

Since neurons maintain the expression pattern of the transcription factors of the NB that generated them (Isshiki et al., 2001), a possible cause of the increase in the number of neurons with the early-born fate in the *svp* mutant is that the parental NB did not switch its expression pattern properly from the HB, KR double-positive state to the KR single-positive state. To address whether *svp* mutants indeed have a defect in this switching in the NB, we examined gene expression patterns in NB7-3 when this NB was producing progeny. In normal development, NB7-3 starts HB expression before producing a GMC and remains HB positive when it has given rise to one GMC but not after two GMCs are generated (Figure 4A). In the *svp* mutant, the early phase of NB7-3 development was normal; NB7-3 started HB expression when it had no progeny, and HB expression was maintained in the NB that generated the first GMC, which also expressed HB (Figures 4Ba and 4Bb). However, NB7-3 continued to expresses HB even after generating two GMCs (Figure 4Bc). Therefore, unlike wild-type embryos, where only the first GMC is HB positive, *svp* mutant embryos produced two GMCs that maintained HB expression. This accounts for the phenotypes observed at the neuron level: overproduction of neurons with the early-born fate. We conclude that the primary cause of the overproduction of neurons of the early-born cell type in the *svp* mutant is the prolonged expression of HB in the NB, causing duplication of the GMC with early-born identity.

The effect of SVP on HB expression in the NB was also observed upon misexpression of *svp*. When *svp* was expressed precociously in NB7-3 using *engrailed-Gal4*, HB expression in this NB or a GMC within its lineage was often absent (29.1%, n = 48) (Figure 4C). We observed NB7-3, which was at a stage before giving rise to a GMC, expressing KR but not HB: a situation never observed in normal embryos. Because SVP is necessary to prevent precocious expression of HB in NB7-3 (Figure 4B), this result indicates that SVP is nec-

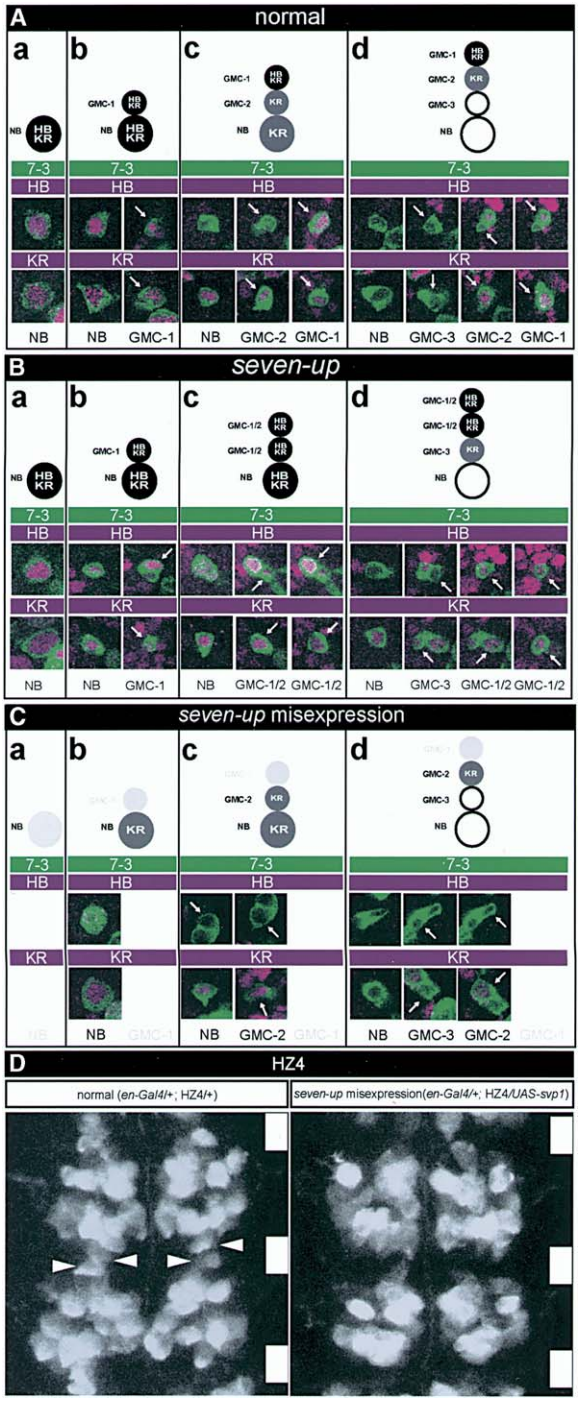

Figure 4. *svp* Regulates HB Expression in the NB

NB7-3 lineage was marked with *eagle-kinesin-lacZ* (green). Expression of HB and KR are shown in magenta. GMCs were identified by double labeling with PROS (not shown), and are indicated by arrows. All images, except for (D), were from stage 11 and 12 embryos. (A) normal; (B) $svp^{e22/e300}$; and (C) *engrailed-Gal4/UAS-svp 1*. (D) Expression of the HZ4, a *lacZ* reporter gene driven by an enhancer element of *hb*, in stage 11 embryo. Expression is extinguished in NBs where *UAS-svp1/engrailed-Gal4* is expressed (arrowhead). White bar on the right indicates the region of misexpression.

