Drosophila Grainyhead specifies late programmes of neural proliferation by regulating the mitotic activity and Hox-dependent apoptosis of neuroblasts

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Summary

The *Drosophila* central nervous system is generated by stem-cell-like progenitors called neuroblasts. Early in development, neuroblasts switch through a temporal series of transcription factors modulating neuronal fate according to the time of birth. At later stages, it is known that neuroblasts switch on expression of Grainyhead (Grh) and maintain it through many subsequent divisions. We report that the function of this conserved transcription factor is to specify the regionalised patterns of neurogenesis that are characteristic of postembryonic stages. In the thorax, Grh prolongs neural proliferation by maintaining a mitotically active neuroblast. In the abdomen, Grh terminates neural proliferation by regulating the competence of neuroblasts to undergo apoptosis in response to Abdominal-A expression. This study shows how a factor specific to late-stage neural progenitors can regulate the time at which neural proliferation stops, and identifies mechanisms linking it to the Hox axial patterning system.

Key words: Grainyhead, Neuroblasts, Apoptosis, Mitotic activity, Neural progenitors, *Drosophila*

Introduction

During development, the time at which a neuron or glial cell is born has an important influence on its fate. A classic example of the link between birth order and cell fate is the inside-first/outside-last order of layer formation in the cerebral cortex (Berry et al., 1964). The particular sequence of cell types generated in this, and some other neural contexts, involves individual progenitors giving rise to distinct types of neuronal and glial progeny at different times (Turner and Cepko, 1987; Wetts and Fraser, 1988; Walsh and Reid, 1995; Reid et al., 1997). Such switching or biasing of progenitor potential with developmental time is known to be influenced by extrinsic signals (McConnell and Kaznowski, 1991; Bohner et al., 1997; Cepko, 1999; Zhang and Yang, 2001). There is also evidence that intrinsic cues provided by transcription factors play important roles in temporally regulating daughter cell fates (reviewed by McConnell, 1995; Edlund and Jessell, 1999; Marquardt, 2003; Pearson and Doe, 2004). Recent evidence indicates that at least one of these factors, the winged-helix repressor Foxg1, functions by actively repressing the competence of cortical progenitors to form the earliest born neuronal types (Hanashima et al., 2004). To ensure that appropriate numbers of each neural cell type are produced, the temporal modulation of cell fates must somehow be coordinated with cell proliferation. At present, the identity of factors controlling the number of progenitor cell divisions, and their relationship to those regulating the changing potential of neural progenitors with time, are far from clear.

Recent studies in the developing central nervous system (CNS) of *Drosophila* have identified a striking example of an expression sequence of transcription factors that modulates neural progenitor potential with time (reviewed by Pearson and Doe, 2004). The progenitors involved, neuroblasts, share two properties with mammalian neural stem cells: they are multipotent and they undergo self-renewing divisions (reviewed by Doe and Bowerman, 2001; Jan and Jan, 2001; Chia and Yang, 2002; Betschinger and Knoblich, 2004). Each asymmetric neuroblast division generates a smaller progenitor, termed a ganglion mother cell (GMC), which usually divides only once to produce two neurons or glia. In the *Drosophila* embryo, dividing neuroblasts express four transcription factors in a characteristic sequence Hunchback→Kruppel→Pdm1→Castor, providing each GMC with a temporal label (Kambadur et al., 1998; Brody and Odenwald, 2000; Isshiki et al., 2001; Novotny et al., 2002; Pearson and Doe, 2003). When combined with the anteroposterior and dorsoventral positional information defining neuroblast type (reviewed by Skeath and Thor, 2003), this temporal tag confers a unique cell identity to each GMC and its progeny. Interestingly, there is also evidence that two members of the temporal transcription factor series may regulate the overall number of divisions that a neuroblast undergoes. For example, neuroblast 7-3 normally stops dividing in the embryo after it has switched to Pdm1 (Nub – FlyBase)-positive status but it can be forced to produce a much larger lineage than normal by persistently expressing either one of the 'early' factors, Hunchback or Kruppel (Isshiki et al., 2001).

The majority of neuroblasts appear to undergo the Pdm1 to

Castor transition in the embryo (Kambadur et al., 1998), and go on to divide numerous times during postembryonic stages, generating large numbers of neurons that will function in the CNS of the adult fly (reviewed by Truman et al., 1993). This raises the issue of whether there are additional temporal transcription factors expressed in neuroblasts during postembryonic stages. In vivo and cell culture studies indicate that the expression of another nuclear protein, Grainyhead (Grh), is first switched on in neuroblasts towards the end of embryonic neurogenesis and thus may follow on from Castor (Bray et al., 1989; Uv et al., 1997; Brody and Odenwald, 2000). Grh is a sequence-specific DNA-binding protein that defines a family of transcription factors conserved from *Drosophila* to mammals (Bray et al., 1989; Dynlacht, 1989; Bray and Kafatos, 1991; Attardi and Tjian, 1993; Uv et al., 1994; Uv et al., 1997; Wilanowski, 2002; Venkatesan et al., 2003). In *Drosophila*, Grh is required for several non-neural developmental processes (Bray and Kafatos, 1991; Huang, 1995; Liaw, 1995; Ostrowski et al., 2002; Hemphala et al., 2003; Lee and Adler, 2004). Although it is known that most neuroblasts continue to express a neural-specific isoform of Grh during postembryonic stages (Bray et al., 1989; Uv et al., 1997; Bello et al., 2003), a specific function in this late context has yet to be identified. Therefore, although Grh has been proposed to be a late member of the temporal transcription factor series (Brody and Odenwald, 2000; Brody and Odenwald, 2002), any supporting evidence that it temporally regulates neuronal fate and/or number has been lacking.

The developmental stage at which a postembryonic neuroblast ceases dividing is one of several crucial control points determining the overall number of progeny that it generates (reviewed by Maurange and Gould, 2005). This endpoint is highly region specific, with neuroblast divisions stopping two days later in the thorax than in the abdomen, correlating with average lineage sizes of ~100 cells for the former but only ~5 cells for the latter. Such anteroposterior differences strongly implicate the conserved axial patterning system encoded by Hox homeodomain proteins (Lewis, 1978; Wakimoto and Kaufman, 1981; McGinnis and Krumlauf, 1992; Mann and Morata, 2000). Although mechanisms linking Hox proteins to neuroblast activity have yet to be identified in most regions, in the abdomen it is known that programmed cell death plays a crucial role. In brief, neuroblasts stop dividing soon after they upregulate Abdominal-A (AbdA), which activates one or more of the three *H99* proapoptotic genes *grim*, *head involution defective* (*hid*; Wrinkled – FlyBase) and *reaper* (*rpr*), and thus triggers their death (White et al., 1994; Bello et al., 2003). Like other Hox proteins, AbdA acts in a highly context-dependent manner (Lohmann and McGinnis, 2002). However, the factors that restrict the competence to undergo this particular AbdA output, apoptosis, to neuroblasts rather than neurons and to late rather than early neuroblasts have yet to be identified.

Here, we analyse the roles of Grh during postembryonic stages and find that it regulates late region-specific patterns of neural proliferation. Characterisation of neural-specific mutations and marked neural clones lacking Grh activity demonstrate that Grh regulates the mitotic activity and apoptosis of neuroblasts. We show that the differential regulation of these neuroblast properties in the thorax and abdomen accounts for the very different proliferation endpoints in these two regions. Genetic analysis reveals that Grh plays two separable roles within abdominal neuroblasts, regulating the duration of the late-phase of AbdA expression and also the competence to respond appropriately to it. Epistasis tests show that the latter competence function of Grh in late-stage neuroblasts is essential for terminating neural proliferation in the abdomen. This study reveals, for the first time to our knowledge, a proliferation stop mechanism linking a late stage-specific neuroblast factor to the Hox axial patterning system.

