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Seven up acts as a temporal factor during two different stages of neuroblast 5-6 development

Jonathan Benito-Sipos[1], Carina Ulvklo[2], Hugo Gabilondo[1], Magnus Baumgardt[2], Anna Angel[2], Laura Torroja[1] and Stefan Thor[2,*]

SUMMARY

Drosophila embryonic neuroblasts generate different cell types at different time points. This is controlled by a temporal cascade of Hb→Kr→Pdm→Cas→Grh, which acts to dictate distinct competence windows sequentially. In addition, Seven up (Svp), a member of the nuclear hormone receptor family, acts early in the temporal cascade, to ensure the transition from Hb to Kr, and has been referred to as a ‘switching factor’. However, Svp is also expressed in a second wave within the developing CNS, but here, the possible role of Svp has not been previously addressed. In a genetic screen for mutants affecting the last-born cell in the embryonic NB5-6T lineage, the Ap4/FMRFamide neuron, we have isolated a novel allele of *svp*. Expression analysis shows that Svp is expressed in two distinct pulses in NB5-6T, and mutant analysis reveals that *svp* plays two distinct roles. In the first pulse, *svp* acts to ensure proper downregulation of Hb. In the second pulse, which occurs in a Cas/Grh double-positive window, *svp* acts to ensure proper sub-division of this window. These studies show that a temporal factor may play dual roles, acting at two different stages during the development of one neural lineage.

KEY WORDS: Temporal genes, COUP-TFI/II, Cell specification, Lineage progression, *Drosophila*

INTRODUCTION

Neural progenitor cells, in both vertebrates and invertebrates, go through temporal competence changes, evidenced by the generation of different classes of neurons and glia at different time points (Okano and Temple, 2009). These programmed changes are likely to be controlled by a combination of both extrinsic and intrinsic cues, and evidence points to the existence of both mechanisms in vertebrates and invertebrates. With respect to intrinsic cues, major progress has been made in the *Drosophila melanogaster* system, in particular in the embryonic ventral nerve cord (VNC). Here, temporal competence changes have been shown to be under control of an intrinsic temporal cascade of transcription factors, the temporal gene cascade (Brody and Odenwald, 2002; Jacob et al., 2008; Pearson and Doe, 2004). This cascade consist of the sequential expression, and function, of the Hunchback (Hb), Kruppel (Kr), Nubbin and Pdm2 (denoted collectively Pdm herein), Castor (Cas) and Grainy head (Grh) transcription factors, in a Hb→Kr→Pdm→Cas→Grh cascade. The precise progression of this cascade is an effect of mutually activating and repressing actions of the factors upon each other. In addition, studies have also identified factors that facilitate this progression, i.e. ‘switching factors’. Here, the *seven up* (*svp*) and *distal antenna*/*distal antenna related* (collectively referred to as *dan* herein) genes have been shown to play important roles in ensuring the switch from Hb→Kr, by suppressing Hb (Kanai et al., 2005; Kohwi et al., 2011; Mettler et al., 2006). Both Svp and Dan display a second wave of expression, but their function here is unknown. Finally, our previous studies have also identified the existence of so-called ‘sub-temporal’ genes, which act downstream of the temporal genes, do not regulate temporal genes, and act to sub-divide larger temporal windows (Baumgardt et al., 2009). However, in spite of the progress in understanding temporal competence changes, it is not clear how neuroblasts switch from one competence window to the next, how window size is controlled and how windows are sub-divided. Moreover, recent mathematical modelling of the temporal cascades, indicate the existence of additional players involved in the temporal competence changes observed in vivo (Nakajima et al., 2010).

To address these issues, we are using the *Drosophila* embryonic thoracic neuroblast 5-6 (NB5-6T) as a model. This neuroblast, which can be readily identified by the specific expression of reporter genes under the control of an enhancer fragment from the ladybird early gene [*lbe*(*K*)] (De Graeve et al., 2004), is generated in each of the six thoracic VNC hemisegments. Each NB5-6T produces a mixed lineage of 20 cells, and the four last cells to be born are a set of four interneurons expressing the Apterous (Ap) LIM-homeodomain transcription factor: the Ap neurons (Baumgardt et al., 2009). The four Ap neurons can be further sub-divided into three different neuronal sub-types: the Ap1/Nplp1 and Ap4/FMRFa neurons, expressing the Nplp1 and FMRFamide neuropeptides, respectively, and the Ap2/Ap3 interneurons (Fig. 1A,B) (Baumgardt et al., 2009; Baumgardt et al., 2007; Benveniste et al., 1998; Park et al., 2004). The birth order of each Ap neuron is stereotyped, and the number of the neuron refers to its birth order. The unique expression of different *lbe*(*K*) reporters, allowing for the selective identification and analysis of this one lineage, combined with the selective expression of Ap, Nplp1 and FMRFa, and several other markers, makes this lineage particularly useful for addressing temporal competence changes with single-lineage and single-cell resolution.

Previously, we resolved the lineage progression of NB5-6T, and determined the expression and function of the temporal and sub-temporal genes within this lineage, with particular emphasis upon Ap neuron generation (Baumgardt et al., 2009). These studies revealed that Ap neurons are generated in a Cas/Grh late temporal

[1]Departamento de Biología, Universidad Autónoma de Madrid, Madrid, 28049, Spain. [2]Department of Clinical and Experimental Medicine, Linkoping University, SE-581 85, Linkoping, Sweden.

*Author for correspondence (stefan.thor@liu.se)

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window, and that both genes are crucial for specification of Ap neurons (Fig. 1B). *grh* plays a highly specific role and is crucial for specification of the last-born neuron, Ap4/FMRFa. *cas* plays a more central role, and activates a number of downstream targets, including genes that determine Ap neuron fate, such as *collier* (*col*; *knot* – FlyBase), and genes that act to sub-divide the Ap window. The latter genes, *squeeze* (*sqz*) and *nab*, were denoted sub-temporal genes, and act to downregulate Col in the later-born Ap neurons. This allows Col to play its two distinct roles in Ap neurons; first specifying a generic Ap neuron fate, and second specifically dictating final Ap1/Nplp1 fate. Thus, downregulation of Col in the Ap2, Ap3 and Ap4/FMRFa neurons is crucial for allowing these alternate Ap neuron fates to be established at later stages. However, although elaborate in their nature, these temporal and sub-temporal cascades fall short of explaining the precision in Ap neuron sub-type specification – the highly stereotyped generation of exactly one Ap1/Nplp1 neuron, followed by two Ap2/3 neurons, and finally the Ap4/FMRFa neuron, i.e. a precise 1-2-1 sub-type specification, at the end of the NB5-6T lineage.