essary and sufficient for suppressing HB expression in this NB. In a small percentage of hemisegments, the NB7-3 lineage marker *eagle-kinesin-lacZ* was also absent (15.0%, n = 48), consistent with the observation that, in some embryos, all cells generated from NB7-3 were missing in stage 15 embryos (16.1%, n = 180) (see previous section).

The augmented expression of HB in the *svp* mutant was observed in many NBs, suggesting that SVP can repress HB in many NBs (Supplemental Figure S2). To investigate the mechanism by which SVP blocks HB expression, we used *hb* reporter gene HZ4, in which a regulatory element located at –8 kb to –12 kb of the *hb* locus is fused to a *lacZ* gene driven by a minimal promoter (Margolis, 1992; Margolis et al., 1995). When *svp* was misexpressed using *engrailed-Gal4* driver, the expression of this *hb* reporter gene was extinguished in NBs residing within the misexpressed region (Figure 4D). This strongly suggests that SVP controls *hb* expression by affecting its transcription.

The Role of Seven-Up in Generating Later Lineage

In addition to the overproduction of neurons with early-born identity, *svp* mutant NB lineages have an additional phenotype: the reduction of late-born neurons (Figures 2B and 3B). Late-born neurons are produced in either the *Kr* mutant or the *hb* mutant (Isshiki et al., 2001), and we found that, even when both HB and KR were inactivated, ZFH-2- or CAS-positive neurons were produced in NB7-3 and NB7-1 lineages (Figures 5D and 6D). Because precocious expression of KR induces PDM expression, Isshiki et al. (2001) proposed that late-born identity is promoted by the expression of KR. This raises the possibility that SVP somehow cooperates with KR to initiate the late-lineage program. To examine how SVP functions in generating late-born progeny, we analyzed the phenotype of the *svp Kr* double mutant.

In the *Kr* mutant, initial expression of HB ceases and late-born neurons are produced normally, simply skipping the KR-positive stage (Isshiki et al., 2001). In the *svp* mutant, about 57% of the NB7-3 lineage produces late-born neuron EW3, expressing ZFH-2 (Figure 3B). In the *svp Kr* double mutant, however, ZFH-2-positive neurons in the NB7-3 lineage were almost completely eliminated (Figure 5E; Supplemental Table S2). Likewise, in NB7-1, the *svp Kr* double mutant hardly contained U neurons that expressed Runt and CAS, characteristics of the late-born U neurons (Figure 6E; Supplemental Table S1). Thus, in both NB lineages, removing KR function strongly enhanced the reduction in the number of late-born progeny seen in the *svp* single mutant.

One mechanism by which SVP and KR could contribute to the generation of the late lineage is that these transcription factors induce expression of a transcription factor, such as PDM and CAS, that specifies the temporal identity following KR. However, at the time when late-born neurons are generated, SVP expression is already downregulated to an undetectable level both in normal NB lineage (Figure 3D) or in the *Kr* mutant (data not shown). It is thus unlikely that the requirement for SVP in activating the late-lineage program is direct.