Materials and methods

Fly strains

Chromosomes were obtained from the Bloomington Stock Center except for *dp, cn, grh^{B37}, bw* (Bray and Kafatos, 1991) and *b, pr, cn, grh^{370}, bw* (Uv et al., 1997). The *hs-abdA* and *UAS-p35 (BH2)* transgenes have been described previously (Hay et al., 1994; Bello et al., 2003). *grh^{370}* hemizygotes refers to *b, pr, cn, grh^{370}, bw/Df(2R)Pcl^{7B}* larvae, distinguished from their balanced siblings carrying *CyO-GFP* using fluorescence microscopy. The controls for *grh^{370}* hemizygous larvae were balanced siblings or *yellow white* larvae. In the *grh^{370}* and *hs-abdA* experiments, *yellow white* flies were used as controls. (*FRT2A, +*), (*FRT2A, Df(3L)H99*) and (*FRT 82B, +*) MARCM clones were generated as described previously (Bello et al., 2003). For this study, the following MARCM stocks were generated for chromosome arms 2R and X: *elav-GAL4^{C155}, hs-FLP; FRTG13, tubP-GAL80; UAS-nlsLacZ, UAS-mCD8::GFP* and *FRT19A, tubP-GAL80, hs-FLP; UAS-nLacZ, UAS-mCD8::GFP/CyO; tubP-Gal4/TM6B*. Two recombinant chromosomes were also constructed: *grh^{370}, hs-abdA* and *FRTG13, grh^{B37}*.

Rearing and staging of larvae

Newly hatched larvae (0 hour) were collected during a 4- or 6-hour time window and raised at 25°C at low density on standard cornmeal/yeast/agar medium supplemented with live yeast. Hence, developmental stages referred to as 48 hour, 63 hour, 72 hour and 96 hour correspond to larval age intervals of 46-50 hours, 60-66 hours, 70-74 hours and 94-98 hours, respectively. For the 104-hour time point, animals were morphologically selected at the white prepupal stage. BrdU-incorporation studies were performed as described (Truman and Bate, 1988), using 0.2 mg/ml BrdU for continuous labelling and 1 mg/ml for pulse labelling with no live yeast supplementation of the medium. As the rate of development of *grh^{370}/Df2RPcl^{7B}* larvae is more variable than that of wild type, both age and morphological criteria were used for staging (Bodenstein, 1994). For MARCM experiments, embryos of the appropriate genotype were collected on yeasted grape juice-agar plates over a 4-hour window. Heat-shock induction of FLP was at 37°C for 90 minutes, using larvae of 4-8 hours in age. For the experiments using *hs-abdA*, *grh^{370}/Df2RPcl^{7B}*, a 1-hour heatshock at 37°C was used and the control larval genotypes were y w; *hs-abdA/CyO* and *grh^{370}/Df2RPcl^{7B}*.

Immunolabelling

Larval tissues were fixed and immunostained, as previously described (Bello et al., 2003). For BrdU staining, larval tissue was treated with 2N HCl as described (Truman and Bate, 1988), or with 50 Units/ml of RQ1 RNase-free DNase (Promega) for 30 minutes at 37°C. The primary antibodies used were: rabbit anti-β-galactosidase (βgal, Cappel) 1:7000, mouse anti-βgal (Promega) 1:1000, rabbit anti-Cas (gift of W. F. Odenwald) 1:2000, rabbit anti-H3p (Upstate Biotechnology) 1:400, rat anti-AbdA (gift of J. Casanova) 1:500, mouse anti-Ubx (FP.3.38, gift of R. White) 1:20, mouse anti-Mira (Mab81, gift of F. Matsuzaki) 1:50, mouse anti-BrdU (G3G4, Developmental Studies Hybridoma Bank) 1:200, mouse anti-Grh

(BF1, gift of S. Bray) 1:3. All fluorescent images were taken using a Leica TCS SP scanning confocal microscope with a pinhole of 1 and represent projections of multiple sections unless otherwise indicated in the figure legend. Clone/lineage sizes were determined from confocal *z* stacks of sections, spaced by ~1 μm. Using ImageJ, cells were counted section-by-section and marked to avoid double counting. Sample sizes, means and standard deviations for all histograms are indicated in the text and figure legends. For other data points, only illustrated by photographic panels, $n \geqslant 4$.

Results

Grh is expressed by postembryonic neuroblasts and GMCs, but not by neurons

This study focuses on neurogenesis from 0 to 104 hours of postembryonic development. The first-instar larva (L1) hatches from the embryo at 0 hours, the L1 to second-instar larva (L2) moult occurs at ~24 hours, the L2 to third-instar larva (L3) moult at ~48 hours, and the L3-to-prepupal transition takes place at ~96 hours.

We first sought to identify all of the postembryonic neural cell types that express Grh and thus have the potential to mediate *grh* functions during late neurogenesis. Individual wild-type neuroblast clones were labelled in the thorax from 0 to 96 hours using the MARCM technique (Lee and Luo, 1999). Consistent with previous studies (Uv et al., 1997; Bello et al., 2003), we observed that Grh protein is expressed by postembryonic neuroblasts. Importantly, labelling single postembryonic clones shows clearly that Grh is never detected in postmitotic adult-specific neurons, although, surprisingly, we do observe it within the nucleus of new-born GMCs (Fig. 1A-C). The expression analysis thus indicates that the potential sites-of-action for Grh are within neural progenitors rather than their postmitotic progeny.

Neural proliferation is regulated by Grh in a segment-specific manner

To determine the function of Grh during postembryonic neurogenesis, we made use of the larval-viable grh^{370} mutation. This introduces a premature stop codon into an alternatively spliced exon such that, although it is not a null allele, it selectively inactivates the Grh protein isoforms that are CNS specific (Uv et al., 1997). We used larvae transheterozygous for grh^{370} and a chromosomal deficiency for *grh* (hereafter termed grh^{370} hemizygotes), and compared these with balanced sibling controls. Phosphorylated Histone-H3 (H3p) labelling at mid-L3 (78 hours) indicates that the frequency of M-phase within the CNS is reduced within the thorax by ~40% but, within the central abdomen, we observe a very different effect, with mitoses continuing past 72 hours, the time at which they would normally cease (Fig. 2A-C and data not shown).

For the thoracic CNS, the basis of reduced proliferation was investigated further by BrdU pulse-labelling cells passing through S-phase from 72-78 hours (Fig. 2D-G). This revealed that grh^{370} hemizygotes display a small but significant reduction in the overall number of BrdU-positive neuroblast lineages in the thorax (Fig. 2D-F). As there is also a small reduction in the total number of Mira-positive neuroblasts within the thorax by this stage (Fig. 2F), we conclude that a limited subset of thoracic neuroblasts are missing by 72 hours, and that most, if not all, of those remaining have divided during the 72- to 78-hour time window. Each BrdU-positive cluster represents one neuroblast lineage that, during the 6-hour pulse, generates an average of 7.1 cells in wild-type larvae (n=52 clusters, s.d.=2.1, Fig. 2G). This average is reduced to 5.3 cells in grh^{370} hemizygotes (n=57 clusters, s.d.=2.4), equating to an increase in asymmetric cell cycle length from ~100 to ~135 minutes (Fig. 2G). Thus, Grh activity promotes the high amount of neural proliferation that is characteristic of the wild-type thorax by two routes. First, it is required for the full complement of neuroblasts during L3, and second, it directly or indirectly promotes the high mitotic activity characteristic of thoracic neuroblast lineages.