To address this issue, we have conducted a large-scale forward genetic screen, using an *FMRFa-EGFP* reporter transgene. One of the mutants identified in this screen, by its loss of *FMRFa-EGFP* expression, was mapped to *svp*. Our analysis of Svp expression demonstrates that it is expressed in two distinct pulses in the NB5-6T lineage. In the early pulse, *svp* plays its previously identified role, i.e. acting as a switching factor by suppressing Hb, thereby allowing for the progression of the temporal cascade during the early parts of the lineage. However, in the latter part of the lineage, as Svp is re-expressed in a second wave throughout the VNC, Svp is expressed specifically in the Ap neurons. Its second pulse of expression is dynamic, initially commencing in all four Ap neurons but rapidly being downregulated in the first- and last-born Ap neurons, Ap1/Nplp1 and Ap4/FMRFa, respectively. Mutant analysis shows that in the second pulse, *svp* acts as a sub-temporal gene, by downregulating Col, thereby allowing for the establishment of alternate Ap cell fates. The second function of *svp* in this lineage – acting as a sub-temporal gene – is similar to the role of *sqz* and *nab*. However, in contrast to *sqz* and *nab*, misexpression of *svp* results not only in ectopic downregulation of Col in the Ap1/Nplp1 neuron, and suppression of this fate, but also in suppression of the last-born neuron fate, Ap4/FMRFa. Thus, *svp* acts to 'gate' the central Ap window (Ap2/3) by suppressing the two cell fates temporally adjacent to this, i.e. the Ap1/Nplp1 and Ap4/FMRFa fates. Hence, *svp* adds insight to the complex regulatory cascades involved in the precise 1:2:1 temporal generation of the three distinct Ap neuron sub-types at the end of NB5-6T lineage.

These studies show that one gene can have dual temporal function in one neural lineage, acting first as a switching factor by regulating canonical temporal genes, and secondly by acting as a sub-temporal gene, 'micro-managing' the Ap window.

MATERIALS AND METHODS

Fly stocks

Fly stocks were maintained at 25°C on standard medium. The following stocks were used: svp^{e22} (also called svp^{1}) (Hiromi et al., 1993); $svpe^{300}$ (also called svp^{2}) (Mlodzik et al., 1990); svp^{11C115} (this work); *UAS-svp1 1.12* (Kramer et al., 1995) containing *svp* type 1 cDNA (Mlodzik et al., 1990); *UAS-svp1.36* (provided by Y. Hiromi, National Institute of Genetics, Mishima, Japan); *FMRFa-EGFP* (C.U. and S.T., unpublished); *ladybird early* fragment K driving *lacZ* (referred to as *lbe*(*K*)*-lacZ*) (provided by K. Jagla, INSERM U.384, Clermont-Ferrand, France) (De Graeve et al., 2004); *ladybird early* fragment K driving *Gal4* (referred to as *lbe*(*K*)*-Gal4*) (Baumgardt et al., 2009); *UAS-nls-myc-EGFP* (referred to as *UAS-nmEGFP*), *UAS-myc-EGFP–farnesylation*, sqz^{Df}, sqz^{ie}, *UAS-sqz* (Allan and Thor, 2003); ap^{md544} (referred to as ap^{Gal4}) (O'Keefe et al., 1998); ap^{rK568} (referred to as ap^{lacZ}) (Cohen et al., 1992); gsb^{01155} (referred to as gsb^{lacZ}) (Duman-Scheel et al., 1997), a marker for neuroblast lineages in rows 5 and 6 (Buenzow and Holmgren, 1995; Duman-Scheel et al., 1997; Gutjahr et al., 1993; Skeath et al., 1995); *elav-Gal4* (provided by A. DiAntonio) (DiAntonio et al., 2001); $cas^{\Delta 1}$ and $cas^{\Delta 3}$ (Mellerick et al., 1992), and *UAS-cas* (Kambadur et al., 1998) (both provided by W. Odenwald, NINDS, National Institutes of Health, Bethesda, USA); grh^{IM} (Nusslein-Volhard et al., 1984). *Df*(*2R*)*Pcl7B* (referred to as grh^{Df}); col^{1}, col^{3} (Crozatier and Vincent, 1999) (provided by A. Vincent, CNRS/Universite Paul Sabatier, Toulouse, France); nab^{SH143}, nab^{R52}, *UAS-nab* (provided by F. J. Díaz-Benjumea, CSIC, Madrid, Spain) (Terriente Felix et al., 2007). Mutants were kept over *CyO, Act-GFP*; *CyO, Dfd-EYFP*; *TM3, Ser, Act- GFP*; *CyO, twi-Gal4, UAS-GFP*; *TM3, Sb, Ser*, *twi-Gal4, UAS-GFP*; or *TM6, Sb, Tb, Dfd-EYFP* balancer chromosomes. As wild type, *OregonR* (*iso2*) was often used. Unless otherwise stated, flies were obtained from the Bloomington Drosophila Stock Center.

Immunohistochemistry

The following antibodies were used: Mouse α-Svp (1:50) (Kanai et al., 2005) (provided by Y. Hiromi); guinea pig α-Col (1:1000); guinea pig α-Dimm (1:1000); chicken α-proNplp1 (1:1000) and rabbit α-proFMRFa (1:1000) (Baumgardt et al., 2007); rat α-Grh (1:1000) (Baumgardt et al., 2009); rabbit α-Nab (1:1000) (Terriente Felix et al., 2007) (provided by F. J. Díaz-Benjumea); rabbit α-Cas (1:250) (Kambadur et al., 1998) (provided by W. Odenwald); guinea pig α-Deadpan (1:1000) (provided by J. Skeath, Washington University School of Medicine, St Louis, USA); Rat monoclonal α-Gsbn (1:10) (provided by R. Holmgren, Northwestern University, Evanston, USA); Rabbit α-Hunchback (1:1000) (provided by R. Pflanz, MPIbpC, Göttingen, Germany); Rat α-Sqz (1:750) (Tsuji et al., 2008) (provided by T. Isshiki, National Institute of Genetics, Mishima, Japan); Rabbit α-β-Gal (1:5000; ICN-Cappel, Aurora, OH, US); Mouse α-myc (1:2000; Upstate/Millipore, Billerica, MA, US); Chicken α-β-Gal (1:1000; Abcam, Cambridge, UK); Mouse α-Eya 10H6 (1:250) (Developmental Studies Hybridoma Bank, Iowa City, IA, US). All polyclonal sera were pre-absorbed against pools of early embryos. Secondary antibodies were conjugated with AMCA, FITC, Rhodamine-RedX or Cy5, and used at 1:200 (Jackson ImmunoResearch, PA, US). Embryos were dissected in PBS, fixed for 25 minutes in 4% PFA, blocked and processed with antibodies in PBS with 0.2% Triton-X100 and 4% donkey serum. Slides were mounted in Vectashield (Vector, Burlingame, CA, US). For embryonic stages 9-12, embryos were stained as whole-mounts, using the same protocol. Embryos were staged according to Campos-Ortega (Campos-Ortega, 1997).

Confocal imaging and data acquisition

Zeiss LSM 5 or Zeiss META 510 confocal microscopes were used to collect data for all fluorescent images; confocal stacks were merged using LSM software or Adobe Photoshop. Where immunolabeling was compared for levels of expression, wild-type and mutant tissue was stained and analysed on the same slide. Statistical analysis was performed using Microsoft Excel, and bar graphs generated using GraphPad Prism software. Statistical Methods Quantifications of observed phenotypes were performed using Student's two-tailed *t*-test, assuming equal variance.

