The *achaete–scute* complex proneural genes contribute to neural precursor specification in the *Drosophila* CNS

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Background: The *Drosophila* central nervous system (CNS) develops from a segmentally reiterated array of 30 neural precursors. Each precursor acquires a unique identity and goes through a stereotyped cell lineage to produce an invariant family of neurons and/or glia. The proneural genes *achaete*, *scute* and *lethal of scute* are required for neural precursor formation in the *Drosophila* CNS, and are expressed in overlapping subsets of 'proneural cell clusters' from which a single neural precursor later develops. Vertebrate *achaete–scute* homologues are expressed early during neurogenesis, and promote neurogenesis, neuronal development and/or differentiation. The *Drosophila* proneural *achaete–scute* genes govern neural precursor formation, but their role in specifying neural precursor identity has not been tested.

Results: Here, we test the role of the *Drosophila achaete–scute* genes in specifying neural precursor identity, focusing on the well characterized CNS MP2 precursor. MP2 delaminates from a cluster of *achaete–scute*-expressing ectodermal cells. In an *achaete–scute* double mutant, MP2 formation was reduced (to 11–14 %) as expected because of the function of proneural genes in promoting neural precursor formation. Surprisingly, we also observed that the developing MP2 precursors were incorrectly specified and acquired traits characteristic of adjacent neural precursors. In rescue experiments, *achaete* or *scute*, but not *lethal of scute*, completely restored the normal MP2 identity.

Conclusions: These results demonstrate that the *achaete–scute* complex genes specify aspects of neural precursor identity in the *Drosophila* CNS. Given the phylogenetically conserved function of these genes, our results raise the possibility that *achaete–scute* homologues may help specify neural precursor identity in other organisms.

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Background

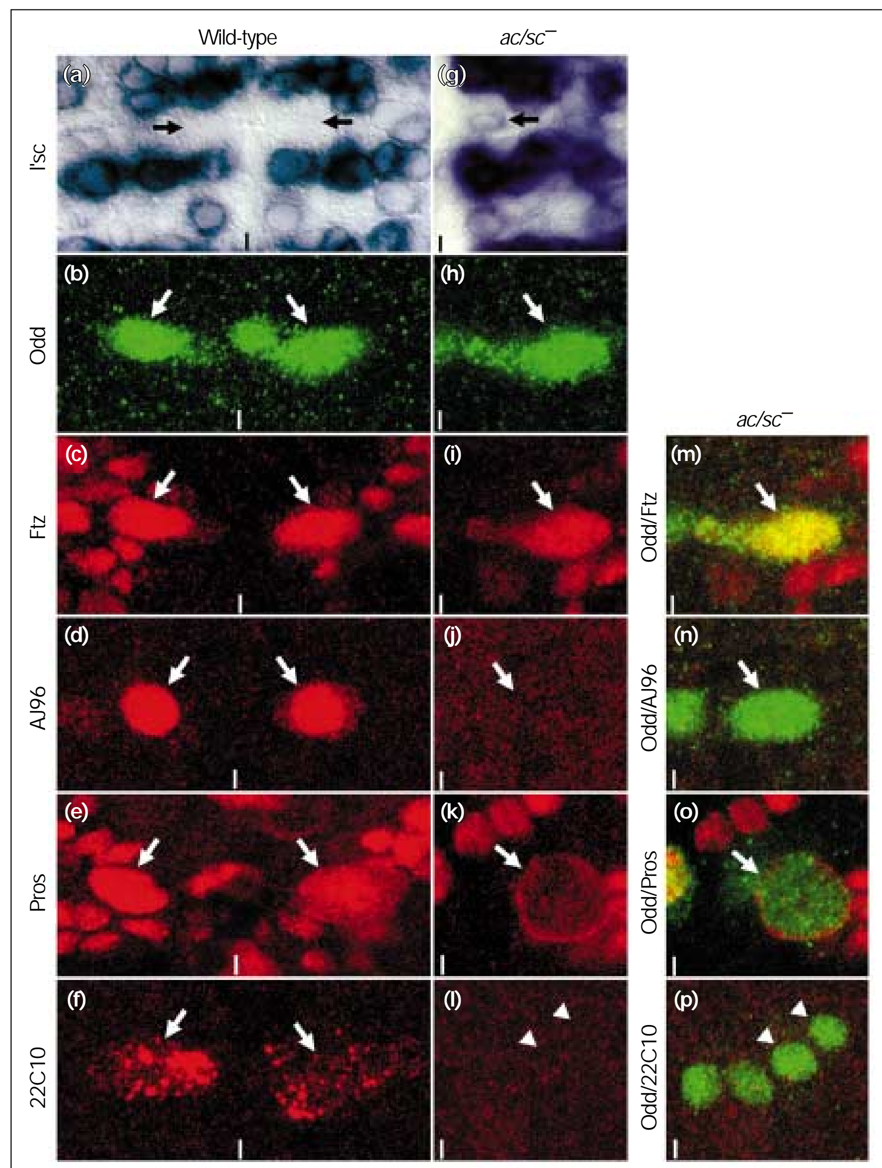
The *achaete–scute* complex (AS-C) consists of the adjacent *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*) proneural genes, and the neural precursor gene *asense*. All of these genes encode basic helix–loop–helix (bHLH)-type transcription factors [1–3]. The *ac*, *sc* and *l'sc* genes are expressed in a stereotyped pattern of 'proneural clusters' within the ectoderm. Within each cluster, a single cell retains proneural gene expression and segregates as a neural precursor, while all other cells lose proneural gene expression and remain in the ectoderm [4–8]. The *asense* gene is not expressed in ectodermal clusters, but is activated in neural precursors [9]. The AS-C proneural genes regulate the time and position of neural precursor formation: loss of AS-C expression results in fewer neural precursors [10], whereas overexpression produces additional neural precursors [9,11]. Homologues of AS-C genes exist in a diverse range of organisms, including hydra, mice and humans, where they are expressed at early stages of neurogenesis [12–18]. Loss-of-function analysis in both mice and *Caenorhabditis elegans*, and mis-expression experiments in *Xenopus*, are consistent with AS-C homologues playing a role in the early steps of neural precursor formation and/or differentiation [16,18–20].

