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

Alain Garces¹ and Stefan Thor²,*

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 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 a well-defined and specific muscle target (Jacobs and Goodman, 1997). The even-skipped (eve) regulator gene is specifically expressed in ISN motoneurons and eve is both necessary and sufficient for ISN motor axon pathfinding (Landgraf et al., 1999). However, eve is expressed in all ISN motoneurons and is cell-autonomously crucial for their axonal exit out of the VNC (Fujikawa et al., 2003). Recent studies reveal that the zinc-finger/homeodomain gene zfh1 is also expressed by ISN motoneurons (Layden et al., 2006). However, zfh1 is expressed by most if not all motoneurons, and important for many motor axons to exit the VNC. Together, these results suggest that regulators other than eve and zfh1 are necessary to explain the specification of each individual ISN motoneuron identity.

To gain further insight into motoneuron specification, we have addressed the role of the Drosophila GATA transcription factor grain (grn). We find that grn is specifically expressed within the ISN motoneuron subclass and plays a crucial role for ISN axon projections. Genetic analysis reveals that the regulatory interplay between eve, grn and zfh1 varies between the different ISN motoneurons. Within the postmitotic aCC motoneuron, these three regulators act in a unique eve–grn–zfh1 genetic cascade that is crucial for the correct specification of aCC identity. Misexpression of zfh1 (Layden et al., 2006) or co-misexpression of eve with grn, can trigger lateral axonal exit from the ventral nerve cord. grn and zfh1 are, furthermore, sensitive to Notch signaling within this ISN motoneuron, whereas they are insensitive to Notch in other ISN motoneurons. These findings reveal the existence of a unique genetic program for the aCC motoneuron fate, consisting of factors expressed by all ISN motoneurons.

MATERIALS AND METHODS

Drosophila stocks

The grn
mutZ 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
mutZ, as previously described (St Pierre et al., 2002). For grn mutant analysis, grn and grn
ZP (Brown and Castelli-Gair Hombria, 2000) were placed over deficiency Df(3R)A1013, and both allelic combinations showed the same pathfinding phenotype and no detectable Gm expression (not shown). For grn misexpression and rescue experiments, we used UAS-grn
#2 (Brown and Castelli-Gair Hombria, 2000). UAS-mEGFP was a c-myc epitope-tagged membrane-targeted EGFP reporter line (Allan et al., 2003). Other lines used were: islet-α-myc-EGFP (S.T., unpublished); RN2-GAL4, CQ2-GAL4, Df(2R)eve, ΔRP2α/CyO,P[wg-lacZ];RN2-GAL4,UAS-rlacZ, Df(2R)eve/CyO,P[wg-lacZ];ΔRP2B (Fujikawa et al., 2003); UAS-eve and eve
D0 (Landgraf et al., 1999), zfh12, zfh123.26 alleles were obtained from R. Lehmann and UAS-zfh1 from the Bloomington stock center. Hb9GAL4, Hb9KK30, UAS-vnd, man212GAL4, spdcGAL4 were provided by J. B. Skeath and H. T. Brothier. UAS-NotchR97D 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.

¹INSERM U 583, INM-Hopital St Eloi, 80 rue Augustin Fliche, 34091 Montpellier Cedex 5, France. ²Division of Molecular Genetics, Department of Physics, Chemistry and Biology, Linkoping University, S-581 83 Linkoping, Sweden.

*Author for correspondence (e-mail: steth@ifm.liu.se)
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 grm 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-Even 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-Zfh1 (Van Doren et al., 2003) (1:5,000), rabbit α-Hb9 (Brohier 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 monoclonal 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; Lin et al., 1995b). We generated Grn-specific antibodies and found that the expression of Grn closely matches the grn<sup>lacZ</sup> and grn<sup>GAL4</sup> reporter expression in these structures (not shown), as well as in the VNC (Fig. 1A1-3, 1E1-3; not shown).

In the VNC, grn expression commences at early stage 12. The position and morphology of grn<sup>lacZ</sup>- and grn<sup>GAL4</sup>-expressing cells suggested a postmitotic and neuronal identity. Using grn<sup>GAL4</sup>/UAS-<sup>�</sup>lacZ, we observed that grn is expressed in a diverse set of interneurons and motoneurons that extend axons along the major axon tracts (Fig. 1F,B). Double labeling with the glial-specific marker Repo showed that, with the exception of one dorsal glial cell per hemisegment (Fig. 1I), Grn (and grn<sup>lacZ</sup> or grn<sup>GAL4</sup>) expression is restricted to neurons. To resolve the identity of grn-expressing neurons further, we assayed for overlap with regulators known to be expressed in restricted sets of neurons, such as isl, lim3, Hb9, zfh1, apterous and even-skipped (eve) (Fig. 1B-D,F-H; not shown). Of these genes, only eve and zfh1 showed apparent overlap with grn, specifically in the intersegmental nerve (ISN) motoneurons: aCC, RP2 and the five Us (U1-5 or CQ) (Fig. 1D,H). The ISN motoneurons are born during early embryogenesis with aCC and RP2 born at stage 9, and the U motoneurons born sequentially during stage 9-11 (Broadus et al., 1995; Doe et al., 1988a; Weigmann and Lehner, 1995). Expression of grn and Grn in ISN motoneurons commences at stage 11-12, subsequent to Eve expression, and expression of grn and Grn is maintained in ISN motoneurons into larval stages (not shown). Thus, grn is expressed in subsets of interneurons, and in a distinct subclass of motoneurons that innervate the dorsal-most muscles in the Drosophila embryo (Fig. 1J).

grain is required for ISN motor axon pathfinding

To determine if grn plays a role in ISN motoneuron specification, 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) (Vactor 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 (Vactor 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-mEGFP<sup>F</sup>) (Allan et al., 2003). In the wild type, RN2-GAL4/UAS-mEGFP<sup>F</sup> 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-mEGFP<sup>F</sup> 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. 2J,L). 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-mEGFP<sup>F</sup> or CQ2-GAL4/UAS-mEGFP<sup>F</sup>) 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,J). 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 the dorsalmost muscles 1 and 9, respectively, the aCC and/or RP2, and at least one of the U motoneurons are known to innervate the dorsalmost muscles 1/9, respectively (Fig. 2A-I,J). 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,J). 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.
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^D19^), 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^{D19} (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.