RESULTS

A screen for genes controlling the specification of the Ap4/FMRFamide neuron, the last-born neuron in the NB5-6T lineage, identifies *seven up*

Previous studies have identified a number of regulatory genes and pathways acting, between stage 12 and 18 hours after egg-laying (hAEL), to specify the Ap neurons (Fig. 1A,B) (Allan et al., 2005; Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Benveniste et al., 1998; Hewes et al., 2003; Miguel-Aliaga et al., 2004; Park et al., 2004). To further understand the development of this lineage and the specification of the Ap neurons, we have

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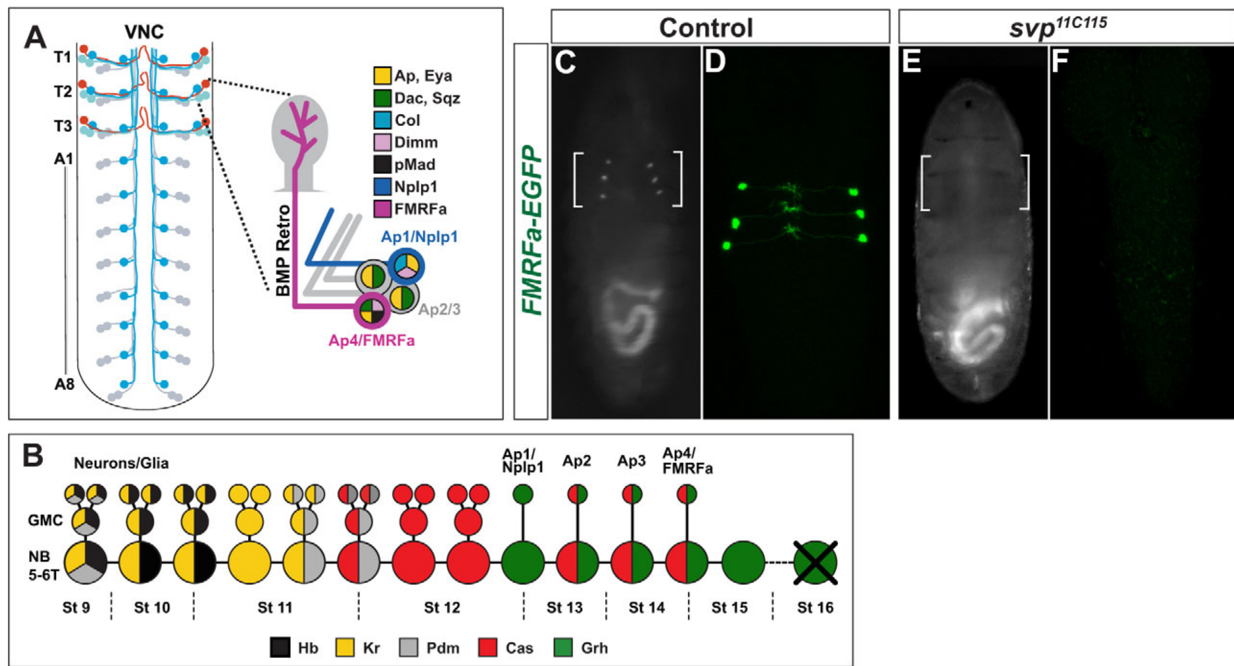

Fig. 1. A genetic screen for *FMRF-EGFP* expression in Apterous neurons identifies *seven up*. (**A**) Previous studies identified several regulatory genes specifically expressed in subsets of Ap neurons, acting to specify their identities (see text for references). (**B**) Model of the NB5-6T lineage based on previous studies (Baumgardt et al., 2009). The four Ap cluster neurons are the last-born neurons, and are generated within a Cas/Grh temporal window. (**C-F**) Expression of *FMRFa-EGFP* in living late embryos (C,E) or dissected VNCs (D,F), in wild type (C,D) and *svp* mutants (E-F). In wild type, EGFP is clearly observed in the six thoracic Ap4/FMRFa neurons. In *svp*, EGFP is completely lost. Genotypes: (C,D) *w^{1118};;FMRFa-EGFP, UAS-mRFP*. (E-F) *w^{1118};;FMRFa-EGFP, UAS-mRFP, svp^{11C115}*.

conducted a forward genetic screen, looking for genes affecting an *FMRFa-EGFP* reporter (C.U. and S.T., unpublished). One mutant identified in this screen, *11C115*, was mapped by deficiency mapping to the *svp* gene (Fig. 1C-F). Several *svp* allelic combinations all displayed a near complete loss of FMRFa expression (see below). For this study we have used *svp^{1}*, which is a strong (amorph) EMS allele (Hiromi et al., 1993; Kerber et al., 1998), and our novel *svp^{11C115}* allele, which also acts as a strong allele.

Seven up is expressed in two pulses in the NB5-6T lineage

Previous studies revealed that Svp is expressed in two pulses during VNC development (Kanai et al., 2005; Kohwi et al., 2011). We also noted a distinct biphasic appearance of Svp expression in the VNC (not shown). In line with previous studies, we found that *svp* mutants displayed an early failure to downregulate Hb in general in the VNC (Fig. 2A,B). In the NB5-6T specifically, we also observed two distinct windows of Svp expression (Fig. 3). The early pulse of Svp expression commences at stage 10, and lasts until stage 11 (Fig. 3A-C). This brief pulse of Svp expression correlates well with a role for *svp* in downregulating Hb, and indeed, we found that *svp* mutants often displayed a failure to downregulate Hb also in the NB5-6T (Fig. 2C,D). However, the failure to downregulate Hb was only partially penetrant, and 64% of hemisegments did not show persistent Hb expression (Fig. 2E).

The second pulse of Svp expression commenced at stage 14 in the neuroblast, and was dynamic during subsequent stages (Fig. 3G-M). At late stage 14, Svp expression was observed in the

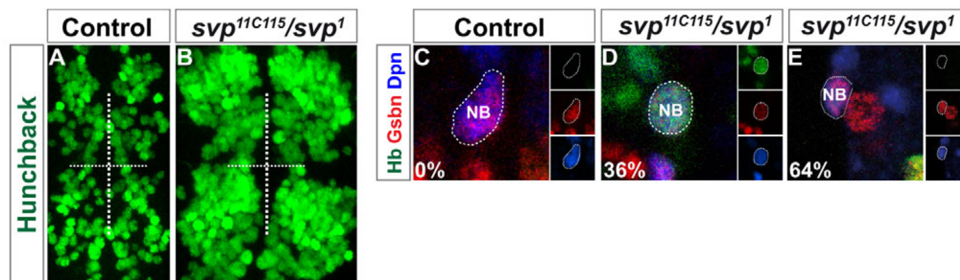

Fig. 2. *seven up* is crucial for downregulating Hunchback in the VNC and in NB5-6T. (**A**,**B**) Hb expression at stage 14, in control (A) and *svp* mutants (B). *svp* mutants show a failure to downregulate Hb in the VNC. (**C-E**) Staining for Hb in NB5-6T at stage 14 in control (C) and *svp* mutants (D-E). The NB5-6T is identified as the anterior- and lateralmost neuroblast within the *gsb^{lacZ}* domain (red), as well as by cell size and staining for Deadpan (blue). At stage 14, Hb is not detected in the control embryo. However, in *svp* mutants, Hb is still present in 36% of hemisegments (D), while being absent in 64% (E). Genotypes: (A,C) *OregonR*. (B,D,E) *svp^{1}/svp^{11C115}*.

