Specification of *Drosophila* aCC motoneuron identity by a genetic cascade involving *even-skipped*, *grain* and *zfh1*

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During nervous system development, combinatorial codes of regulators act to specify different neuronal subclasses. However, within any given subclass, there exists a further refinement, apparent in *Drosophila* and *C. elegans* at single-cell resolution. The mechanisms that act to specify final and unique neuronal cell fates are still unclear. In the *Drosophila* embryo, one well-studied motoneuron subclass, the intersegmental motor nerve (ISN), consists of seven unique motoneurons. Specification of the ISN subclass is dependent upon both *even-skipped* (*eve*) and the *zfh1* zinc-finger homeobox gene. We find that ISN motoneurons also express the GATA transcription factor Grain, and *grn* mutants display motor axon pathfinding defects. Although these three regulators are expressed by all ISN motoneurons, these genes act in an *eve*→*grn*→*zfh1* genetic cascade unique to one of the ISN motoneurons, the aCC. Our results demonstrate that the specification of a unique neuron, within a given subclass, can be governed by a unique regulatory cascade of subclass determinants.

**KEY WORDS:** Axon pathfinding, Even-skipped, Grain, Neuronal fate specification, Combinatorial code, *Drosophila*

**INTRODUCTION**

During the past decade, motoneuron specification has been intensely studied and work from both invertebrates and vertebrates has shown that motoneuron subclass identity is determined by combinatorial transcription factor codes (Briscoe and Ericson, 2001; Shirasaki and Pfaff, 2002; Thor and Thomas, 2002). However, how individual identities, within a related pool of motoneurons, are determined is still not understood. In the abdomen of the developing *Drosophila* embryo, reiterated sets of ~80 motoneurons are generated in each segment of the ventral nerve cord (VNC). These motoneurons project along distinct nerves to innervate peripheral target muscle fields and, based upon their peripheral axonal projections, they are typically grouped into six well-defined classes (Landgraf et al., 1997). The motor nerve innervating the dorsal-most muscle field, the intersegmental nerve (ISN), contains axons from seven well-defined motoneurons; the aCC, RP2 and the five U motoneurons, each with identities, within a related pool of motoneurons, are determined is dependent upon both *even-skipped* (*eve*) and the *zfh1* zinc-finger homeobox gene. We find that ISN motoneurons also express the GATA transcription factor Grain, and *grn* mutants display motor axon pathfinding defects. Although these three regulators are expressed by all ISN motoneurons, these genes act in an *eve*→*grn*→*zfh1* genetic cascade unique to one of the ISN motoneurons, the aCC. Our results demonstrate that the specification of a unique neuron, within a given subclass, can be governed by a unique regulatory cascade of subclass determinants.

**MATERIALS AND METHODS**

**Drosophila stocks**

The *grn* mutant allele *l(3)05930* was identified in a survey of the BDGP *lacZ* collection (Spradling et al., 1999) for lines with restricted expression pattern in the embryonic VNC. *grn*GAL4 was generated by P element conversion of *grn*mut*, as previously described (St. Pierre et al., 2002). For *grn* mutant analysis, *grn* and *grn*mut* (Brown and Castelli-Gair Hombría, 2000) were placed over deficiency *Df(3R)dsx3*, and both allelic combinations showed the same pathfinding phenotype and no detectable *Grn* expression (not shown). For *grn* misexpression and rescue experiments, we used *UAS-grn#2* (Brown and Castelli-Gair Hombría, 2000). *UAS-mEGFP* is a c-myc epitope-tagged membrane-targeted EGF reporter line (Allan et al., 2003). Other lines used were: *islet-α-myc-EGFP* (S.T., unpublished); *RN2-GALE, CQ2-GAL4, Df(2R)eve,ARP2A/Cyo.P[wg-lacZ];RN2-GAL4, UAS-rlacZ, Df(2R)eve/Cyo.P[wg-lacZ];ARP2B* (Fujikawa et al., 2003); *UAS-eve* and *eve*GAL4 (Landgraf et al., 1999), *zfh1*, *zfh1*mut*, *zfh1*mut*26* alleles were obtained from R. Lehmann and *UAS-zfh1* from the Bloomington stock center. *Hb9GAL4, Hb9KK30, UAS-vnd, man*GAL4*+, spdcGAL4* were provided by J. B. Skeath and H. T. Brothier. *UAS-NotchICD* was obtained from S. Artavanis-Tsakonas.