In the abdominal CNS, BrdU-pulse labelling showed that cells in grh^{370} hemizygotes remain actively engaged in the cell cycle after the 72-hour time point when they would normally stop dividing (Fig. 2D,E). This observation is consistent with the previous H3p staining. We then labelled all neural cells born during the postembryonic phase using continuous BrdU treatment from 0 hours onwards. At 96 hours, grh^{370} hemizygous larvae display three abdominal rows of labelled lineages corresponding to the positions of the ventromedial (vm), ventrolateral

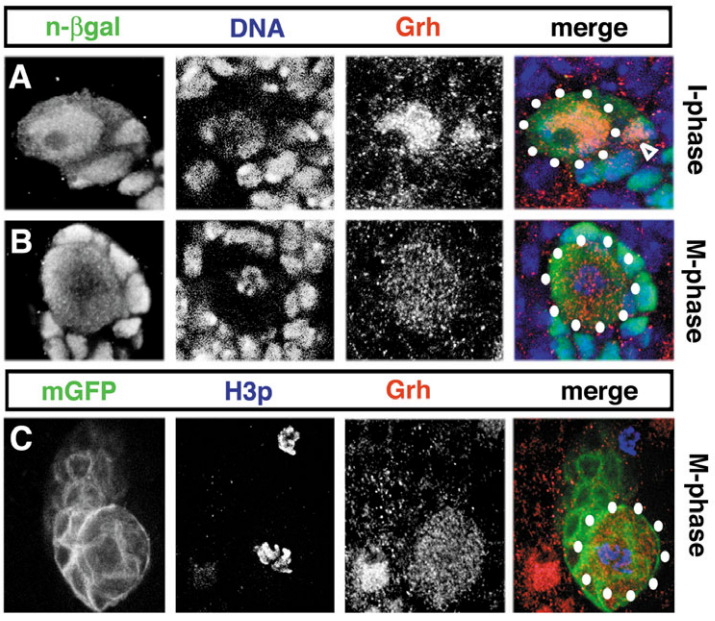

Fig. 1. Neural Grh expression is restricted to progenitors and its distribution is cell-cycle dependent. (A-C) Wild-type thoracic MARCM clones marked in green with nuclear β-galactosidase (n-βgal) or membrane-tethered GFP (mGFP) under *tubulin-GAL4* control. Expression of Grh (red) is observed in neuroblasts (large dotted circles) and GMCs (arrowhead), but not in adult-specific neurons. (A) In interphase (I-phase) neuroblasts, Grh is excluded from the nuclelus and co-localizes with DNA (blue) in a crescent. (B,C) In mitotic (M-phase) neuroblasts, Grh is evenly distributed throughout the cell and does not specifically co-localise with DNA (blue in B) or phosphorylated histone H3 (H3p, blue in C), and is evenly distributed throughout the cell. In this and all subsequent figures, the neuroblast is indicated by a dotted circle.

(vl) and dorsolateral (dl) wild-type neuroblast lineages (Truman and Bate, 1988). Combined cell counts of the vm and vl lineages show that both are larger than the wild-type average count of four cells (72 hours: n=19 lineages, mean=3.9, s.d.=1.5; 96 hours: n=18, mean=4.2, s.d.=1.5), containing an average of 7.4 cells by 72 hours (n=14, s.d.=2.4) and 13.7 cells by 96 hours (n=18, s.d.=2.4, Fig. 3A-F; see Fig. S1 in the supplementary material). Although part of this 3.5-fold increase in vm/vl lineage size is likely to involve increased cell cycle speed, this is difficult to quantitate as the corresponding neuroblasts in *grh*^{370} hemizygotes also prematurely exit from the period of non-proliferation (quiescence) that separates embryonic from postembryonic neurogenesis (data not shown). Interestingly, *grh*^{370} hemizygotes also generate some supernumerary postembryonic lineages that occupy ectopic positions outside the vm, vl and dl rows, often located close to the midline (Fig. 3B, see Discussion). Together, these abdominal labelling studies demonstrate that *grh* is required for timely cessation of postembryonic neurogenesis. In its absence, mitotic activity persists for at least 24 hours longer than normal and, for the vm/vl lineages, we showed that this is associated with a 3.5-fold increase in cell number.

In summary, these genetic and expression analyses show that Grh is not only expressed in postembryonic neural progenitors but that it also regulates their proliferation. Grh acts in a pro-proliferative manner in the thorax but is anti-proliferative in the abdomen. As it is similarly expressed in both segment types, we infer that at least some of its downstream targets are likely to be regulated in a segment-specific manner.

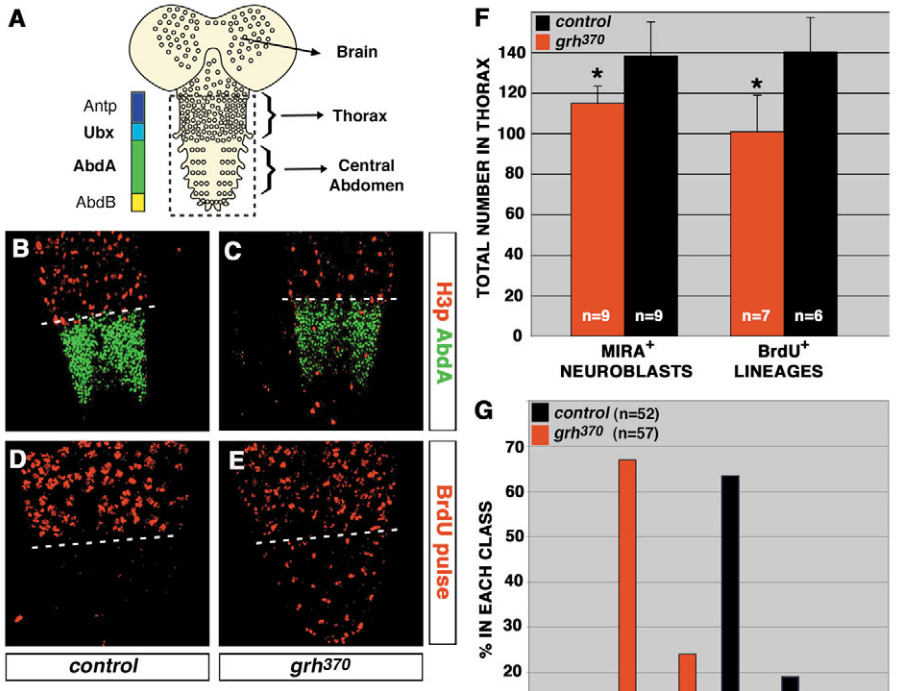

Fig. 2. Grh regulates neural proliferation in a segment-specific manner. (A) Cartoon showing the distribution of neuroblasts (circles) in the postembryonic CNS; the thorax and central abdomen are boxed. The major domains of expression of the Hox genes Antennapedia (Antp, blue), Ultrabithorax (Ubx, cyan), Abdominal-A (AbdA, green) and Abdominal-B (AbdB, yellow) are shown. Anterior is to the top in panels A-E. (B-E) Single confocal sections of the thoracic (above dotted line) and abdominal (below dotted line) regions of the CNS isolated from 78-hour larvae. (B,C) H3p/AbdA labelling of balanced control (B) and *grh*^{370} hemizygous (C) larvae showing that loss-of-*grh* activity reduces the mitotic index in the thorax but leads to ectopic mitoses in the abdomen. (D,E) 72-78-hour BrdU-pulse labelling of wild-type control (D) and *grh*^{370} hemizygous (E) larvae showing that removing neural *grh* function leads to fewer thoracic cells progressing through S-phase but that numerous ectopic S-phases occur in the abdomen. (F) Histogram showing the total thoracic number of Miranda-positive cells at 72 hours (Mira^{+} neuroblasts), or lineages incorporating BrdU from 72-78 hours (BrdU^{+} lineages). In both cases, a reduction (star indicates significance at P=0.002) is observed in *grh*^{370} hemizygotes (for Mira: n=9 larvae, mean=114.6, s.d.=8.6; for BrdU: n=7, mean=100.7, s.d.=18) relative to balanced siblings (for Mira: n=9, mean=138.1, s.d.=16.8; for BrdU: n=6, mean=140.1, s.d.=16.9). (G) Histogram showing that the number of cells per thoracic lineage incorporating BrdU during the 72- to 78-hour window is higher for wild-type control larvae (n=52 lineages, mean=7.1, s.d.=2.1) than for *grh*^{370} hemizygotes (n=57, mean=5.3, s.d.=2.4).