In the adult *Drosophila* peripheral nervous system (PNS), the AS-C proneural genes promote external sensory organ precursor formation. The *atonal* gene, which encodes another bHLH protein, promotes the formation of internal sensory organ precursors [21]. Furthermore, ectopic *atonal* expression can reprogram external sensory organs into internal sensory organs [21]. On the other hand, all three AS-C proneural genes are interchangeable in their ability to form all of the different types of external sensory organs; neither *ac*, *sc* nor *l'sc* appear to generate distinct types of external sensory organs [11,22]. These results suggest the *achaete–scute* genes do not contribute to the diversity of neural precursor fates. In the *Drosophila* central nervous system (CNS), *ac*, *sc* and *l'sc* are expressed in overlapping proneural clusters in the neuroectoderm from which neural precursors arise, at the time neural precursor identity is established [7,8]. This has led to the speculation that they

influence the identity of neural precursors [4]. Here, we test the role of the *achaete–scute* genes in specifying neural precursor identity within the *Drosophila* CNS.

The first 10 CNS neural precursors consist of nine neuroblasts and one MP2; the two types of precursors can be distinguished by cell-division orientation, time of mitosis, and six molecular markers [23,24]. MP2 forms early in neurogenesis from a cluster of ectodermal cells expressing *ac* and *sc*. MP2 and neuroblasts share a number of features common to all neural precursors: they delaminate from proneural clusters in the neuroectoderm, enlarge in size, contain bilobed nuclei surrounded by basophilic cytoplasm, express the *snail* and *hunchback* (*hb*) genes and localize the Numb protein to their basal cortex at mitosis [25,26]. Nonetheless, MP2 and neuroblasts can be distinguished easily by a number of criteria. First, MP2 divides once to produce a pair of neurons; neuroblasts produce multiple progeny [27]. Second, MP2 divides about 1.5 hours after formation; neuroblasts divide within 20 minutes of formation. Third, MP2 expresses the zinc-finger protein Odd-skipped (Odd), the homeodomain protein Fushi-tarazu (Ftz), the enhancer trap line AJ96 and the membrane protein 22C10, but not the proneural gene *l'sc*; in contrast, all neuroblasts express *l'sc*, but do not express Ftz, AJ96, 22C10 or Odd (except for two neuroblasts, which express Odd very transiently) (Fig. 1) [7,24]. Fourth, MP2 exhibits nuclear Prospero (Pros) protein and distributes Pros to the

Figure 1



MP2 is incorrectly specified in *ac/sc*⁻ embryos. **(a–f)** Single segments of wild-type embryos and **(g–p)** single hemi-segments of *ac/sc*⁻ embryos labeled for (a,g) *l'sc* transcript, (b,h) Odd, (c,i) Ftz, (d,j) AJ96-lacZ, (e,k) Pros, (f,l) 22C10. **(m–p)** Merged images of panels showing Odd expression (green) and other markers (red) shown for Ftz (m), AJ96 (n), Pros (o) and 22C10 (p). Anterior, up; ventral midline, small line; MP2, arrow; dMP2 and vMP2, arrowhead. (a–f) In wild-type embryos, MP2 does not express *l'sc* transcript (a) but does express Odd (b), Ftz (c), AJ96-lacZ (d), nuclear Pros (e) and 22C10 (f). (g–p) In *ac/sc*⁻ embryos, 'MP2' expresses *l'sc* ectopically ~20 % of the time (g), expresses *odd* and *ftz* normally (h,i,m), but does not express AJ96-lacZ (j,n) or 22C10 (l,p) and mis-localizes Pros to the cortex ~32 % of the time (k,o). In panels (l,p), 'MP2' has divided but neither dMP2 nor vMP2 express 22C10 (arrowheads).