**Quantification of pathfinding phenotypes**

ISN motor axonal projections were scored at embryonic stage 16/17 in A2-A6 abdominal hemisegments using anti-Fasciclin 2, *RN2-GAL4/UAS-mEGFP* or *CQ2-GAL4/UAS-mEGFP*. Phalloidin-Texas Red (Molecular Probes) was used to visualize the musculature.

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Antibody production and staining of embryos

grn cDNA encoding amino acids 1-166 was cloned into pGEX-2T (Amersham) for protein expression and purification (J. Castelli-Gair Hombria, unpublished). Fusion protein was used to immunize rabbits and rats (Covance). Grn antibodies were used at 1:200 and their specificity was verified by the absence of staining in grn mutants. Immunolabeling was carried out as previously described (Thor et al., 1999). The following antibodies were used: α-c-Myc 9E10 (1:50), α-Fas2 1D4 (1:50), α-Eve skipped 2B8 (1:5) and α-β-gal 40-1a (1:10) (all from Developmental Studies Hybridoma Bank). Rabbit α-β-gal (Cappel; 1:5,000), rabbit α-pMad (Tanimoto et al., 2000) (1:2,000), rabbit α-Zh1 (Van Doren et al., 2003) (1:5,000), rabbit α-Hb9 (Briohier and Skeath, 2002) (1:500) and rabbit α-Vnd (Shao et al., 2002) (1:1,000). Double-labeled images were false colored for the benefit of color-blind readers. Prior to use, the polyclonal α-β-gal, -pMad, -Hb9, -Vnd and -Grn antibodies were pre-absorbed against early-stage wild-type embryos.

RESULTS

grain is expressed in subsets of developing motoneurons and interneurons

To identify genes controlling motoneuron specification, we analyzed the expression patterns of a number of lacZ enhancer trap lines, surveying for lines with expression in the embryonic VNC (see Materials and methods). One line that showed restricted expression in subsets of cells in the VNC is an insertion in the grain (grn) gene. grn encodes a GATA transcription factor previously shown to control cell rearrangements in the developing leg imaginal disc and in the posterior spiracle (Brown and Castelli-Gair Hombria, 2000).

Previous studies revealed that grn expression commences at the cellular blastoderm stage, and rapidly becomes localized to the dorsal part of the embryo, being most prominent in the procephalic region. From stage 11, expression is evident in the posterior spiracles, in the midgut and in a patch of cells in the lateral ectoderm (Brown and Castelli-Gair Hombria, 2000). From stage 11, expression is evident in the posterior spiracle (Brown and Castelli-Gair Hombria, 2000).