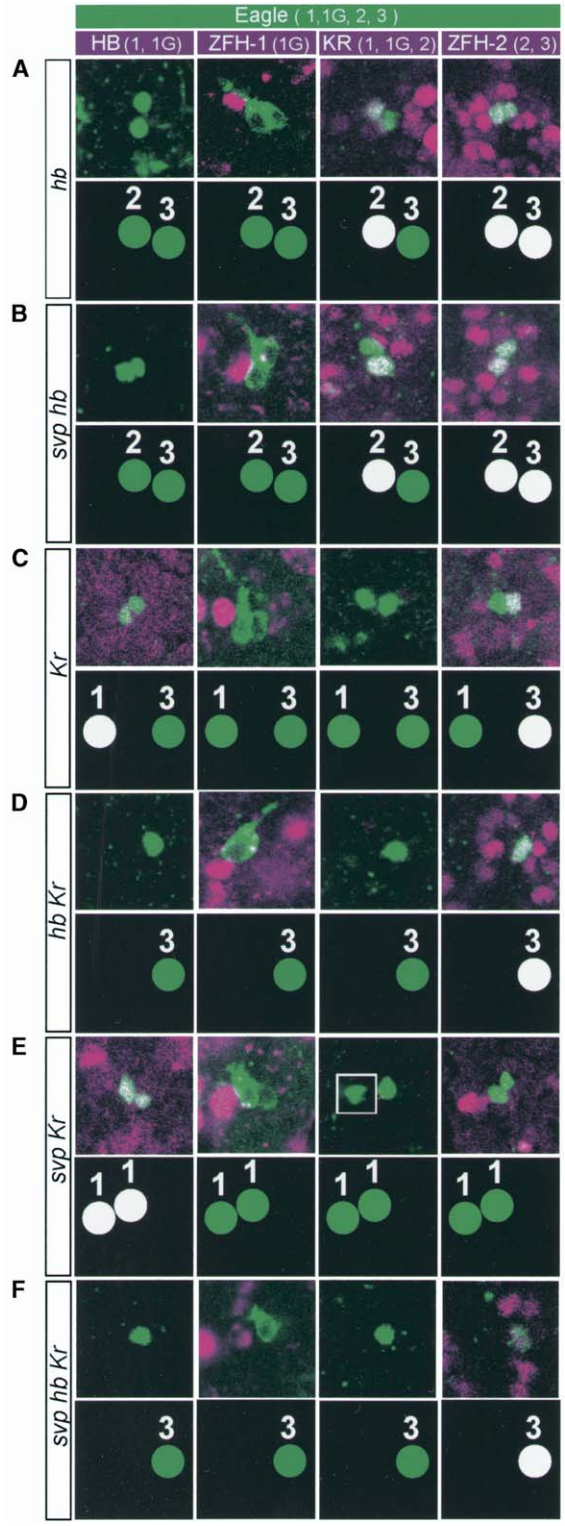

Figure 5. The Role of SVP in Generating Late-Born Neurons in NB7-3 Lineage

NB7-3 lineage was marked with Eagle expression or *eagle-kinesin-lacZ* (green). Shown in magenta are expression patterns of markers indicated at the top of each column. All embryos are at stage 15. (A) $hb^{14F/FB}$; (B)$svp^{e22/e300}$ $hb^{14F/FB}$; (C) $Kr^{1,\ CD}$; (D) hb^{14F} $Kr^{1,CD}$; (E) svp^{e22} $Kr^{1,CD}$; and (F) svp^{e22} hb^{14F} $Kr^{1,CD}$. Dotted line indicates the midline. In all panels, the bottom panel shows a schematic representation of the typical phenotype for the particular genotype. Numbers in the schematic figure show neuronal identities (1: EW1, 2: EW2, and 3: EW3), inferred from the expression patterns of markers.

Another interpretation of the phenotype of the *svp Kr* double mutant is that the removal of KR generated a role of SVP to mediate the switch from HB to a transcription factor specifying the late lineage, in a manner similar to its function in the HB-to-KR switch. In this scenario, SVP does not have a specific function in inducing the expression of a transcription factor for the late-lineage program, but acts by shutting off HB expression so that the late program can initiate. Since HB can repress *pdm* expression (Isshiki et al., 2001; Kambadur et al., 1998), prolonged expression of HB caused by the loss of SVP may have blocked PDM expression and the generation of the subsequent lineage.

To test whether the effect of the loss of SVP function on the late-lineage program is due to the prolonged expression of HB, we removed HB function in the *Kr svp* double mutant. As described above, in the *Kr svp* double mutant, neither the NB7-3 lineage nor the NB7-1 lineage produced late-born neurons expressing ZFH-2 or CAS (Figures 5E and 6E). However, in the *hb Kr svp* triple mutant, NB7-3 lineage restored production of one ZFH-2-positive cell, which likely corresponds to the late-born neuron EW3 (Figure 5F; Supplemental Table S2). Similarly, the NB7-1 lineage in the *hb Kr svp* triple mutant generated 2.9 (n = 32) ZFH-2-positive cells, some of which expressed Runt and CAS (Figure 6F; Supplemental Table S1). Thus, removing HB function restored the ability of this NB to produce U3, U4, and U5 neurons with late-born identity, which were absent in the *Kr svp* double mutant. Likewise, removing HB function in the *svp* mutant background also rescued the reduction of late-born neurons in NB7-3 and NB7-1 lineages (Figures 5B and 6B; Supplemental Tables S1 and S2). We conclude that the primary function of SVP is to control the HB-to-KR switch, and this switching allows the initiation of the late-lineage program by removing the HB-mediated repression of transcription factors responsible for the late part of the NB lineage.

