scratch, a pan–neural gene encoding a zinc finger protein related to snail, promotes neuronal development

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The Drosophila scratch (scrt) gene is expressed in most or all neuronal precursor cells and encodes a predicted zinc finger transcription factor closely related to the product of the mesoderm determination gene snail (sna). Adult flies homozygous for scrt null alleles have a reduced number of photoreceptors in the eye, and embryos lacking the function of both scrt and the pan–neural gene deadpan (dpn), which encodes a basic helix–loop–helix (bHLH) protein, exhibit a significant loss of neurons. Conversely, ectopic expression of a scrt transgene during embryonic and adult development leads to the production of supernumerary neurons. Consistent with scrt functioning as a transcription factor, various genes are more broadly expressed than normal in scrt null mutants. Reciprocally, these same genes are expressed at reduced levels in response to ectopic scrt expression. We propose that scrt promotes neuronal cell fates by suppressing expression of genes promoting non-neuronal cell fates. We discuss the similarities between the roles of the ancestrally related scrt, sna, and escargot (esc) genes in regulating cell fate choices.

[Key Words: scrt; sna; pan–neural; Drosophila; transcription factor; zinc finger; neurogenesis]

Received May 26, 1995; revised version accepted August 9, 1995.
transcription factor closely related to the product of the
mesoderm determination gene sna. Elimination of sct
function results in a mild reduction of photoreceptor cell
number in escaping adult flies but does not obviously
disrupt morphogenesis of the embryonic nervous sys-
tem. However, double mutant embryos lacking both sct
and dpn display a significant loss of neurons. This neu-
ronal loss phenotype is evident early during neurone
sis as the number of neuroblasts is reduced in sct; dpn
double mutant embryos. Conversely, ubiquitous expres-
sion of sct in transformed flies carrying sct under the
control of a heat shock promoter (HS–sct flies) leads to
the production of extra neurons during embryogenesis
and adult neurogenesis. The increased number of neu-
rons in HS–sct embryos may be attributable in part to
the precocious appearance of neuronal precursor cells.
Finally, we provide evidence that sct functions to re-
press expression of various target genes including the
non-neuronal Drosophila Egf-Receptor [Egf-r] gene. These
data suggest that sct functions in combination with
dpn to promote neuronal differentiation by repress-
ing expression of genes promoting non-neuronal fates.
We compare the roles of sct and dpn in neurogenesis to
that of sna in mesoderm formation.

Results
Identification of sct, a new gene expressed
in all neuronal precursors

Several enhancer trap lines recovered in a screen for pat-
terned gene expression (Bier et al. 1989) express lacZ in a
pan–neural pattern (Bier et al. 1992). Among these pan–
neural insertions, two map cytologically to the 64A re-

dion of the P-element insertion sites was isolated by plas-
mid rescue. An antisense digoxigenin-labeled RNA
probe made from one of the rescued genomic fragments
hybridized in situ to a transcript expressed in the pre-
picted pan–neural pattern [Fig. 1; see Materials and
methods for details of cloning]. We have named this
flanking gene sct on the basis of one of the phenotypes
exhibited by survivors homozygous for a deletion of the
coding sequences [scarred facets in the eye] and on the
presence of five zinc-finger domains [Fig. 3B] in the en-
coded protein [see below]. sct transcripts are first detect-
able in a single row of ectodermal cells flanking the
ventral midline [Fig. 1A] during the early germ-band-
extended stage [stage 8]. sct-expressing cells then de-
laminate and contribute to the first of three rows [r1–r3]
of S1 neuroblasts in the CNS [Fig. 1B,C]. sct is subse-
quently expressed in all S1 neuroblasts as well as in neu-
roblasts formed during later rounds of segregation. sct
continues to be expressed throughout the neuronal lin-
eage in cells likely to be the immediate progeny of neu-
roblasts, the ganglion mother cells [GMCs], and then in
postmitotic neurons. sct expression differs from that of
dpn in that dpn is not expressed in GMCs [Bier et al.
1992]. This is illustrated in Figure 1D, where sct expres-
sion in GMCs obscures the rosette pattern of neuroblasts
expressing dpn in a late germ-band-extended embryo
(Fig. 1E). Similarly, in the PNS sct is first expressed in
primary SMCs [Fig. 1D], then in cells likely to be sec-
ondary precursor cells [Fig. 1F], and finally in postmitotic
neurons [Fig. 1G]. Neuronal expression of lacZ derived
from sct enhancer trap insertions persists long enough
to label all identified neurons of the PNS [Fig. 1G,H].

We have used double label in situ hybridization
[O’Neill and Bier 1994] to compare the pattern of sct
expression to that of dpn and sna. This analysis reveals
that in the embryo most or all cells of the nervous sys-
tem expressing sct also express dpn [data not shown],
consistent with the view that these two genes are ex-
pressed in most or all primary neuronal precursor cells.
The onset of sct and dpn expression appears to vary
among neuroblasts. sct and dpn expression appears to vary
among neuroblasts. Subsequently, however, dpn ex-
pression precedes sct in the CNS and in SMCs of the
PNS. In contrast, sct and sna have only partially over-
lapping expression patterns. Most S1 neuroblasts express
sna before expressing sct, but many neuronal progeny
expressing sct do not express sna. Also, sna is expressed
in the support cells of chordotonal organs [CHO],
whereas postmitotic sct expression is restricted to the
neuron. Similarly, double labeling of dpn and sna reveals
that many neuronal precursors in the CNS and PNS do
not express sna at early stages of neuronal precursor for-
mation [data not shown]. Finally, sna differs from sct
and dpn in that it is expressed in embryonic dorsal imagi-

nal disc primordia and in abdominal histoblasts [Al-
berg et al. 1991].