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To determine if grn plays a role in ISN motor axon pathfinding, we analyzed motor axon projections in grn mutants. In Drosophila embryos, motor axonal projections are stereotyped and can be revealed using an antibody directed against the surface molecule Fasciclin 2 (Fas2) (Victor et al., 1993). The aCC and U1 motor axons are known to innervate the dorsalmost muscles 1 and 9, respectively, while the RP2 and U2 motor axons innervate the dorsal muscles 2 and 10 respectively (Fig. 1J) (Jacobs and Goodman, 1989; Johansen et al., 1989; Landgraf et al., 1997). Fas2 reveals the high reproducibility of these projections in the wild-type embryo (Victor et al., 1993) (Fig. 2A; 100% innervation, n=96; throughout the text, n refers to the numbers of hemisegments counted). In grn mutants, we find that the ISN motor axons are stalled at muscles 2/10, leading to a near complete loss of innervation of the dorsal-most muscles 1/9 (12% innervation; n=136) (Fig. 2B). To better resolve the grn pathfinding phenotype we used both an aCC/RP2-specific and a U-specific GAL4 driver line (RN2-GAL4 and CQ2-GAL4, respectively) (Fujioka et al., 2003; Landgraf et al., 2003) and expressed a membrane targeted EGFP reporter (UAS-mEGFPF) (Allan et al., 2003). In the wild type, RN2-GAL4/UAS-mEGFPF clearly visualizes the peripheral projections of aCC and RP2 onto muscles 1 and 2 (arrow and arrowhead, respectively, in Fig. 2D), as well as their terminal processes (Fig. 2G). In grn mutants, muscle 2 is innervated with near wild-type frequency, but, by contrast, muscle 1 is innervated in only 15% of hemisegments (n=146) (Fig. 2E,K). Using CQ2-GAL4/UAS-mEGFPF in grn mutants, we observed a similar phenotype – apparently normal innervation of muscles 2/10 but only 18% muscles 1/9 innervation (n=88) (Fig. 2L,K). In addition, using Fas2, RN2-GAL4 or CQ2-GAL4 as markers, we noticed aberrant projections onto muscle 8 (Fig. 2B,E,H). We quantified this phenotype using RN2-GAL4 or CQ2-GAL4, and found that whereas control embryos (RN2-GAL4/UAS-EGFPF or CQ2-GAL4/UAS-EGFPF) displayed no innervation of muscle 8 (0%; n=87 and n=72, respectively), grn mutants displayed frequent innervation of muscle 8. This phenotype was observed more often with RN2-GAL4 than with CQ2-GAL4 as marker (35%; n=140 versus 21%; n=146). In affected hemisegments, we observed a grossly normal pattern of axonal projections to the dorsal muscles 2/10 (Fig. 2B,E,H). This indicates that aCC and/or RP2, and at least one of the U motoneurons project aberrantly to muscle 8. These results show that grn is crucial for proper motor axon pathfinding of ISN motoneurons.

grain acts cell-autonomously in ISN motoneurons

Although grn is expressed in ISN motoneurons, it is also expressed in a patch of ectodermal cells in the lateral body wall that underlie the SNa muscle field, muscles 21-24 (Brown and Castelli-Gair Hombria, 2000) (not shown). In grn mutants, we observe a partially penetrant muscle patterning phenotype, evident as an imprecise insertion of muscles 21-24 into the body wall (Fig. 2A-F,I,J). Although the ISN motoneurons do not normally innervate this muscle field, it still raised the concern that the motor axon pathfinding defect observed in grn mutants may not result from a cell-autonomous role for grn in ISN motoneurons. To address this issue, we used the RN2-GAL4 and CQ2-GAL4 drivers to provide grn activity in aCC/RP2 and U1 motoneurons, respectively. We find that RN2-GAL4 efficiently rescues grn mutant axon pathfinding (100% muscle 1/9 innervation; n=88) (Fig. 2C,K). By contrast, the CQ2-GAL4 driver only partially rescued the grn phenotype; 54% of muscles 1/9 (n=132) (Fig. 2K). Together, these results show that grn acts cell-autonomously in ISN motoneurons to ensure proper axon pathfinding to the dorsal-most muscles (Fig. 2L,M).
An eve–grn–zfh1 regulatory cascade in the aCC motoneuron