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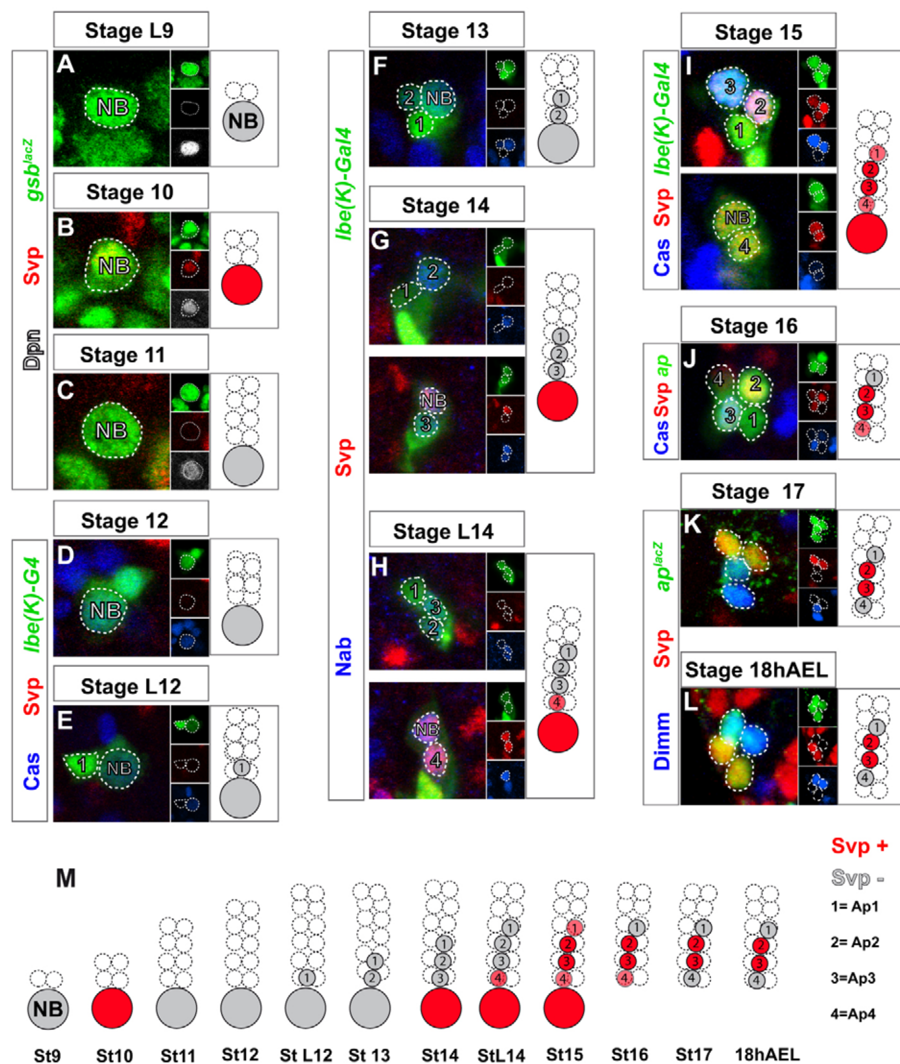

Fig. 3. Seven up is expressed in two distinct windows of NB5-6T development. The expression of Svp (red) within NB5-6T during embryonic development. To the right are side-view graphic representations of the lineage. In G-I, images are composed from confocal stacks subdivided into two sub-stacks, from dorsal (up) to ventral (down). Anterior is up in all images. (**A-C**) NB5-6T is identified as the anterior- and lateralmost neuroblast within the *gsb*^{lacZ} domain (green), as well as by cell size and staining for Deadpan (white) (A-C). (**D-I**) Alternatively, NB5-6T is identified by reporter gene expression driven from NB5-6-specific lbe(K) enhancer. Ap1, Ap2, Ap3 and Ap4 neurons are identified by different levels of Nab or Cas (blue) and Col (not shown) (Baumgardt et al., 2009). (**J-L**) During later stages, Ap1, Ap2, Ap3 and Ap4 neurons are identified by expression of *ap*^{lacZ} (green), different levels of Cas staining (J; blue), Dimm (K and L; blue) and Col (not shown) (Baumgardt et al., 2009). The early pulse of Svp expression commences at stage 10 (B), but is rapidly lost, and not detectable in the neuroblast until late stage 14 (G), when the late pulse of Svp expression commences. The second pulse is dynamic; first observed in the newly born Ap4/FMRFa neuron and at stage 15 in all four Ap neurons (I). From stage 16, Svp is no longer evident in Ap1 (J), and at stage 17 is also lost from Ap4 (K). However, Ap2 and Ap3 express Svp until the end of the embryogenesis (L). (**M**) Cartoon depicting the expression of Svp in the NB5-6T lineage. Genotypes: (A-C) *gsb*^{lacZ}/+; (D-I) *lbe*(*K*)*-Gal4*, *lbe*(*K*)*-Gal4*/*UAS-nmEGFP*; (J-L) *ap*^{lacZ}/+.

newly born Ap4 cell and at stage 15 in all four Ap neurons. At stage 16, expression was lost from Ap1, and at stage 17 from Ap4, whereas Ap2 and Ap3 maintained Svp expression until end of embryogenesis (Fig. 3G-M). Thus the second pulse of Svp expression fits well with a role in controlling Ap neuron specification.

seven up mutants display defects in Apterous neuron differentiation

Mutations in *svp* resulted in a complete loss of *FMRFa-EGFP* expression (Fig. 1E,F). To further validate this finding, we analysed expression of the propeptide for the FMRFa neuropeptide itself, and observed a complete loss also of FMRFa in the lateral thoracic areas (Fig. 4B,F). By contrast, the anterior SE2 FMRFa neurons, which are generated by a different neuroblast, displayed an increase in cell numbers (Fig. 4B,F) (Losada-Perez et al., 2010). To address whether or not Ap neurons were generated in *svp* mutants, we used the Eyes absent (Eya) marker, a selective marker for Ap neurons at late embryonic stages (Miguel-Aliaga et al., 2004). In *svp* mutants, we found two very different outcomes. In one subset of hemisegments, we found a complete loss of Eya expression (29%; *n*=102 hemisegments) (Fig. 4A,E). By contrast, in another subset of hemisegments, we found that Ap neurons were indeed generated in *svp* mutants, and in fact we observed a prevalent increase in Ap neuron numbers, from 4 to ~6 cells (71%; *n*=102 hemisegments; Fig. 4E,P). These numbers correlate well with the number of mutant hemisegments where downregulation of Hb has not occurred properly (Fig. 2D,E).

Focusing on the hemisegments where Ap neurons could be identified by their Eya expression, we analysed expression of FMRFa and Nplp1 and found that they were also strongly affected in Eya-expressing hemisegments, but in opposite ways: FMRFa was typically absent whereas Nplp1 was often ectopically expressed (Fig. 4I,J,L,M,P). Next, we analysed expression of the Dimmed (Dimm) basic-helix-loop-helix transcription factor, a key regulator of the general neuropeptide cell identity, which is normally expressed in both the Ap1/Nplp1 and Ap4/FMRFa neuropeptide neurons (Allan et al., 2005; Baumgardt et al., 2007; Hewes et al., 2003; Park et al., 2004). In Eya-expressing hemisegments, we found that *svp* mutants displayed ectopic Dimm expression (Fig. 4K,N,P).