To determine whether sct might play a role in adult
neurogenesis we examined sct expression in the CNS of
third-instar larvae and in imaginal discs. We observed
that sct is expressed in neuronal cells in the wing disc
[Fig. 1I], in developing neurons in pupal wings [data not
shown], in many cells posterior to the morphogenetic
furrow in the eye–antennal disc [Fig. 1J], in a pattern
resembling that of neuronal precursor cells in the leg
that express the pan–neural gene a sense [data not
shown; Brand et al. 1993; Domínguez and Campuzano
1993], and in many cells in the third-instar larval brain
and ventral nerve cord [data not shown]. Similar to the
situation in embryos, sct and sna have overlapping but
distinct neuronal expression patterns in imaginal discs:
sna is expressed late in neurons of everting wing discs
but not earlier in neuronal progenitors and is absent dur-
ing larval stages of eye disc development.

sct encodes a predicted five zinc finger protein
with homology to sna

The genomic fragment including a pan–neural transcrip-
tion unit was used to probe Drosophila genomic and em-
byronic cDNA libraries [see above and Materials and
methods]. Genomic clones were recovered and restric-
tion mapped [Fig. 2], and several cDNAs were isolated
and sequenced [see Materials and methods]. A digoxige-
nin-labeled probe made from a cDNA containing a com-
plete open reading frame hybridizes to a transcript ex-
Figure 1. Pan-neural expression of *scrt* during embryogenesis. The pattern of *scrt* expression was determined by in situ hybridization using an anti-sense digoxigenin-labeled *scrt* genomic probe containing the complete *scrt* transcription unit, which includes one or more introns. A similar expression pattern is observed using a cDNA probe with the exception that nuclear transcripts are not as intensely labeled prior to cytoplasmic translocation of the mature message. In this and subsequent figures, anterior is to the left and dorsal is to the top, unless indicated otherwise, and the ventral midline is indicated by an arrowhead. Embryos are staged according to Campos-Ortega and Hartenstein (1985). [A] A ventral view of *scrt* expression in ectodermal cells in the region giving rise to the inner (r1) row of SI neuroblasts prior to delamination (~5-5½ hr). [B] A ventral view of *scrt* expression in cells comprising the full SI wave of neuroblasts in an early germ-band-extended embryo (~5½ hr). Neuroblast rows are numbered r1−r6. [C] *scrt* expression in an embryo at the same stage shown in A viewed in sagittal section. The ectoderm (e), mesoderm (m), and neuroblasts (n) are indicated. [D] A ventral view of *scrt* expression in a late germ-band-extended embryo (~6½ hr). The majority of neuroblasts have delaminated by this stage. CNS labeling is solid by this stage (arrow), differing from the ring-like staining of neuroblasts observed with *dpn* at the same stage (see E). Note the two PNS precursor cells located dorsal to the CNS [bracket]. [E] *dpn* expression in neuroblasts at the same stage shown in D. This embryo has been flattened more than the embryo in D during mounting, leading to an artifactual appearance of a broader CNS. At this stage, *dpn* is expressed only in neuroblasts arranged in a ring (or rosette) with an unlabeled center [arrow]. The first two PNS precursor cells that express *dpn* at this stage are out of focus. [F] *scrt* expression in most or all PNS precursors of a germ-band-retracting embryo (~8½–9 hr). [G] Pattern of β-galactosidase expression in dorsal (d), lateral (l), ventral prime (v'), and ventral (v) clusters of PNS neurons in germ-band-retracted embryos carrying a *scrt P-lacW* enhancer trap insertion. [Top] The embryo is viewed laterally; [bottom] the embryo is viewed ventrolaterally. [H] A diagram of PNS cells, slightly modified from those of Ghysen et al. (1986), Bodmer and Jan (1987), and Hartenstein (1988) to account for the incomplete migration of neurons, is shown for reference. [I] *scrt* expression in a third-larval instar wing imaginal disc. [J] *scrt* expression in a third-instar larval eye-antenna imaginal disc. The morphogenetic furrow is denoted by an arrowhead.

pressed in the same pan-neural pattern as the genomic fragment used to screen the cDNA library. A Northern blot hybridized with the same cDNA probe reveals a single transcript of 6.5 kb present in 4- to 12-hr embryos but absent in 0- to 4-hr embryos [data not shown], consistent with the expression pattern observed by whole-mount in situ hybridization.

One of the longest cDNA clones contains a single long open reading frame predicting a protein of 664 amino acids [Scrt] with five zinc fingers near its carboxyl terminus [Fig. 3A]. The predicted amino acid sequences of each of these zinc fingers show similarity to each other as well as to each of the five zinc fingers of the *Sna* protein, which is required for mesoderm determination,
and of the Escargot (Esc) protein, which controls polyplody during imaginal disc growth (Fuse et al. 1994). In the most conserved stretch of 46 amino acids, Scrt exhibits 76% identity (and 87% similarity permitting only the most conservative changes) with Sna. Each zinc finger in Scrt most resembles its counterpart in the Sna and Esc proteins. Fingers 3 and 4 exhibit particularly high degrees of sequence similarity with runs of 12/13 and 13/14 identical residues between Scrt and Sna. This high degree of sequence identity extends to proteins encoded by various vertebrate sna homologs (Fig. 3B).

**scrt collaborates with dpn to promote neurogenesis**

To determine the function of the scrt gene we selected for white minus transposase-induced revertants of one of the P-lacW insertions into the scrt locus. Among these revertants, five imprecise jump-out excisions (scrt10) of the P-lacW element were recovered (scrt106, scrt1011, scrt1012, scrt1016, and scrt1017). Homozygous scrt1011, scrt1012, and scrt1017 escapers have a subtle but completely penetrant rough eye, frequently have scratched facets near the edge of the eye, hold their wings vertically under mild CO2 anesthesia, and are so weak and uncoordinated that they often get stuck in the food immediately after eclosion. Similarly, each of the scrt10 chromosomes in trans to large deletions of the 64A region [Df(3L)HR277 and Df (3L)A466] show the same phenotypes. The scratched eye phenotype consists of black scars near the anterior margin of the eyes resembling scratched eye, a mutation that maps nearby in the 64C–E region [Lindsley and Grell 1968]. The five scrt10 revertants fail to complement each other, but do complement the scratched eye mutation. The recovery of five independent transposase-induced revertants causing similar eye defects suggests that a nearby gene contributes to normal eye development.

Southern blot analysis of the scrt10 revertants indicates that they lack varying amounts of genomic DNA, including part or all of the scrt transcription unit (Fig. 2). Consistent with these findings, scrt expression is undetectable by in situ hybridization in embryos homozygous for the scrt1011, scrt1016, and scrt1017 mutations and is greatly reduced in scrt1006 homozygous mutants. Because each of these transposase-induced lesions is associated with overlapping local deletions of the scrt transcription unit, we conclude that the complementation group defined by these revertants corresponds to the scrt gene.

