cause of repair of the chromosomal double-strand breaks (5). Similar results were obtained with Eco RI (Fig. 3, C and D).

Cleavage of the host chromosome after plasmid loss was demonstrated directly by analysis of total DNA by pulsed-field gel electrophoresis (Fig. 3E). With the Pae R7 r^+m^+ plasmid, chromosome degradation was visible 2 hours after the temperature shift and became more extensive later, whereas no degradation was visible with the r^-m^+ control.

Gingeras and Brooks (3) cloned the Pae R7 r^+m^+ genes in pBR322 and showed that r^+m^- clones were both viable and stable and were phenotypically r^- during virus infection. To explain this apparent inconsistency with our results, we suggest that these earlier experiments were detecting an altered Pae R7 restriction enzyme activity, as the authors themselves hypothesized (3).

The same mechanism that ensures the stability of type II rm genes on a plasmid should also enforce the retention and functional integrity of the genes when they are chromosomally encoded. Failure to maintain the expression of an rm gene pair could kill the cell if the modification enzyme became sufficiently dilute before loss of effective restriction enzyme activity. Thus a pair of rm genes can be regarded as a parasite, symbiont, or "selfish" unit that enforces its retention in hosts whose chromosomes bear the specific sites it recognizes.

As an explanation for the widespread occurrence, diversity, and specificity of rm gene pairs, the "selfish gene" hypothesis is no less plausible than the "cellular defense" hypothesis. Both hypotheses are consistent with the finding that r and m genes are tightly linked and widely distributed. The diversity and high degree of specialization in sequence recognition, however, seem more readily explained as a consequence of adaptation to uncontested ecological niches, similar to competitive exclusion among species. In particular, the existence of rare cutter restriction enzymes is more consistent with the selfish gene hypothesis than with the cellular defense hypothesis.

Lethal behavior that affords survival value in the long run is fixed as programmed cell death during evolution. The mechanism of programmed cell death described here is formally similar to that of the post-segregational killing or addiction systems of plasmids and the postfertilization killing systems of maternal-effect selfish genes (6), although different in detail.

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Modulation of Chemotropism in the Developing Spinal Cord by Substance P

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Developing axons find their targets through direct contact with cues in the extracellular environment and in response to gradients of diffusible factors. The floor plate, a neuroepithelial structure, guides developing commissural axons in the spinal cord by release of chemoattractants. Floor plate cells express neurokinin-1 receptors, and a transiently appearing subpopulation of commissural axons contains substance P, the neuropeptide ligand for this receptor. Substance P increases the amount of axon outgrowth from dorsal horn explants cocultured with floor plate explants. Results of experiments with embryonic rats suggest that substance P released from pioneering neuronal pathways may regulate the release of chemoattractants from floor plate cells.

Diffusible factors released by cellular targets support and guide developing neurites. One example of this process occurs in the spinal cord, in which commissural axons are guided by factors released by the floor plate. The floor plate lies in the ventral midline of the neural tube. It is thought to have an early role in dorsoventral patterning of the neural tube and in the induction of motor neurons, and it is thought to become later an important intermediate target for developing commissural axons (*1*, *2*). In the rat, commissural axons originate from the dorsal neural tube, pass ventrally and medially to the motor neuronal column, and cross at the ventral midline, where they then turn sharply and adopt a rostral trajectory (*1*–*3*). In vitro it has been demonstrated that commissural fiber outgrowth from dorsal explants occurs only in the presence of the floor plate and that the orientation of neurite outgrowth from dorsal explants is crucially dependent on the relative position of the floor plate (*1*, *3*). We now present evidence that release of chemoattractants from the floor plate may be influenced by neuropeptides released from a subset of commissural neurons.