nuclei of both daughter cells (see Figs 1e and 2a,e–g); neuroblasts localize Pros to their cell cortex and distribute Pros asymmetrically at mitosis to the smaller, basally located ganglion mother cell (see Fig. 2a) [28].

We took advantage of the specific molecular and morphological traits of MP2 to assay MP2 identity in *Drosophila* embryos that lack *ac/sc* function. In *ac/sc* double mutant embryos, a precursor forms in the MP2 position approximately 11–14 % of the time. In the absence of *ac* and *sc*, this precursor fails to activate a set of genes appropriate for MP2 and acquires traits characteristic of neuroblasts. In rescue experiments, we found that *ac*, *sc* and *l'sc* were similarly able to promote neural precursor formation in the MP2 position to near wild-type levels; however, only *ac* and *sc* completely rescued the MP2 identity. These results suggest that different proneural genes play different roles in specifying neural precursor identity within the *Drosophila* CNS.

Results and discussion

MP2 is mis-specified and acquires neuroblast-like traits in the absence of *ac* and *sc*

To assay the effects of removing *ac* and *sc* function on the formation and identity of MP2, we used a deletion (*Df (1)y^{3PL}sc^{8R}*) mutation that completely removes the *ac* gene together with the enhancers that drive *sc* expression in MP2 and its proneural cluster [29]. In this background, *ac* and *sc* transcripts are not detectable in either MP2 or its proneural cluster [29]. In embryos homozygous for *Df (1)y^{3PL}sc^{8R}* (hereafter called *ac/sc*$^-$ embryos), a neural precursor formed in the MP2 position 11–14 % of the time, as assayed by expression of the Odd and Snail proteins (Table 1); this decrease in MP2 formation is expected because of the known role of *ac–sc* genes in promoting neuroblast formation [10]. All neuroblasts adjacent to MP2 did not express *ac* or *sc*, and all formed normally in *ac/sc*$^-$ embryos. Thus, the defects we observe in the MP2 identity in *ac/sc*$^-$ embryos (see below) likely reflect a cell-autonomous requirement for *ac/sc* function within MP2.

We analyzed the neural precursors that form in the MP2 position in *ac/sc*$^-$ embryos (hereafter termed ‘MP2’) to score for defects in MP2 identity. If the *ac* and *sc* proneural genes play no role in specifying neural precursor identity, then the precursors at the MP2 position in *ac/sc*$^-$ embryos should have the normal MP2 identity. Conversely, if *ac* and *sc* are required to specify neural precursor identity, then these precursors should not acquire the normal MP2 identity. In wild-type embryos, MP2 expressed AJ96, Ftz, Odd and 22C10, and had nuclear Pros, but did not express *l'sc* (Fig. 1a–f; Table 1). In the absence of *ac/sc*, when ‘MP2’ formed (as detected by Odd expression), it exhibited clear defects in specification: it rarely expressed AJ96 (10 %, *n* = 7/69) or 22C10 (6 %, 3/52), and occasionally expressed *l'sc* (20 %, 23/113), albeit at a reduced intensity relative to neuroblasts (Fig. 1g–p; Table 1). ‘MP2’ always expressed Ftz (26/26), as in wild-type embryos (Fig. 1h; Table 1). These results are consistent with a partial transformation of MP2 identity to a novel Odd$^+$, Ftz$^+$, AJ96$^-$, 22C10$^-$ neural precursor, and suggest that *ac* and *sc* help specify MP2 identity. Alternatively, the mere absence of the AJ96 and 22C10 markers could indicate a block in MP2 differentiation.