eve is expressed in a small subset of transiently identified GMC (ganglion mother cell) and derived aCC, RP2 and U motoneurons. Studies show that eve is both necessary and, at least in part, sufficient for dorsal motor axon projections (Landgraf et al., 1999). Given that eve and grn show similar mutant phenotypes in dorsally projecting motoneurons, we wanted to address whether these two genes regulate each other or act at the same genetic level. As eve-null mutants display severe segmental defects, a temperature-sensitive (ts) allele (eve^ts19), was previously used to study the role of eve in motoneuron specification (Landgraf et al., 1999). However, recent studies have shown that the eve ts allele does not completely remove eve function in ISN motoneurons. Using a sophisticated strategy, Fujioka et al. have succeeded in restoring eve function in all eve-expressing cells, except in the aCC and RP2 neurons, in an otherwise eve-null background (Fujioka et al., 2003). Using this ‘composite’ eve allele, eve^mosaic (denoted eve mosaic herein), we reproduced the recently described aCC/RP2 eve-null phenotype; a failure of these two motoneurons to project out of the VNC (Fig. 3A,B,F,G). This is coupled both with ectopic expression of the Hb9 homeobox gene and loss of Grn expression within these cells. In aCC, these effects...
are highly penetrant and observed at several stages, whereas in RP2 the effects are partly penetrant at stage 12 and almost absent at stage 15 (Fig. 3C-E,H-J). However, in grn mutants, we did not observe any evidence of Eve downregulation in aCC, RP2 or U motoneurons (Fig. 5A,B,D,E; not shown). We also addressed whether grn is important for repressing Hb9 in these motoneurons, but found no evidence for ectopic expression of Hb9 in aCC (or in RP2) in grn mutants (Fig. 5G,H).

Zfh1, a Zn-finger-homeodomain protein, has been reported to be expressed in aCC and RP2, as well as in many other motoneurons (Lai et al., 1991). Recent analysis of zfh1 reveals that is indeed expressed in all identifiable motoneurons, and genetic analysis reveals that it is necessary for proper motor axon pathfinding (Layden et al., 2006). In stage 15 embryos, we find that Zfh1 expression is dependent both upon eve and grn, but only in aCC and not in RP2 (Fig. 4A-E, Fig. 5D,E). As expected, when grn function is rescued (RN2-GAL4/UAS-grn; grn), Zfh1 expression is restored in aCC (Fig. 5I). In line with the notion that eve and grn act upstream of zfh1, Eve or Grn expression is unaffected in zfh1 mutants (Fig. 5C,F).

Drosophila motoneurons depend upon a target-derived BMP signal for proper maturation (Aberle et al., 2002; Marques et al., 2002). Consistent with the failure of aCC and RP2 axons to exit the VNC in eve mosaic mutants, we observe a complete loss of pMad staining in both aCC and RP2 (0% pMad in aCC and RP2; n=32) (Fig. 4F,G), indicating that these neurons are unable to receive the peripheral BMP retrograde signal. By contrast, in grn and zfh1 mutants, where aCC and RP2 still project into the periphery, we detect wild-type staining for pMad (100% pMad in aCC and RP2; n=46 and n=48, respectively) (Fig. 5A-C). These observations indicate that in grn and zfh1, ISN motoneurons maintain a ‘generic’ motoneuronal identity and further indicate that embryonic activation of the BMP pathway does not rely on the establishment of functional contacts between motoneurons and their proper muscle targets.

Within the aCC motoneuron, we are thus able to place these three genes in an eve→grn→zfh1 regulatory cascade, with the added complexity that eve also acts to suppress Hb9. By contrast, there is only partial crossregulation between eve, grn, zfh1 and Hb9 in the RP2 motoneuron.
eve and grain play additional roles outside of the eve-grn-zfh1 regulatory cascade

Do eve and grn act solely in the eve-grn-zfh1 regulatory cascade to specify aCC motoneuron identity, or do these regulators play additional roles during aCC specification? To address this question, we attempted to rescue the motoneuron pathfinding phenotype of eve mutants with UAS-grn, and, similarly, to rescue grn mutants with UAS-zfh1 (using in both cases RN2-GAL4). First, we find that grn does not rescue the eve phenotype in aCC; a failure of aCC to project its axon out of the VNC and activate Zfh1 expression (Fig. 6A-E). Second, we find that UAS-zfh1 can only partially rescue the grn motoneuron phenotype; muscle 1/9 innervation is increased to 34% (n=136) compared with the more severe (12%) grn mutant phenotype (Fig. 2F,K).