We found that *svp* mutants display two separate effects in the NB5-6T lineage. At early stages, the downregulation of the first temporal factor, Hb, often fails. At later stages, this failure of proper temporal progression leads to a failure to specify Ap neurons, as evident by the loss of Eya expression in one-third of hemisegments. However, in the other two-thirds of hemisegments,

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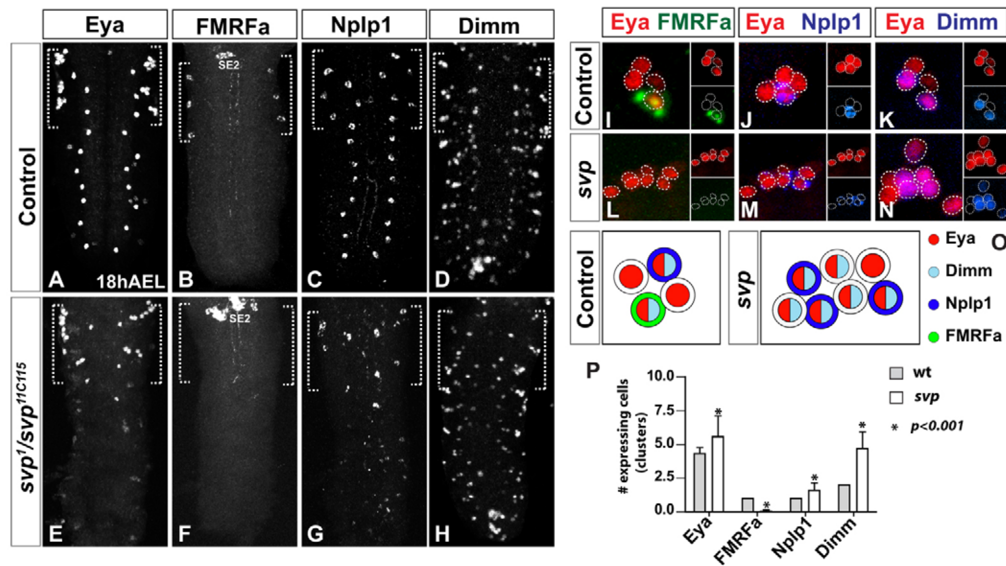

Fig. 4. *seven up* is critical for Apterous neuron specification. (**A-N**) Expression of the Ap cluster determinants Eya and Dimm, and of the terminal identity markers FMRFa and Nplp1, in control and *svp* mutants: stage 18hAEL embryonic VNCs (anterior up; brackets outlining the three thoracic segments). (**O**,**P**) The observed phenotypes are summarized in the cartoon (O) and in a graphical representation of the quantified results (P) [$n \geq 10$ VNC in all genotypes; asterisks indicate significant difference compared with control (Student's *t*-test, $P<0.001$)]. (A,E) Expression of Eya reveals that the Ap cluster is not generated in some thoracic hemisegments in *svp* mutants. However, in the cases in which the cluster is born, it displays an increase in the number of cells when compared with control (E,L-P). Staining against FMRFa shows a complete loss in *svp* mutants (B,F,L,O,P) in Ap4/FMRFa neurons (brackets). By contrast, FMRFa expression in the more anterior and medial SE2 neurons, generated by a different neuroblast (Losada-Perez et al., 2010), is not lost in *svp,* but rather upregulated in additional cells (B,F). Nplp1 is observed in additional cells in the Ap clusters, as revealed by proNplp1 staining (G,M,O,P). Expression of Dimm reveals ectopic Dimm-positive neurons within the Ap cluster (N-P). Genotypes: (A-D,I-K) *OregonR*; (E-H,L-N) svp^{1}/svp^{11C115}.

Ap neurons are indeed generated. But there are three apparent phenotypes at these later stages: we often observe one to two extra Ap neurons; there are extra Ap1/Nplp1 neurons; and there is a loss of the Ap4/FMRFa cell fate (Fig. 4O).

seven up positively and negatively controls Apterous neuron determinants

To address the role of *svp* in the Ap window in more detail, we analysed expression of a number of other genes crucial for proper Ap neuron specification. These included the temporal genes *cas* and *grh*, and the sub-temporal genes *sqz* and *nab* (Baumgardt et al., 2009; Terriente Felix et al., 2007). We observed a weak effect upon Cas expression, with a small numerical loss (Fig. 5A,G,M). Grh expression was somewhat weaker but was numerically unaffected (Fig. 5B,H,M). Thus both of these temporal genes are still expressed in *svp* mutants. Of the sub-temporal factors, Sqz was largely unaffected, although it was expressed in an occasional extra cell (Fig. 5C,I,M). By contrast, Nab was completely lost from Ap neurons in *svp* (Fig. 5D,J,M).

Next, we analysed the expression of the Ap neuron determinant Col, which has a dynamic expression pattern in the NB5-6T lineage (Baumgardt et al., 2009). In wild type, Col is initially expressed in all four newly born Ap neurons, and it plays a critical role in activating Ap and Eya. This leads to transient specification of a generic Ap neuron fate. Subsequently, there is a crucial downregulation of Col during stage 17-18hAEL in the Ap2, Ap3 and Ap4/FMRFa neurons, whereas expression of Col is maintained in Ap1/Nplp1 throughout larval stages. In the Ap1/Nplp1 neurons, *col* plays a crucial multi-step feedforward role, activating Nplp1 and specifying the Ap1/Nplp1 fate (Baumgardt et al., 2007). Conversely, downregulation of Col in the Ap2, Ap3 and Ap4 cells allows for the establishment of alternate terminal cell fates (Baumgardt et al., 2009). Analysing Col expression in *svp* mutants, we found that the initial activation of Col was unaffected, and Col expression was observed in all newly born Ap neurons (Fig. 5E,K,M). However, the subsequent downregulation of Col did not occur in *svp* mutants, and Col was observed in three to four cells in *svp* embryos (Fig. 5F,L,M). Thus, the principal effects in *svp* mutants are a loss of Nab expression, and a failure to downregulate Col (Fig. 5N).

Combined with the analysis of the expression of the Nplp1 and FMRFa neuropeptides, as well as of the Dimm regulator, these findings are consistent with an expansion of the early Ap window, the Ap1/Nplp1 cell fate, in *svp* mutants. This notion is furthermore supported by the highly restricted expression of Svp: initially being expressed in all four Ap neurons, and gradually restricted to the central Ap window cells, Ap2 and Ap3.

seven up misexpression suppresses cell fates in both the early and late Apterous window

The *svp* mutant analysis suggests that *svp* acts to suppress Col in the Ap2 and Ap3 neurons at later stages of Ap neuron differentiation, thereby preventing the feedforward action of *col*, which would otherwise result in Ap1/Nplp1 terminal cell fate. In addition, the loss of FMRFa in *svp* may be interpreted as *svp* having an important role also in specifying the last-born Ap neuron cell fate, Ap4/FMRFa. However, since there is an apparent expansion of the early Ap window in *svp* mutants, the loss of FMRFa may merely reflect a secondary effect. To address these issues further, we misexpressed *svp* using a late postmitotic driver, ap^{Gal4}, a driver that commences at stage 16: i.e. after all four Ap neurons are born and have acquired their early generic cell identity