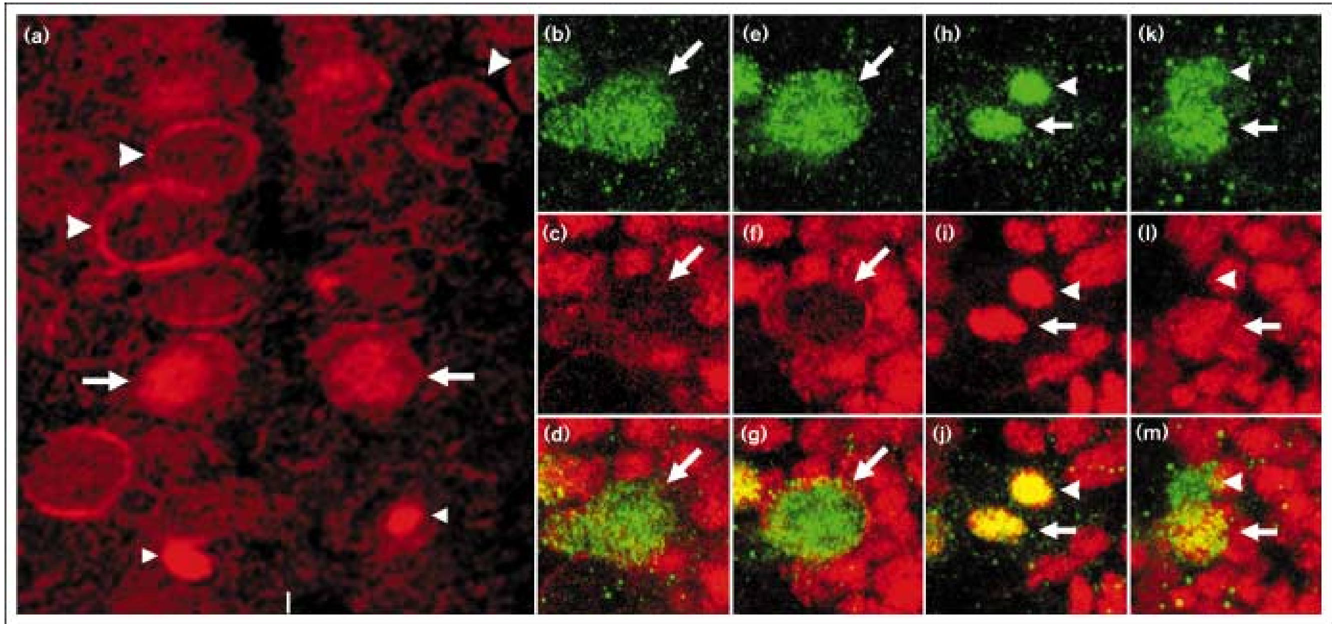
Table 1

***ac* and *sc*, but not *l'sc*, completely rescue MP2 specification in *ac/sc*$^-$ embryos.**

	Proneural genes expressed				
	Wild-type	*ac/sc*$^-$	*ac/sc*$^-$ + *ac*	*ac/sc*$^-$ + *l'sc*	*ac/sc*$^-$ + *sc*
MP2 formation*					
snail	99 % (123/124)	14 % (10/79)	99 % (111/112)	94 % (275/292)	ND
hb	ND	ND	ND	95 % (99/104)	ND
Odd	100 % (106/106)	11 % (97/884)	99 % (305/308)	59 % (376/671)	92 % (212/230)
MP2 markers					
Odd	100 % (106/106)	†	99 % (305/308)	59 % (376/671)	92 % (212/230)
Ftz	100 % (112/112)	100 % (26/26)	98 % (277/282)	56 % (196/352)	ND
AJ96	99 % (103/104)	10 % (7/69)	98 % (439/450)	33 % (98/296)	ND
22C10	96 % (110/114)	6 % (3/52)	95 % (462/486)	35 % (74/214)	93 % (145/156)
Pros nuclear	100 % (101/101)	68 % (88/129)	100 % (378/378)	56 % (185/328)	98 % (155/158)
Neuroblast-specific markers					
Pros cortical	0 % (0/101)	32 % (41/129)	0 % (0/378)	26 % (33/126)‡	0 % (0/158)
l'sc	0 % (0/101)	20 % (23/113)	1 % (2/204)	N/A	ND

* MP2 formation: percentage of hemisegments in which a precursor in the MP2 position expressed the indicated gene. All assays were performed prior to MP2 division, except for analysis of 22C10 expression, which was assayed as, or just after, MP2 divided (stage 11). ND, not determined; N/A, not applicable. † MP2 markers: in wild-type, *ac*-rescue, *sc*-rescue and *l'sc*-rescue embryos, the percentage of hemisegments in which a precursor in the MP2 position expressed the indicated gene is shown. In *ac/sc*$^-$ embryos, Odd expression was used to identify ‘MP2’ and thus the percentage of Odd-positive ‘MP2’ precursors that expressed the indicated gene is shown. ‡ Percentage of hemisegments in which ‘MP2’ localized Pros to its cortex or divided prematurely.

