The Development of Crustacean Limbs and the Evolution of Arthropods

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Arthropods exhibit great diversity in the position, number, morphology, and function of their limbs. The evolutionary relations among limb types and among the arthropod groups that bear them (insects, crustaceans, myriapods, and chelicerates) are controversial. Here, the use of molecular probes, including an antibody to proteins encoded by arthropod and vertebrate *Distal-less* (*Dll* and *Dlx*) genes, provided evidence that common genetic mechanisms underlie the development of all arthropod limbs and their branches and that all arthropods derive from a common ancestor. However, differences between crustacean and insect body plans were found to correlate with differences in the deployment of particular homeotic genes and in the ways that these genes regulate limb development.

The unbranched limbs of extant adult insects are located on the head and on the three thoracic segments. Crustaceans have more diverse body plans, with variable numbers of thoracic and abdominal segments, and they usually bear limbs on abdominal segments as well as on the head and thoracic segments. Crustaceans also have more diverse limb morphologies; their limbs may be uniramous (unbranched or branched distally), biramous (branched from a proximal limb element), or phyllopodous (multiply branched) (*1*). Each branch is specialized for functions such as walking, swimming, feeding, and respiration.

Two types of theories have been put forth concerning the origins and diversity of arthropod limbs. The first, which has many variations, suggests that branches can be added (*2*) or subtracted (*3*–*5*) during the development of the primary proximodistal axis of the limb. The second type of theory, based on the fossil *Tesnusocaris*, proposes that branched crustacean limbs evolved from the fusion of two adjacent segments with unbranched limbs to form a diplosegment (*6*). These theories play critical roles in debates surrounding arthropod phylogeny (*5*, *7*–*9*). For example, the morphological diversity of insect and crustacean limbs has been cited as evidence that arthropods could not have shared a common ancestor (*8*). Conversely, similarities between fossil insect limbs and the limbs of living crustaceans have been used to argue for a common ancestry (*5*). To investigate the relations among arthropods and arthropod limbs, we examined which aspects of limb development are regulated in other arthropods as they are in insects.

In the insect *Drosophila melanogaster*, limb development is regulated at three levels (*10*). The first level is the formation of a limb primordium. A subset of the anterior cells of each limb primordium expresses *wingless* (*wg*), whereas the posterior cells express *engrailed* (*en*); *wg* is thought to position primordia along the anteroposterior axis of the embryo. Without *wg*, the primordia are not formed (*11*). How limb primordia are positioned along the dorsoventral axis is unknown. The second regulatory level is the outgrowth of a proximodistal axis. The earliest gene to be specifically activated in *Drosophila* limb primordia is *Distal-less* (*Dll*) (*12*). *Dll* is not expressed uniformly throughout the primordia but is primarily limited to presumptive distal structures (*13*) that do not form in the absence of *Dll* (*14*). Ectopic expression of *Dll* in insects has been correlated with the formation of extra proximodistal axes, that is, branched limbs (*15*). Thus, multiply branched crustacean limbs may have resulted from variation in the regulation of genes such as *Dll*. The third regulatory level involves homeotic genes encoded by the Bithorax and Antennapedia

