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14. The binding of ^{125}I-labeled insulin or ^{125}I-labeled IL-4 was measured as described [J. Lowenthal *et al.*, *J. Immunol.* **140**, 456 (1988)]. Briefly, aliquots of cells were incubated at 4°C for 2 hours in the presence of labeled factor in a volume of 200 μl. Cell-bound ^{125}I-insulin or ^{125}I-IL-4 was separated from unbound factor by centrifugation through an oil gradient. Nonspecific binding was measured in the presence of 100-fold excess of unlabeled factor and subtracted from the total bound counts per minute. The mean of cell-bound counts per minute at plateau from triplicate samples was then used to determine the receptor number per cell.
15. Electroporation of 32D cells was done as described [J. H. Pierce *et al.*, *Science* **239**, 628 (1988)]. The murine IL-4 receptor cDNA was inserted into the LTR-2 expression vector containing an SV40-driven *gpt* selectable marker [P. P. Di Fiore *et al.*, *Cell* **51**, 1063 (1987)]. The human insulin receptor expression vector was cotransfected with an SV2*neo* vector. The expression vector with rat IRS-1 cDNA contains a histidinol resistance marker (*7*). Transfected populations were selected by emergence in growth medium containing 80 μM mycophenolic acid and the addition of hypoxanthine, aminopterin, thymidine (HAT), or geneticin (750 μg/ml), or 10 μM histidinol, respectively, for each protein expressed.
16. Flow cytometric analysis was done on FACScan (Becton-Dickinson San Jose, CA). The IL-4 receptor monoclonal antibody (mAb) (1 μg/ml) (Immunex Seattle, WA) and the human α-subunit insulin receptor mAb (Amersham) were used at a concentration of 1 μg/ml and a 1:100 dilution, respectively. Phycoerythrin-conjugated goat antibodies to rat or mouse (both at 1:100) from Caltag were used as secondary antibodies. Rabbit antipeptide sera specific for the IL-4 receptor (*12*) or insulin receptor (*7*) (1:500 dilutions) were used to immunoblot 300 μg of protein from cell lysates as described.
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Neuroblast Specification and Formation Regulated by *wingless* in the *Drosophila* CNS

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The *Drosophila* central nervous system (CNS) develops from a heterogeneous population of neural stem cells (neuroblasts), yet the genes regulating neuroblast determination remain unknown. The segmentation gene *wingless* is regionally expressed in the neuroectoderm from which neuroblasts develop. A conditional *wingless* mutation is used to inactivate CNS function without affecting segmentation. The stripe of wingless-expressing neuroectoderm generates apparently normal neuroblasts after wingless inactivation; however, adjacent anterior and posterior neuroectoderm requires wingless nonautonomously for subsequent neuroblast determination and formation. Loss of wingless results in the absence or duplication of identified neuroblasts, highlighting its role in generating neuroblast diversity in the CNS.

The insect CNS is composed of hundreds of neurons, each expressing a unique combination of neurotransmitters, ion channels, receptors, and cell surface molecules. The generation of neuronal diversity begins immediately after gastrulation, when the superficial ventral cells of the embryo form a neuroectoderm. Within this neuroectoderm, individual cells enlarge and delaminate inwards to produce a stereotyped, subectodermal array of 30 unique neural precursor cells (neuroblasts; NBs) (*1*). Each NB divides according to an invariant cell lineage to generate an average of five ganglion mother cells (GMCs); subsequently, each GMC produces a characteristic pair of neurons (*2*). Genes regulating NB formation (*3–5*) and GMC and neuronal determination (*6–9*) have been identified; however, genes controlling NB determination have yet to be described.

