



An Interneuronal Chemoreceptor Required for Olfactory Imprinting in *C. elegans*

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Science **309**, 787 (2005);
DOI: 10.1126/science.11114209

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forward: Modern primates are predisposed to learn to fear spiders and snakes because such preparedness conferred a selective advantage to our ancestors over conspecifics that were not thus prepared (11). A similar argument has previously been made for the superior conditioning effect observed to angry in comparison with happy faces, emphasizing the evolutionary relevance of the face as a means of signaling threat (25). The evolutionary interpretation for the racial outgroup bias found in experiment 2 is more nuanced. The differentiation of *Homo sapiens* into what modern humans recognize as distinct races occurred relatively recently in human evolutionary history, by some estimates within the past 100,000 to 200,000 years (26). Critically, it is believed that this differentiation occurred precisely because of the mass migration and consequent geographic isolation of different human lineages, meaning that natural selection could not have specifically prepared whites to fear blacks and blacks to fear whites. However, humans might have evolved a more general preparedness to fear others who were dissimilar to them or who otherwise appeared not to belong to their social group because such individuals were more likely to pose a threat (27, 28). If a general preparedness to fear dissimilar others did indeed evolve, then present-day members of another race, with their physical differences and common categorization as belonging to an outgroup, could activate such a mechanism and produce the robust conditioning effect observed in experiment 2.

In other words, because of its relatively recent emergence as an important dimension in human social interaction, race inherently cannot be the basis of the outgroup preparedness result. Instead, it is likely that sociocultural learning about the identity and qualities of outgroups is what provides the basis for the greater persistence of fear conditioning involving members of another group. Most notably, individuals acquire negative beliefs about outgroups according to their local cultures, and few reach adulthood without considerable knowledge of these prejudices and stereotypes (14, 29, 30). It is plausible that repeated exposure to information about outgroups might prepare individuals to fear newly encountered outgroup members.

Further research will pinpoint the generality and the interpretation of the outgroup bias in aversive conditioning. For now, our finding that close, intergroup contact may reduce this bias suggests that individual experiences can play a moderating role. Millennia of natural selection and a lifetime of social learning may predispose humans to fear those who seem different from them; however, developing relationships with these different others may be one factor that weakens this otherwise strong predisposition.

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12. Materials and methods are available as supporting material on Science Online.
13. A mixed analysis of variance (ANOVA) conducted for acquisition trials revealed that participants exhibited greater CRs to outgroup than ingroup faces [$F(1, 71) = 4.03, P < 0.05$], an effect not qualified by participant race [$F(1, 71) = 0.85, NS$]. Likewise, a mixed ANOVA conducted for extinction trials revealed greater CRs to outgroup than ingroup faces [$F(1, 71) = 5.59, P < 0.05$], an effect not qualified by participant race [$F(1, 71) = 1.70, NS$]. In other words, participants acquired stronger CRs to outgroup relative to ingroup faces, a difference that remained pronounced during extinction.
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Supporting Online Material

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Materials and Methods

SOM Text

Fig. S1

Tables S1 to S5

References

13 April 2005; accepted 20 June 2005

10.1126/science.1113551

An Interneuronal Chemoreceptor Required for Olfactory Imprinting in *C. elegans*

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Animals alter their behavioral patterns in an experience-dependent manner. Olfactory imprinting is a process in which the exposure of animals to olfactory cues during specific and restricted time windows leaves a permanent memory ("olfactory imprint") that shapes the animal's behavior upon encountering the olfactory cues at later times. We found that *Caenorhabditis elegans* displays olfactory imprinting behavior that is mediated by a single pair of interneurons. To function in olfactory imprinting, this interneuron pair must express a G protein-coupled chemoreceptor family member encoded by the *sra-11* gene. Our study provides insights into the cellular and molecular basis of olfactory imprinting and reveals a function for a chemosensory receptor family member in interneurons.