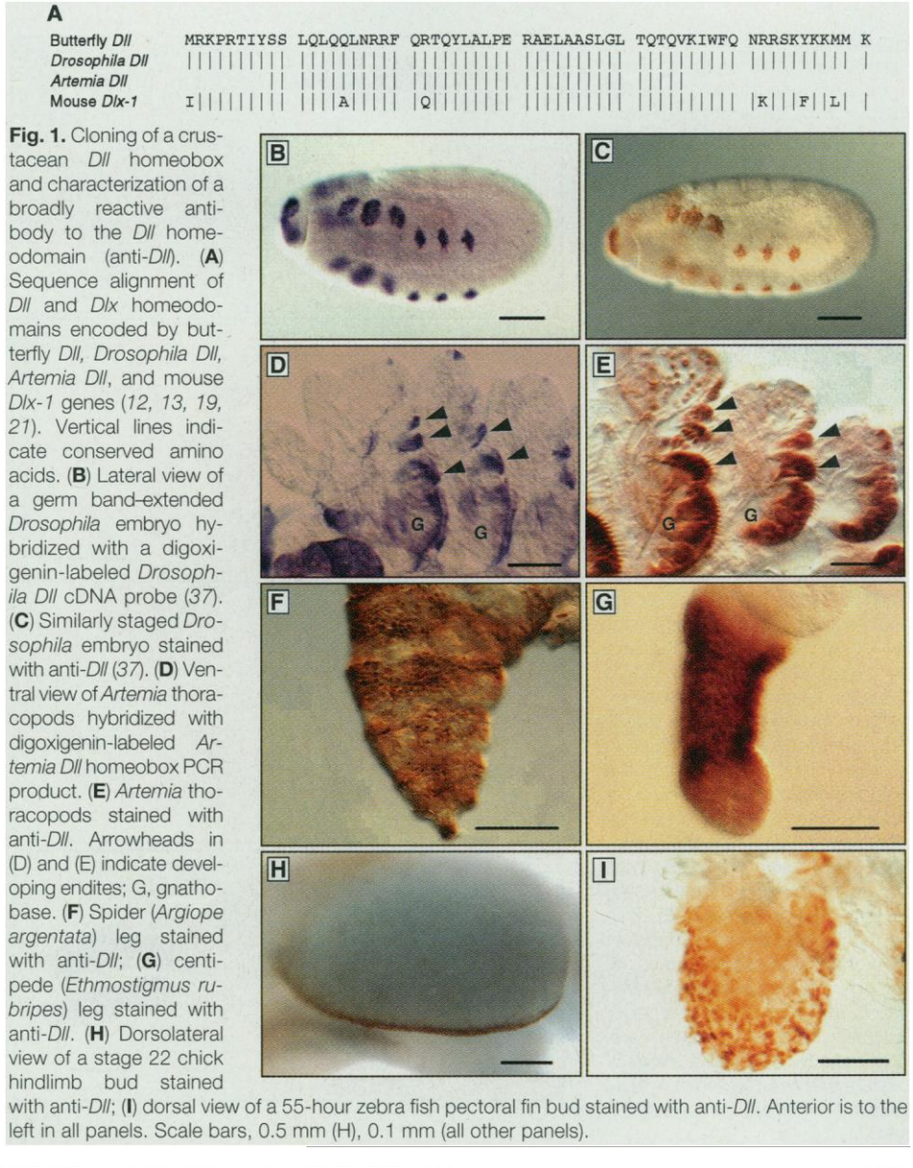

Fig. 1. Cloning of a crustacean *Dll* homeobox and characterization of a broadly reactive antibody to the *Dll* homeodomain (anti-*Dll*). (**A**) Sequence alignment of *Dll* and *Dlx* homeodomains encoded by butterfly *Dll*, *Drosophila Dll*, *Artemia Dll*, and mouse *Dlx-1* genes (*12*, *13*, *19*, *21*). Vertical lines indicate conserved amino acids. (**B**) Lateral view of a germ band–extended *Drosophila* embryo hybridized with a digoxigenin-labeled *Drosophila Dll* cDNA probe (*37*). (**C**) Similarly staged *Drosophila* embryo stained with anti-*Dll* (*37*). (**D**) Ventral view of *Artemia* thoracopods hybridized with digoxigenin-labeled *Artemia Dll* homeobox PCR product. (**E**) *Artemia* thoracopods stained with anti-*Dll*. Arrowheads in (D) and (E) indicate developing endites; G, gnathobase. (**F**) Spider (*Argiope argentata*) leg stained with anti-*Dll*; (**G**) centipede (*Ethmostigmus rubripes*) leg stained with anti-*Dll*. (**H**) Dorsolateral view of a stage 22 chick hindlimb bud stained with anti-*Dll*; (**I**) dorsal view of a 55-hour zebra fish pectoral fin bud stained with anti-*Dll*. Anterior is to the left in all panels. Scale bars, 0.5 mm (H), 0.1 mm (all other panels).