The revertant carrying the largest deletion of scrt genomic sequences, scrt1011, lacks all cDNA-coding sequences of the scrt gene and therefore represents a null mutation. Although homozygotes for this small deletion show low viability, they do eclose as adults under favorable conditions [see above] and are fertile in outcrosses. Embryos homozygous for the scrt1011 deletion exhibit no detectable morphological defects when examined carefully with a variety of tissue-specific markers including early neuronal precursor markers [e.g., ASC-T3, dpn, sna, Hunchback (Hb), and Prospero [Pros]], late postmitotic neuronal markers [embryonic lethal abnormal visual system (elav), anti-HRP, mAb22C10, Even-skipped (Eve), and cho, a chordotonal organ neuron-specific marker], epidermal markers [orthodenticle (otd), Enhancer of split (E(spl)lm7), Serrate (Ser), tramtrack (ttk), and rho], and early and late muscle markers [nautilus and mbA6D9] [data not shown]. Similarly, embryos homozygous for the scrt1014, scrt1016, and scrt1017 mutations appear morphologically normal as do embryos trans-heterozygous for any of the five scrt10 alleles in combination with either Df(3L)HR277 or Df (3L)A466 [data not shown].

As embryos lacking scrt function appear morphologically normal, we tested the possibility that scrt might collaborate with other genes to promote embryonic neurogenesis. Thus, we examined nervous system formation in embryos lacking other pan–neural genes in addition to

**Figure 2.** Molecular map of the scrt locus. Restriction map of the genomic region encompassing the scrt locus. Locations of a single and a double head-to-head P-lacW insertion site are indicated above the map. The region hybridizing to the longest scrt cDNA is shown immediately below the map. The extents of various scrt10 deletions are also indicated below the map as well as the limits of the genomic phage inserts covering the scrt locus.
The predicted protein sequence derived from conceptual translation of cDNA sequences. Several clones obtained from Kuvar and Zinn embryonic cDNA libraries were sequenced, including one clone containing the full scrt open reading frame [Zinn library]. Alignment of zinc finger domains of Scrt, Drosophila Sna [Boulay et al. 1987], Xenopus Sna [Sargent and Bennett 1990], Drosophila Escargot [Whitely et al. 1992], and Chicken Slug [Nieto et al. 1994]. Other Sna-related proteins not shown in the alignment include those isolated from zebrafish (Hammerschmidt and Nüsslein-Volhard 1993; Thisse et al. 1993) and mouse (Nieto et al. 1992, Smith et al. 1992). Boxed positions indicate amino acids for which a strong consensus sequence exists among the various zinc fingers. Stars indicate the invariant positions defining the larger family of zinc finger proteins to which the Sna/Scrt/Esc subgroup belongs. Boldface type indicates an amino acid residue that is most frequent at a given position within a particular zinc finger repeat. Italicized residues are similar to the consensus amino acid for that position. The GenBank accession number for the scrt sequence is U36477.
ditions, animals homozygous null for each of these genes occasionally complete development and eclose. In contrast, animals homozygous null for both genes never hatch and frequently exhibit a dramatic reduction of the nervous system [Fig. 4B,E,H]. In the PNS of dnp\(^1\); scrt\(^{011}\) double mutant embryos, where individual neurons can be identified, we consistently observed a reduction in the number of neurons labeled with mAb22C10 [Fig. 4, cf. B and A], anti-HRP [data not shown], and cho [Fig. 4; cf. E and D]. In addition, axon projections are frequently disorganized in dnp\(^1\); scrt\(^{011}\) double mutant embryos. dnp\(^1\); scrt\(^{011}\) double mutant embryos also exhibit serious CNS defects such as missing and disorganized longitudinal and commissural axon tracks labeled with anti-HRP and loss of CNS neurons including a fraction of those expressing Eve [data not shown]. CNS defects are evident early during neurogenesis as there are fewer Hb-expressing cells contributing to the SI wave of neuroblasts than in wild type embryos [Fig. 4, cf. H and G].

Ectopic expression of scrt generates extra neurons

The loss of neurons in embryos double mutant for scrt and dnp described above and the reduction in photoreceptor number in scrt single mutants [see below] suggests that scrt promotes neuronal development. To test this hypothesis further we examined the consequence of ectopic scrt expression during embryogenesis. A HS–scrt construct in which the scrt-coding region is under control of the hspl0 promoter in the P[hs–CaSpeR] vector [Bang and Posakony 1992] was introduced into flies by P-element-mediated transformation (Spradling 1986). The consequence of brief ubiquitous expression of scrt following heat induction was examined at a variety of developmental stages. A 1-hr heat shock beginning at 3–5 hr of embryogenesis leads to an increase in cells staining with mAb22C10. Cuticle mounts of embryos heat-shocked at these times occasionally display duplicated external structures such as hairs or pits [data not shown]. More frequently, we observe missing cuticular structures, such as campaniform sensilla, which may correspond to the transformation of secondary outer support cell precursors into inner neuronal/glial precursors [see adult data described below]. Multiplication of mAb22C10 staining cells in the region of the developing chordotonal organs is a very penetrant phenotype of HS–scrt embryos [Fig. 4C]. The additional cells have a CHO neuronal identity [Fig. 4, cf. F and D]. In addition, the lateral CHO clusters are often misplaced, and PNS axons follow abnormal pathways. HS–scrt embryos also have CNS defects. For example, the number of Hb-expressing cells is greater than in wild-type embryos of comparable age [Fig 4, cf. I and G]. The increase in the number of Hb-expressing neuroblasts results in part from a precocious appearance of primary neuronal precursor cells. Thus, Hb–, scute– [T4], achaete– [T5], and dnp-expressing neuroblasts first appear during early stages of germ-band extension in HS–scrt embryos [Fig. 4K,L; dnp–4½ hr], more than an hour before neuroblast delamination or expression of neuroblast markers in wild-type embryos [Fig. 1B, scrt; Fig. 4, dnp]. The very earliest time when subectodermal dnp-expressing cells can be observed in HS–scrt embryos is at the beginning of germ-band extension, when the most terminal stripe of dnp pair–rule expression is still visible [data not shown]. Reciprocally, expression of dnp in proneural-like ectodermal patches prior to neuroblast delamination (Bier et al. 1992) is foreshortened in HS–scrt embryos. These patches have nearly disappeared before germ-band extension is >50% complete [Fig. 4K,L]. dnp and Hb expression in PNS precursors is also observed earlier than usual as SMCs are visible during the beginning of the extended germ-band stage when the SI stage of neuroblast delamination normally commences [Fig. 4I, bracket]. This precocious expression of neuronal precursor markers is not a result of heat shock treatment, as heat-induced wild-type embryos express Hb and dnp on schedule relative to morphological criteria such as extent of germ-band elongation [data not shown]. It is possible that ectopic expression of scrt slows germ-band extension rather than accelerating neuroblast formation. We believe this to be unlikely, however, because the complex pattern of cells expressing rho and otd do so in step with the morphological measures of germ-band extension [data not shown]. In the case of rho, the heat shock treatments were effective in these experiments because rho expression in tracheal pits was specifically reduced [see below]. In addition, the fact that the proneural-like expression of dnp is not shifted to an earlier period but, rather, is significantly foreshortened suggests that the cells that normally would express dnp in the proneural pattern have prematurely made the neuroblast versus epidermal cell late choice.