Using an antibody specific for the neurokinin-1 (NK1) receptor, the receptor for substance P, we have localized receptor protein throughout the rostrocaudal extent of the rat floor plate by embryonic day (E) 13 (Fig. 1A) (*4*). Immunoreactivity was seen only at rostral levels at E12 and was absent by postnatal day (P) 10. With immunofluorescence, we identified a group of sub-

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stance P–positive commissural neurons toward the midline in intermediate and dorsal regions of the spinal cord (Fig. 1, B through E). These substance P–positive cells were visible by E13, and axons could be traced from these neurons toward and across the floor plate (Fig. 1, C and D). This group of neurons was distinct both in time of origin and position from previously described commissural neurons, which originate from cell bodies on the dorsal and lateral margins of the spinal cord and stained heavily for the cell surface adhesion molecule TAG1 (*1*, *2*) (Fig. 2). Immunopositive substance P neurons contained large bright fluorescent granules reminiscent of neurosecretory cells (Fig. 1E). Their axons were of extremely fine diameter (Fig. 1D) and did not cross the floor plate after E16; possible explanations of this observation include a down-regulation of substance P expression by these neurons, a retraction of their axons, or selective cell death of this population. Substance P–positive axons were not seen to ascend in the ventral funiculus. This observation may imply that this population of fibers terminated at the floor plate or that peptide concentrations had fallen below detectable amounts once fibers had crossed the midline.

Coupling of the NK1 receptor to an intracellular effector system in dissociated floor plate cells was established by monitoring intracellular calcium concentration ($[Ca^{2+}]_i$) with the Ca^{2+} indicator fura-2 (*5*) (Fig. 3). The resting intracellular $[Ca^{2+}]_i$ values were 50 to 100 nM, with no evidence for spontaneous changes in $[Ca^{2+}]_i$ or Ca^{2+} spikes; these values for $[Ca^{2+}]_i$ are similar to the $[Ca^{2+}]_i$ in other cell types (*6*). Bath application of 10 to 1000 nM substance P or the selective NK1 receptor agonist [Sar^9,$Met(O_2)^{11}$]substance P (*7*) for 120 s (Fig. 3, A and B) produced a rapid and reversible increase in $[Ca^{2+}]_i$. The response was concentration-dependent; 1 nM [Sar^9,$Met(O_2)^{11}$]substance P had little effect, whereas 10, 100, and 1000 nM [Sar^9,$Met(O_2)^{11}$]substance P produced an increase in $[Ca^{2+}]_i$. The selective neurokinin-receptor agonist [β-Ala^8]-neurokinin A 4-10 (*8*) and the neurokinin-receptor agonist senktide (100 nM) were inactive (Fig. 3A). The selective rat NK1 receptor antagonist RP67580 (*7*) (50 nM) blocked the response to submaximal concentrations of substance P (Fig. 3, B and C). This antagonism could be overcome by a 100-fold increase in the concentration of substance P. RP67580 had the same blocking effect when [Sar^9,$Met(O_2)^{11}$]substance P was used as the agonist.

We next considered the hypothesis that substance P could have an effect on the release of chemoattractants from floor plate cells. The floor plate will promote the directed growth of a distinct population of dorsally situated commissural neurons when cocultured with dorsal spinal cord explants in collagen gels (*1*, *2*). We therefore dissected pieces of dorsal cord from the lumbar cord of E13–E14 embryos and cocultured them with floor plate explants in collagen gels in the presence or absence of the substance P stable analogs GR73632 or [Sar^9,$Met(O_2)^{11}$]substance P (Fig. 4) (*3*). In the first set of experiments, care was taken to exclude as much of the endogenous subpopulation of substance P–containing commissural neurons as possible. This effort was monitored by immunostaining whole mounts of explants for substance P and TAG1 immunoreactivity after completion of the experiment.

The number of axon fascicles growing toward the dorsal explant was counted after 36 hours in culture (Fig. 4B). In the presence of the stable NK1 agonist GR73632 (30 μM) or the more selective rat NK1 agonist [Sar^9,$Met(O_2)^{11}$]substance P (1 μM), there was a significant increase (35.3 and 108%, respectively) in the number of fascicles growing from the dorsal explant toward the floor plate (Fig. 4A). This effect was blocked by addition of specific NK1 receptor antagonists (Fig. 4A). There was no effect of antagonist alone on outgrowth in cocultures. TAG1-positive commissural axons were abundant within these axon fascicles (Fig. 4C). The length of axon fascicles was unaffected by treatment with NK1 agonists. Similarly, we stained cocultures with the monoclonal antibody (mAb) 3A10, which stains many TAG1-negative fibers; 3A10-positive fibers grew both toward and away from the floor plate explant but were not influenced by addition of NK1 agonists to the culture medium. At 36 hours, no outgrowth was seen from dorsal