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complexes (BX-C and ANT-C) and controls limb number and limb pattern. The BX-C gene products Ultrabithorax (Ubx) and abdominal A (abdA) regulate limb number (*16*) by repressing genes such as *Dll* (*17*). After primordia are formed, particular homeotic genes determine their adult morphologies (*18*). Here, we used molecular probes to examine the cellular and genetic components of arthropod limb development and of crustacean body plan organization.

We isolated a *Dll* homeobox fragment by the polymerase chain reaction (PCR) from the crustacean *Artemia franciscana* (Fig. 1A) (*19*) and used it as an in situ probe to localize sites of *Dll* transcription (Fig. 1D). The conservation of *Dll* and *Dlx* homeodomains among arthropods and vertebrates (Fig. 1A) (*12*, *13*, *20*–*24*) enabled us to make a polyclonal antibody that would recognize *Dll* and *Dlx* proteins from multiple organisms (*25*). This antibody recognizes the native *Dll* protein in *Drosophila* embryos and does not stain cells in which *Dll* is not expressed (Fig. 1, B and C). The antibody detects the same pattern of *Dll* expression in developing *Artemia* thoracic limbs as detected by a digoxigenin-labeled *Artemia Dll* homeobox probe (Fig. 1, D and E). The developing limbs of chelicerates and myriapods, two other extant arthropod groups, also express the *Dll* antigen (Fig. 1, F and G). Because at least three of the six classes of cloned vertebrate *Dlx* genes (*20*–*24*) are expressed in the apical ectodermal ridge (AER) of developing limbs (*26*), we stained chick limb buds and zebra fish fin buds to test whether the antibody could also recognize *Dlx* homeodomains (Fig. 1, H and I). The observed nuclear AER staining suggests that the antibody recognizes the homeodomain encoded by at least one vertebrate *Dlx* gene.

We used the *Dll* antibody to analyze biramous limb formation in the crustacean *Mysidopsis bahia* and phyllopodous limb formation in *Artemia*. Each branch of the biramous *Mysidopsis* limbs (Fig. 2, A, B, D, E, and F, and Fig. 3, A through D), each outgrowth of the *Artemia* phyllopodous limbs (Fig. 4, B and C), and the developing mandibles of these species express *Dll*. However, the ontogeny of the *Dll* expression pattern differs among limb types. The differences observed are both spatial and temporal, and they probably reflect regulatory changes that occurred during the evolution of various limb morphologies.

Each branch of the thoracic biramous limbs of *Mysidopsis* arises from an independent cluster of *Dll*-expressing cells. Although the initial activation of *Dll* is somewhat imprecise (*27*), *Dll* expression rapidly resolves to two clusters of cells per half segment, each of which begins to form a proximodistal outgrowth (Fig. 2B). Subsequently, nearby cells express *Dll* and contribute to the emerging limbs (Fig. 2, D and E). At these intermediate stages it is difficult to tell whether there are one or two fields of *Dll*-expressing cells. Later, the inner and outer branches express *Dll* in separate and nonoverlapping patterns (Fig. 2F).

In contrast, both branches of the cephalic biramous limbs of *Mysidopsis* arise from a single field of *Dll*-expressing cells (Fig. 3, A and B), whereas the two branches of the abdominal limbs arise sequentially from contiguous fields of *Dll*-expressing cells (Fig. 3, C and D). These data suggest that biramous limbs are formed in more than one way. Two separate outgrowths can form close to one another, either simultaneously (as in the thoracic limbs) or sequentially (as in the abdominal pleopods). Alternatively, as seen in the antennae, a single proximodistal outgrowth can form, which subsequently splits lengthwise to generate two branches.

In the phyllopodous thoracic limb of *Artemia*, *Dll* is deployed sequentially in each of the developing outgrowths. First, *Dll* is activated in the endopod, then in the gnathobase, and subsequently in the four endites as they develop (Fig. 4, B and C). Because *Dll* expression marks only distal limb elements, it is possible that the multiple sites of *Dll* expression in phyllopodous and biramous limbs are contained within a single limb primordium.