Olfactory imprinting, which occurs in contexts as diverse as homing behavior in salmon and neonatal attachment in mammals, is a learned

olfactory response whose defining features are that the olfactory memory is long-lasting and can only be acquired during a defined developmental time window or during a specific physiological state (*I*). These features distinguish it from other learned olfactory responses, such as olfactory adaptation, which can occur at many distinct developmental or physiological states and usually lasts for a limited amount of time. However, the cellular and molecular basis of olfactory imprinting is poorly understood.

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To assess whether olfactory imprinting exists in *C. elegans*, we exposed worms to specific odorants over defined developmental time windows and then assayed odorant attraction in adult worms. Odorant attraction is classically assayed by quantifying the number of animals in a population capable of migrating up a

gradient of a defined olfactory cue (2). To increase the sensitivity of the odorant attraction assays, we did not restrict ourselves to determining the number of animals that had accumulated at the source of an olfactory cue after a given time period but rather chose to closely monitor the kinetics of the migratory

behavior of animals in an olfactory gradient. Specifically, we recorded the position of animals in the olfactory gradient at several distinct time points, thus allowing us to calculate a “migration index” (fig. S1) (3). This migration index is an indicator of the speed and efficiency with which animals can respond to olfactory cues.

We found that preexposure of worms to the odorant benzaldehyde (BA) at a specific developmental stage significantly improved the ability of adult worms to migrate toward a BA source presented at moderately attractive concentrations (Fig. 1, A and B) (table S1). We express the impact of preexposure to an olfactory cue as an olfactory “imprinting index,” which we define as the difference between the migration indices of preexposed (“imprinted”) and non-preexposed (“naïve”) animals. For example, the migration index in a BA gradient is 2.3 ± 0.23 for naïve worms and 4.5 ± 0.22 for imprinted worms ($P = 0.0001$; Fig. 1B), which translates into an imprinting index of 2.27 ± 0.24 (Fig. 1A).

Preexposure to BA must occur at a specific developmental window, coinciding with the first larval stage (Fig. 1A), therefore defining this learned olfactory behavior as olfactory imprinting. Odorant exposures before hatching or after the L1 stage produced no significant imprint.

Sensory inputs such as the presence of food profoundly affect egg-laying rate (4). We found that the presence of the olfactory cue BA also strongly affected egg-laying rate (fig. S2). Notably, the dose response values for the effect of BA on egg laying and odorant attraction (migration index) were strongly correlated. We therefore tested whether olfactory imprinting also affects egg-laying rate. Upon encountering BA in the adult stage, BA-imprinted adult wild-type worms laid up to twice as many eggs per hour as naïve worms (fig. S3). Olfactory imprinting therefore leads to a sensitization of two distinct motor outputs, locomotion, and egg laying. We note that the locomotory output of olfactory imprinting (i.e., the enhanced performance of imprinted animals in odorant attraction assays) also correlates with the reproductive state of the animal, because imprinted larval or pre-reproductive adult animals showed no enhanced response in odorant attraction assays (5).

The BA odorant is sensed by the AWC sensory neuron class (2). Two other AWC-sensed olfactory cues, isoamylalcohol (IA) and citronellol (CI), are similarly able to leave an olfactory imprint (Fig. 1B). In contrast, diacetyl, which is sensed by the AWA neuron class, is unable to leave an imprint (5). Because BA and IA are both sensed by the AWC sensory neurons, we tested whether imprints could be generated in an odorant-specific manner within one olfactory neuron class. Imprinting of animals with IA (or CI)