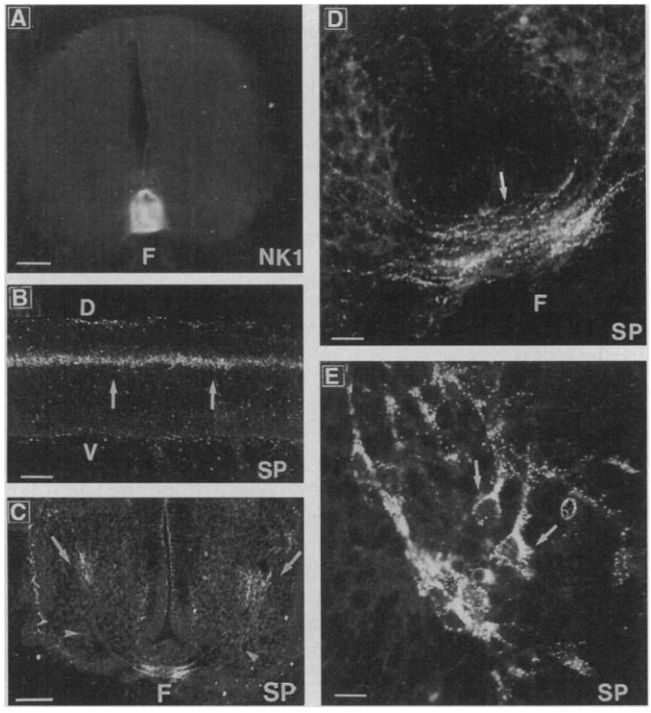

Fig. 1. Substance P and NK1 receptor in embryonic rat spinal cord. At E13, the NK1 receptor is expressed by the floor plate while the endogenous ligand for this receptor, substance P, is contained within a subpopulation of commissural axons. (The day of sperm positivity is considered E0.) (**A**) Floor plate (F) of the lumbar spinal cord with NK1 receptor immunofluorescence. Scale bar, 60 μm. (**B**) Sagittal section of the thoracic spinal cord stained for substance P and showing a band of immunoreactive neuronal cell bodies within the dorsal (D) half of the spinal cord (arrows). V, ventral cord. Scale bar, 150 μm. (**C**) Transverse section of the lumbar spinal cord stained for substance P, showing positive fibers crossing the midline at the floor plate and originating from a dorsally situated group of labeled neurons (arrows). Some fine substance P–positive axons can be seen passing ventrally to the floor plate (arrowheads). Scale bar, 80 μm. (**D**) Substance P–positive fibers crossing at the floor plate seen in (C). Scale bar, 10 μm. (**E**) Substance P–positive neuronal cell bodies containing large immunofluorescent granules and giving rise to commissural axons. Scale bar, 20 μm.

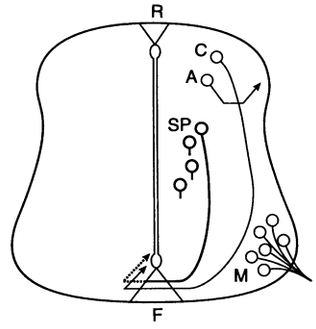

Fig. 2. The E13 rat lumbar spinal cord, showing the location of commissural axons, motor neurons (M), floor plate (F), roof plate (R), and association neurons (A). There are two populations of commissural axons—those derived from a dorsally located superficial population of neurons (C) and a more ventral and medial population that expresses the peptide substance P (SP). Substance P–positive neurons may terminate at the floor plate or enter the ventral funiculus.

explants when they were grown either without the floor plate, as has been previously demonstrated (*1*), or without the floor plate but in the presence of NK1 agonist.