Although considerable controversy has surrounded the issue of whether insect mandibles are derived from only proximal or from both distal and proximal limb elements, it has been generally accepted that crustacean mandibles derive from only proximal structures (*2*, *4*, *5*, *7*, *8*). The expression of *Dll* in the developing mandibles of both *Mysidopsis* and *Artemia* (Figs. 2A and 4A) was therefore unexpected. However, it is the distal elements of embryonic and larval crustacean mandibles that

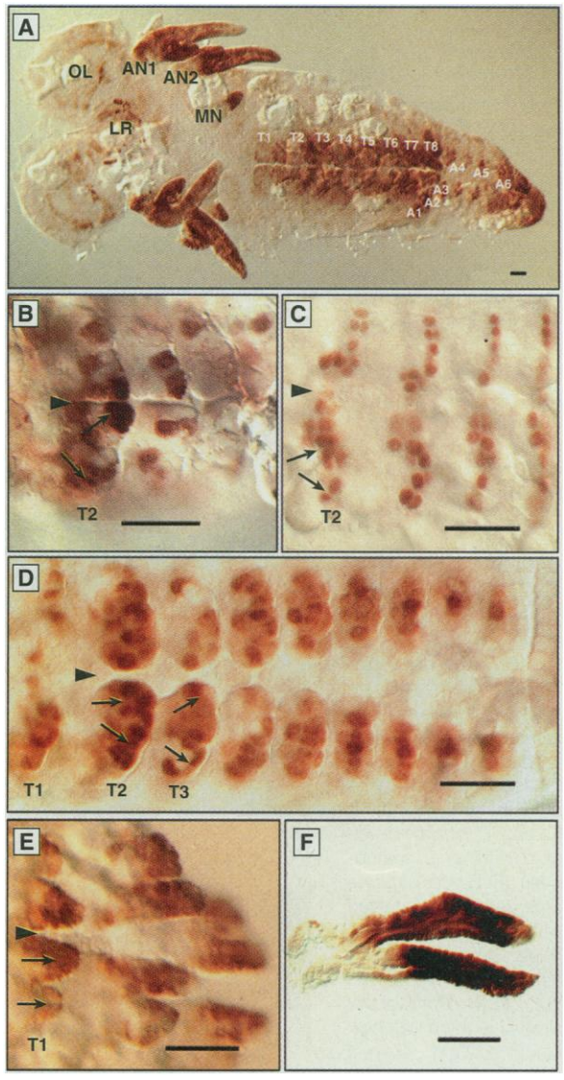

Fig. 2. Ontogeny of the *Dll* expression pattern in biramous thoracic limbs. Arrowheads mark the ventral midline; arrows indicate inner (ventral) and outer (lateral) limb branches. (**A**) Ventral view of a *Mysidopsis bahia* embryo midway through embryogenesis stained with anti-*Dll*. OL, optic lobe; LR, labrum; AN, antenna; MN, mandible; T, thoracic segment; A, abdominal segment. At later stages, both the first and second maxillae also express Dll (not shown). (**B**) Early embryonic thoracic segments with *Dll* expressed in developing rudiments of inner and outer limb branches. *Dll* expression is activated imprecisely, with expression subsequently restricted to the limb-forming cells. Because the segments are only four cells wide when *Dll* is activated, regulation of *Dll* by diffusible factors (such as *wingless* and *hedgehog*) could account for imprecise *Dll* activation. (**C**) Similarly staged embryos stained with anti-inv4D9. The *en* stripe is continuous between the inner and outer branches. (**D**) Slightly older embryo with *Dll* expressed in large amounts in the presumptive distal regions of the inner and outer thoracic limb branches. Development proceeds in an anterior-to-posterior direction, and hence limbs that are more anterior are more advanced developmentally. (**E**) Thoracic limbs from midstage *Mysidopsis* embryo stained with anti-*Dll*. (**F**) A single biramous thoracic *Mysidopsis* limb from an older embryo, illustrating discontinuous *Dll* expression in the two branches; the inner branch is at the top. Anterior is to the left in all panels. Scale bars, 0.1 mm.

express *Dll*, and these cells do not contribute to the adult structures (*8*, *28*). Our findings, in conjunction with the interpretation of fossil insect mandibles as derived from proximal structures (*5*) and the lack of *Dll* expression in insect mandibles (*12*, *13*), are most consistent with the view that insects and adult crustaceans have a common mandibular architecture and a common arthropod ancestor. *Dll* may have been expressed in the mandible of a common ancestor of crustaceans and insects, and this expression may have been lost entirely from insects, with only a vestige remaining in the embryonic and larval crustaceans.