Grh maintains the mitotic activity of thoracic neuroblasts

We next identified which type of neural progenitor is affected by the pro-proliferative function of Grh in the thorax. *grh* loss-of-function MARCM clones were induced at 0 hours and, to ensure that no residual zygotic Grh activity remained, we used the *grh*^{B37} null allele that disrupts all isoforms of Grh (Bray and Kafatos, 1991; Uv et al., 1997). Consistent with the previous analysis of *grh*^{370} hemizygous larvae, thoracic *grh*^{B37} clones display a reduction in clone size that is already apparent at 72 hours (Fig. 4A,B). By 96 hours, the mean size of *grh*^{B37} clones is 28.6 cells (n=43 clones, s.d.=12.7), approximately half the corresponding value of 57.3 cells for wild-type clones (n=61, s.d.=15.7, Fig. 4C,D). At 72 hours, ~40% of *grh*^{B37} mutant clones contain a single large cell that expresses the neuroblast marker Miranda (Mira, data not shown), but, whereas 37% of wild-type neuroblasts (n=56) are H3p-positive, none of the *grh*^{B37} neuroblasts analysed (n=51) were observed in M phase (Fig. 4A,B). As this *grh*^{B37} null phenotype is stronger than the reduced neuroblast number and frequency of mitoses observed in 72-78 hour *grh*^{370} larvae, it is likely that the *grh*^{370} allele retains a low-level of neural Grh activity. Importantly, the large but inactive neuroblast observed in *grh*^{B37} clones at 72 hours is completely absent by 96 hours (n=43 clones, Fig. 4C). Together, the *grh*^{370} and *grh*^{B37} analyses indicate that the pro-proliferative role of Grh in the thorax is mediated, at least in part, by maintaining the neuroblast in a mitotically active state.

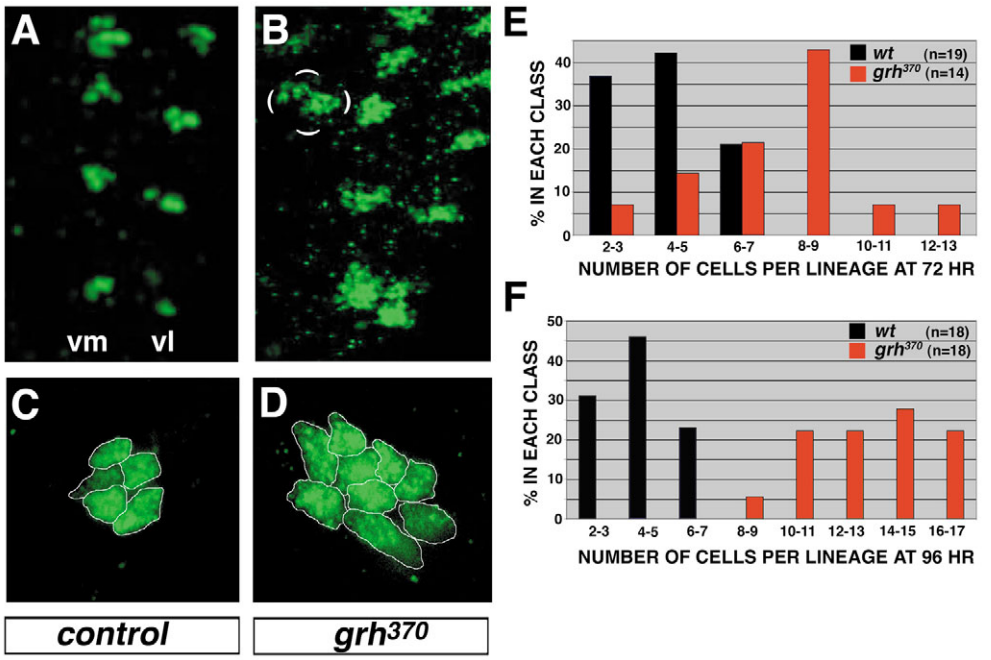

Fig. 3. Abdominal neuroblast lineages lacking Grh activity proliferate for longer than normal. (A,B) Four to five abdominal segments labelled by continuous BrdU treatment from 0-96 hours, showing rows of vm and vl clusters from wild-type control larvae (A) and corresponding but enlarged clusters in grh^{370} hemizygotes (B). The hemizygotes also show BrdU-positive clusters in ectopic abdominal locations (outlined). (C,D) Confocal projections of one vl cluster containing six cells from a balanced control larva (C) and part of one vm cluster, where nine out of a total of 14 cells are shown from a grh^{370} hemizygous larva (D; see also Fig. S1 in the supplementary material). BrdU-labelled nuclei are outlined in white. (E,F) Histograms comparing the number of cells generated per vm/vl lineage (from 0 hours onwards) in wild-type larvae and grh^{370} hemizygous larvae at 72 hours (E) and 96 hours (F). Average lineage size remains at four cells from 72-96 hours in wild-type larvae (72 hours: n=19 lineages, mean=3.9, s.d.=1.5; 96 hours: n=18, mean=4.2, s.d.=1.5) but in grh^{370} hemizygotes it increases from 7.5 to 14 cells (72 hours: n=14, mean=7.4, s.d.=2.4; 96 hours: n=18, mean=13.7, s.d.=2.4).

Grh does not repress Ubx expression in thoracic neuroblasts

The different *grh* phenotypes in the thorax and abdomen indicate that *grh* must be acting upstream, downstream or in a parallel pathway to the Hox axial patterning system (see the Introduction). As Grh is expressed similarly by postembryonic neuroblasts in the thorax and abdomen, it is unlikely to be a downstream target of Hox proteins in this context. In the case of neuroblasts in the thorax, it is also unlikely that Hox proteins act in parallel with Grh, as neither Antp nor Ubx is expressed in neuroblasts at the relevant stages, and the average size of thoracic neuroblast clones lacking the larval functions of *Ubx*, or both *Ubx* and *abdA*, is not significantly affected (Bello et al., 2003). Moreover, Ubx and Antp misexpression experiments indicate that thoracic neuroblasts must avoid expressing thoracic Hox proteins if they are to continue dividing during normal postembryonic development (Bello et al., 2003). To explore the remaining potential genetic relationship within the thorax, namely that Grh acts upstream of Hox genes, we focused on the posterior thorax, where Ubx is expressed in a subset of adult-specific neurons (Bello et al., 2003). A time course of expression within marked wild-type postembryonic lineages shows that Ubx is activated in adult-specific neurons at late stages and in a progressive manner.