The dMP2 peptidergic neurons project posteriorly in the VNC (Hidalgo and Brand, 1997) and exit the VNC to innervate the hindgut (Miguel-Aliaga and Thor, 2004). dMP2 neurons do not express Eve, Grn or Zfh1 (Fig. 6F; not shown). Recent studies show that misexpression of zfh1 in dMP2 neurons can potently trigger lateral axonal exit from the VNC (45% lateral exit) (Layden et al., 2006). To test whether misexpression of eve and/or grn can similarly alter axonal projections of dMP2 neurons, we misexpressed them alone and in combination. We find that although eve can trigger lateral VNC exit at low frequency (5.5%; n=36; Fig. 6H), grn has no such effect (0%; n=28). By contrast, co-misexpression of eve and grn leads to a high frequency of lateral exit (40.5%; n=84; Fig. 6G,H). To our surprise, the combinatorial misexpression of eve and grn alters axon pathfinding without any obvious sign of ectopic Zfh1 expression (Fig. 6G). Thus, misexpression of either zfh1 alone or of eve/grn together, can act equally well in triggering dMP2 lateral axonal exit. These rescue and misexpression results indicate that although eve and grn act in an eve-grn-zfh1 regulatory cascade within aCC, both genes play additional roles to ensure proper aCC identity.

The eve-grn-zfh1 regulatory cascade and integration of the Notch pathway

In the aCC neuron, grn and zfh1 are positively regulated by eve. aCC and its sibling, the pCC interneuron, is a well-studied sibling pair. The pCC neuron also expresses Eve, as well as the Nkx-family member vnd (ventral nervous system defective) (McDonald et al., 1998). Using eve mosaic mutants, we find that Vnd expression in pCC is completely dependent upon eve (Fig. 4H,I). Thus, eve acts in both sibling cells to regulate different downstream genes in each neuron; grn and zfh1 in aCC, and vnd in pCC. Studies have shown that the aCC versus pCC cell fate decision is dependent upon Notch signaling, with pCC being dependent upon Notch activation (Skeath and Doe, 1998). Although Eve expression in aCC and pCC...
does not respond to alterations in the Notch pathway, expression of both Zfh1 and Vnd in these siblings has been shown to be sensitive to Notch signaling (Lear et al., 1999). To address whether grn also responds to Notch activity in the aCC/pCC cell pair, we analyzed grnlacZ, grnGAL4 and Grn expression in two mutants affecting the Notch pathway, sanpodo (spdoG104) and mastermind (maml(2)04615). spdo facilitates N signaling specifically during asymmetric cell divisions, and mutants permit normal N signaling during early neurogenesis (O’Connor-Giles and Skeath, 2003). Likewise, mam is needed for nuclear events downstream of N signaling, but has a maternal contribution (Skeath and Doe, 1998). This allows, in both cases, for the examination of N function at later stages of neuronal development. In spdo and mam mutants, we find activation of both Grn (and grnGAL4) expression in pCC (Fig. 7E,F,H). As previously reported, we find that Vnd expression is lost in pCC (Fig. 7A,B,B’/H11032). Conversely, ectopic Notch activation in aCC, using the RN2-GAL4 driver to express the intracellular (activated) UAS-NotchICD transgene (Doherty et al., 1996), produces the reverse phenotype: de-repression of Vnd in aCC (but not in RP2) and repression of grn in aCC and RP2 (Fig. 7C,G,I). Thus, in the eve grn zfh1 regulatory cascade, only grn and zfh1 respond to Notch signaling. We next asked whether grn was sufficient to activate aCC-specific or to suppress pCC-specific genes, respectively? Although grn is necessary for Zfh1 expression in aCC, we find that misexpression of grn in pCC neither suppresses Vnd nor activates Zfh1 (Fig. 8A-C; not shown). This is in agreement with the fact that we never observed Vnd expression in aCC in grn mutants (data not shown). Likewise, using RN2-GAL4/UAS-vnd, we asked whether vnd was sufficient to suppress aCC-specific markers but find that vnd cannot suppress Grn expression in aCC (Fig. 8D-F).