We also cocultured floor plate with larger pieces of spinal cord that contained both commissural neurons and more ventrally placed substance P–containing neurons. If the release of endogenous substance P from the tissue were augmenting the release of chemoattractant from the floor plate, then addition of NK1 antagonist should reduce the number of fascicles growing toward the floor plate. This was indeed the case. Explants of spinal cord contained immunohistochemically identified TAG1 and substance P–positive neurons with processes that extended out into the collagen matrix. In the presence of NK1 antagonist (10 nM) there was a 34% reduction in the number of fascicles growing toward the floor plate (10.2 ± 0.6) compared to that in control cocultures (15.5 ± 0.8) or those incubated with inactive enantiomer RP68651 (15.8 ± 2.7; n = 10 in each group, $P < 0.001$). Taken together, our results suggest that release of chemoattractants from floor plate cells can be modulated by substance P released from a subset of commissural neurons.

In the lumbar spinal cord, the substance P–containing neurons reach the floor plate between E12 and E13, just before most commissural fibers have crossed the midline (*3*). This result suggests that the peptide could then increase the concentration gradient of diffusible chemoattractant released by the floor plate and could maximize directed growth of commissural axons (*2*, *9*). It is also possible that this subpopulation may release substance P considerably earlier as a means of nonsynaptic communication (by volume transmission or diffusion) with the floor plate. Substance P may be involved in the growth and differentiation of neurons and other cell types and may also control the secretion of active substances and ions from innervated tissues (*10*). We cannot rule out a role for substance P–regulated floor plate factors in the growth and differentiation of other pathways in the spinal cord (*11*), although we failed to find evidence for an increase in the number of