scrt promotes adult neurogenesis

Because a rough eye is the most penetrant loss-of-function scrt phenotype, we examined the effects of loss of scrt and ubiquitous scrt expression in the eye. Eyes of scrt\(^{011}\)/scrt\(^{011}\) homozygotes and of flies homozygous for the HS–scrt construct that had been heat-shocked for 1 hr during the late third-larval instar were dissected, fixed, and sectioned. Flies homozygous for the scrt\(^{011}\) deletion have a mild, but highly penetrant, rough eye phenotype. In superficial sections of the eye the pattern of lenses and interommatidial bristles is relatively normal [data not shown]. In deeper sections passing through the apical regions of photoreceptor cells we frequently observe ommatidia with fewer than seven photoreceptor cells. In addition, many of the photoreceptors appear shrunken relative to wild type, the number of pigment cells is reduced, and the ommatidial units are often broken and abnormally spaced. The shrunken cell phenotype becomes progressively more obvious in deeper sections where large empty spaces are observed between ommatidia [Fig. 5, cf. B and A].

In contrast to the loss of photoreceptor cells observed in scrt\(^{011}\) homozygous eyes, ommatidia of heat-shocked HS–scrt flies frequently have eight rather than seven photoreceptor cells [Fig. 5C]. This extra cell may be a
Figure 4. Embryonic scret phenotypes. The neuronal loss phenotype of dprt; scret double mutant embryos was observed in collections of dprt/+; scrt^+/+; TM6AbdA-lacZ and Df(2L)193A/Cyotz-lacZ; scrt^+/+; TM6AbdA-lacZ balanced stocks [Df(2L)193A eliminates the dprt gene]. Only double mutant embryos without any β-galactosidase protein expression exhibited the loss of neuron phenotype. The same phenotype was also observed in unmarked outcrosses of dprt^+/+, scrt^+/- crossed to Df(2L)193A/+; scrt^+/- flies in which double mutant embryos were scored by the absence of scret and dprt RNA expression [data not shown]. (A) A wild-type germ-band-retracted embryo (~14 hr) stained with the mAb22C10 antibody. The arrows in A–C point to the location of the lateral CHO cluster. (B) A dprt^+; scrt^+/- double homozygous mutant embryo stained with mAb22C10 has reduced numbers of labeled cells in each cluster. (C) A HS-scret embryo heat-shocked for 1 hr at 5–6 hr of development and aged for 7 hr at 25°C has an increased number of mAb22C10-labeled cells. The lateral CHO cluster indicated by the arrow is magnified (inset) to show 10 CHOs (wild-type embryos have 6 CHOs in this region). (D) A wild-type embryo hybridized with a CHO neuron-specific probe [cho; L. Ditmer and L.J. Deftos, unpubl.). The large arrow indicates the position of the lateral CHO cluster (containing 6 ± 0 neurons; n = 10 hemisegments), the small arrow indicates the position of the two ventral CHOs (see diagram in Fig. 1H for the relative position of these cells with respect to other neurons). Arrows point to the same positions in D and E. (E) A dprt^+; scrt^+/- double homozygous mutant embryo hybridized with the cho probe. Note the complete loss of ventral CHOs and the severe reduction in number in lateral CHOs (average = 3 neurons ± 1, n = 50 hemisegments). (F) cho expression in a HS-scret embryo heat-shocked for 1 hr at 5–6 hr of development and aged for 7 hr at 25°C. Note the increased number of labeled cells in both lateral (average = 8 neurons ± 1; n = 50 hemisegments) and ventral clusters. These clusters are also no longer located in stereotyped positions. (G) Expression of Hb protein in neuroblasts of a wild-type embryo (~5½ hr) in which the SI wave of neuroblasts has delaminated and the first S2 neuroblasts have formed [at this stage Hb is detectable in an average of 37 ± 1 cells per 4 hemisegments, n = 24 hemisegments]. Neuroblast rows r1–r3 also are indicated. (H) Hb expression in a dprt^+; scrt^+/- double mutant embryo at the same stage as shown in G. Fewer cells express Hb (average = 27 ± 3 cells per 4 hemisegments; n = 24 hemisegments) indicating that neuroblasts are either missing or abnormal. (I) Hb expression in a HS-scret embryo heat-shocked for 1 hr at 4 hr of development and aged for 1 hr at 25°C. More Hb-positive cells are observed than in wild-type embryos at a similar stage of germ-band elongation (average = 52 ± 5 cells per 4 hemisegments; n = 32 hemisegments). (J) A ventral view of dprt expression in SI neuroblasts in a wild-type germ-band-extended embryo. The arrow marks the posterior end of the germ band that is in contact with the back of the head. (K) A ventral view dprt expression in a HS-scret embryo that was heat-shocked for 1 hr at 3 hr of development and aged for 1 hr at 25°C. The arrow marks the posterior end of the germ band that has not progressed beyond 50% elongation. (L) dprt expression in a HS-scret embryo similar to that shown in K but viewed in sagittal section. The arrow marks the posterior end of the germ band. (e) Outer ectodermal layer; (n) neuroblast layer; (m) inner mesoderm layer.
particular photoreceptor cell that is duplicated, an accessory cell converted to a neuronal fate, or a misplaced R8 cell. scrt also collaborates with dpn during photoreceptor development as well as during embryogenesis (see above) because $dpn^{+/+}; scrt^{1011}/scrt^{1011}$ have an enhanced rough eye phenotype and have a more severe loss of photoreceptor phenotype than typically observed for $scrt^{1011}/scrt^{1011}$ alone (data not shown). This interaction between scrt and dpn is noteworthy, as eyes of homozygous $dpn$ flies appear normal (data not shown).