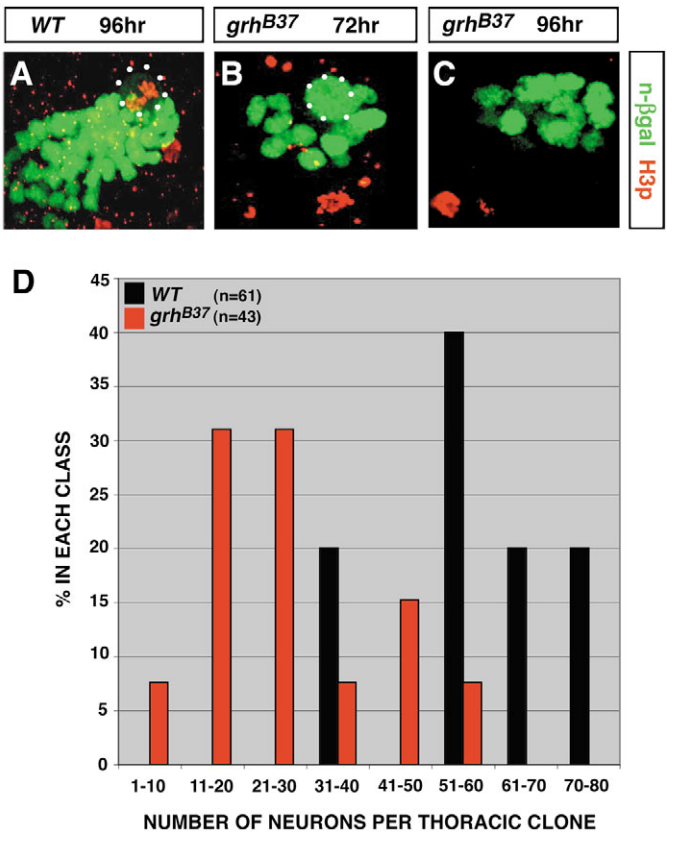

Fig. 4. Grh is required to maintain a large mitotically active neuroblast at late stages. (A-C) Single thoracic MARCM clones marked with n-βgal driven by *elav-GAL4* and labelled for M-phase with H3p. Wild-type clones contain many neurons and the large neuroblast frequently undergoes mitosis at 96 hours (A), whereas grh^{B37} homozygous clones contain less neurons and the neuroblast ceases mitosis by 72 hours (B) and is no longer evident by 96 hours (C). (D) Histogram showing that the average number of adult-specific thoracic neurons at 96 hours in grh^{B37} clones (n=43 clones, mean=28.6, s.d.=12.7) is approximately half the wild-type value (n=61, mean=57.3, s.d.=15.7).

Beginning at ~72 hours, activation sweeps from the early-born postembryonic neurons to their later-born siblings, and, by 104 hours, Ubx is expressed in many adult-specific neurons in most lineages of the posterior thorax (Fig. 5A-D). Importantly, however, Ubx remains excluded from neuroblasts and GMCs at 96 hours and 104 hours, indicating that the late-larval upregulation is restricted to postmitotic progeny. This pattern is complementary to that of Grh and we note that the *grh* phenotype mimics the neuroblast loss obtained by misexpressing Ubx within thoracic neuroblasts (Bello et al., 2003). We therefore tested whether Grh is required to repress Ubx expression in neuroblasts. However, this appears not to be the case, as Ubx expression remains excluded from the mitotically inactive neuroblast of grh^{B37} clones at 72 hours (Fig. 5E). Moreover, Grh activity is not essential for Ubx activation in at least some adult-specific neurons at 96 hours and 104 hours (Fig. 5F,G). The results thus far strongly suggest that, although Grh plays a thorax-specific function in maintaining the mitotic activity of neuroblasts, this is independent of a cell-intrinsic input from thoracic Hox genes.

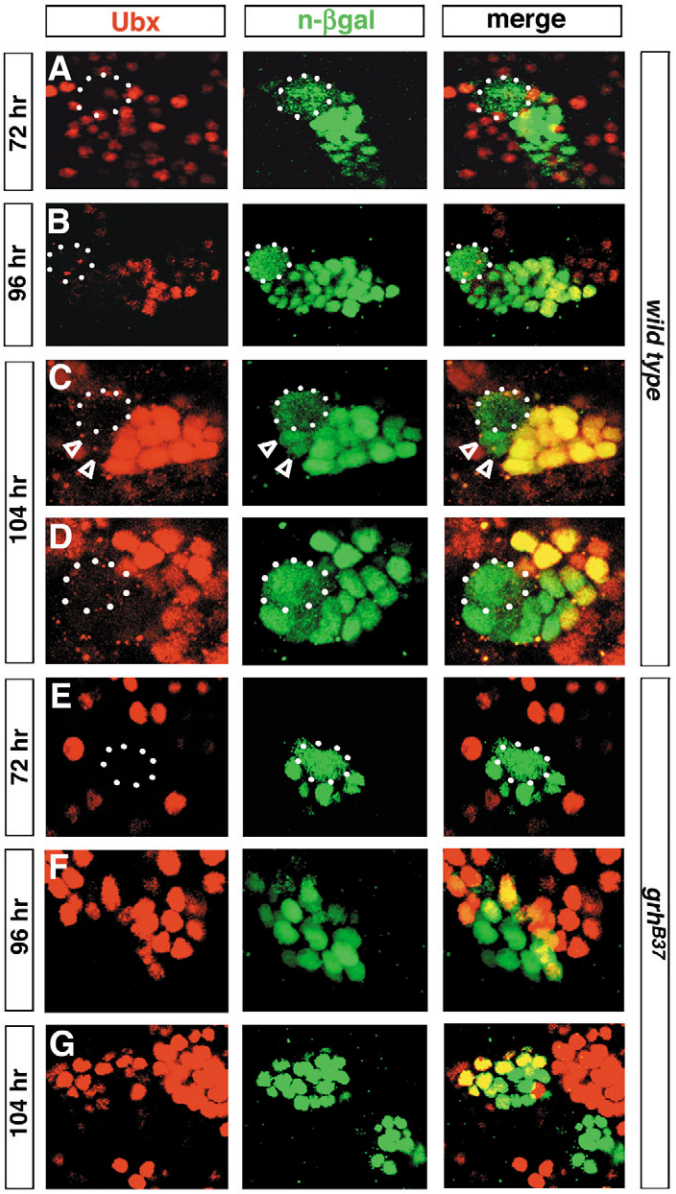

Fig. 5. Repression of Ubx in postembryonic neuroblasts does not require larval Grh activity. All panels show the pattern of Ubx expression in MARCM neuroblast clones in the posterior thorax (n-βgal driven by *elav-GAL4*) at the stages indicated on the left. (A-D) Wild-type clones do not express Ubx in the neuroblast or GMCs at any of the stages shown, but it is progressively upregulated within adult-specific neurons. At 72 hours, few if any cells are Ubx positive (in A, projected sections produce false overlap), by 96 hours early-born adult-specific neurons become Ubx positive (B), and, by 104 hours, the majority of neurons in many of the lineages become Ubx positive, but not the GMCs (arrowheads) or neuroblasts (C,D). (E-G) grh^{B37} mutant clones do not express Ubx in the neuroblast or adult-specifc neurons at 72 hours (E). By 96 (F) and 104 hours (G), although clone size is small and the large neuroblast is absent, Ubx is upregulated in a subset of adult-specific neurons in some but not all clones (in G, two clones are shown).