The segment polarity class of genes are the best candidates for genes controlling NB specification (*10*). Many of these genes are regionally expressed in the neuroectoderm, and mutations often result in abnormal CNS development (*11*). One example is the *wingless* (*wg*) gene, which encodes a secreted glycoprotein that is required to specify the fate of epidermal cells (*12–14*) and is also required for the development of the *even-skipped*–expressing (eve$^+$) RP2 motoneuron in the CNS (*11*). The CNS phenotype could be due to changes in the fate of neuroectodermal cells, NBs, GMCs, the RP2 neuron, or cell death at any of these stages. Here we use a temperature-sensitive *wg* mutation and eight molecular markers that label specific subsets of neuroectodermal cells, NBs, GMCs, and neurons to determine the role of *wg* during CNS development.

The first 10 NBs form about 30 min after gastrulation [early stage 9; staging according to (*15*)]. These S1 NBs form an orthogonal array of four rows (2/3, 4, 5, 6/7) and three columns (medial, intermediate, and lateral) in each hemisegment (Fig. 1). A second phase of NB formation occurs 40 min later (mid–stage 9), when the five S2 NBs enlarge and delaminate (Fig. 1). The *wg* gene is transcribed specifically in row 5 neuroectoderm and NBs (*1*), and the secreted wg protein can be detected in the adjacent row 4 and row 6/7 neuroectoderm and NBs (*12, 14*). To determine the CNS function of *wg*, we assayed expression of our molecular markers in embryos homozygous for the temperature-sensitive wg^{IL114} mutation grown at the restrictive temperature. These embryos have an epidermal and CNS phenotype indistinguishable from that of embryos homozygous for the null wg^{CX4} allele (*16*) and will be referred to as wg$^-$ embryos.

Wild-type and wg$^-$ embryos have identical patterns of all markers in S1 NBs at early stage 9 (*17*). This result suggests that *wg* is not required for the formation or initial specification of S1 NBs and is consistent with previous data showing that loss of *wg* does not affect the formation of several identified S1 NBs (*18*).

The first CNS phenotype in wg$^-$ embryos is an alteration in the neuroectodermal pattern of runt and the enhancer trap line 5953 at early stage 9 (S1 NB stage). Changes in runt and 5953 patterns are documented in stage 10 embryos because the staining is more intense in older embryos; however, the alterations shown reflect changes seen at early stage 9. In wild-type embryos, runt is observed in neuroectoderm of row 2/3 (Fig. 1A). In wg$^-$ embryos, runt expression expands into row 4 (but not into row 5 or 6/7), indicating that *wg* nonautonomously represses runt expression specifically in row 4 neuroectoderm (Fig. 1B). In wild-type embryos, 5953 is expressed in row 2/3, in a patch of cells in row 4 (where NB 4-2 will delaminate), and in a patch of cells in row 5 (Fig. 1C). In wg$^-$ embryos, there is a selective loss of 5953 expression in row 4 (Fig. 1D), but no change

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in expression in rows 2/3 and 5. These data show that wg nonautonomously represses runt and activates 5953 in row 4 neuroectoderm prior to the delamination of NB 4-2.

Altered gene expression in row 4 neuroectodermal cells foreshadows defects in NB 4-2 formation and fate in wg^- embryos (Fig. 1, E to J). NB 4-2 does not delaminate in 80% of the hemisegments ($n = 318$) and when present expresses markers characteristic of NB 3-2 (Fig. 1, E to J). Subsequently, NB 4-2 produces a runt-positive, even-negative GMC characteristic of the NB 3-2 lineage (Fig. 1, G and H). Taken together, all NB and GMC markers reveal a consistent transformation of NB 4-2 to the NB 3-2 fate in wg^- embryos (Fig. 2). We favor the hypothesis that wg protein acts nonautonomously to control the fate of adjacent row 4 neuroectodermal cells; altered specification of these cells results in a lack of NB formation or the production of an incorrectly specified NB.

To test whether *wg* function is required solely in neuroectodermal cells to control NB 4-2 development, we transiently inactivated the temperature-sensitive wg^{IL114} protein before, during, or after NB 4-2 formation (Fig. 3). We assayed NB 4-2 development by scoring for the presence of the eve^+ RP2 neuron, which is derived from NB 4-2 (*1*). Our results show that wg activity is necessary and sufficient for NB 4-2 specification and formation prior to NB 4-2 delamination, when only neuroectodermal cells exist at the 4-2 position (Fig. 3). This suggests that NB 4-2 identity is acquired in one or more neuroectodermal cells prior to NB delamination.