We performed a series of timed heat induction experiments to identify specific developmental stages when restricting scrt expression to the developing nervous system is essential (Table 1). Thus, heat shocks adminis-
Table 1. Adult phenotypes from heat shocks during imaginal development

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<th>Phenotype</th>
<th>Stage: (Stage) The time that the first of two 45-min heat shocks was begun (the two heat shocks were separated by 1 hr).</th>
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Larvae or pupae of various stages were collected onto wet Whatman filter paper in petri dishes and heat-shocked at 38°C by floating in a circulating water bath. Data are expressed as the percentage of animals showing the phenotype out of the total number scored.

*d. thorax* Dorsal thorax

**[Stage] The time that the first of two 45-min heat shocks was begun (the two heat shocks were separated by 1 hr). [hr] Hours after puparium formation, where 0 hr = white prepupal stage; 1i, 2i, and 3i = first-, second-, and third-instar larvae; f = feeding, and w = wandering.**

- Missing posterior crossvein: 94 24 64 67 100 89
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Loss of function and misexpression of scrt have opposite effects on target gene expression suggesting that scrt acts as a transcriptional repressor

As scrt is likely to encode a transcription factor we tested the effects of altered scrt function on the expression of various marker genes during embryogenesis. The Efg-r gene is one example of a potential scrt target gene. Normally, Efg-r is expressed in the mesodermal and ectodermal cell layers of the germ-band-extended embryo [Kamermeyer and Wadsworth 1987; Zak et al. 1990, Katzen et al. 1991, Zak and Shilo 1992, Sturtevant et al. 1993].
1994) but is excluded from the neuroblast layer (Fig. 6A). In *scrt* null animals, however, ectopic *Egf-r* expression is observed at low levels in most neuroblasts and at high levels in occasional neuroblasts (data not shown). Ectopic expression of *Egf-r* in the neuroblast layer is significantly stronger and more penetrant in *dpn*; *scrt* double mutant embryos (Fig. 6B), whereas neuron specific markers (e.g., Hb, *sna, Pros, anti-HRP, Eve, mAb22C10, and *cho*) are expressed normally in the reduced number of neuronal cells. *otd*, another marker normally expressed only in epidermal cells during germ band extension, also is ectopically expressed in neuroblasts in *dpn*; *scrt* double mutant embryos (data not shown). Reciprocally, heat-induced HS-*scrt* animals exhibit a dramatic loss of *Egf-r* staining in the ectoderm, although mesodermal expression remains unaffected (Fig. 6C). Similarly, expression of both *rho* and *Ser* is repressed in developing tracheal cells in heat-induced HS-*scrt* embryos, whereas other epidermal components of these gene expression patterns remain unaffected (data not shown). Epidermal expression of *ttk*, however, is not obviously affected in either *dpn*; *scrt* double mutant embryos or in heat-induced HS-*scrt* animals (data not shown). Other than an increase in the number of neuronal cells in HS-*scrt* heat-induced embryos (as assayed by various markers; see above), non-neuronal cells such as

Figure 6. Alterations in gene expression patterns because of loss of function or misexpression of *scrt*. (A) Pattern of *Egf-r* expression in a wild-type germ-band-extended embryo. *Egf-r* is expressed strongly in both the ectoderm (e) and in the mesoderm (m) but is present at much lower levels in the neuroblast layer. (B) *Egf-r* expression in a *dpn*; *scrt* homozygous double mutant extends into the neuroblast layer. (C) *Egf-r* expression in a HS-*scrt* embryo that received a 1-hr heat shock at 4–5 hr of development and was allowed 1 hr of recovery at room temperature prior to fixation and staining. Note that virtually all epidermal staining is eliminated. Similarly heat-shocked control embryos have the wild-type pattern of *Egf-r* expression, although at slightly reduced levels. (D) *Egf-r* expression in a wild-type eye disc. Anterior is to the left and dorsal is at the top. Note that staining is most intense at the morphogenetic furrow but also is expressed at significant levels posterior to the furrow. (Inset) The EGF-R protein pattern in the dorsal portion of the disc. (E) *Egf-r* expression in a *scrt* mutant eye disc. Labeling posterior to the furrow is significantly elevated relative to wild type [arrow]. This is most evident in the pattern of EGF-R protein expression (inset). (F) *Egf-r* expression in a HS–*scrt* eye disc following 1 hr of heat shock and 45 min of recovery. *Egf-r* expression is reduced significantly, particularly posterior to the morphogenetic furrow. Uninduced HS–*scrt* eye discs or heat-induced wild-type eye discs have the wild-type pattern of *Egf-r* expression. (G) *scabrous* expression in a wild-type eye disc. Typically, three rows of cells in and mutant eye disc is expanded in the posterior direction to approximately five rows of labeled cells posterior to the furrow. (H) *scabrous* expression in a HS–*scrt* eye disc following 1 hr of heat shock and 45 min of recovery is reduced to one to two rows of labeled cells posterior to the furrow. (I) *hh* is expressed in a domain posterior to the furrow in a wild-type eye disc. Expression in the more differentiated region of the disc has faded by this time generating a relatively unstained posterior domain [arrow]. (K) *lh* expression in a *scrt* mutant eye disc is expanded in the posterior direction to include nearly all clusters virtually eliminating the unstained posterior domain. (L) *hh* expression in a HS–*scrt* eye disc following 1 hr of heat shock and 45 min of recovery is reduced relative to wild type. The unstained posterior domain is larger than in comparable wild-type discs. Expression in the antennal portion of the disc is less affected by HS–*scrt* expression, providing an internal control for staining efficiency. (M) *hairy* expression in a wild-type eye disc. Note that much of the *hairy* expression domain is anterior to the furrow. (N) *hairy* expression in a *scrt* mutant eye disc is significantly expanded in the anterior direction relative to wild type. (O) *hairy* expression in a HS–*scrt* eye disc following 1 hr of heat shock and 45 min of recovery is reduced relative to wild type.
epidermal or muscle cells do not express inappropriately any neuronal marker tested.