In summary, we have shown that Notch signaling acts downstream of, or in parallel to, eve to restrict grn and zfh1 to aCC, and vnd to pCC. However, these determinants are not involved in cross-repressive interactions within these post-mitotic sibling cells (Fig. 9). We furthermore find that although both aCC and RP2 express eve, grn and zfh1, their regulatory interactions differ between aCC and RP2.

**DISCUSSION**

**Specification of unique motoneuron identities**

During motoneuron generation, combinatorial codes of regulators act to specify important aspects of subclass identity (Briscoe and Ericson, 2001; Shirasaki and Pfaff, 2002; Thor and Thomas, 2002). However, within any given subclass, there exists a further refinement, apparent in *Drosophila* and *C. elegans* at single-cell resolution. Our findings suggest that unique motoneuron identities may be defined by the unique interplay between subclass determinants (i.e. eve/grn/zfh1 in the ISN subclass). Our findings, combined with previous studies of the aCC/pCC and RP2/RP2sib pairs (Doe et al., 1988b), reveal a remarkable difference in the genetics of aCC and RP2 specification. A summary of the specification of these cells is presented in Fig. 9 and highlights how a unique genetic cascade allows for the specification of the aCC...
Motoneuron specification in *Drosophila*

**Fig. 5.** In *grn* mutants, loss of Zfh1 expression is restricted to the aCC motoneuron. Stage 15 wild-type (A,D,G), *grn* mutant (B,E,H), Zfh1 mutant (C,F) and *grn* rescue (I) (using RN2-GAL4/UAS-*grn*; *grn*+/−) embryos stained for Eve and pMad (A-C), Eve and Zfh1 (D-F) or Grn and Zfh1 (I). (G,H) RN2-GAL4/UAS-*mEGFP* embryo stained with α–Hb9. (A-C) pMad staining in *grn* and Zfh1 mutants appears unaffected within aCC and RP2. (D-F) In *grn* mutants, Zfh1 expression is not detectable in the aCC motoneuron, but RP2 maintains Zfh1 expression. Grn expression is not affected in aCC or RP2 in Zfh1 mutants. (G,H) Hb9 expression is unaffected in *grn* mutants. (I) In *grn* rescue experiments, Zfh1 expression is restored in aCC showing the cell autonomous effect of *grn* on Zfh1 expression in this motoneuron. Arrowheads and asterisks indicate aCC and RP2, respectively.

**Fig. 6.** *eve* and *grain* play additional roles outside of the *eve*→*grn*→Zfh1 cascade. (A-E) Stage 15 embryo stained for Grn (A,B) β-Gal (A-D) and Zfh1 (A,C,E). B-E are identical to A but with different combinations of color channels to facilitate the observation of Grn and Zfh1 expression in aCC (arrows) and RP2 (arrowheads). *grn* is unable to rescue eve mosaic mutants (UAS-grn, eve mosaic; RN2-GAL4, UAS-α-LacZ), evident as a failure of aCC and RP2 to project axons out of the VNC, and of aCC to express Zfh1. (F-H) Stage 15 embryo stained for Myc and Zfh1, expressing only UAS-*EGFP* (F), UAS-*eve* (G) or co-misexpressing both *eve* and *grn* (H). (F) In the control, dMP2 axons project posteriorly in the longitudinal connective and never exit the VNC laterally (n=62). (G) Ectopic *eve* triggers lateral VNC exit, but only in 5% of hemisegments. (H) Ectopic *eve* and *grn* (UAS-*eve*, UAS-*grn*, dMP2-GAL4; UAS-*EGFP*) triggers lateral VNC exit in 40% of hemisegments (n=84). There is no evidence of Zfh1 expression in dMP2 neurons (yellow circles), in the control (F) or in the misexpression backgrounds (G). Arrowheads indicate dMP2 axons exiting the VNC.
motoneuron. But why do these three genes act in a unique fashion in aCC, and why is grn and zfh1 sensitive to Notch specifically in this ISN motoneuron? One explanation may be that the differential input from upstream regulators, such as Ftz, Pdm1, Hkb and Pros (McDonald et al., 2003), acts to modify the genetic interactions between eve, grn and zfh1. Another possibility is that the relative level of each factor plays an important role in dictating different cellular fates. Studies of the related Isl1 and Isl2 LIM-homeobox genes suggest that their involvement in motoneuron subclass specification is not primarily the result of the unique activity of each gene, but rather by the combined ‘generic’, tightly temporally controlled, Isl1 and Isl2 levels (Thaler et al., 2004). Similarly, the different expression levels of the transcription factor Cut have been shown to play instructive roles during the specification of neuronal cell identities within the PNS (Grueber et al., 2003). We have also noticed different levels of expression of Grn and Zfh1; while Grn is strongly expressed in aCC and weakly in RP2, Zfh1 expression shows an opposite distribution. It is tempting to speculate that these levels may be instructive for ISN motoneuron specification.