The temperature-shift experiments also show that the *wg* requirement for NB 4-2 specification can be separated from its requirement for epidermal development. Transient loss of wg function between 30 and 90 min (at 18°C) after gastrulation results in loss of virtually all of the eve^+ RP2 neurons, without altering the segmental array of denticles secreted by the epidermis (Fig. 3, F and G), although there may be subtle alterations in epidermal fate that do not disrupt the denticle pattern. In contrast, transient activation of *wg* function during this same interval permits virtually normal development of the eve^+ RP2 neurons, in spite of a nearly null epidermal phenotype (Fig. 3, H and I). Although *wg* is required initially for NB 4-2 development and subsequently for epidermal development, we expect that the temperature-sensitive period (TSP) for later forming wingless-dependent NBs will overlap with the TSP for epidermal development.

There are a total of 30 NBs per hemiseg-

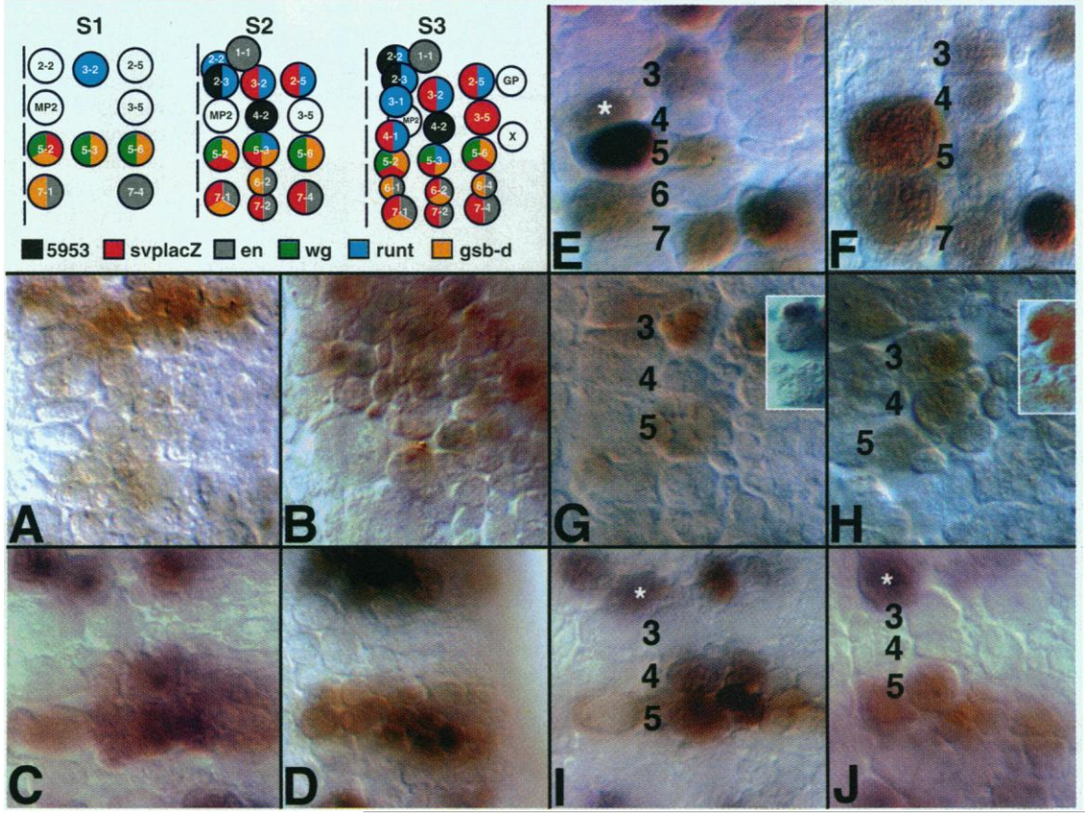

Fig. 1. (Upper left schematic) Molecular markers used to assay NB identity at S1, S2, and S3 stages of neurogenesis (embryonic stages early 9, 9, and 10, respectively). Antibodies were used to detect the protein products of the *wg, engrailed* (*en*), *runt, hunchback* (*hb*), and *gooseberry-distal* (*gsb-d*) genes or β-galactosidase from the enhancer trap lines 5953 and H162 (svplacZ). Ventral midline, dashed line; anterior, top. The four rows of S1 NBs are 2/3, 4, 5, and 6/7 from anterior to posterior (anterior at top); from left to right, the three columns are medial, intermediate, and lateral. (**A** to **J**) The *wg* gene nonautonomously controls neuroectodermal and NB gene expression. For clarity, we focus specifically on intermediate column NBs (indicated by numbers just to the left of each NB as follows: 3, NB 3-2; 4, NB 4-2; 5, NB 5-3; 6, NB 6-2; 7, NB 7-2), although several differences in medial or lateral NBs are noted. One hemisegment is shown per panel with the ventral midline at left, anterior at top. All embryos are at stage 10 (S3 NBs) unless noted otherwise. (A and B) Pattern of runt neuroectodermal expression. (A) In wild-type embryos, runt protein is localized to row 2/3. (B) In wg^- embryos, runt expression expands posteriorly into row 4. (C and D) Enhancer