Discussion

SVP Regulates Temporal Identity of NBs and Their Progeny

In *Drosophila* CNS development, NBs generate a variety of cell types by transcription factor switching, thereby producing progeny with different identities upon each division (Isshiki et al., 2001). We showed that, in the *svp* mutant, NB7-3 did not switch its expression pattern from the HB, KR double-positive state to the KR single-positive state until one division after the normal transition period. This prolonged expression of HB resulted in overproduction of HB-positive neurons exhibiting characteristics of early-born neurons. The timing of the expression of SVP protein in NB7-3 coincides with the transition in the expression of HB to KR, and precocious expression of SVP caused the loss of HB expression within the lineage. These results indi-

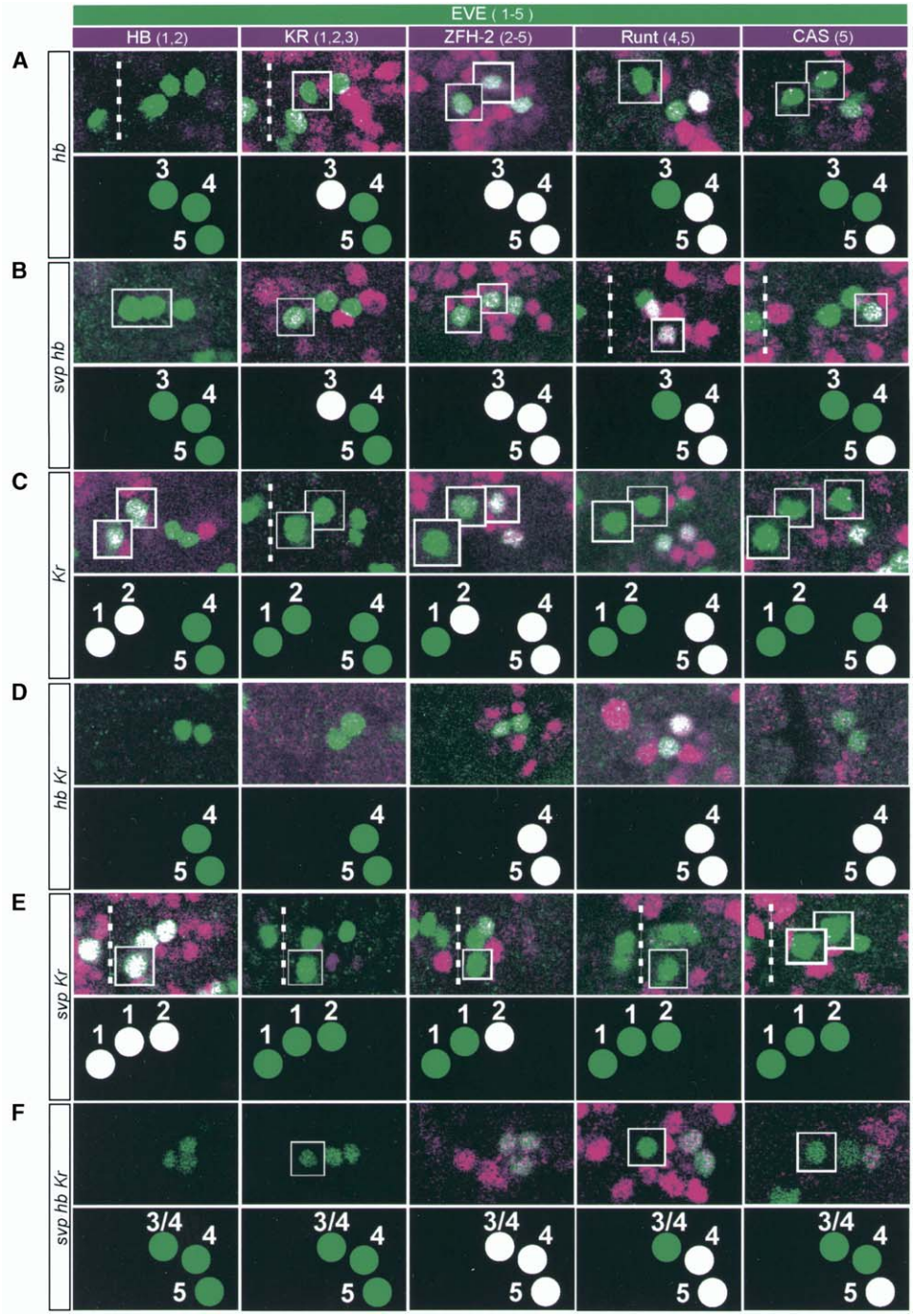

Figure 6. The Role of SVP for the Generation of Late-Born Neuron in NB7-1

U neurons generated from NB7-1 were marked with EVE (green). Shown in magenta are expression patterns of markers indicated at the top of each column. The genotypes of mutants used are the same as those presented in Figure 5. The numbers in the schematic figure show the identities of individual U neurons (1: U1, 2: U2, etc.). All preparations are from stage 15 embryos.

cate that SVP has an instructive role in determining the period of HB expression in the NB and the proper generation of neuronal diversity. While our work places SVP upstream of HB, how the expression of SVP itself is regulated is not well understood. In *hb* mutant embryos, SVP is still expressed transiently at the time that NB7-3 produces its first GMC (Supplemental Figure S3). Thus, it is unlikely that the temporal delay of SVP expression with respect to HB is due to a negative feedback loop in which HB induces its own repressor.