Cross-repressive interactions and Notch signaling specify neural fates
In the VNC, we observe mutually exclusive expression between Grn and Hb9 (and Islet) in different subsets of interneurons and motoneurons. Cross-inhibitory interactions between eve and Hb9 has been shown to contribute to their mutually exclusive expression patterns, and functional studies demonstrate that eve and Hb9 regulate axonal trajectories of dorsally and ventrally projecting axons, respectively (Broihier and Skeath, 2002; Doe et al., 1988b; Fujioka et al., 2003; Landgraf et al., 1999). These observations are reminiscent of the cross-repressive interactions between classes of regulators that act to determine, refine and maintain distinct progenitor domains along the dorsoventral axis of the vertebrate neural tube (Briscoe et al., 2000). We have shown that eve is important for proper grn and zfh1 expression in aCC, but not in RP2. These results are consistent with previously reported observations that the requirement for eve in axonal guidance is somewhat more stringent in aCC than in RP2, leading the authors to propose that there may be different target genes for Eve in these two motoneurons (Fujioka et al., 2003).

Zfh1 expression was previously shown to depend upon Notch signaling activity in the aCC/pCC sibling pair as mutations in spdo or mam, members of the Notch signaling pathway, lead to de-repression of Zfh1 in pCC (Skeath and Doe, 1998). Using the same allelic combinations, we also observed de-repression of grn in pCC. Whether or not grn is directly suppressed by the Notch pathway remains to be seen, but it is interesting to note that in vertebrates, gata2/3 have been identified as targets of Notch during the differentiation of specific hematopoietic lineages (Amsen et al., 2004; Kumano et al., 2001).

aCC, RP2 and U motoneurons – several pioneers for ISN?
Within the ISN subclass, the aCC motoneuron pioneers the ISN to innervate the dorsal-most muscle, muscle 1 (Jacobs and Goodman, 1989; Sanchez-Soriano and Prokop, 2005; Thomas et al., 1984). A
number of genetic and cell-ablation studies have convincingly shown that aCC plays an instructive pioneer role and guides the follower U motoneurons along the ISN nerve (Fujikawa et al., 2003; Lin et al., 1995a; Sanchez-Soriano and Prokop, 2005). Our results lend support for the proposed instructive role of aCC in ISN formation. However, our studies indicate that aCC may not be essential for ISN formation. First, using RN2-GAL4 to visualize aCC and RP2, we frequently find (35% of hemisegments) aberrant innervation of muscle 8 in grn mutants. However, we simultaneously observe an axonal projection at the vicinity of the dorsal muscles 2/10. In grn mutants, zfh1 expression is specifically lost in aCC but maintained in RP2. Given the role for zfh1 in motor axon pathfinding, we propose that aberrant innervation of muscle 8 in grn mutants, is caused by aCC but not by RP2, and that RP2 pathfinds normally to the muscles 2/10. If so, RP2 may function as a pioneer motoneuron for muscle 2 and project there without the aCC axon. Second, although the rescue of grn mutants using RN2-GAL4 is complete, we do find that using CQ2-GAL4 to specifically rescue U motoneurons does lead to a partial rescue (54% muscles 1/9 innervated compared with 15% in grn mutants). Thus, even in the absence of aCC pioneer function, the Us (presumably U1) can still project to the dorsal-most muscles. This is in line with previous studies showing that in eve aCC/RP2 mosaic mutants and in aCC/RP2 cell ablation experiments, there is still partial innervation of muscle 1/9 (Fujikawa et al., 2003; Lin et al., 1995a; Sanchez-Soriano and Prokop, 2005).