trap line 5953 (black) and wg (orange) neuroectodermal expression. (C) In wild-type embryos, 5953 is expressed in row 2/3 and a cluster spanning rows 4 and 5; wg is at high levels in row 5. (D) In wg^- embryos, there is a selective loss of 5953 expression in row 4 (arrowhead); inactive wg protein remains in row 5. (E and F) Pattern of svplacZ NB expression at late S2 stage (all S2 NBs plus NB4-1). (E) In wild-type embryos, svplacZ is expressed in intermediate column NBs 3-2, 5-3, and 7-2, but not in NB 4-2 and NB 6-2. Other svplacZ-positive NBs include NB 4-1 (asterisk), NB 7-4, NB 5-2, and NB 2-5. (F) In wg^- embryos, NB 4-2 expresses svplacZ, and NB 6-2 and NB 4-1 never form. Expression in other NBs is unaltered. (G and H) Pattern of runt NB and GMC expression. (G) In wild-type embryos, runt is detected at high levels in NB 3-2 and low levels in NB 5-3, but is not present in NB 4-2. The runt protein is also strongly expressed in GMC3-2a, but not in GMC4-2a (inset). (H) In wg^- embryos, runt protein is expressed at high levels in NB 3-2 and NB 4-2, and at low levels in NB 5-3. Both GMC3-2a and GMC4-2a express runt at high levels (inset). (I and J) Enhancer trap line 5953 (black) and wg (orange) NB expression in late stage 10 embryos (early S4 NB pattern). (I) In wild-type embryos, 5953 is expressed in NBs 2-1, 2-3 (asterisk), 2-4, and 4-2. (J) In wg^- embryos, NB 4-2 does not express 5953; no change is seen in NB 2-3 (asterisk), NB 2-1, and NB 2-4 (out of focus). The wg protein remains detectable in all row 5 NBs.

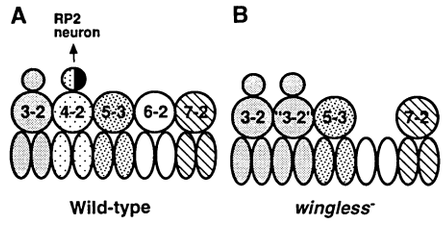

Fig. 2. The secreted wg protein generates NB diversity. (**A**) Wild-type embryos have five unique intermediate column NBs per hemisegment. Anterior, left; internal, top. NB 3-2 expresses svplacZ and runt (dark stipple); NB 4-2 expresses 5953 (light stipple) and generates the eve$^+$ GMC4-2a (black) and RP2 neuron; NB 5-3 expresses wg, runt, and svplacZ (medium stipple); NB 6-2 expresses en (white); and NB 7-2 expresses en and svplacZ (cross-hatching). (**B**) The wg$^-$ embryos are usually missing NB 4-2 and NB 6-2, owing to the misspecification of neuroectodermal cells adjacent to the wg$^+$ stripe. When NB 4-2 forms (20%) it appears transformed into a duplicate NB 3-2, on the basis of gene expression in the NB and its GMC progeny.