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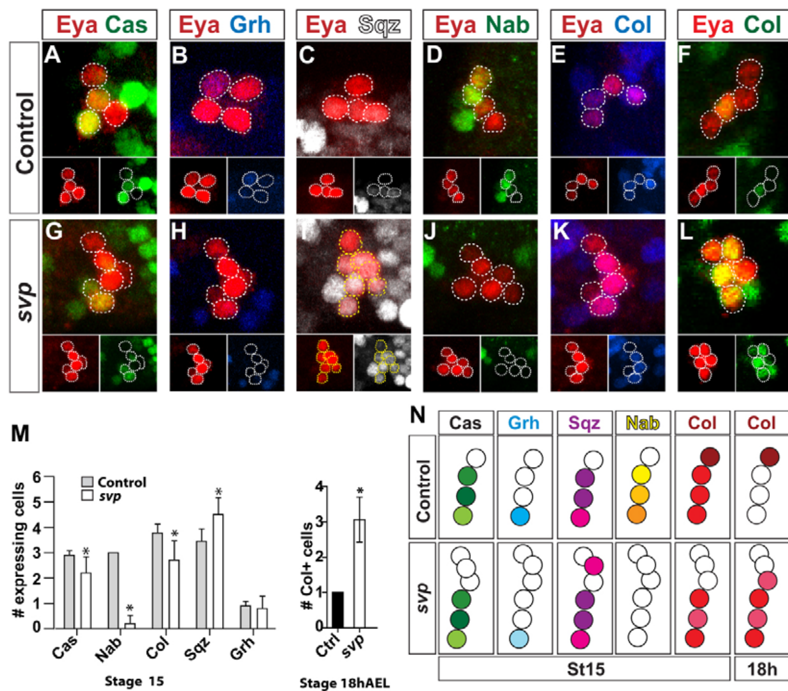

Fig. 5. *seven up* both positively and negatively controls several Apterous neuron determinants. (**A-L**) Expression of Ap neuron determinants Cas, Grh, Sqz, Nab and Col, in control and *svp* mutant thoracic hemisegments, at stage 16 (A-E,G-K) or 18hAEL (F,L). Eya was used to visualize Ap neurons. (**M**) Quantification of the observed phenotypes, at stage 15 (left) and 18hAEL (right) ($n \geq 10$ hemisegments; asterisks (*) denote significant difference compared with control (Student's *t*-test, $P<0.05$). (**N**) Cartoon summarizing the phenotypes of the quantified results. Cas is expressed in three out the four Ap neurons in control (A), and its expression appears reduced in *svp* (G,M,N). Expression of Grh is limited to one Ap neuron in both control (B) and *svp* mutant embryos, although this cell expresses weakly in the mutant Ap neurons (H). Sqz is detectable in three of the Ap neurons in control (C) and is not lost in *svp* mutants. Nab is expressed in three Ap cells in the control (D). However, Nab staining is not detectable in *svp* mutants (J). Col is detected in all neurons of the Ap cluster in control at stage 16 (E), but it is absent from some cells in *svp* mutants (K,M,N). At stage 18hAEL, Col expression is restricted to one cell in the Ap cluster in the control (F), whereas it is expressed in additional cells in *svp* mutants (L,M.N). Genotypes: (A-F) *OregonR*; (G-L) svp^{1}/svp^{11C115}.

(Allan et al., 2003). We found that misexpression of *svp* from this postmitotic driver had strong effects upon Ap1/Nplp1 neuron terminal differentiation, with a complete loss of Dimm and Nplp1 expression (Fig. 6B,D,F). We also noted a partial loss of Col expression from the Ap1/Nplp1 neuron (Fig. 6A,C,F). Strikingly however, we also observed a loss of expression of Dimm from the Ap4/FMRFa neuron, as well as loss of the FMRFa neuropeptide (Fig. 6D,F). Similar results were obtained using the *elav-Gal4* driver (Fig. 6E).

The *svp* gain-of-function phenotypes are in line with the loss-of-function phenotypes with respect to the role of *svp* in repressing the Ap1/Nplp1 cell fate. However, *svp* is also normally downregulated from the Ap4/FMRFa cells, and misexpression of *svp* led also to suppression of the Ap4/FMRFa fate.

The late expression of Seven up is controlled by the late temporal genes *castor* and *grainy head*

It is currently not known how the first pulse of Svp expression is controlled. To address the activation of Svp in the second pulse, we analysed Svp expression in the pertinent mutant backgrounds. These studies revealed crucial input from both the *cas* and *grh* temporal genes. In *cas* mutants, Svp expression was lost in NB5-6T at all embryonic stages (Fig. 7A-C,J).

By contrast, in *cas* mutants there was no apparent global loss of Svp expression in the VNC (not shown). However, the highly dynamic expression of Svp in many lineages coupled with the dynamics of Cas expression and function does not enable us to rule out a regulatory connection between *cas* and *svp* in other neuroblast lineages. In *grh* mutants, Svp expression was delayed, particularly in the neuroblast (Fig. 7D-F,J). By contrast, mutants for the *sqz* and *nab* sub-temporal genes, as well as the *col* determinant displayed no numerical loss of Svp, although there was reduced intensity of Svp expression in *nab* and *col* (Fig. 7G-K). Thus, the primary upstream regulator of Svp expression in the second pulse within NB5-6T is *cas* and *grh* (Fig. 7L).

DISCUSSION

We find that Svp is expressed in two pulses and plays two different roles in the NB5-6T lineage (Fig. 8). Initially, Svp is expressed briefly in the early part of this lineage, where it acts to control the

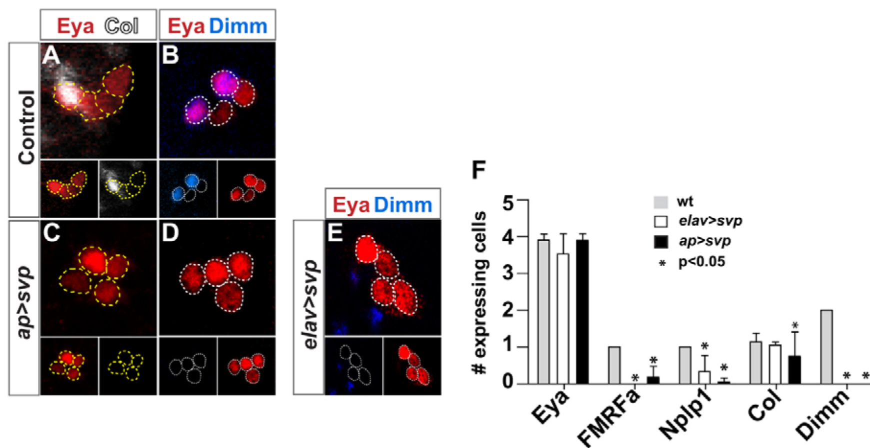

Fig. 6. Misexpression of *seven up* blocks the Ap1/Nplp1 and Ap4/FMRFa cell fates. (**A-D**) Expression of Eya and Dimm in the Ap cluster at 18hAEL in control and *svp* misexpression from the postmitotic driver ap^{Gal4}. (A) In control, one of the four Ap cluster cells (Ap1/Nplp1) expresses Col. (C) Postmitotic expression of *svp* reduces Col expression. (B) In control, the Ap1 and Ap4 neurons express Dimm. (D) In *svp* misexpression, Dimm is completely lost from both neurons. (**E**) Misexpression of *svp* from the earlier driver *elav-Gal4* results in similar suppression of Dimm. (**F**) Quantification of the observed phenotypes ($n \geq 30$ hemisegments in all genotypes; asterisks (*) denote significant difference compared with control (Student's *t*-test, $P<0.001$). Genotypes: (A-B) $ap^{Gal4}/+$; (C-D) $ap^{Gal4}/UAS\text{-}svp$.