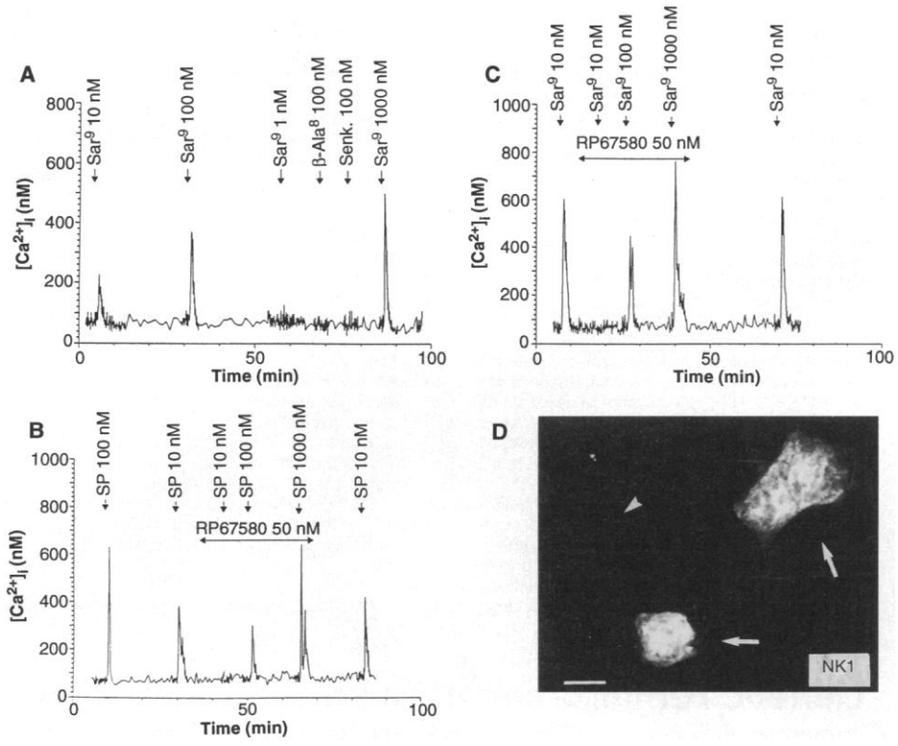

Fig. 3. Increased $[Ca^{2+}]_i$ in dissociated floor plate cells after activation of NK1 receptors with substance P. Substance P (SP), [β-Ala^8]-neurokinin A 4-10 (β-Ala^8), and [Sar^9,Met(O$_2$)^{11}]substance P (Sar^9) were applied in a bath to the preparation for 120 s at the arrow (concentrations indicated). (**A**) [Sar^9,Met(O$_2$)^{11}]substance P was able to increase $[Ca^{2+}]_i$, whereas [β-Ala^8]-neurokinin A 4-10 and senktide (Senk.) did not increase $[Ca^{2+}]_i$. (**B** and **C**) Blockade of the response to substance P (B) and [Sar^9,Met(O$_2$)^{11}]substance P (C) by RP67580. The actions of the antagonist reversed on washout. RP67580 was applied for ~5 min before one of the agonists was tested; (C) shows that on its own RP67580 had no action on $[Ca^{2+}]_i$. (**D**) Immunostaining with antibody to NK1 (*3*). Large, regularly shaped cells were labeled (arrows). Glial cells and fibroblasts (arrowhead) were not labeled. Scale bar, 25 μm.

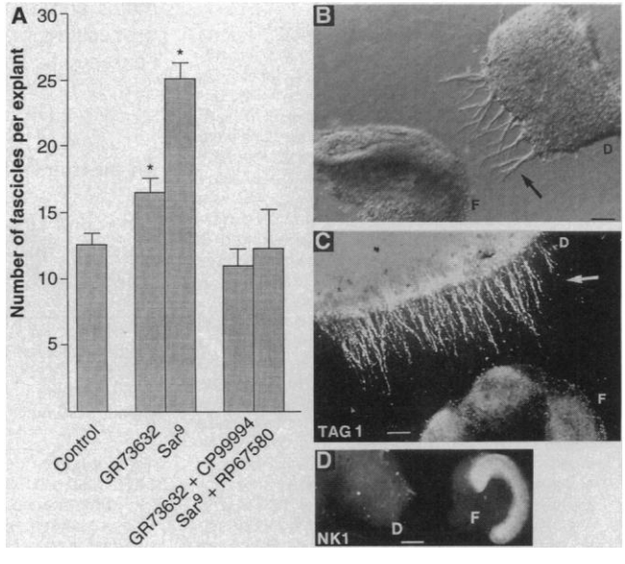

Fig. 4. Facilitation of the outgrowth of commissural axons toward the floor plate in vitro by addition of substance P. Three-dimensional collagen gels were used to coculture dorsal cord explants with floor plate for 36 hours before fixation. (**A**) Effect of substance P on the outgrowth of axon fascicles (control, n = 63). GR73632 (30 μM) was added to the culture medium every 12 hours (n = 65) and antagonized with the specific NK1 antagonist CP99994 (3 μM) added simultaneously (n = 28). A greater effect was seen with [Sar^9,Met(O$_2$)^{11}]substance P (1 μM) added every 12 hours (control, n = 15; agonist-treated, n = 15), which was fully antagonized by the NK1 antagonist RP67580 (10 nM, n = 5). This agonist has 10 to 100 times greater affinity for the rat receptor than does GR73632. Dorsal cord explants alone in the presence of agonist (n = 14) did not show commissural neurite outgrowth. Data were analyzed with Dunn's test for multiple comparisons; the agonist treatment was statistically significant at $P < 0.05$ (error bars indicate SEM; asterisks indicate statistically significant differences from the control). (**B**) Fascicles of axons (arrow) growing from a dorsal cord explant (D) toward a floor plate explant (F) in collagen gel matrix. Scale bar, 80 μm. (**C**) Agonist-stimulated coculture immunostained en bloc with the antibody TAG1 (mAb 4D7), which specifically labels commissural axons (*1*, *7*). TAG1-positive fascicles of axons (arrow) were always directed toward the floor plate. Scale bar, 60 μm. (**D**) A coculture fixed after 36 hours and stained with antibody to NK1 to show that immunoreactivity is still fully visible within the floor plate and not within the dorsal cord explant. Scale bar, 150 μm.

TAG1-negative axon fascicles. Similarly, a major change in the expression of adhesion factors has also been reported: down-regulation of TAG1 expression as the commissural neurons cross the floor plate (*2*). The arrival of substance P–containing axon terminals at the floor plate may modulate or initiate this change; in such a mechanism, release of substance P onto the floor plate would cause floor plate secretion of factors taken up by axons of passage (*12*). Here, we suggest that during development, substance P initiates or may modulate programs of gene expression in floor plate cells, which may in turn influence and guide the growth and development of neuronal pathways within the brain and spinal cord.