---

**Fig. 2. grain is required for ISN motor axon projections.**

Stage 16 embryos stained with α-Fas2 (green in A–C,G), RN2-GAL4 driving UAS-mEGFP (green in D–H), CO2-GAL4 driving UAS-mEGFP (green in I) and Phalloidin-TX (magenta in A–F, H–I). Arrows and arrowheads indicate axons terminals contacting dorsal (2 and 10) and dorsal-most (1 and 9) muscles, respectively. (A) In wild type, the ISN nerve innervates muscles 2/10 and 1/9 (arrowheads). (B) In grn mutants, ISN fails to innervate muscles 1/9, but axonal projections are seen contacting muscles 2/10. Bracket denotes a partially penetrant muscle patterning phenotype, evident as an imprecise insertion of muscles 21-24 into the body wall. (C) In grn rescue (RN2-GAL4/UAS-grn; grn+/-) ISN innervates muscles 2/10 and 1/9 as in wild type. (D) In control, RN2-GAL4/UAS-EGFP reveals muscle 1 innervation by aCC and muscle 2 innervation by RP2. (E) In a grn mutant background, RN2-GAL4/UAS-EGFP reveals that although muscle 1/9 is not innervated by aCC, axon terminals from aCC and/or RP2 contact muscles 2/10. (F) zfh1 can partially rescue grn mutants (RN2-GAL4/UAS-zfh1; grn+/-) and the lack of muscle 1 innervation (arrowheads) is less severe than in grn mutant. (G) Overlap between RN2-GAL4/UAS-EGFP (green) and α-Fas2 (magenta) revealing axons terminals for aCC and RP2. This reporter allows for a precise analysis of aCC and RP2 terminals in the periphery. (H) In grn mutants, 36% of hemisegments (n=69) show ectopic innervation of muscle 8 together with defasciculation of aCC and RP2 motor axons (see also oblique arrow in E). (I) In control, CO2-GAL4/UAS-EGFP reveals muscle 9 innervation by U1 and muscle 10 innervation by other U motoneurons. (J) In a grn mutants, CO2-GAL4/UAS-EGFP reveals that muscle 9 is not innervated (by U1), while U axon terminals contact muscles field 2/10. (K) Quantification of muscles 1/9 innervation in different genetic backgrounds. (L,M) Schematic showing the grn mutant phenotypes (M) compared to wild type (L).
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 is necessary for grain expression and for Hb9 repression in both aCC and RP2 motoneurons.

Stage 12 (A-D) or stage 15 (F-I) eve^{GR101}A heterozygote (eve mosaic+) (A,C,F,H) and eve^{GR101}B homozygote mutant (eve mosaic) (B,D,G,I) embryos. Arrows and arrowheads indicate aCC and RP2, respectively (visualized using RN2-GAL4/UAS-t-LacZ). (A,C,F,H) eve mosaic+/RN2-GAL4/UAS-t-LacZ showing that Grn is expressed in aCC and RP2 at stage 12 and stage 15, while Hb9 is not. (B,D) In stage 12 eve mosaic mutant, Hb9 is derepressed in aCC and RP2 while Grn expression is not detectable in aCC but maintained in RP2. (G,I) In stage 15 eve mosaic mutants, Hb9 remains derepressed in aCC and partly in RP2, while Grn expression is not detectable in aCC but maintained in RP2. At this stage, Grn expression in RP2 appears even stronger in eve mosaic compared with wild type. (E,J) Quantification of these phenotypes.
1450 RESEARCH ARTICLE

**Development 133 (8)**

**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 grain 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 R12-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-mEFP 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–r 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). 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/gata3 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 (Fujisaka 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 and 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 (Fujisaka 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 (Karins 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; Tsarovina 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 (Muruya 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 (Karins 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 Hh9, and their respective orthologs in *Drosophila*, have maintained similar functions throughout evolution (Broihier and Skeath, 2002; Fujioka et al., 2003; Thor and Thors, 2002).

This study was initiated while we were at the Department of Neurobiology, Harvard Medical School, and we are grateful for the support from our former colleagues there. We thank J. Castelli-Gair Hombria, M. Fujioka, J. B. Skeath, M. Nirenberg, R. Lehmann, S. Artavanis-Tsakonas, P. ten Dijke, the colleagues there. We thank J. Castelli-Gair Hombria, M. Fujioka, J. B. Skeath, M. Nirenberg, R. Lehmann, S. Artavanis-Tsakonas, P. ten Dijke, the Strategic Research Foundation and by the Swedish Royal Academy of Sciences Doe for helpful comments on the manuscript. This work was funded by grants from NIH (RO1 NS39875-01), from the Freudenberger Scholarship Fund at Harvard Medical School, by the Swedish Research Council, by the Swedish Strategic Research Foundation and by the Swedish Royal Academy of Sciences to S.T., and by the Fondation Recherche Médicale (FRM) to A.G. 

**References**


Motoneuron specification in Drosophila