Because scrt null and HS–scrt mutant animals have opposite defects in photoreceptor number and because scrt normally is expressed in photoreceptor cells of the developing eye [Fig. 1], we also examined expression of various characterized markers during eye development in these mutants. Digoxigenin-labeled RNA probes for various genes were hybridized to developing eye imaginal discs of wild-type animals (with or without heat induction), scrt"" homozygotes, and HS–scrt flies (heat-shocked for 1 hr prior to fixing and staining of discs). For each marker, labeling of heat-shocked and non-heat-shocked wild-type discs was indistinguishable [data not shown].

In the absence of scrt, the number of cells in the differentiating field of ommatidia-expressing Egf-r RNA is increased [Fig. 6, cf. E and D]. This is reflected by an increase of Egf-r mRNA (detected by in situ hybridization) and by elevated EGF-R protein [revealed by antibody staining, insets in Fig. 6D,E]. Conversely, when scrt is overexpressed via heat shock, Egf-r RNA levels are reduced [Fig. 6F].

To examine events within, behind, and in advance of the furrow we probed eye discs for expression of genes such as scabrous (sca), hedgehog (hh), decapentaplegic (dpp), and hairy (Fig. 6G–O). For both the sca and hh probes, loss of scrt function results in an increased number of cells in the differentiating field behind the furrow expressing the marker [Fig. 6, cf. H and G for sca; and Fig. 6, cf. K and J for hh]. Conversely, ectopic expression of scrt results in fewer cells expressing sca [Fig. 6, cf. I and G] or hh [Fig. 6, cf. L and J]. dpp expression in scrt"" or HS–scrt eye discs, however, is indistinguishable from wild type.

Expression of hairy is also altered in response to scrt. Loss of scrt function leads to increased levels of hairy RNA in cells immediately anterior to the furrow [Fig. 6, cf. N and M], whereas ectopic scrt leads to dramatically reduced levels of hairy RNA [Fig. 6O]. The loss-of-function effect must be indirect, as hairy expression is altered anterior to the morphogenetic furrow, where cells normally do not express scrt.

**Discussion**

**scrt promotes neuronal development**

A variety of evidence suggests that the normal function of the scrt gene is to promote neuronal development. Consistent with a role in specifying neuronal cell fates, scrt expression is restricted to the nervous system at all developmental stages examined. Although embryos homozygous for null scrt alleles appear morphologically normal and can survive to adulthood if cultured with care, adult scrt"" escapers have slightly roughened eyes reflecting a reduction in photoreceptor number. Deeper within the eye the scrt"" mutant phenotype is much more severe, with large spaces separating broken ommatidial clusters. In addition, there are genetic interactions between scrt and dpn, another pan-neural gene, which aggravate the adult eye phenotype. The synergistic action of dpn and scrt is particularly striking in dpn"", scrt"" homozygous double mutant embryos that have significant reductions in neuron number. Consistent with loss-of-function scrt phenotypes leading to neuron loss, ectopic expression of scrt leads to the production of supernumerary neurons during embryogenesis and development of the adult nervous system. These data suggest that scrt normally plays a role in promoting neurogenesis but that additional genes (e.g., dpn) act in parallel with scrt. The strong genetic interaction between scrt and dpn provides evidence that dpn is also likely to play a role in promoting nervous system development.

**scrt represses expression of target genes**

scrt encodes a zinc finger protein related to the products of the sna and esc genes, which have been shown to repress expression of various target genes [Kosman et al. 1991; Leptin et al. 1991; Rao et al. 1991; Ip et al. 1992; Fuse et al. 1994]. We have examined the expression of a variety of neuronal and non-neuronal markers in mutants lacking scrt and in HS–scrt individuals induced to express scrt ubiquitously to determine whether scrt might function analogously as a repressor of non-neuronal genes. These experiments have identified several potential scrt target genes such as the Egf-r gene. Ectopic Egf-r expression is sparsely observed in the neuroblast layer of scrt mutant embryos. This phenotype is enhanced in dpn"", scrt"" double mutant embryos suggesting that dpn also contributes to repression of Egf-r expression in the nervous system. Egf-r also is expressed at higher than normal levels in developing photoreceptor cells in scrt"" eye discs. Reciprocally, Egf-r is strongly down-regulated in epidermal cells when scrt is expressed ubiquitously during embryogenesis or adult development.

In general, loss of scrt function leads to ectopic expression of potential target genes, whereas, reciprocally, ubiquitous scrt expression leads to a reduction in expression of these genes. Because scrt functions to promote the formation of neurons at the level of cell fate specification, we propose that scrt represses transcription of genes such as Egf-r that promote the establishment of non-neuronal cell fates. In contrast, expression of all neuronal markers we have examined is normal in scrt"", dpn"", scrt"", and HS–scrt embryos. These data are consistent with Scrt acting like Sna and Esc to repress expression of target genes. However, more potential direct target genes must be identified and the cis-acting elements of putative scrt responsive genes must be analyzed for functional scrt repressor binding sites to establish direct repression as a mechanism of scrt action.

Whereas some of the effects that we observe on gene expression patterns may be attributable to the direct action of scrt as a transcription factor, the anterior expansion of hairy expression in scrt"" eye discs must be indirect because cells anterior to the furrow normally do not express scrt. It is possible that the expansion of hh.
expression posterior to the furrow in scrt^{iso} mutants plays some role in mediating this effect, although existing data support models in which Hh diffusing over a short distance induces expression of dpp, which in turn encodes a long-range signal to promote furrow progression [Heberlein et al. 1993; Ma et al. 1993]. Interestingly, a recent role for hairy in combination with extramacrochaete (emc) in eye development suggests that these negative regulators of neurogenesis function to retard progression of the furrow [Brown et al. 1995]. Thus, scrt expression posterior to the furrow may promote indirectly furrow progression by suppressing expression of signals required for activating genes such as hairy ahead of the furrow, which slow furrow progression. The precocious appearance of neuroblasts and primary PNS precursor cells in HS–scrt embryos similarly could be explained by models in which scrt functions normally to initiate neurogenesis, perhaps by repressing expression of genes that antagonize neurogenesis.

It is worth noting that pan–neural expression of several vertebrate genes depends on repression of these genes in non-neuronal cells by a factor belonging to a large subfamily of zinc finger proteins that includes scrt and sna [Chong et al. 1995; Schoenherr and Anderson 1995]. Thus, nervous system specific gene expression depends on two forms of negative regulation: (1) repression of non-neuronal genes in the nervous system, and (2) repression of nervous system-specific gene expression in non-neuronal tissues. This suggests that repression must be considered on par with activation as a general mechanism for achieving nervous system specific gene expression.