SVP is a well-conserved nuclear receptor whose human homolog, COUP, has been shown to act as a transcriptional repressor (Mangelsdorf and Evans, 1995). Because a reporter gene that contains only an en-

hancer element of the *hb* gene also responded to SVP, SVP can affect *hb* expression at the level of its transcription. It is thus possible that SVP directly represses *hb* transcription by binding to its *cis*-element. Interestingly, misexpression of SVP in postmitotic neurons did not affect their HB expression, consistent with the observation that the regulatory mechanism of HB expression differs between the NBs and their progeny (Isshiki et al., 2001). The repressor activity of SVP on *hb* expression likely requires other factors that are present in precursor cells of neurons.

In *svp* mutant embryo, augumented expression of HB was seen in many NBs, resulting in overproduction of early-born neurons in at least three NB lineages. This suggests that SVP may have a common function in many NB lineages regulating *hb* expression. However, of 30 NBs within each hemisegment, four do not express *svp* (Doe, 1992). Indeed, in an *svp*-negative NB1-1 lineage, the number of the early-born neurons aCC and pCC in *svp* mutant embryo is normal (data not shown). How do these NBs generate birth-order-dependent progeny without *svp* expression? Since some NBs are known to start their lineage without expressing HB (Isshiki et al., 2001), they may not need SVP to regulate HB expression. Indeed, we found that *svp*-negative NB6-1, which expresses CAS at the time of formation (Cui and Doe, 1992; Isshiki et al., 2001) never expresses HB (data not shown). It is also possible that there are other factors or mechanisms to regulate *hb* expression. In the nematode *C. elegans hb* homolog *lin57/hbl-1*, which controls developmental timing as a heterochronic gene, is regulated by a micro RNA that binds its 3′UTR (Abrahante et al., 2003; Lin et al., 2003). As *Drosophila hb* 3′UTR contains putative micro RNA binding sites, transcription factor switching in *Drosophila* NBs might also be regulated posttranscriptionally by micro RNAs.

While the overproduction of HB-positive neurons is consistent with the idea that prolonged expression of HB in *svp* mutant NBs causes production of supernumerary GMC-1s, examination of postmitotic neurons reveals that the number of neurons with particular identity does not always correspond to duplicated GMC-1s. In the NB7-3 lineage, GMC-1 divides to produce two neurons, EW1 and GW, whereas GMC-2 gives rise to EW2 neuron and its sibling which undergoes programmed cell death (Isshiki et al., 2001; Lundell and Hirsh, 1998; Lundell et al., 2003; Novotny et al., 2002). In *svp* mutant, two EW1 neurons were present consistent with duplicated GMC-1, but we observed only one GW-like neuron. Likewise, when HB was misexpressed in the NB7-3 lineage, not all GMCs that were transformed toward GMC-1 produced GW neurons (Novotny et al., 2002). These data suggest that the fate of postmitotic progeny from GMCs are dependent not only on the birth-order identity of GMCs determined by transcription factors such as HB and KR, but is also influenced by signals that come from outside of the NB lineage. Since the decision for the sibling of the EW2 neuron to undergo cell death depends on the activation of Notch signaling, it is possible that signals for Notch activation originate outside the NB7-3 lineage, and are not affected by genetic manipulations altering the birth-order identity of the GMCs (Lundell et al., 2003; Spana and Doe, 1996).