Figure 2



Mutant *ac/sc*– embryos have cortical localization of Pros in 'MP2' characteristic of neuroblasts. **(a,h–j)** Wild-type embryos or **(b–g,k–m)** *ac/sc*– embryos labeled for (a,f,i,l) Pros, (b,e,h,k) Odd or (d,g,j,m) double-labeled for Pros (red) and Odd (green) protein expression. Anterior is up. All panels show single hemisegments (ventral midline at left) except (a) which shows a full segment (ventral midline, small line). Panels (b–g) show apical (b–d) and basal (e–g) sections through the identical 'MP2' precursor. (a) In wild-type embryos, MP2 has nuclear Pros protein (arrows), whereas all neuroblasts localize Pros protein to their cortex (arrowheads) where it is distributed upon division exclusively to the basally located ganglion mother cell (small arrowheads). (b–g) In *ac/sc*– embryos, 32 % of all 'MP2s' localize Pros protein exclusively to their basal (e–g) but not to their apical cortex (b–d). (h–j) Normally, MP2 distributes Pros protein equally to both its basal daughter cell, dMP2 (arrows), and to its apical daughter cell, vMP2 (arrowheads). (k–m) In *ac/sc*– embryos, when 'MP2' localizes Pros to its cortex it distributes Pros exclusively to its basal daughter cell, dMP2 (arrow), but not its apical daughter cell, vMP2 (arrowhead).

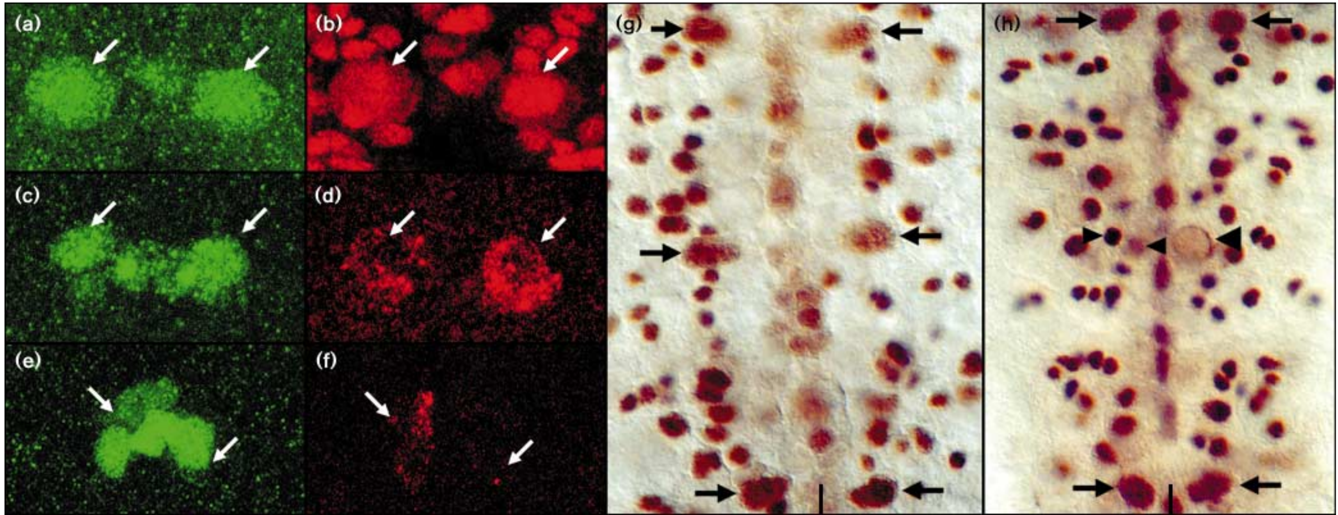
To distinguish between a change in identity and an arrest in MP2 differentiation, we examined the subcellular localization of Pros protein and followed 'MP2' cell divisions in *ac/sc*– embryos. Many 'MP2s' showed nuclear Pros protein, indicative of the normal MP2 identity (68 %, 88/129); however, a significant fraction localized Pros to the cortex (32 %, 41/129; Fig. 1k,o). Cortical Pros was always localized asymmetrically to the dorsal (basal) side of 'MP2' (Fig. 2b–g). When 'MP2' divided, cortical Pros was inherited exclusively by the basal daughter cell (19/19; Fig. 2k–m). The asymmetric cortical Pros localization in 'MP2' followed by the exclusive segregation of Pros to the basal daughter cell of 'MP2' is characteristic of all neuroblasts, and clearly differs from the typical MP2 pattern of nuclear Pros with equal distribution to its siblings (Fig. 2h–j) [28]. Both the novel cortical Pros localization (characteristic of neuroblasts) and the observation that 'MP2' divides to produce neurons regardless of whether it accumulates Pros in its nucleus (data not shown) or cortex (Fig. 2), reveal a change in the identity of MP2 rather than a block in its differentiation in *ac/sc*– embryos. These results, taken together with the altered gene expression in MP2 in *ac/sc*– embryos, demonstrate that the presence of *ac* and *sc* contributes to the identity of MP2.