The role of known pan–neural genes in neurogenesis

Data described above suggest that scrt and dpn collaborate to repress expression of non-neuronal genes in neuroblasts. Although DNA binding has not yet been demonstrated for Scrt, the amino acid sequence in the DNA-binding zinc finger region is highly similar to Sna, which does bind specific DNA sequences and has been shown to behave as a repressor [Ip et al. 1992]. Similarly, the bHLH region of Dpn is closely related to Hairy, which binds functionally important sequences in the achaete promoter to repress gene expression [Van Doren et al. 1994; Ohshako et al. 1994]. Recently, Dpn also has been found to bind DNA [K. Wallace and H. Vaessin, in prep.]. Likewise, E(spl)m8, another protein with a bHLH region related to Hairy, binds DNA and this activity is required to mediate the E(spl)^{D} phenotype [Tietze et al. 1992]. Sna has been shown to function as a repressor over short distances ([<]100–150 bp; Ip et al. 1992; Gray et al. 1994). Dpn, Hairy, and E[spl] proteins repress transcription of target genes by recruiting Groucho through an interaction with the carboxy-terminal WRPWM motif [Ohshako et al. 1994; Paroush et al. 1994; Van Doren et al. 1994], although it remains to be determined whether this takes place over short or long distances (>1 kb). Thus, it is possible that scrt and dpn collaborate through different mechanisms to repress expression of target genes. In this case, the partial redundant function of these two genes would not be attributable to one gene substituting for the other as has been observed in the case of the structurally related genes comprising the AS-C, the E(spl) complex, and the myogenic family of bHLH-encoding genes.

Other known pan–neural genes may collaborate to establish neuronal fates by different mechanisms. For example, asense [Gonzalez et al. 1989; Brand et al. 1993; Dominguez and Campuzano 1993] is likely to function as an activator of neuronal genes, pros [Doe et al. 1991; Vaessin et al. 1991] turns off expression of pan–neuronal genes such as dpp in GMCs [Vaessin et al. 1991], sca [Mlodzik et al. 1990] encodes a secreted factor that inhibits neighboring non-neuronal cells from adopting neuronal fates, and cyclin A [Lehner and O'Farrell 1989] most likely functions to regulate neuron-specific cell cycle progression because maternal cyclin stores have largely disappeared by the time these late embryonic cell divisions take place. Thus, neuron-specific gene expression appears to be accomplished by a combination of negative transcription factors such as Scrt and Dpn repressing non-neuronal gene expression, and positive factors such as Asense and the recently identified vertebrate bHLH protein NeuroD [Lee et al. 1995], which activate expression of neuron-specific genes. These neuron autonomous functions in combination with lateral inhibition of neighbors mediated by secreted factors such as Sea represent three of the most obvious mechanisms by which pan–neural genes might function to promote primary neuronal precursor fates. Expression of these primary precursor genes is then terminated by pros, which permits these cells to move on to the next developmental stage of neurogenesis. The diversity of pan–neural gene function and the regulation of pan–neural genes by different primary upstream regulators [Bier et al. 1992; Jarman et al. 1993; Ip et al. 1994; Vaessin et al. 1994; Emery and Bier 1995], suggests that the neuronal tissue type identity is established by distinct parallel functions rather than by a single orchestrating master gene.

Neurogenesis versus myogenesis

It is noteworthy that cells devoted to forming mesoderm and neuronal tissues express the highly related Scrt and Sna zinc finger proteins in tissue-specific patterns. A global role in establishing a common tissue identity also has been proposed for Esc, which participates in distinguishing imaginal diploid cells from other differentiated larval cells that become polyploid [Hayashi et al. 1993; Fuse et al. 1994]. A collaboration between bHLH proteins and zinc finger proteins may be an important parallel between formation of the mesoderm and the nervous system. In the mesoderm, Sna directly represses the expression of nonmesodermal target genes such as rho, which participates in specification of the neuroectoderm, and the bHLH protein encoded by twist [Twi] acts as an activator of mesoderm-specific genes. In the nervous system, bHLH genes of the AS-C (including the pan–neural asense gene) are required for activation of
neuronal genes whereas \( \text{scrt} \) and \( \text{dpn} \) directly or indirectly repress expression of the epidermal \( \text{Egf-r} \) gene. Whereas single mutant phenotypes of \( \text{asense} \), \( \text{scrt} \), and \( \text{dpn} \) are subtle, the combined action of negative and positive transcription factors during neurogenesis may be analogous to that of \( \text{Sna} \) and \( \text{Twi} \) during myogenesis. It will be interesting to determine whether a protein in the \( \text{Dpn} \), \( \text{Hairy} \), and \( \text{E[spl]} \) repressor subclass of WRPW bHLH proteins also contributes to myogenesis, perhaps by collaborating with \( \text{Sna} \) to repress expression of neuroectodermal genes.

Materials and methods

Fly stocks

Pan–neural insertions of the \( \text{P-lacW} \) vector into the 64A locus were generated in the course of the enhancer trap screen of Bier et al. (1989). \( \text{dpn} \) mutant stocks have been described previously (Bier et al. 1992). All genetic markers and chromosome balancers used are described in Lindsley and Grell (1968) and Lindsley and Zimm (1992). DJ(3L)A466 was kindly provided by Rob Jackson (Worcester Foundation, Shrewsbury, MA). Unless specifically stated, stocks were obtained from either the Bloomington Indiana or Bowling Green Ohio Stock Centers.