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4. Rat embryos or newborn animals (ages E10 to P10) were fixed by immersion in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 2 hours, transferred overnight to PB containing 30% sucrose, and sectioned at 80 to 100 μm on a sliding microtome. Free-floating sections were incubated in primary antisera to substance P or NK1 receptor diluted 1:1000 in PB with 0.1% Triton X-100 (Sigma) added for 2 to 3 days at 4°C with agitation, washed in PB for 1 hour, incubated in biotinylated antibodies to rabbit immunoglobulin G (Vector-Elite), and washed in fluorescein isothiocyanate–labeled avidin (Vector) diluted 1:200 in incubation mix for a further 2 hours in the dark. All sections were examined and photomicrographs taken on an MRC 600 confocal microscope. The antibody to NK1 was raised against a peptide corresponding to a 15–amino acid portion (residues 393 to 407) of the intracellular COOH-terminus of the rat substance P receptor. The polyclonal antiserum did not recognize neurokinin-2 or neurokinin-3 receptor proteins [S. R. Vigna *et al.*, *J. Neurosci.* **14**, 834 (1994)]; the polyclonal substance P antiserum recognized both substance P and neurokinin A [J. I. Nagy and S. P. Hunt, *Neuroscience* **7**, 89 (1982)]. However, a second antibody that recognized only substance P (Incstar) gave similar results. Controls were performed by adsorption of antibody with 10 μg of native peptide per milliliter of diluted antiserum, which abolished all positive staining.
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$$[Ca^{2+}] = K_d \times \beta\left(\frac{R - R_{min}}{R_{max} - R}\right)$$

where K_d is the dissociation constant for fura-2, R is the fluorescence ratio, R_{min} is the fluorescence ratio in 0 mM Ca^{2+}, R_{max} is the fluorescence ratio in 20 mM Ca^{2+}, and β is the proportionality coefficient. Values of R_{min}, R_{max}, and β were obtained with solutions of fura-2 (25 μM in 125 mM KCl, 20 mM CaCl, 10 mM Hepes, and 1 mM Mg^{2+}, pH 7.0, 30°C) in which Ca^{2+} was buffered to known concentrations with 10 mM EGTA. Floor plate cells (n = 61), representing approximately 25% of the total population of cells, were analyzed; they were identified by correlation with parallel NK1 immunohistochemically stained preparations and had a square or rectangular appearance. Fibroblasts were characteristically large and flat with extensive lamellipodia, whereas oligodendrocytes stained positively with an antibody to Gal-C (C. De Felipe, R. D. Pinnock, S. P. Hunt, data not shown).
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Genetic Feminization of Brain Structures and Changed Sexual Orientation in Male *Drosophila*

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The neural basis of sexual orientation in *Drosophila* was studied by the production of males with regionally feminized brains. Such flies express the female form of the sex determination gene *transformer* in a limited number of neurons under the control of *GAL4* enhancer trap inserts. This method facilitated the creation of lines with a stable pattern of feminization. In tests of sexual preferences, flies that were feminized in a portion of the antennal lobes or in a subset of the corpora pedunculata (mushroom bodies) courted both males and females. These two brain structures, both of which are involved in olfactory processing, may function in the recognition of sex-specific pheromones, in the control of sex-specific behaviors, or both.

In the fruit fly *Drosophila melanogaster*, most aspects of sexual behavior are genetically controlled (*1*, *2*). Sexual recognition of females by males largely relies on sexually dimorphic contact pheromones (*3*), and performance of male courtship requires genotypically male cells in higher centers of the nervous system (*4*).

To understand the neural basis of sexual orientation in *Drosophila*, we generated male flies with different patterns of regional feminization of the nervous system by using a *GAL4* enhancer trap strain (*5*). The random insertion of the *GAL4* transposon leads to a line of flies that express *GAL4* in a stable tissue-specific manner that is dependent on the enhancer gene next to which the transposon is inserted. When each *GAL4* line is crossed with a line that contains the reporter feminizing gene *up-*

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