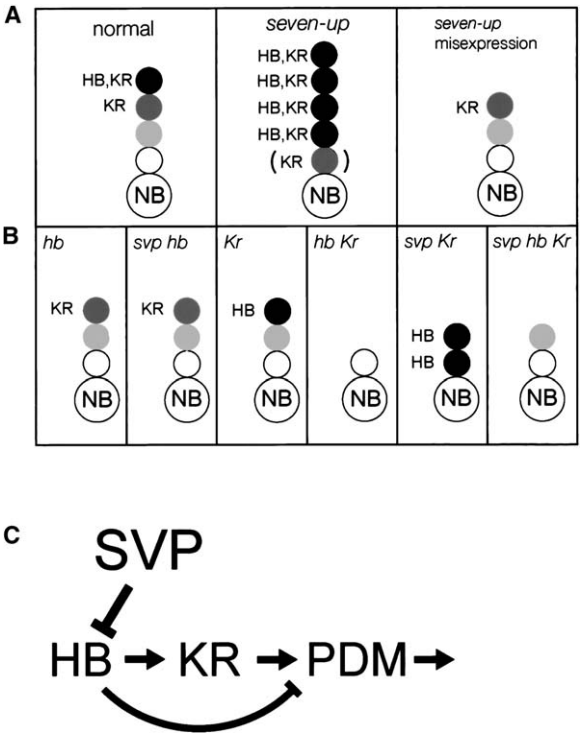

Figure 7. The Role of SVP in the Temporal Switching of the NB Fate

(A) A summary of *svp* function for the NB lineage development. Loss or gain of SVP function alters HB expression in the NB, resulting in the increase or the decrease in the number of GMCs with early-born identity.

(B) The loss of late-born neurons (light gray shading and open circles) seen in *svp* mutant and *svp Kr* double mutant is restored by deleting HB.

(C) SVP functions to terminate HB expression at the proper time, so that NB can switch to the KR-positive state.

SVP Modifies the Stem Cell Program to Introduce Birth-Order-Dependent Neuronal Diversity

In addition to the increase in the number of early born neurons, *svp* mutant embryos display another phenotype, the reduction of late-born neurons that express ZFH-2 (NB7-3), Runt, and CAS (NB7-1). This phenotype was dramatically enhanced when KR was inactivated, freezing the lineage such that only HB-positive cells were produced. One interpretation of this phenotype is that SVP somehow cooperates with KR to generate the late part of the lineage. In fact, to our knowledge, this is the only known genetic situation in which loss of gene function eliminates the late born neurons. However, because this phenotype was completely suppressed by removing HB, we favor the idea that SVP does not have a direct role in activating the transcription factors that specify the late-born identity, but rather acts through repressing HB, which can repress PDM expression (Figure 7C). Thus the apparent requirement of SVP in the generation of the late-born neurons deduced from the *svp* loss-of-function phenotype is due to its primary function in mediating the HB-to-KR switch, whose failure secondarily blocks the initiation of the late lineage program. Our results also show that the late lineage can be produced in the absence of HB and KR (and SVP),

suggesting that it may be the "default" state. It is possible that primitive lineage consisted only of the late lineage program, to which SVP was recruited to add the early program involving HB and KR, thereby generating the birth-order-dependent neuronal diversity.

Experimental Procedures

Drosophila Strains

Two alleles of *svp, svp^{e22}* (also called *svp^{1}*) and *svp^{e300}* (also called *svp^{2}*) (Mlodzik et al., 1990) were used, with identical results. To inactivate *hb* or *Kr* function in the CNS, we used *hb^{14F}/hb^{FB}* and *Kr^{CD}; Kr^{1}* strains (Hulskamp et al., 1994; Isshiki et al., 2001; Romani et al., 1996), which are referred to simply as *hb* mutant and *Kr* mutant, respectively. For *hb* mutants, only abdominal segments were examined. *svp* misexpressipn by the GAL4/UAS system (Brand and Perrimon, 1993) was done using *UAS-svp1 1.12* (Kramer et al., 1995) containing *svp* type 1 cDNA (Mlodzik et al., 1990) and *engrailed-Gal4* (gift of A. Brand) or *elav-Gal4* C155 (Lin and Goodman, 1994) drivers. SVP expression in NB progeny was traced using an *svp* enhancer trap strain AE127 (Heberlein et al., 1991). The NB7-3 lineage and RP neurons were marked with the *eagle-kinesin-lacZ* (Higashijima et al., 1996) and *Lim-3A-TauMyc* (Thor et al., 1999), respectively. Transcriptional regulation of *hb* was monitored using HZ4, an *hb*-reporter gene in which a 4 kb regulatory element is placed in front of an *hsp70* minimal promoter-*lacZ* fusion gene (Margolis, 1992). Homozygous mutant embryos were identified using the following balancer chromosomes bearing a *lacZ* transgene: *TM3,Ubx-lacZ*, *TM3, ftz-lacZ*, and *Cyo, en11*.