ac and *sc*, but not *l'sc*, completely rescue MP2 identity in *ac/sc*– embryos

To determine whether the loss of *ac/sc* caused the change in MP2 identity, we used an *ac* minigene that precisely recapitulates the endogenous expression pattern of *ac* in MP2 (G. Panganiban, J.B.S. and S.B. Carroll, unpublished observations). The *ac* minigene rescued MP2 formation (99 %) as well as the normal expression of Odd, Ftz, nuclear Pros, AJ96 and 22C10 in MP2 (Fig. 3a–d; Table 1). Thus, the replacement of *ac* alone completely restored the normal MP2 identity. To determine whether *sc* alone could also rescue MP2 identity in *ac/sc*– embyros, we used the GAL4 system to express high levels of *sc* in 'MP2' [30]. We found that *sc* expression was also sufficient to rescue the formation of a normally specified MP2 precursor (Table 1).

In wild-type embryos, *l'sc* is expressed in all neuroblasts, but not in MP2. To determine whether *l'sc* gene function could replace the function of *ac/sc* within MP2, we used the GAL4 system to mis-express high levels of *l'sc* in 'MP2' [30]. If *ac*, *sc* and *l'sc* perform identical roles during neural precursor specification, then ectopic *l'sc* expression should rescue the formation of a normally specified MP2

Figure 3



ac, but not *l'sc*, completely rescues MP2 identity in *ac/sc*⁻ embryos. Odd **(a,c,e)**, Pros **(b)** and 22C10 **(d,f)** expression in single segments from *ac/sc*⁻ embryos that express either *ac* (a–d) or *l'sc* (e,f) in MP2. **(g,h)** Pros expression in three consecutive segments from wild-type (g) and *ac/sc*⁻ embryos that express *l'sc* in MP2 (h). Anterior, up; ventral midline, small line. In *ac/sc*⁻ embryos that contain the *ac* minigene all MP2s (arrows, (a–d)) express nuclear Pros protein (arrows, (b)) and ~95 % express 22C10 (arrows, (d)). In *ac/sc*⁻ embryos in which GAL4-mediated expression of *l'sc* rescues 'MP2' formation, 35 % of all 'MP2s' express 22C10, but do so at a reduced level (arrows, (e,f)). Furthermore, in this background, 26 % of 'MP2s' localize Pros to the cortex (large arrowhead, (h)) or divide prematurely (small arrowheads in (h) indicate progeny of premature 'MP2' division) and 56 % of 'MP2s' exhibit nuclear Pros as per wild-type embryos (arrows, (g,h)).

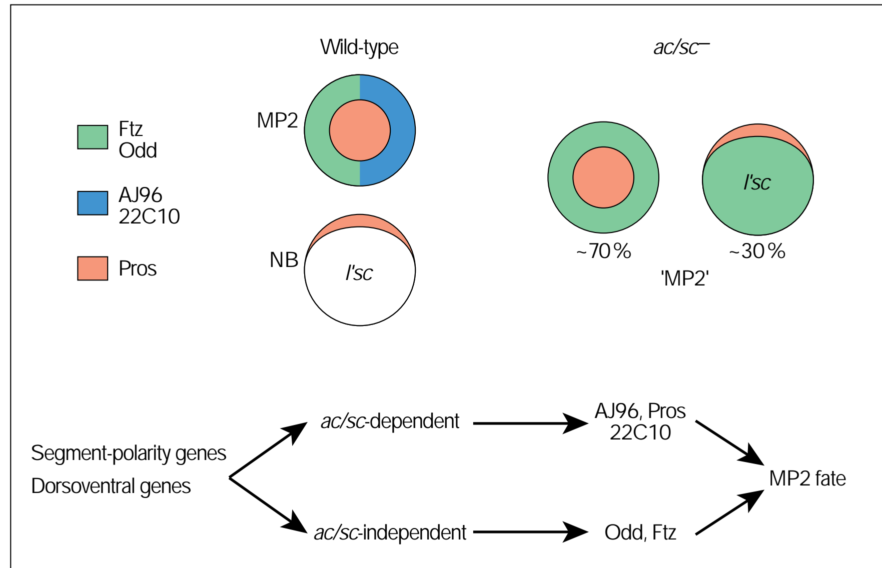
precursor. Conversely, if *l'sc* performs a different role than *ac* and *sc* during neural precursor specification, then *l'sc* should promote the formation of an incorrectly specified 'MP2' precursor. We found that ectopic *l'sc* gene expression rescued the formation of a precursor in the MP2 position; however, this precursor was specified incorrectly (Table 1). Only ~60 % of the precursors expressed the normal MP2 markers Ftz, Odd or nuclear Pros (Table 1), and only ~30 % activated the MP2 markers AJ96 or 22C10 (Fig. 3e,f; Table 1). The level of both AJ96 and 22C10 expression in MP2 was reduced significantly relative to wild-type embryos or to *ac/sc*⁻ embryos rescued with *ac* or *sc*. Moreover, 'MP2' precursors that did not exhibit strong nuclear Pros expression appeared to localize Pros to the cortex and/or divided soon after formation (Fig. 3h; Table 1) — both traits are characteristic of neuroblasts but not of MP2. Thus, the function of *l'sc* was not interchangeable with that of *ac/sc* within MP2, and the specification of MP2 was similar in embryos lacking *ac/sc* compared with those lacking *ac/sc* plus ectopic *l'sc* expression. This suggests that *l'sc* does not promote neuroblast-like traits strongly at the expense of MP2-specific traits. Our results show that the altered MP2 identity results primarily from the lack of *ac* and *sc* function, but we cannot rule out that ectopic *l'sc* expression may enhance the transformation of MP2 towards a neuroblast-like fate.