Grh is required for the maintenance of active thoracic neuroblasts

We have argued that the mechanism leading to cessation of mitotic activity and subsequent loss of the neuroblast in grh^{B37} thoracic clones is likely to be independent of Hox proteins. In principle it could involve a non-self-renewing division, entry into a quiescent state or programmed cell death. To test the latter possibility, $elav\text{-}GAL4^{C155}$, a pan-neural driver expressed in postembryonic neuroblasts and their progeny (Lin and Goodman, 1994; Bello et al., 2003), was used to express the P35 inhibitor of cell-death effector caspases (Hay et al., 1994) within grh^{B37} MARCM clones. Such grh^{B37}; *UAS-p35* clones contain a large neuroblast at 96 hours in over 40% of cases (n=31 clones), compared with 0% in grh^{B37} clones lacking P35 expression (n=43, Fig. 6A,B,D). However, the rescued neuroblast often appears somewhat larger than normal and it fails to generate a wild-type clone size (Fig. 6B-D). Residual caspase activity is likely to underlie the failure to rescue completely the presence of grh^{B37} neuroblasts, as control experiments indicate that the apoptotic pathway in neuroblasts is less efficiently blocked by the *UAS-p35* transgene than by loss of *H99* proapoptotic genes (data not shown). We do not yet know why those 40% of grh^{B37}; *UAS-p35* neuroblasts that are rescued still fail to generate a normal clone size: perhaps incomplete suppression of caspase activity is incompatible with normal cell-cycle progression, or it may be that *grh* has a more direct input into the cell cycle that is independent of caspases. Either way, the *UAS-p35* experiments suggest that the disappearance of *grh* mutant neuroblasts in the thorax is via apoptosis. Together with the previous thoracic experiments, these results demonstrate clearly that Grh stimulates neural proliferation in the thorax by promoting the continued mitotic activity of the neuroblast at late stages.

Abdominal neuroblasts remain Grh-positive until their last asymmetric division

We next addressed the mechanism-of-action of Grh in abdominal neural lineages. The previous finding that Grh may promote survival of neuroblasts in the thorax, raises the possibility that neuroblast death in the abdomen might involve a transition from Grh-positive to Grh-negative status at mid-L3. In apparent support of this notion, we previously observed that Grh expression ceases to be detectable in all three

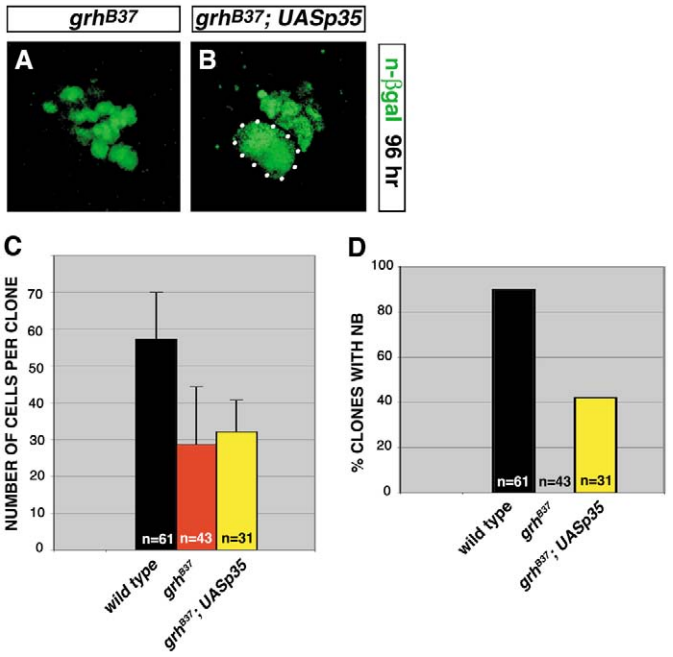

Fig. 6. Grh may prevent apoptosis of thoracic neuroblasts at late stages. (A,B) Single confocal sections of grh^{B37} (A) and grh^{B37}; *UAS-P35* (B) MARCM clones, showing that inhibiting cell-death caspases rescues a large neuroblast at 96 hours. (C) Histogram showing MARCM clone size for wild type (with *elav-GAL4*, mean=57.3, *n*=61, s.d.=12.7), grh^{B37} (with *elav-GAL4*, *n*=43, mean=28.6, s.d.=15.7) and grh^{B37}; *UAS-P35* (with *elav-GAL4*, *n*=31, mean=32, s.d.=8.6). Although *elav-GAL4* driving the *UAS-P35* transgene rescues the neuroblast in grh^{B37} clones at 96 hours, it does not significantly increase clone size. (D) Histogram showing the percentage of MARCM clones containing a large neuroblast at 96 hours (sample sizes and genotypes are as in C). *elav-GAL4* driving the *UAS-p35* transgene rescues the presence of a large neuroblast in >40% grh^{B37} clones.

abdominal lineages just prior to the appearance of TUNEL labelling (Bello et al., 2003). To resolve whether abdominal neuroblasts switch off Grh expression prior to apoptosis, we generated abdominal neuroblast clones deficient for *H99* proapoptotic genes. These continue to divide for at least 24 hours after they would normally have died (Bello et al., 2003) and we now find that, during this 'extra time', they remain Grh-positive (Fig. 7A). This indicates that levels of Grh protein become downregulated only as a consequence of activation of the cell death pathway. Importantly, these results rule out the possibility that a late switch from Grh-positive to Grh-negative status provides the trigger for initiating *H99* proapoptotic gene activity and subsequent neuroblast apoptosis.

Neuroblast apoptosis in the abdomen requires Grh

To determine the cellular basis for extended neural proliferation in the abdomen of grh^{370} hemizygotes, we visualised neuroblasts at late stages using Mira labelling. We find that balanced control larvae no longer possess neuroblasts within the AbdA domain by 96 hours (Fig. 7B). This result is consistent with previous studies and reflects the earlier burst of neuroblast apoptosis at ~72 hours (Bello et al., 2003). By contrast, grh^{370} hemizygotes display numerous persistent neuroblasts within the AbdA domain at 96 hours (Fig. 7C). Consistent with the previous BrdU-labelling experiments, many persistent neuroblasts fall within the expected vm, vl and dl positions, but we also find some ectopic Mira-positive progenitors. The failure of abdominal neuroblasts to undergo apoptosis at ~72 hours in *grh* mutants mimics the phenotype of *H99* clones (Bello et al., 2003) and indicates that Grh is required for timely abdominal neuroblast apoptosis. Our earlier finding that clone size increases from 72 hours to 96 hours, strongly suggests that persistent grh^{370} neuroblasts remain active from mid L3 to late L3 and that their supernumerary divisions account, at least in part, for the extended period of neural proliferation. As with persistent *H99* neuroblasts, the fate of grh^{370} neuroblasts in the abdomen during pupal and adult stages is not yet clear.

Grh is required for maintenance, but not initial activation, of AbdA neuroblast expression

Persistence of mitotically active abdominal neuroblasts in grh^{370} hemizygotes after 72 hours not only mimics the *H99* phenotype but also that associated with loss of function of *abdA* (Bello et al., 2003). This raises the possibility that *grh* might regulate the L3 phase of AbdA expression that is required to trigger neuroblast apoptosis (Bello et al., 2003). We first determined the temporal relationship between wild-type AbdA expression and the onset of neuroblast apoptosis. Abdominal neuroblasts become TUNEL-positive at ~72 hours (Bello et al., 2003), and, at this stage, we find that they still continue to express AbdA despite Mira expression now being weak and punctate (Fig. 7D). This abnormal Mira pattern suggests that AbdA expression continues until after the first stages of neuroblast apoptosis have been initiated. We estimate that AbdA expression is maintained for ~9 hours after the previously described expression at 63 hours (Bello et al., 2003), but, given the method of larval staging (see Materials and methods), the precise duration could lie anywhere within a 4- to 14-hour window. Blocking cell death in *H99* clones further supports the notion that there is no downregulation of AbdA prior to apoptosis, as, in this artificial context, many abdominal neuroblasts continue expressing AbdA at 96 hours (Fig. 7E). AbdA expression is also observed in a subset of neurons positioned close to the neuroblast, consistent with expression being maintained in the late subset of progeny generated after the neuroblast first becomes AbdA positive. These results reveal that wild-type AbdA expression, once switched on in neuroblasts, is not downregulated prior to the onset of apoptosis.