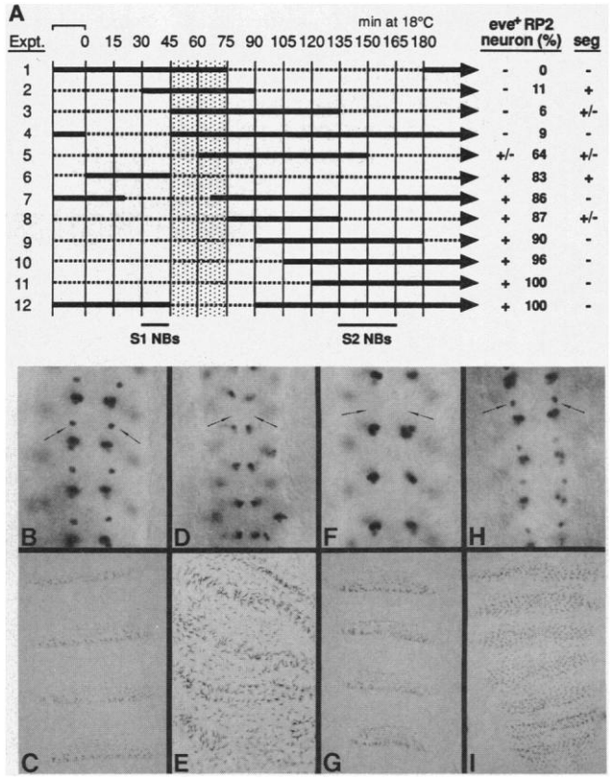

Fig. 3. The *wg* gene is required for NB 4-2 specification prior to NB 4-2 delamination independently of its requirement in the epidermis for segmentation. (**A**) Progeny of *wg*^{IL114}/CyO flies were dechorionated and hand-picked during a 10-min interval at gastrulation (t = 0) and grown at 18°C (permissive temperature, dashed line) or 27°C (nonpermissive temperature, solid line). All time points were adjusted to correspond to development at 18°C. Development of NB 4-2 is assayed by the production of an eve$^+$ RP2 neuron (column at right; n > 66 for each experiment); epidermal development is assayed by formation of segmental grooves (column at far right) (*23*). The stage of NB development is indicated at the bottom of the panel (*24*). (**B** and **C**) Wild-type embryos have one eve$^+$ RP2 neuron per hemisegment in the CNS [arrows, (B)] and a normal denticle pattern (C). (**D** and **E**) *wg*^{IL114} embryos grown entirely at nonpermissive temperature specifically lack the eve$^+$ RP2 [arrows, (D)] and have a severely disrupted denticle pattern (E). (**F** and **G**) *wg*^{IL114} embryos grown at permissive temperature except for a brief interval of nonpermissive temperature prior to NB 4-2 formation [line 2 in (A)] lack eve$^+$ RP2 neurons [arrows, (F)], yet form a normal denticle array (G). (**H** and **I**) The *wg*^{IL114} embryos grown at nonpermissive temperature except for a brief interval of permissive temperature prior to NB 4-2 formation [line 12, (A)] have virtually normal eve$^+$ RP2 neurons [arrows, (H)], yet have a severely disrupted array of denticles (I).