Histology

The following antibodies were developed and provided by D. Kosman and J. Reinitz (Kosman et al., 1998): rat anti-HB (1:300), guinea pig anti-HB (1:300), guinea pig anti-KR (1:300), guinea pig anti-EVE (1:200), and guinea pig anti-Runt (1:200). The Developmental Studies Hybridoma Bank (DSHB) (University of Iowa, Department of Biological Sciences), developed under the auspices of the NIHD, provided the following monoclonal antibodies: anti-Prospero MR1A (developed by C.Q. Doe) (1:2), anti-Myc 9E10 (developed by J.M. Bishop) (1:2), and anti-β-galactosidase 40-1a (developed by J. Sanes). Anti-Eagle monoclonal antibody (1:5), rat anti-ZFH2 (1:400) (Lundell and Hirsh, 1992), rabbit anti-Castor (1:2,000) (Mellerick et al., 1992), mouse anti-ZFH1 (1:5) (Lai et al., 1991), and rabbit anti-EVE were generous gifts from C.Q. Doe, M. Lundell, W.F. Odenwald, Z.C. Lai, and M. Frasch, respectively. Mouse anti-SVP (1:1,000), guinea pig anti-β-galactosidase (1:500), and rat anti-β-galactosidase (1:500) were generated by immunizing animals with bacterially expressed (SVP corresponding to residues 102–543 of the SVP type 1 protein) or commercially available (β-galactosidase) proteins. The specificity of the SVP antiserum was verified using *Df(3R)kar3Q*, a chromosomal deletion uncovering the *svp* locus. The Tyramide Signal Amplification System (Perkin Elmer) was employed when using anti-Eagle, anti-ZFH1, and anti-SVP. Secondary Antibodies were from Jackson Immuno Research. Images were taken using Zeiss PASCAL confocal microscope.

Figure Preparation

Figures were prepared according to "Barrier-free presentation that is friendly to colorblind people" (Okabe, M., and Ito, K., http://jfly.iam.u-tokyo.ac.jp/color/).

Supplemental Data

Supplemental Data associated with this article can be found online at http://www.developmentalcell.com/cgi/content/full/8/2/203/DC1/. The supplemental data set contains three figures and two tables.

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References

Abrahante, J.E., Daul, A.L., Li, M., Volk, M.L., Tennessen, J.M., Miller, E.A., and Rougvie, A.E. (2003). The *Caenorhabditis elegans* hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. Dev. Cell *4*, 625–637.

Bossing, T., Udolph, G., Doe, C.Q., and Technau, G.M. (1996). The embryonic central nervous system lineages of Drosophila melanogaster. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. Dev. Biol. *179*, 41–64.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Broadus, J., Skeath, J.B., Spana, E.P., Bossing, T., Technau, G., and Doe, C.Q. (1995). New neuroblast markers and the origin of the aCC/pCC neurons in the Drosophila central nervous system. Mech. Dev. *53*, 393–402.

Brody, T., and Odenwald, W.F. (2000). Programmed transformations in neuroblast gene expression during Drosophila CNS lineage development. Dev. Biol. *226*, 34–44.

Cui, X., and Doe, C.Q. (1992). ming is expressed in neuroblast sublineages and regulates gene expression in the Drosophila central nervous system. Development *116*, 943–952.

Doe, C.Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the Drosophila central nervous system. Development *116*, 855–863.

Doe, C.Q., and Goodman, C.S. (1985). Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. Dev. Biol. *111*, 206–219.

Elstob, P.R., Brodu, V., and Gould, A.P. (2001). spalt-dependent switching between two cell fates that are induced by the Drosophila EGF receptor. Development *128*, 723–732.

Heberlein, U., Mlodzik, M., and Rubin, G.M. (1991). Cell-fate determination in the developing Drosophila eye: role of the rough gene. Development *112*, 703–712.

Higashijima, S., Shishido, E., Matsuzaki, M., and Saigo, K. (1996). eagle, a member of the steroid receptor gene superfamily, is expressed in a subset of neuroblasts and regulates the fate of their putative progeny in the Drosophila CNS. Development *122*, 527–536.

Hoshizaki, D.K., Blackburn, T., Price, C., Ghosh, M., Miles, K., Ragucci, M., and Sweis, R. (1994). Embryonic fat-cell lineage in Drosophila melanogaster. Development *120*, 2489–2499.

Hulskamp, M., Lukowitz, W., Beermann, A., Glaser, G., and Tautz, D. (1994). Differential regulation of target genes by different alleles of the segmentation gene hunchback in Drosophila. Genetics *138*, 125–134.

Isshiki, T., Pearson, B., Holbrook, S., and Doe, C.Q. (2001). Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell *106*, 511–521.

Kambadur, R., Koizumi, K., Stivers, C., Nagle, J., Poole, S.J., and Odenwald, W.F. (1998). Regulation of POU genes by castor and hunchback establishes layered compartments in the Drosophila CNS. Genes Dev. *12*, 246–260.

Kerber, B., Fellert, S., and Hoch, M. (1998). Seven-up, the Drosophila homolog of the COUP-TF orphan receptors, controls cell proliferation in the insect kidney. Genes Dev. *12*, 1781–1786.

Kosman, D., Small, S., and Reinitz, J. (1998). Rapid preparation of a panel of polyclonal antibodies to Drosophila segmentation proteins. Dev. Genes Evol. *208*, 290–294.