We next analysed the time course of AbdA expression in grh^{370} hemizygotes. Mira/AbdA double labelling reveals that AbdA is expressed in interphase neuroblasts at 63 hours, as normal (Fig. 7F). Also similar to in wild type, AbdA is expressed in M-phase (H3p-positive) neuroblasts at this time (Fig. 7G). However, whereas AbdA expression is maintained until 96 hours in *H99* neuroblasts, this is not the case for grh^{370} hemizygous larvae at 96 hours (Fig. 7H). We conclude that *grh* is not essential for initiating the late larval phase of AbdA expression in neuroblasts, but that it is required to maintain it.

Grh provides the competence for neuroblasts to undergo AbdA-dependent apoptosis

To test whether rescuing the AbdA maintenance deficit in

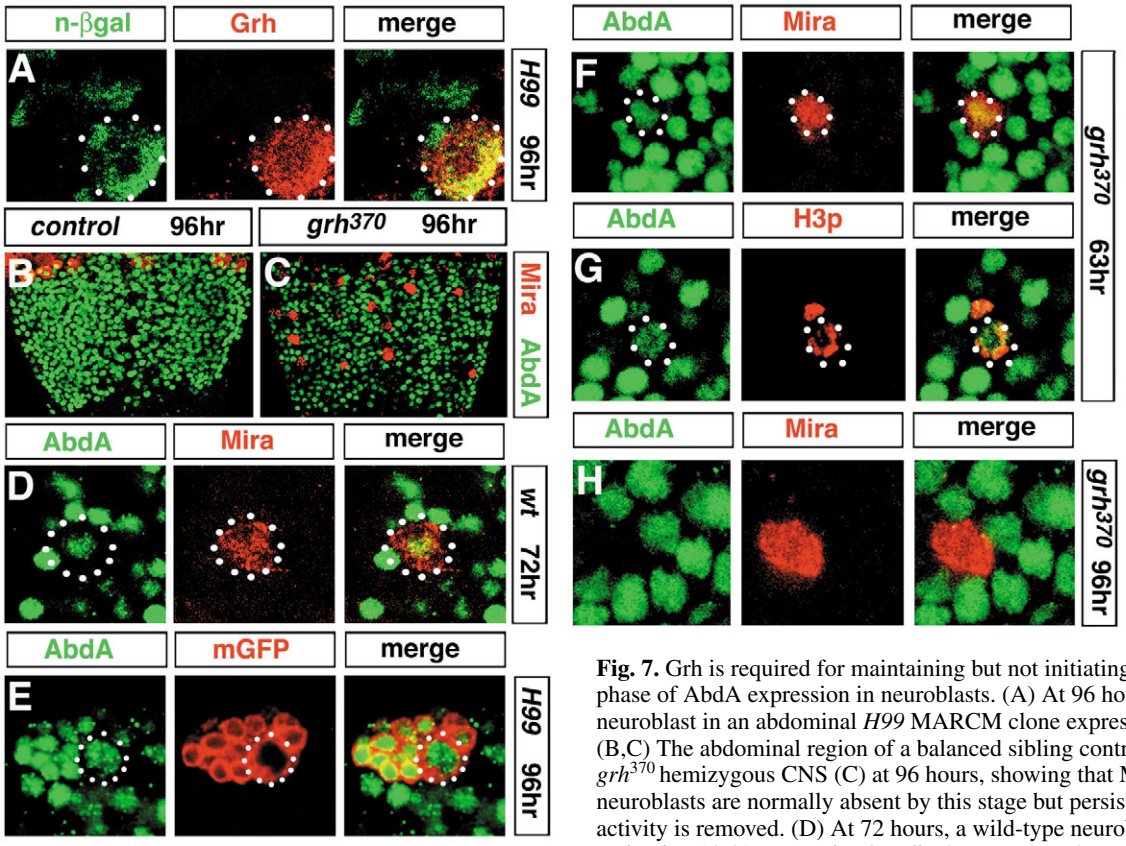

Fig. 7. Grh is required for maintaining but not initiating the late phase of AbdA expression in neuroblasts. (A) At 96 hours, a neuroblast in an abdominal *H99* MARCM clone expresses Grh. (B,C) The abdominal region of a balanced sibling control (B) and grh^{370} hemizygous CNS (C) at 96 hours, showing that Mira-positive neuroblasts are normally absent by this stage but persist when Grh activity is removed. (D) At 72 hours, a wild-type neuroblast maintains AbdA expression but displays weak and punctate Mira labelling, suggestive of the early stages of apoptosis. (E) At 96 hours, a neuroblast and its last-born progeny in an abdominal *H99* MARCM clone maintain AbdA expression. (F,G) At 63 hours, Mira-positive interphase (F) and H3p-positive M-phase (G) neuroblasts in grh^{370} hemizygotes express AbdA as normal. (H) At 96 hours, a Mira-positive neuroblast from a grh^{370} hemizygous CNS fails to maintain AbdA expression.

grh^{370} hemizygotes would be sufficient to restore neuroblast apoptosis, we made use of an *hs-abdA* transgene. In the first set of experiments, *hs-abdA* activity was transiently induced at the 63-hour time point to prolong the endogenous phase of AbdA expression in grh^{370} hemizygotes. However, we failed to observe any abdominal neuroblast apoptosis (data not shown). In a second series of experiments, *hs-abdA* expression was induced at 48 hours, a protocol known to provide sufficient AbdA activity to stop all abdominal neuroblast divisions prematurely (Bello et al., 2003). In a wild-type background, we find that this early-induction protocol leads to sustained ectopic AbdA expression and the associated elimination of all Mira-positive neuroblasts by 72 hours (Fig. 8A,B). In sharp contrast, neuroblasts in a grh^{370} hemizygous background are resistant to the induction of apoptosis by *hs-abdA* (Fig. 8C). Strikingly, they continue to express AbdA for at least 24 hours after heat-shock induction, yet are not eliminated by programmed cell death (Fig. 8D). Together, the abdominal experiments demonstrate that Grh is a terminal neuroblast factor that restricts postembryonic lineage size by promoting the proapoptotic subfunction of AbdA in two distinct ways. First, it regulates the duration of the AbdA burst, and, second, it installs the competence to undergo an apoptotic response to it. The grh^{370}, *hs-abdA* epistasis tests clearly separate these two Grh activities by showing that, although AbdA maintenance may be necessary, it is not sufficient and, importantly, that AbdA competence is essential for neuroblast apoptosis.