Limb morphology and the pattern of *Dll* expression differ between groups of crustacean segments. The best candidates for regulating the spatial pattern of *Dll* expression along the main body axis are the homeotic genes. We therefore examined the expression of BX-C proteins and their relation to *Dll* expression in *Artemia* and *Mysidopsis*. Because the antibody we used recognizes the products of both the *Ultrabithorax* (*Ubx*) and *abdominal A* (*abdA*) genes of the BX-C (*29*), we refer to them collectively. *Ubx/abdA* and *Dll* are coexpressed in the primordia of the developing thoracic limbs of *Artemia* (Fig. 4D) and in the developing abdominal pleopods of *Mysidopsis* (Fig. 4E), whereas in insects *Ubx* and *abdA* repress the abdominal limb primordia (*17*, *30*). The anterior boundary of *Ubx/abdA* expression lies at the anterior thorax in *Artemia*, which corresponds to the morphological transition between thoracopods and maxillae (Fig. 4D) (*31*). Similarly, the posterior boundary of *Ubx/abdA* expression in *Mysidopsis* lies at the posterior of the fifth abdominal segment (Fig. 4E), corresponding to the transition between pleopod-bearing segments and segments without appendages. The correspondence between homeotic gene expression patterns and limb morphologies suggests that crustacean homeotic genes, like those of insects, regulate the different morphologies of limbs arising in different body regions.

Each branch of the disparate arthropod limbs examined here expresses *Dll*. This finding implies that similar patterning mechanisms are used to establish the main limb axis and its branches. For multiply branched limbs, the cells that give rise to the branches express *Dll* before they form outgrowths. In addition, *Dll* expression and limb formation in crustaceans and insects are only initiated at the boundary between the anterior and posterior compartments of a segment (Fig. 3E). This position encompasses *en*-expressing cells and the cells just anterior to them (Fig. 2C) (*27*, *32*). We therefore propose that modulation of the dorsoventral positioning of groups of *Dll*-expressing cells accounts for the branching patterns of arthropod limbs as well as for the

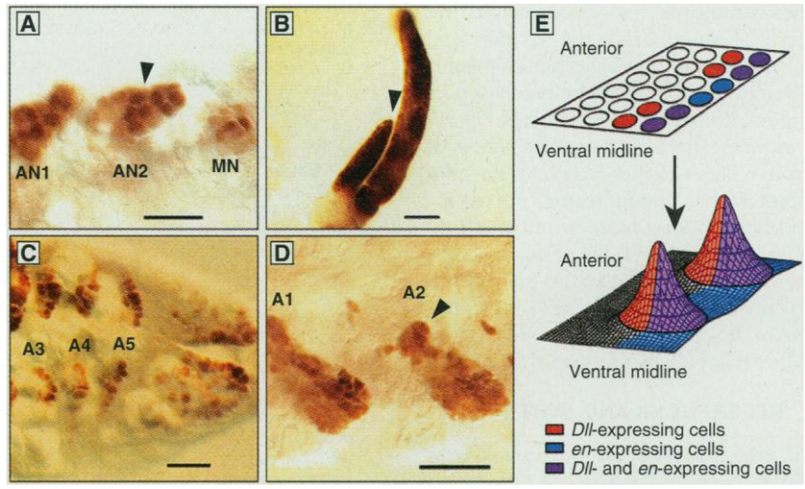

Fig. 3. *Dll* is expressed in both branches of all biramous limb types. The two branches of the second antenna develop from a single field of *Dll*-expressing cells (**A**), which splits longitudinally to give rise to the separate branches (**B**). Arrowheads indicate the forming distal tips of each branch. (**C**) The outer branch of the abdominal pleopod arises from a group of cells along the posterior margin of the first through fifth abdominal segments. (**D**) The inner branch (arrowhead) of the abdominal pleopod arises after the outer branch has formed from cells expressing large amounts of *Dll*. Abbreviations are as in Fig. 2. Anterior is to the left in all panels. Scale bars, 0.1 mm. (**E**) Schematized view of biramous limb formation. Representations are of the left half of a developing *Mysidopsis* thoracic segment, viewed ventrally. Both branches of a biramous crustacean limb arise from *Dll*-expressing cells in the posterior half of the segment. Independent clusters of *Dll*-expressing cells, differing in their dorsoventral position within the segment, extend from the body wall to form the proximodistal axes of the two developing limb branches.