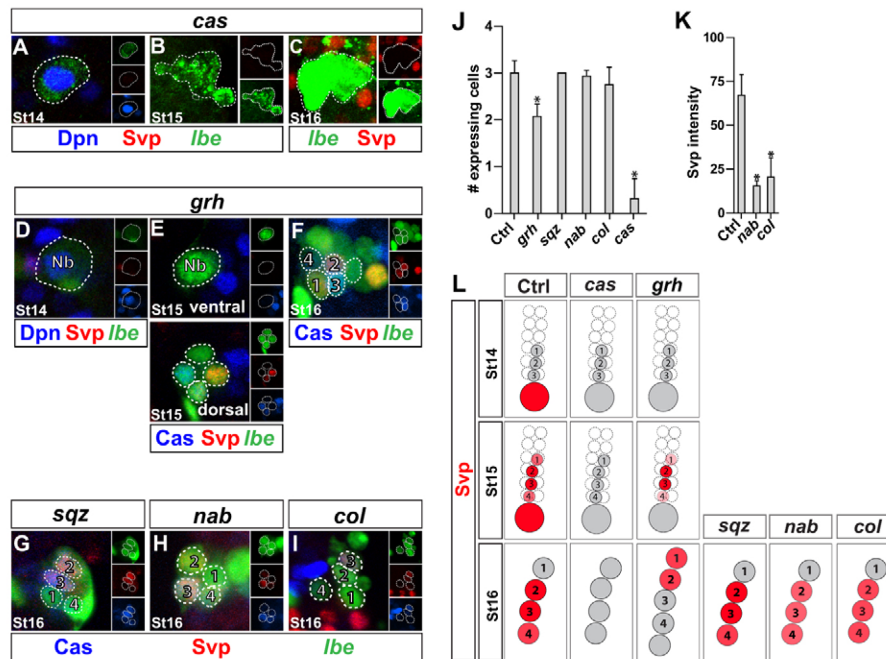

Fig. 7. The temporal genes *castor* and *grainy head* control Seven up expression during late NB5-6T lineage development. Svp expression in mutants for Ap neuron determinants. (**A-C**) In *cas* mutants, Svp expression is completely lost at all embryonic stages. (**D-F**) In *grh* mutants, the onset of Svp expression in the neuroblast at stage 14 is delayed (D-E), and is not apparent until stage 16 (F). However, expression of Svp in Ap neurons commences on schedule (E-F). (**G**) Svp expression is unaffected in *sqz* mutants. (**H**,**I**) In *nab* and *col* mutants, Svp is expressed in the proper cells, but weaker than in control. (**J**) Quantification of Svp expression ($n \geq 10$ VNC in all genotypes). Asterisks (*) denote significant difference compared with control (Student's *t*-test, $P<0.001$). (**K**) Quantification of staining intensity of Svp in control, *nab* and *col* mutants ($n \geq 10$ VNCs).The asterisk (*) denotes that staining intensity of Svp is significantly affected both in *nab* and *col* mutants (Student's *t*-test, $P<0.001$). Wild type and mutant VNCs were stained and analysed on the same slide. (**L**) Cartoon summarizing our findings. See text for details. Genotypes: (A-C) *lbe(K)-lacZ; $cas^{\Delta 1}/cas^{\Delta 3}$*; (D-F) *$grh^{IM}/grh^{Df}$, lbe(K)-Gal4/UAS-nmEGFP*; (G) *lbe(K)-Gal4/UAS-nmEGFP; sqz^{ie}/sqz^{Df2411}*; (H) *lbe(K)-Gal4/UAS-nmEGFP; nab^{SH143}/nab^{R52}*; (I) *col^{1}/col^{3}; lbe(K)-Gal4/UAS-nmEGFP.*

downregulation of the first temporal factor, Hb. Subsequently, Svp is expressed in the late part of this lineage, in the Ap window, in a highly dynamic fashion: initiated in all four Ap neurons, to be downregulated in the first- and last-born Ap cells. In the second expression phase, Svp acts to suppress Col and Dimm, thereby preventing the first-born Ap neuron fate, Ap1/Nplp1, from being established in the subsequently born Ap2 and Ap3 neurons. Misexpression studies further indicate that Svp also suppresses the last-born Ap neuron fate, Ap4/FMRFa, from being established in Ap2/3.

The early role of Seven up: a temporal 'switching factor'

Previous studies of Svp demonstrated that it is expressed in a brief pulse in the majority of early embryonic neuroblasts, where it acts to suppress Hb, thereby allowing for the switch to the next stage of temporal competence (Kanai et al., 2005; Mettler et al., 2006). Recently, studies have identified additional factors involved in the downregulation of Hb: the pipsqueak-domain proteins Distal antenna and Distal antenna-related (herein referred to collectively as 'Dan') (Kohwi et al., 2011). Dan is expressed somewhat earlier than Svp, and is also maintained in a longer pulse. *svp* and *dan* do not regulate each other, and although they can be activated by ectopic *hb* expression, neither Svp nor Dan expression is lost in *hb* mutants. This raises the intriguing questions of how Svp and Dan are activated during early stages of lineage progression, and how they become downregulated at the appropriate stage.

Another interesting complexity with respect to Svp expression and function pertains to the fact that the Hb window is of different size in different lineages. For example, in NB6-4T and NB7-3, Hb is downregulated in the neuroblast immediately after the first division (Isshiki et al., 2001; Novotny et al., 2002), whereas in NB5-6T, Hb expression is evident during three divisions (Baumgardt et al., 2009). In line with this, we do not observe Svp expression in NB5-6T until stage 10, when the neuroblast has already gone through two rounds of division (Fig. 3). How the on- and offset of Svp, and perhaps Dan, expression is matched to the specific lineage progression of each unique neuroblast lineage, to thereby allow for differing Hb window sizes, is an interesting topic for future studies.

The late role of Seven up: a 'sub-temporal' factor

We find that Svp is re-expressed in the NB5-6T lineage in a second pulse. In contrast to the early pulse of Svp expression, where there is no evidence for temporal genes controlling Svp, we do find that the second pulse of Svp expression is regulated by the temporal genes *cas* and *grh*. However, we do not find that *svp* is important for the expression of Cas or Grh. Instead, we find that *svp* participates in the sub-division of the Cas/Grh temporal window, i.e. the Ap window. Based upon the idea that Svp is regulated by temporal genes, and acts to sub-divide a broader temporal window, it could be referred to as a 'sub-temporal' factor in the latter part of the NB5-6T lineage.

The expression of Svp is dynamic also in the second pulse of expression, commencing in the neuroblast at stage 14 – after the three first Ap neurons are born – and being maintained in the