Discussion

Grh regulates the proliferative properties of late-stage neuroblasts

This study has shown that a conserved transcription factor, Grh, defines proliferative properties of neural progenitors that are characteristic of late developmental stages. It has segment-specific activity that accounts for the highly regionalised patterns of postembryonic neural proliferation. It is also possible that Grh, acting within the neuroblast or the GMC, regulates the fates of adult-specific neurons, but this is difficult to resolve as few suitable markers are available. It is thus not yet clear whether this study has identified a component of a new early-late system dedicated to regulating neuroblast proliferation, or whether Grh is an additional member of the Hunchback→Kruppel→Pdm1→Castor series regulating both proliferation and neuronal fate. Either way, we have identified multiple molecular mechanisms acting downstream of Grh and shown how these enable it to regulate late neuroblast activity in a segment-specific manner, despite being expressed in a pan-neuroblast pattern.

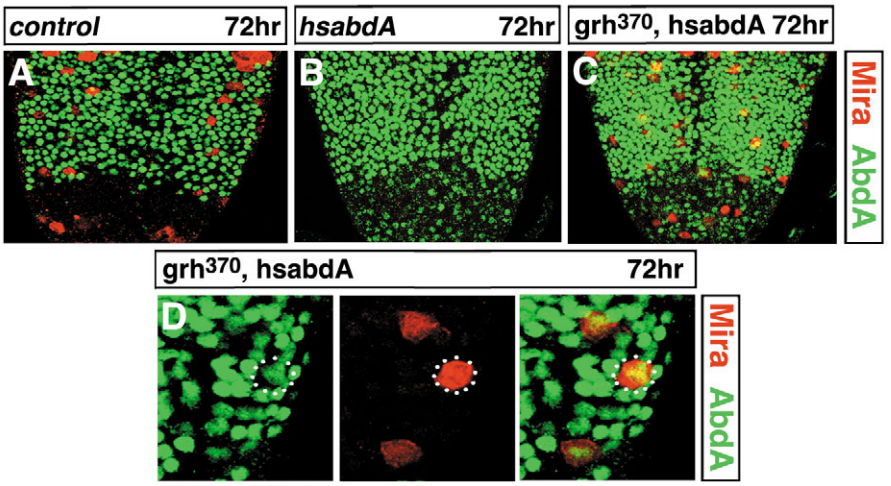

Fig. 8. *grh* mutant neuroblasts are insensitive to AbdA-induced apoptosis. (A-D) Abdominal region of balanced control (A), *hs-abdA* (B) and grh^{370}, *hs-abdA* (C) larvae that received a heat shock at 48 hours and were labelled for Mira and AbdA at 72 hours. Ectopic AbdA expression from the *hs-abdA* transgene persists for at least 24 hours after heat shock and is observed posterior to the endogenous AbdA domain. The exogenous AbdA activity is sufficient to eliminate all Mira-positive neuroblasts from the abdomen of *hs-abdA* but not grh^{370}, *hs-abdA* larvae. (D) A single confocal section of a grh^{370}, *hs-abdA* larva showing three central abdominal neuroblasts that have expressed AbdA for 24 hours after heat-shock induction but have remained resistant to apoptosis.

Thoracic neuroblasts require Grh for mitotic activity at late stages

Thoracic neuroblasts normally continue dividing into pupal stages, stopping at ~120 hours, by which time ~100 adult-specific neurons have been generated (Truman and Bate, 1988). By compromising *grh* function, we observed that neurogenesis ceases two days prematurely, at ~72 hours. This limits the average size of neuroblast clones to ~30 cells, indicating that Grh is required to generate 70% of all adult-specific neurons in the thorax.

We provided four lines of evidence that the underlying basis for premature cessation of thoracic proliferation in *grh* mutant clones is reduced mitotic activity of the neuroblast, most probably followed by Hox-independent apoptosis. First, although *grh* mutant neuroblasts are present at 72 hours they are mitotically inactive. Second, by 96 hours, no recognisable *grh* mutant neuroblasts remain. Third, inhibiting cell-death effector caspases by misexpressing P35 rescues the loss of *grh* mutant neuroblasts. And fourth, although misexpression of Hox proteins in thoracic neuroblasts induces apoptosis (Bello et al., 2003), Ubx, the resident Hox protein of the posterior thorax, remains excluded from *grh* mutant neuroblasts at 72 hours. Importantly, the role of Grh in maintaining mitotically-active neuroblasts is not a general 'housekeeping' function but is specific for their age. Thus, wild-type neuroblasts in the early embryo are Grh-negative yet viable and actively dividing. This observation suggests that the late switch to Grh-dependency involves additional factors. These could be intrinsic to the neuroblast or provided by a glial-cell niche (reviewed by Maurange and Gould, 2005). Consistent with the niche idea, neuroblast divisions within the postembryonic brain require DE-cadherin-dependent interactions between glia and neural cells (Dumstrei et al., 2003).

Grh is required to stop abdominal neuroblast divisions

In the central abdomen, we previously found that, at 72 hours, many neuroblasts downregulate Grh and become TUNEL positive (Bello et al., 2003). In the present study, when the neuroblast death pathway was blocked in *H99* clones, Grh expression continued in mitotically active neuroblasts long after the 72-hour stage. This indicates that abdominal neuroblasts remain in Grh-positive mode during their final division and that Grh is only downregulated after the onset of apoptosis. Moreover, we showed that loss of Grh activity leads to the failure of neuroblasts to undergo apoptosis. As these persistent neuroblasts not only survive but also remain actively engaged in the cell cycle, they generate a 3.5-fold excess of cells within each abdominal neuroblast lineage. Together, these findings identify Grh as a terminal neuroblast factor that is an essential component of the abdomen-specific 'stop' programme.

Grh installs the competence for AbdA-dependent neuroblast apoptosis

Two different interactions with the Hox gene AbdA underlie the dramatic reversal of Grh function from pro-proliferative in the thorax to anti-proliferative in the abdomen. First, Grh acts upstream of AbdA to maintain its late phase of expression, and, second, it functions in parallel with AbdA to activate apoptosis. Although the functional significance of *grh*-dependent AbdA maintenance is not clear, it may be that efficient neuroblast apoptosis requires AbdA levels to remain high for a significant proportion of the interval separating initial AbdA upregulation and the TUNEL-positive stage. More definitively, we used epistasis tests to show that Grh, acting in parallel with AbdA activity, is essential for abdominal neuroblast apoptosis. Thus, when the AbdA-maintenance deficit was rescued using *hs-AbdA*, neuroblast death remained blocked. As AbdA is not required to activate neuroblast Grh expression, Grh and AbdA must work in parallel to activate apoptosis. Together with the finding that AbdA is required to activate *H99* gene activity (Bello et al., 2003), our study demonstrates that inputs from Grh and AbdA are both essential to activate proapoptotic genes and thus trigger neuroblast apoptosis. Whereas the late upregulation of AbdA provides a timing cue to schedule the onset of apoptosis, the much broader phase of Grh expression defines the period of neuroblast competence to respond appropriately to it.

The restricted temporal pattern of Grh expression ensures that competence to undergo AbdA-dependent apoptosis, rather than some other AbdA-dependent output, is only installed at late stages. Consistent with this, neuroblasts in the early embryo that are AbdA positive but Grh negative go on to

generate substantial embryonic lineages. Low levels of expression from *UAS-grh* transgenes (data not shown) make it difficult to test whether Grh is sufficient to confer apoptotic competence to these early embryonic neuroblasts. In the late embryo, however, neuroblasts have already switched on Grh, and, within the central abdomen, all but three undergo *abdA*-dependent death (Truman and Bate, 1988; Bray et al., 1989; White et al., 1994; Prokop et al., 1998). Our observation that reduced neural *grh* function leads to supernumerary postembryonic neuroblasts positioned outside the vm, vl and dl rows, raises the possibility that Grh is required for all developmentally programmed neuroblast apoptosis.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/17/3835/DC1

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