Kramer, S., West, S.R., and Hiromi, Y. (1995). Cell fate control in the Drosophila retina by the orphan receptor seven-up: its role in the decisions mediated by the ras signaling pathway. Development *121*, 1361–1372.

Lai, Z.C., Fortini, M.E., and Rubin, G.M. (1991). The embryonic expression patterns of zfh-1 and zfh-2, two Drosophila genes encoding novel zinc-finger homeodomain proteins. Mech. Dev. *34*, 123–134.

Lin, D.M., and Goodman, C.S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron *13*, 507–523.

Lin, S.Y., Johnson, S.M., Abraham, M., Vella, M.C., Pasquinelli, A., Gamberi, C., Gottlieb, E., and Slack, F.J. (2003). The C elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. Dev. Cell *4*, 639–650.

Lo, P.C., and Frasch, M. (2001). A role for the COUP-TF-related gene seven-up in the diversification of cardioblast identities in the dorsal vessel of Drosophila. Mech. Dev. *104*, 49–60.

Lundell, M.J., and Hirsh, J. (1992). The zfh-2 gene product is a potential regulator of neuron-specific dopa decarboxylase gene expression in Drosophila. Dev. Biol. *154*, 84–94.

Lundell, M.J., and Hirsh, J. (1998). eagle is required for the specification of serotonin neurons and other neuroblast 7–3 progeny in the Drosophila CNS. Development *125*, 463–472.

Lundell, M.J., Lee, H.K., Perez, E., and Chadwell, L. (2003). The regulation of apoptosis by Numb/Notch signaling in the serotonin lineage of Drosophila. Development *130*, 4109–4121.

Mangelsdorf, D.J., and Evans, R.M. (1995). The RXR heterodimers and orphan receptors. Cell *83*, 841–850.

Margolis, J.S. (1992). Regulation of Drosophila gap segmentation gene hunchback. PhD thesis, University of California at San Diego, La Jolla, California.

Margolis, J.S., Borowsky, M.L., Steingrimsson, E., Shim, C.W., Lengyel, J.A., and Posakony, J.W. (1995). Posterior stripe expression of hunchback is driven from two promoters by a common enhancer element. Development *121*, 3067–3077.

McConnell, S.K. (1995). Constructing the cerebral cortex: neurogenesis and fate determination. Neuron *15*, 761–768.

Mellerick, D.M., Kassis, J.A., Zhang, S.D., and Odenwald, W.F. (1992). castor encodes a novel zinc finger protein required for the development of a subset of CNS neurons in Drosophila. Neuron *9*, 789–803.

Mlodzik, M., Hiromi, Y., Weber, U., Goodman, C.S., and Rubin, G.M. (1990). The Drosophila seven-up gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. Cell *60*, 211–224.

Novotny, T., Eiselt, R., and Urban, J. (2002). Hunchback is required for the specification of the early sublineage of neuroblast 7–3 in the Drosophila central nervous system. Development *129*, 1027–1036.

Romani, S., Jimenez, F., Hoch, M., Patel, N.H., Taubert, H., and Jackle, H. (1996). Kruppel, a Drosophila segmentation gene, participates in the specification of neurons and glial cells. Mech. Dev. *60*, 95–107.

Schmid, A., Chiba, A., and Doe, C.Q. (1999). Clonal analysis of Drosophila embryonic neuroblasts: neural cell types, axon projections and muscle targets. Development *126*, 4653–4689.

Sen, A., Reddy, G.V., and Rodrigues, V. (2003). Combinatorial expression of Prospero, Seven-up, and Elav identifies progenitor cell types during sense-organ differentiation in the Drosophila antenna. Dev. Biol. *254*, 79–92.

Skeath, J.B., and Thor, S. (2003). Genetic control of Drosophila nerve cord development. Curr. Opin. Neurobiol. *13*, 8–15.

Spana, E.P., and Doe, C.Q. (1996). Numb antagonizes Notch signaling to specify sibling neuron cell fates. Neuron *17*, 21–26.

Thor, S., Andersson, S.G., Tomlinson, A., and Thomas, J.B. (1999). A LIM-homeodomain combinatorial code for motor-neuron pathway selection. Nature *397*, 76–80.

Urbach, R., and Technau, G.M. (2003). Molecular markers for identified neuroblasts in the developing brain of Drosophila. Development *130*, 3621–3637.

Urbach, R., Schnabel, R., and Technau, G.M. (2003). The pattern of neuroblast formation, mitotic domains and proneural gene expression during early brain development in Drosophila. Development *130*, 3589–3606.

Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N.P., and Perlmann, T. (2003). Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature *423*, 555–560.