In the absence of *ac* and *sc*, does 'MP2' switch its identity to that of an identified neuroblast? The best candidates are neuroblasts 1–1, 4–2 and 5–2, which form adjacent to MP2. The progeny of neuroblasts 1–1 and 4–2 express the *even-skipped* (*eve*) gene, whereas neuroblast 5–2 expresses the *seven-up* (*svp*) gene [24]. To determine whether 'MP2' assumed the identity of one of these neuroblasts, we assayed for ectopic *eve* and *svp* expression. We never observed ectopic *eve* (n > 1000) or *seven-up* (n > 250) expression in *ac/sc*⁻ embryos. Thus, 'MP2' does not fully assume the identity of a neighboring neuroblast. This is consistent with the fact that 'MP2' retains some normal MP2-specific characteristics (Ftz and Odd expression). The ability of 'MP2' to express these genes independently of *ac* and *sc* function indicates that 'MP2' has retained at least part of its normal identity.

Conclusions

Our results show that the proneural *ac* and *sc* genes contribute to the specification of neural precursor identity in the *Drosophila* CNS. Similar results have been obtained independently in the laboratory of Fernando Jimenez [31]. In particular, we have demonstrated that at least two inputs specify the fate of the MP2 CNS precursor. The *ac* and *sc* proneural genes are required for several aspects of MP2 fate (AJ96, 22C10 expression and Pros nuclear localization), but a second pathway is necessary for other aspects of MP2 identity (Ftz and Odd expression). We speculate that the segment-polarity and dorsal–ventral genes are at the top of the genetic hierarchy controlling both *ac/sc*-dependent and independent aspects of MP2 identity (Fig. 4). For example,

Figure 4



(a) Loss of *ac/sc* function changes MP2 identity. Dorsal is to the top. MP2 normally expresses Odd, Ftz, AJ96, and 22C10 and localizes Pros protein to the nucleus. In *ac/sc*⁻ embryos, 'MP2' expresses Odd and Ftz but neither AJ96 nor 22C10. In addition, ~20 % of the time 'MP2' ectopically expresses *l'sc* and ~30 % of the time localizes Pros protein to the cortex. As all neuroblasts express l'sc and localize Pros to the cortex, these alterations represent a shift of the 'MP2' precursor towards a 'neuroblast-like' fate. **(b)** Combinatorial model for the specification of neural precursor identity in the *Drosophila* embryonic CNS. We propose that the activities of the segment-polarity and dorsal–ventral genes govern both *ac/sc*-dependent and independent aspects of MP2 identity. Complete specification of MP2 identity requires both pathways: *ac* and *sc* activate AJ96, 22C10 and Pros in MP2, and *ac/sc*-independent factors regulate Ftz and Odd expression in MP2.

in embryos mutant for the segment-polarity *gooseberry* genes, there is a nearly complete transformation of neuroblast 5–2 into MP2, with *ac* and *sc* expression replacing that of *l'sc* in neuroblast 5–2 and its proneural cluster [32]. The activities of the segment-polarity and dorsal–ventral genes along the anteroposterior and dorsoventral axes, respectively, would activate different sets of genes, of which *ac* and *sc* are one example, within neural precursors. These genes then act combinatorially to specify neural precursor identity (Fig. 4).