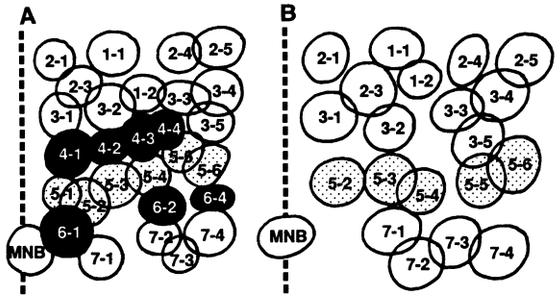

Fig. 4. The *wg* gene is nonautonomously required for the development of adjacent anterior and posterior NBs. Camera lucida drawing of the NBs at S5 (stage 11) in wild-type (**A**) and wg$^-$ embryos (**B**). NB identity is based on engrailed expression, cell morphology, and cell position relative to other NBs. Development of the *wg*-expressing row 5 NBs (stipple) are not affected by loss of wg, nor are adjacent S1 NBs, but the seven adjacent S2-S5 NBs (black) are either missing or incorrectly determined. The engrailed-positive NBs in rows 7 and 1 develop normally.

ment: five express wg and seven form adjacent to wg$^+$ neuroectoderm during NB stages S2 to S5 (*1*). The wg protein has no apparent role in the formation or specification of the wg$^+$ row 5 NBs, but is absolutely required for the specification or formation of all seven adjacent NBs (Fig. 4) (*19*). To understand the fate of the missing NBs, we focused on NB 4-2. The absence of NB 4-2 is not due to a delay in formation or cell death; NB 4-2 or its characteristic eve$^+$ progeny are not observed at any stage of development, and cell death in the CNS is not observed until the end of stage 11, much later than the normal time of NB 4-2 formation (*17*). Another possibility is that *wg* is required to trigger cell division in the adjacent neuroectodermal cells, in a manner similar to its role of regulating cell division in the Malpighian tubule anlage (*20*), and that the lack of NB formation is the result of a paucity of neuroectodermal cells. This explanation is unlikely because we observe normal NB formation in *string* mutant embryos in which neuroectodermal cells never divide (*21*). We expect that the wg signal modulates NB formation by increasing or decreasing the activity of proneural or neurogenic proteins, respectively. The *wg* gene is directly involved in the specification or formation of nearly one-quarter of all NBs that generate the CNS.

The role of *wg* in CNS development has superficial similarity to the role of the murine ortholog *wnt-1* during neurogenesis. In *Drosophila*, *wg* is expressed in a stripe of neuroectoderm, and loss of function mutations result in the nonautonomous reduction in number and type of NBs. In the mouse, *wnt-1* is regionally expressed in the CNS, where it is nonautonomously required for the development of a large portion of the midbrain (*22*). Whether fundamental molecular similarities exist between the CNS function of *wg* and *wnt-1* genes remains to be determined.

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19. The percentage of row 4 and row 6 NBs present in wg^{IL114} homozygous embryos is as follows: NB 4-1, 0% (n = 34); NB 4-2, 20% (n = 318); NB 4-3, 0% (n = 34); NB 4-4, 3% (n = 34); NB 6-1, 4% (n = 78); NB 6-2, 0% (n = 298); and NB 6-4, 0% (n = 78). The percentage of row 6 NBs present in wg^{CX4} homozygous embryos is as follows: NB 6-1, 9% (n = 66); NB 6-2, 0% (n = 66); and NB 6-4, 1.5% (n = 66).
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23. Cuticles from homozygous mutant embryos in (E) and (I) were picked by their non–wild-type cuticle phenotype; cuticles from mutant and wild-type embryos from the experiment shown in line 2 of (A) were indistinguishable, and one is represented in (G). Homozygous wg^{IL114} embryos scored for the eve^+ RP2 were identified by the loss of eve^+ dorsal mesoderm cells near the amnioserosa.
24. For each experiment shown in Fig. 3A, the staged embryos were divided into three groups: one-third were fixed at time shift began and one-third were fixed at the time shift concluded (to score the NB pattern); the remaining third were fixed at 14 hours of development (to score for the eve^+ RP2 neuron and epidermal segmentation).
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