Isolation of transposase-induced \( \text{scrt}^{\text{ret}} \) revertants

Transposase-induced revertants of the single element \( \text{scrt} \) enhancer trap \( \text{P-lacW} \) insertion were isolated by the following scheme. Flies homozygous for the viable single element \( \text{P-lacW} \) insertion into \( \text{scrt} \) locus were crossed to a stock of the genotype \( \text{yw; P-lacW} \) \( \text{A2-3} \) Single locus were crossed to a stock of the genotype \( \text{yw; H^{TM6}} \) females (only one male per vial in vials. Progeny from these crosses were screened for \( \text{w} \) (non-\( \text{yw; H^{TM6}} \) females) were crossed to the \( \text{yw; P-lacW} \) \( \text{A2-3} \) male progeny from this cross were crossed to the \( \text{yw; H^{TM6}} \) \( \text{TM6} \) females in vials. Progeny from these crosses were screened for \( \text{w} \) (non-\( \text{yw; } \text{Sb} \)) jump-out (\( \text{scrt}^{\text{ret}} \)) male revertants. Single \( \text{scrt}^{\text{ret}} \) males then were crossed to \( \text{yw; H^{TM6}} \) \( \text{TM6} \) females (only one male per vial was mated to assure that independent events were recovered), and balanced (\( \text{w} \) \( \text{Sb} \))/\( \text{yw; scrt}^{\text{ret}} \)/\( \text{TM6} \) stocks were established. Homozygous viable revertant chromosomes were discarded, whereas lethal or semilethal \( \text{scrt}^{\text{ret}} \) revertants were retained. DNA was prepared from escaping flies homozygous for the \( \text{scrt}^{\text{ret}} \), \( \text{scrt}^{\text{ret}^1} \), \( \text{scrt}^{\text{ret}^2} \), \( \text{scrt}^{\text{ret}^3} \), and \( \text{scrt}^{\text{ret}^5} \) semi-lethal revertant chromosomes and DNA lesions in these stocks were mapped with respect to the genomic walk and to the longest \( \text{scrt} \) cDNA clone by Southern blot analysis [see below].

Antibody staining of embryos

Antibody staining was performed according to Bier et al. (1990) and Sturtevant et al. (1993).

In situ hybridization to RNA in sections or whole-mount embryos

In situ hybridization to whole-mount embryos using antisense RNA probes was performed according to O’Neill and Bier (1994).

Isolation of \( \text{scrt} \) genomic and cDNA clones

DNA flanking the site of \( \text{P} \)-element insertion was cloned by plasmid rescue (Pirrotta et al. 1986) using genomic DNA isolated from flies carrying the double \( \text{P} \)-element insertion [see Fig. 2]. Digoxigenin-labeled DNA probes synthesized to rescued fragments corresponding to both \( \text{P} \)-element ends were hybridized in situ to whole-mount embryos. The probe synthesized to a rescued fragment containing 8 kb of flanking genomic DNA 3’ to the \( \text{P} \)-element insertion site (relative to the direction of \( \text{scrt} \) transcription) hybridized to a pan–neural transcript, whereas the 1.8-kb probe containing 5’ rescued DNA did not hybridize to any transcript. The 8-kb rescued fragment was labeled with \(^{32}\text{P} \) phosphate and used as a probe to screen a lambda genomic library and the Kuvav E7 cDNA library. A partial cDNA containing the 3’ portion of an open reading was recovered from the Kuvav library, which in turn was used to screen the Kau Zinn embryonic library, from which two longer cDNAs clones were isolated. These two cDNAs were sequenced on both strands. The longer of these two cDNAs represents an incompletely spliced RNA precursor molecule, and the other (3084 bp long) contains the full open reading frame encoding a predicted 664-amino-acid \( \text{Scrt} \) protein. Overlapping genomic phage covering the \( \text{scrt} \) transcription unit were also isolated and restriction mapped. Breakpoints in various \( \text{scrt}^{\text{ret}} \) revertants were mapped to this walk by Southern blot analysis. Deletion of the full cDNA-coding region in \( \text{scrt}^{\text{ret}^1} \), \( \text{scrt}^{\text{ret}^2} \), and \( \text{scrt}^{\text{ret}^5} \) mutants also was confirmed by in situ hybridization with cDNA and genomic probes. In the case of \( \text{scrt}^{\text{ret}^5} \), embryos were also collected from \( \text{scrt}^{\text{ret}^5}/\text{TM6}/\text{lacZ} \) flies. As expected, embryos lacking the \( \text{TM6}/\text{lacZ} \) balancer did not label with the \( \text{scrt} \) probe.

Construction of the \( \text{scrt} \) HS vector

The region of \( \text{scrt} \) cDNA clone between a BamHI site at nucleotide 530 (74 nucleotides upstream from the initiator ATG of the predicted \( \text{Scrt} \) protein) and the \( \text{Hin}^c \) site at nucleotide 2752 (157 nucleotides downstream of the termination codon) was subcloned into Bluescript. The \( \text{Hin}^c \) site was then changed to an \( \text{XbaI} \) site with linkers so that the \( \text{scrt} \) insert was flanked at both ends with \( \text{XbaI} \) sites (an endogenous \( \text{XbaI} \) site is present in the Bluescript polylinker immediately upstream of the \( \text{BamHI} \) site). The 2.2-kb \( \text{XbaI} \) fragment containing the full \( \text{scrt} \) open reading frame and minimal 5’- and 3’-untranslated sequences then was inserted into the \( \text{P}[^{\text{hs-CaSpeR1}}] \) vector (Bang and Posa­kony 1992). Both sense (\( \text{HS-\text{scrt}} \) and antisense (\( \text{HS-anti-scrt} \)) orientations of the \( \text{scrt} \) insert were recovered in \( \text{P}[^{\text{hs-CaSpeR1}}] \), and these constructs were injected into fly embryos according to standard methods (Spradling 1986). Several transformant lines were isolated and tested for phenotype following heat shock. The three HS-\( \text{scrt} \) sense lines tested all generate similar adult bristle phenotypes [described in text].

Other molecular techniques

Plasmid rescue from minipreps of fly DNA was performed according to Pirrotta (1986). Other cloning techniques followed standard procedures, as in Maniatis et al. (1982).

Acknowledgments

We thank Yuh Nung Jan and Lily Jan, in whose laboratory this work was initiated, Larry Zipursky for comments on the manuscript, and Kathryn S. Burton for assembling the figures. We thank Jason W. O’Neill, Brian Bihs, Robya Tuma, and James Lowery for technical assistance. We thank Dr. Leonard Deftos for supplying the \( \text{cho} \) chordotonal organ marker and Benny Shilo for the anti-\( \text{EGF-R} \) antibody. This work was supported by National Institutes of Health grant RO1-NS29870-01, and re­search grant S-FY92-1175 from the March of Dimes Birth De­fects Foundation. E.B. was supported by funds from the
McKnight Neuroscience Foundation, Sloan Foundation, and an American Cancer Society Junior Faculty Award.

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