Taken together, our results suggest that the proneural genes of the AS-C help specify neural precursor identity in the *Drosophila* CNS. Recent evidence suggests that the role of the AS-C genes during neural precursor development is conserved phylogenetically. Furthermore, phenotypic analysis of *MASH1* (mammalian *ac/sc* homolog 1) mutant mice and *in vitro* studies suggest that *MASH1* controls many aspects of the autonomic neuronal phenotype [33]. These results in mice and our results in *Drosophila* raise the possibility that the AS-C genes contribute to the diversification of neural precursor fates throughout the animal kingdom.

Materials and methods

Fly strains and genetics

Wild-type patterns of gene expression were examined in a *ry*^{506} background. The following fly lines, all of which are homozygous viable unless indicated, were used: *Df(1)y*^{3PL}*sc*^{8R} [29]; *scabrous*-GAL4 (kindly provided by M. Mlodzik); UAS-*l'sc* (kindly provided by F. Jimenez), which contains homozygous second and third chromosomes, each containing two independent insertions of the UAS-*l'sc* transgene); the AJ96 enhancer trap line (generously provided by C. Goodman [34]); and two strains, pG4(63-1) and pG4(65-1)/CyO-*ftz lacZ*, each carrying an independent second chromosomal insertion of the *ac*-minigene (kindly provided by G. Panganiban).

For MP2 rescue experiments – *ac*-rescue embryos – *ac/sc*⁻ virgin females that carried a second chromosome homozygous for pG4(63-1) were crossed to *ac/sc*⁻ males which carried pG4(65-1)/CyO-*ftz lacZ* on their second chromosome. Embryos resulting from the cross were fixed and stained for one of the six markers and β-galactosidase. Embryos that lacked β-galactosidase expression in the *ftz* pattern were scored for the various markers in the MP2 position. To express *l'sc* ectopically in MP2 in *ac/sc*⁻ embryos, we used a second chromosomal *scabrous-Gal4* insertion which activates genes placed under the *Gal4* upstream activating sequence (UAS) throughout the neuroectoderm. As effectors, we used insertions of the *l'sc* coding sequence under the control of GAL4-binding sites (UAS-*l'sc*). *scabrous-Gal4* drives *l'sc* expression within the region where MP2 will form in wild-type embryos by stage 8 at levels comparable to or higher than the endogenous *l'sc* gene (data not shown). *ac/sc*⁻ virgin females homozygous for UAS-*l'sc* on the second and third chromosomes were crossed to *ac/sc*⁻ males heterozygous for the *scabrous-Gal4* driver. The resulting embryos were fixed and stained for the appropriate markers. One half of the these embryos carry both the *scabrous-Gal4* driver and the UAS-*l'sc* effectors. In all experiments, approximately one-half of the embryos exhibited near wild-type levels of 'MP2' formation, whereas the remainder exhibited significantly reduced 'MP2' formation (to ~10 %) indicative of *ac/sc*⁻ embryos. Only embryos that exhibited near wild-type 'MP2' formation were scored for the presence or absence of various markers in the MP2 position.

Immunohistochemistry and RNA in situ *analysis*

Standard immunohistochemical [29] and double-label immunofluorescent [28] techniques were used to stain fixed embryos with the following antibodies at the indicated dilution: rabbit anti-Odd (provided by E. Ward and D. Coulter; used at a 1:1000 dilution); mouse anti-Ftz (1:500); mouse anti-β-Gal (Promega; 1:1000); mouse anti-Pros MR1A (1:4); and 22C10 (provided by the laboratory of S. Benzer; 1:10). RNA *in situ* experiments were performed as described in [29].

Quantitation of gene expression in neural precursors in the MP2 position

In all experiments, except for those using *ac/sc*⁻ embryos, immunohistochemically stained embryos of the appropriate genotype were inspected for the presence or absence of each marker in MP2. The numbers presented indicate the total number of MP2s expressing the indicated marker over the total number of hemisegments scored. For *ac/sc*⁻

embryos, the significant decrease in MP2 formation made this method intractable. For these embryos, we used double-label confocal microscopy and the anti-Odd antibody in conjunction with either of the anti-Pros, FTZ, 22C10 or β-galactosidase antibodies to quantitate the fraction of Odd-positive 'MP2s' that also expressed each of the four markers. For *ac/sc*– embryos, the numbers presented indicate the number of Odd-positive 'MP2s' co-expressing each marker over the total number of Odd-positive 'MP2s' scored. To obtain an accurate estimate of the percent of *l'sc* positive 'MP2s' that formed in *ac/sc*– embryos, we scored 1029 hemisegments and observed 23 *l'sc* positive 'MP2s'. To approximate the total number of 'MP2s' that formed in 1029 hemi-segments, we multiplied 1029 by the frequency at which an 'MP2' formed in a hemisegment in *ac/sc*– embryos (0.11). Thus, out of approximately 113 'MP2s', 23 or 20 % expressed *l'sc* ectopically.

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