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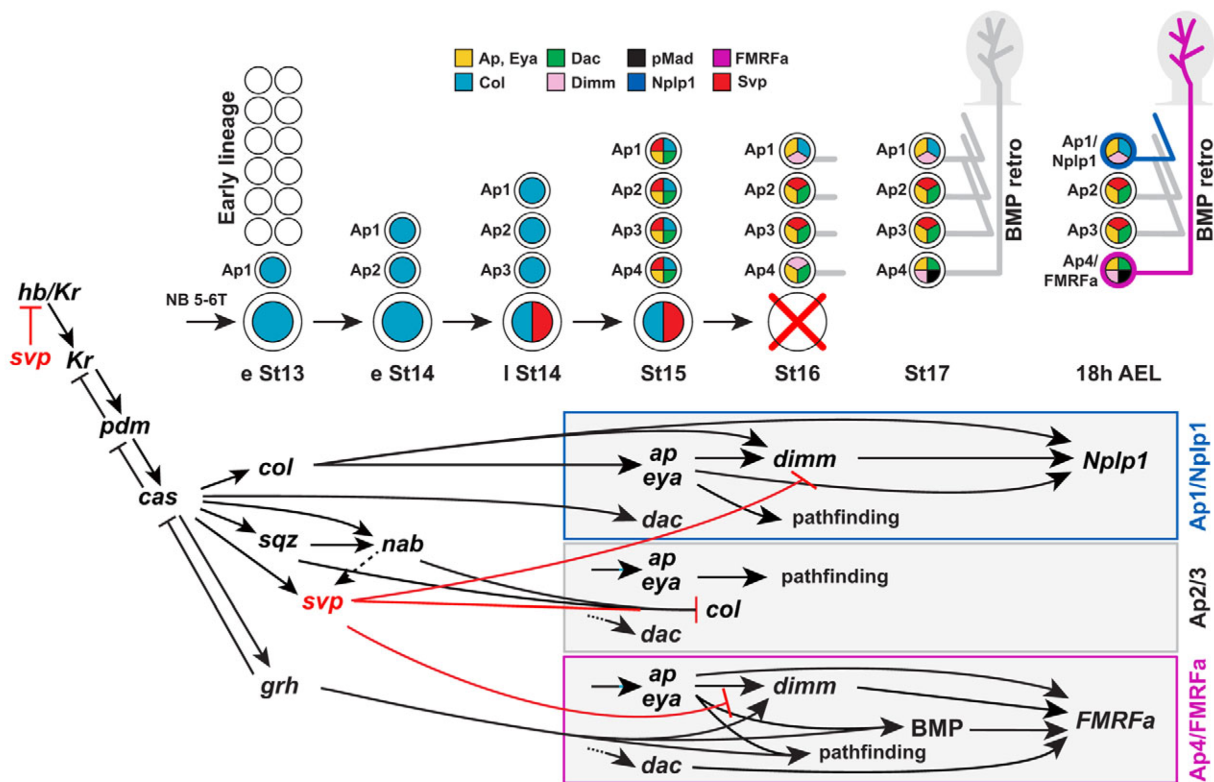

Fig. 8. Model of NB5-6 lineage development and the role of *seven up*. The NB5-6T lineage generates an early lineage of neurons and glia (middle), and undergoes the typical temporal progression during these stages. Here, Svp acts to ensure the proper downregulation of Hb (left). As the lineage progresses into the Cas/Grh window, generation of the four Ap neurons commences, with the precise orchestra of regulatory gene expression playing out in the neuroblast and in the developing Ap neurons (top). Svp (red) is re-expressed during these stages, and shows dynamic expression, being turned on in the neuroblast first, in all four Ap neurons, and downregulated and maintained only in the Ap2 and Ap3 neurons. During this second phase of Svp expression, our findings indicate that *svp* acts to ensure that the Ap2/3 cell fate is established in the middle Ap window, by suppressing Col and Dimm.

neuroblast until it exits the cell cycle at stage 15. At late stage 14 and 15, Svp expression becomes evident in all four Ap neurons, but it is rapidly downregulated from Ap1 and Ap4 during stages 16 and 17 (Fig. 3). Svp is, however, maintained in the Ap2 and Ap3 neurons into late embryogenesis. The role of *svp* in the Ap window appears to be to ensure proper specification of the Ap2/3 interneurons, generated in the middle of the Ap window. This is achieved by *svp* suppressing the first- and last-born Ap neuron fates: the Ap1/Nplp1 and Ap4/FMRFa fates. With regard to the suppression of the Ap1 fate, one important role for *svp* is to suppress Col expression in Ap2/3. Importantly, the temporal delay in Svp expression when compared to Col – commencing two stages after Col in the Ap neurons – allows for *col* to play its critical early role in Ap neuron specification: activating *ap* and *eya* (Fig. 8). The timely suppression of Col in Ap2/3 is mediated also by *sqz* and *nab* (Baumgardt et al., 2009), and the loss of Nab expression in *svp* mutants may be a contributing factor to the failure of Col downregulation in *svp*. However, the potent function of *svp* in suppressing Ap1/Nplp1 fate when misexpressed postmitotically from ap^{Gal4} does not appear to require Nab, as Nab is not ectopically expressed in these experiments (not shown). Thus, *svp* may act via several routes to prevent Ap1/Nplp1 fate from being established in the Ap2/3 cells: by suppressing Col and by activating Nab.

Regarding the second role of *svp* in the Ap window – the suppression of the Ap4/FMRFa fate – it is less clear what the target(s) may be. However, a common denominator for both the Ap1/Nplp1 and the Ap4/FMRFa neurons is the expression of Dimm. Dimm, a basic-helix-loop-helix protein, is a critical determinant of the neuropeptidergic cell fate, and also controls high-level neuropeptide expression in many neuropeptide neurons (Hewes et al., 2003; Park et al., 2008). Both *svp* loss and gain of function results in robust effects upon Dimm expression in the NB5-6T lineage, indicating that Dimm is an important target for *svp*. However, *dimm* mutants show only reduced levels of FMRFa expression (Hewes et al., 2003), and thus *svp* is likely to regulate additional targets to prevent the Ap4/FMRFa cell fate in the Ap2/3 neurons.

Another interesting phenotype in *svp* mutants, pertaining to the second pulse of Svp expression in the NB5-6T lineage, is the finding of one to two extra Ap neurons. This indicates that the NB5-6T neuroblast undergoes one to two extra rounds of division, and that the expression of Svp in the neuroblast during stage age 14-16 is important for precise cell cycle exit. Interestingly, the other temporal (*cas* and *grh*) and sub-temporal (*sqz* and *nab*) genes acting in the latter part of the NB5-6T lineage also play roles in controlling cell cycle exit. Moreover, studies of neuroblast cell cycle exit in other neuroblasts, both embryonic and postembryonic, have also shown roles for *grh* and *svp* in these decisions (Maurange et al., 2008; Tsuji et al., 2008). Thus, a picture is emerging in which late temporal and sub-temporal genes may be broadly involved in controlling timely cell cycle exit of many neuroblasts.

How global are the roles of *seven up*?

The early role of *svp*, in its first expression pulse, is to suppress Hb expression. Svp is expressed transiently by most if not all neuroblasts, and the regulation of Hb also appears to be a global event. Similarly, the second pulse of Svp expression has been observed in many lineages, although the role for *svp* in this later pulse was hitherto unknown. Our findings of a role for *svp* as a sub-temporal gene in the latter part of the NB5-6T lineage indicates that *svp* may play such roles in many lineages. However, it should be noted that we do not observe global changes in Col, Dimm and Eya expression in the embryonic central nervous system (CNS) (not shown). Thus, unlike the more universal role of *svp* in regulating Hb during the first pulse, the putative sub-temporal function of the second pulse of *svp* expression in other lineages must be highly context-dependent and involve other targets.

In mammals, the *svp* orthologues *COUP-TFI* and *–II* are expressed dynamically in the developing CNS (Qin et al., 2007; Yamaguchi et al., 2004). Functional studies reveal a number of important roles for *COUP-TFI/II* during nervous system development, and mutant mice display aberrant neuro- and gliogenesis, accompanied by axon pathfinding defects (Qiu et al., 1997; Yamaguchi et al., 2004). Intriguingly, recent studies have revealed that *COUP-TFI/II* acts in a temporal manner to control the timing of generation of sub-classes of neurons and glia in the developing mouse brain (Naka et al., 2008; Tomassy et al., 2010). Given that the other genes described in this study are also conserved, it is tempting to speculate that temporal and sub-temporal cascades similar to those outlined here are also used in the mammalian CNS during development.

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Competing interests statement

The authors declare no competing financial interests.

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