The eve→grn→zfh1 genetic cascade contra other roles for eve and grn

We find that grn is part of an eve→grn→zfh1 transcriptional cascade crucial for specification of aCC motoneuron identity. However, the failure of grn to rescue eve, and of zfh1 to completely rescue grn, combined with the misexpression results, indicate additional roles for both eve and grn. These roles could be either in the regulation of other aCC determinants and/or in the regulation of genes directly involved in aCC axon pathfinding. Although we are unaware of obvious candidates for additional aCC determinants, recent studies point to a candidate axon pathfinding gene. The Drosophila unc-5 gene encodes a netrin receptor and is expressed in subsets of neurons in the VNC (Keleman and Dickson, 2001). Misexpression of unc-5 is sufficient to trigger ectopic VNC exit in subsets of interneurons (Allan et al., 2003; Keleman and Dickson, 2001). Recent studies now show that unc-5 is specifically expressed in eve motoneurons, and that eve is necessary, but only partly sufficient for unc-5
expression (Labrador et al., 2005). In line with these findings, we find that whereas single misexpression of eve or grn in dMP2 neurons has very minor effects, co-misexpression of eve and grn can efficiently trigger dMP2 lateral axonal exit. This combinatorial effect of eve/grn occurs without apparent activation of zfh1. However, misexpression of zfh1 can also trigger dMP2 lateral exit (Layden et al., 2006). Thus, these genes appear to be able to act in an independent manner to trigger VNC exit, but in a highly context-dependent manner. A speculative explanation for not only the mutant and rescue results, but also these misexpression results, would be that all three regulators are needed for robust and context-independent activation of axon pathfinding genes such as, for example, unc-5.

Evolutionary conservation of GATA gene function
grn encodes a GATA Zn-finger transcription factor and is the ortholog of the closely related vertebrate gata2 and gata3 genes. In vertebrates, gata2/3 are expressed in overlapping domains in the nervous system, but relatively little is known about their function. Expression data and evidence from gene targeting suggest an involvement in neurogenesis, neuronal migration and axon projection (Karls et al., 2001; Nardelli et al., 1999; Pandolfi et al., 1995; Pata et al., 1999). A role in specifying neuronal subtypes within the context of neural tube patterning is emerging (Karunaratne et al., 2002; Zhou et al., 2000) and recently a role for gata2/3 during 5-HT neuron development has been reported (Craven et al., 2004; Tsarvina et al., 2004; van Doorninck et al., 1999). The role of gata3 in the development of the inner ear has been of particular interest, and in humans, mutations in this gene have been linked to HDR syndrome, deafness and renal defects (Muroya et al., 2001; Van Esch et al., 2000). In the mouse, gata3 is expressed in auditory but not vestibular ganglion neurons during development (Lawoko-Kerali et al., 2002; Rivolta and Holley, 1998). The mouse gata3 mutant shows auditory ganglion neuron loss and inefficient nerve misrouting, revealing that gata3 regulates molecules associated with neural differentiation and guidance (Karls et al., 2001). These vertebrate studies, combined with our results, suggest that gata2/3 genes, similar to other transcription factors specifying neuronal identities, such as islet1/2, evx1/2 or Hb9, and their respective orthologs in Drosophila, have maintained similar functions throughout evolution (Broihier and Skeath, 2002; Fujioka et al., 2003; Thor and Thomas, 2002).

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