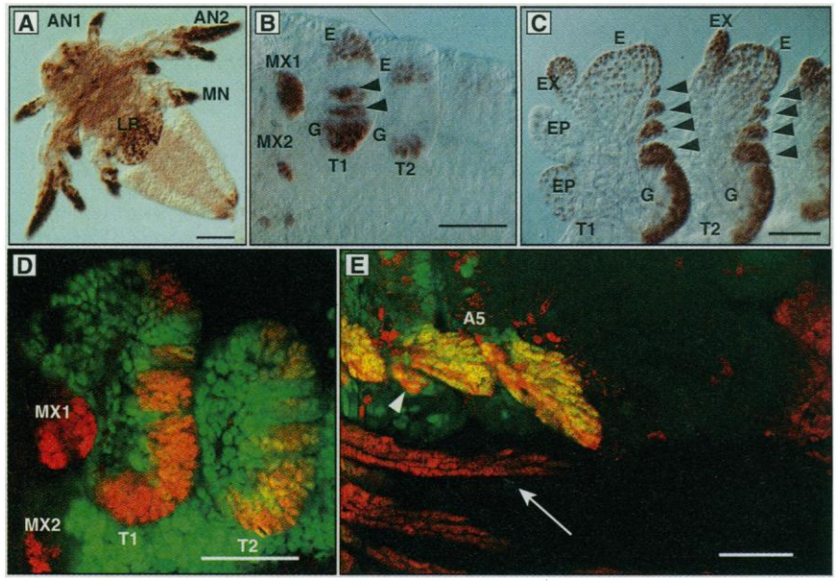

Fig. 4. *Dll* is expressed in all branches of phyllopodous limbs and is not repressed by BX-C gene products in the thorax or abdomen. (**A**) *Dll* expression in a newly hatched *Artemia* nauplius larva. (**B**) *Dll* expression in the endopod (E) and gnathobase (G) of slightly older naupliar thoracic appendages. *Artemia* development also proceeds in an anterior-to-posterior direction; limbs that are more anterior are more advanced developmentally. At this stage, *Dll* expression is detected in the two medial endites (arrowheads) of the T1 but not the T2 limb. (**C**) *Dll* expression in still older naupliar thoracic limbs. By this stage, *Dll* also is expressed in all four endites (arrowheads), the exopod (EX), and the epipods (EP). (**D**) *Artemia* and (**E**) *Mysidopsis* embryos stained with both anti-*Dll* (red) and anti-*Ubx/abdA* (green). In the young naupliar thoracic limbs of *Artemia* (D), *Dll* and *Ubx/abdA* are coexpressed (yellow) and the anterior extent of the *Ubx/abdA* domain lies between the maxilla and the first thoracic segment. In the developing pleopods of *Mysidopsis* (E), *Dll* and *Ubx/abdA* are coexpressed (yellow) and the posterior limit of the *Ubx/abdA* domain is the posterior of the fifth abdominal segment. The developing inner branch is indicated by an arrowhead. The arrow points to the outer branch of a T8 leg, which at this stage expresses *Dll* but not *Ubx/abdA*. Anterior is to the left in all panels. MX, maxilla; other abbreviations are as in Fig. 2. Scale bars, 0.1 mm.

presence of multiple limbs (for example, the two legs of *Tesnusocaris* or the leg and wing of an insect) on a single hemisegment. Modulation may occur by the regulation of the original dorsoventral position of *Dll* expression or by the migration of a subset of cells from a larger cluster (*33*). Our data are consistent with a common origin of all arthropod limbs from a more primitive structure that also expressed *Dll*. This structure was probably unjointed, like the lobopods of modern onychophorans (*1*, *34*). An examination of whether and where *Dll* is expressed in lobopods would test this view.

