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

Jonathan Benito-Sipos¹, Carina Ulvklo², Hugo Gabilondo¹, Magnus Baumgardt², Anna Angel², Laura Torroja¹ and Stefan Thor²,*

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 subdivided into three different neuronal sub-types: the Ap1/Nplp1 and Ap4/FMRFamide 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 FMRFamide, 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...
window, and that both genes are crucial for specification of Ap neurons (Fig. 1B). ghr 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 initial 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 subtype 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 sqz. Our analysis of Svp expression demonstrates that it is expressed in two distinct pulses in the NB5-6T lineage. In the early pulse, sqz 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, sqz acts as a sub-temporal gene, by downregulating Col, thereby allowing for the establishment of alternate Ap cell fates. The second function of sqz in this lineage – acting as a sub-temporal gene – is also crucial for specification of sqz and nab. However, in contrast to sqz and nab, misexpression of sqz 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, sqz 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, sqz adds insight to the complex regulatory cascades involved in the precise 1:2:1 temporal generation of the three distinct Ap neuron subtypes 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: sqz123 (also called sqz1) (Hiromi et al., 1993); sqz125 (also called sqz2) (Mlodzik et al., 1990); sqz125Cas (this work); UAS-sqz1.12 (Kramer et al., 1995) containing sqz type 1 cDNA (Mlodzik et al., 1990); UAS-sqz1.36 (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, sqz125, sqz125Cas (Allan and Thor, 2003); ap1254 (referred to as ap1254) (O’Keefe et al., 1998); ap1258 (referred to as ap1258) (Cohen et al., 1992); gsh1155 (referred to as gsh1155) (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 Di Antonio) (Di Antonio et al., 2001); cas11 and cas31 (Mellerick et al., 1992), and UAS-Cas (Kambadur et al., 1998) (both provided by W. Odenwald, NINDS, National Institutes of Health, Bethesda, USA); ghr12 (Nusslein-Volhard et al., 1984). Df(2R)Pic7B (referred to as ghr12), col2, colf (Crozier and Vincent, 1999) (provided by A. Vincent, CNRS/Universe Paul Sabatier, Toulouse, France); nab51111, nab51112, UAS-nab (provided by F. J. Diaz-Benjumea, CSIC, Madrid, Spain) (Terriente Felix et al., 2007). Mutants were kept over CyO, Act-GFP, CyO, Df;elav-GFP, TM3, Ser, Act-GFP, CyO, twi-Gal4, UAS-GFP, TM3, Sb, Ser, twi-Gal4, UAS-GFP; or Tm6, Sb, Tb, Df;elav-GFP balancer chromosomes. As a 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. Diaz-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-Gsβn (1:10) (provided by R. Holmgren, Northwestern University, Evanston, USA); Rabbit α-Hunchback (1:1000) (provided by R. Pflanz, MPIbPC, Göttingen, Germany); Rat α-Spz (1:750) (Tsujii et al., 2008) (provided by T. Ishikita, National Institute of Genetics, Mishima, Japan); Rabbit α-β-Gal (1:5000); ICN-Cappel, Aurora, OH, US); Mouse α-myelin (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 5 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-Alia et al., 2004; Park et al., 2004). To further understand the development of this lineage and the specification of the Ap neurons, we have
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 svp1, which is a strong (amorph) EMS allele (Hiromi et al., 1993; Kerber et al., 1998), and our novel svp11C115 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. 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) w1118;;FMRFa-EGFP, UAS-mRFP. (E,F) w1118;;FMRFa-EGFP, UAS-mRFP, svp11C115.

**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 gsblacZ 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) svp1/0 svp11C115.
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 NBS-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,
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). 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 (Fig. 5B,H,M). Thus, the principal effects in *svp* mutants are a loss of *Nab* expression, and a failure to downregulate Col (Fig. 5N).

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 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. 5E,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. 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≥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*/*svp^1CI199*.
We found that misexpression ofsvp 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
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 (≥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 (≥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<sup>K</sup>3/; (D-F) grh<sup>MM</sup>/grh<sup>Df</sup>, lbe(K)-Gal4/UAS-nmEGFP; (G) lbe(K)-Gal4/UAS-nmEGFP; sqz<sup>ZDf417</sup>; (H) lbe(K)-Gal4/UAS-nmEGFP; nab<sup>Ik</sup>/nab<sup>Is2</sup>, (I) col<sup>/col</sup>; lbe(K)-Gal4/UAS-nmEGFP.

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
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 andeya (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 apColtf2 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.

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.
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 NBS-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, Dmm 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.

Competing interests statement

The authors declare no competing financial interests.

References