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19. *Artemia* and *Mysidopsis Dll* homeodomain fragments were amplified by means of reverse transcription (RT)–PCR, essentially according to the methods of (*23*). Modifications included the use of random primers and Superscript II (Gibco-BRL) for first-strand complementary DNA (cDNA) synthesis. The 5′ and 3′ primers used to amplify the *Artemia* homeodomain encoded the amino acids Lys-Pro-Arg-Thr-Ile-Tyr and Lys-Ile-Trp-Phe-Gln-Asn, respectively; the primer sequences were 5′-CCGAATTCAARCC-NMGNACNATHTA-3′ and 5′-CCGGATCCRTTYT-GRAACCADATYTT-3′ (where R = A or G; N = A, C, G, or T; M = A or C; H = A, C, or T; Y = C or T; and D = A, G, or T). PCR conditions were 5 cycles with an annealing temperature of 43°C and 30 cycles with an annealing temperature of 50°C. PCR reaction products were cloned into the TA cloning vector (Invitrogen) and sequenced (*35*).
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25. The polyclonal antibody was made as follows: Rabbits were immunized 12 times over 7 months with a 200-amino acid butterfly *Dll* peptide, which comprised the homeodomain and NH_2-terminal sequences (*13*), expressed in pET23a (Novagen) with a COOH-terminal histidine tag, and purified on nickel-agarose (Novagen or Qiagen). The *Drosophila* and butterfly homeodomains are identical (*13*), yet antibodies from these animals recognized only the butterfly *Dll* protein. The antibodies therefore were not directed against the homeodomain. The rabbits were subsequently boosted with the 61–amino acid butterfly homeodomain (Fig. 1A), which was also expressed in pET23a and purified on nickel-agarose beads. After one boost with the homeodomain, the *Dll* expression pattern was observed in *Drosophila* embryos stained with crude sera. The *Dll* antibodies were affinity-purified on a column of homeodomain protein coupled to Actigel (Sterogene) and eluted with Actisep (Sterogene) (*36*).
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37. In situ hybridizations were carried out as described (*13*). However, *Artemia* were sonicated in phosphate-buffered saline with 0.1% Tween-20 after xylene treatment and before postfixation. Optimal sonication was determined to be the point at which 30 to 50% of the embryos were visibly disintegrated. Detailed in situ and immunostaining protocols are available on request.

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Selective Opioid Inhibition of Small Nociceptive Neurons

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Opioid analgesia, the selective suppression of pain without effects on other sensations, also distinguishes between different types of pain: severe, persistent pain is potently inhibited by opioids, but they fail to conceal the sensation of a pinprick. The cellular basis for this specificity was analyzed by means of patch-clamp experiments performed on fluorescently labeled nociceptive neurons (nociceptors) that innervate rat tooth pulp. Activation of the μ opioid receptor inhibited calcium channels on almost all small nociceptors but had minimal effect on large nociceptors. Somatostatin had the opposite specificity, preferentially inhibiting calcium channels on the large cells. Because persistent pain is mediated by slow-conducting, small nociceptors, opioids are thus likely to inhibit neurotransmitter release only at those primary synapses specialized for persistent pain.

Nociceptors are primary sensory neurons that are specialized to detect tissue damage and to evoke the sensation of pain (*1*). Their cell bodies are located in peripheral ganglia together with other sensory neurons, and their axons form synapses in the central nervous system that are targets for opioids (*2*). Each neuron in a sensory ganglion transduces a particular sensory modality. To be able to identify this modality in dissociated tissue culture, we fluorescently labeled nociceptors in vivo by placing crystals of a lipid soluble dye, $DiIC_{18}$, in the tooth pulp of rats. Through retrograde transport back to the cell body, $DiIC_{18}$ identifies the neurons projecting to tooth pulp (*3*). Pain is the only sensation perceived by humans when any type of physiologic stimulus is applied specifically to tooth pulp (*4*). Thus, labeling the cells that innervate tooth pulp identifies a nearly pure population of nociceptors, sensory neurons

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