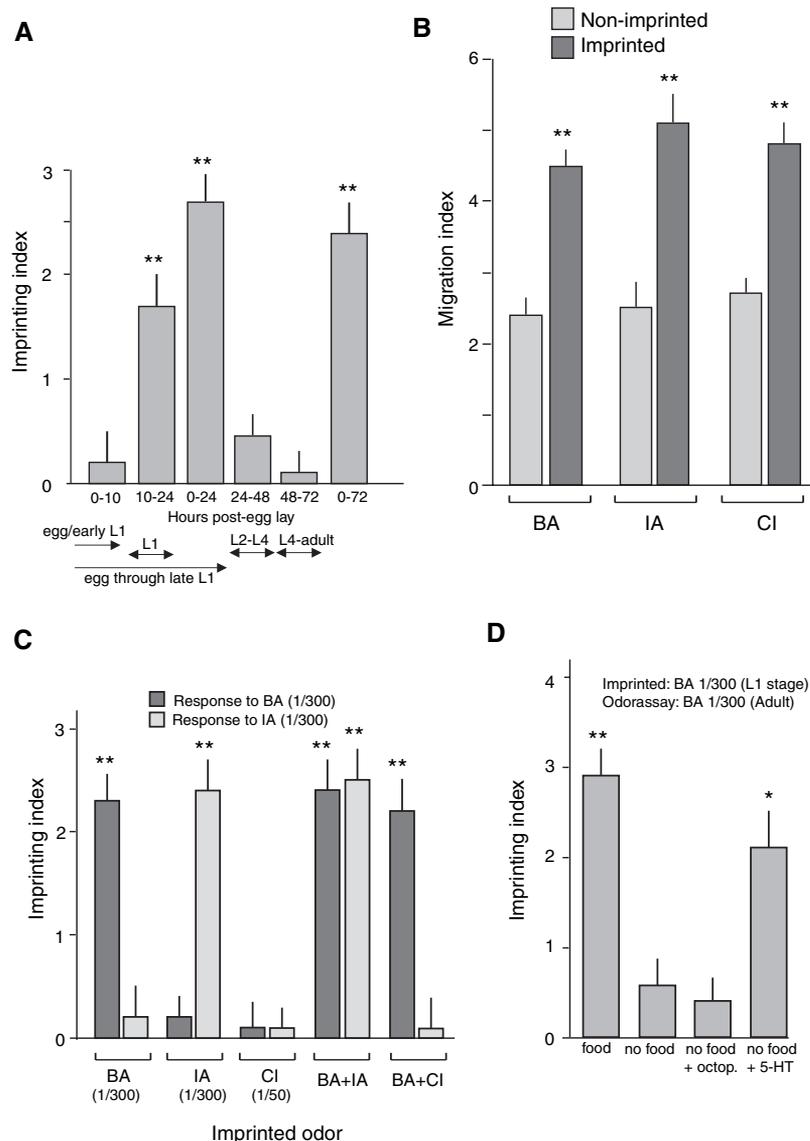


Fig. 1. *C. elegans* displays odor-specific olfactory imprinting. (A) Stage-specific olfactory imprinting with BA. Developing worms were exposed to BA diluted 1/300 in water at different developmental stages. Migration indices of 5-day-old animals were determined at an odorant concentration of 1/300. Each assay was done five times with 20 animals each. Interaction analysis with the two-way analysis of variance (ANOVA) test indicated a highly significant ($**P < 0.001$) impact of olfactory response depending on both olfactory imprinting and the development time course of imprinting. (B) Imprinting with BA, IA, and CI. The odorant concentrations for imprinting and for the attraction assay that followed were 1/300 for BA and IA and 1/50 for CI. The assays were done 10 times for BA, 7 times for CI, and 4 times for IA, with 20 worms tested in each assay. $**P \leq 0.005$, comparing imprinted and nonimprinted worms. (C) Olfactory imprinting is odorant-specific. Each assay was done three to five times with 20 animals each. Animals were imprinted at the L1 stage. The P value ($**P < 0.01$) refers to the significance of the comparison of the migration indices of naïve and imprinted worms (i.e., the imprinting index). See table S1 for assays at different odorant concentrations. (D) Odorant imprinting is food-dependent. BA (1/300) was presented during the imprinting period (L1 stage) in the presence or absence of food with or without 3 mM serotonin ($n = 4$, 20 animals each) or 3 mM octopamine ($n = 3$). $**P < 0.0001$, comparing food to no food; $*P < 0.05$, comparing no food to no food + 5-HT (serotonin).

did not affect the response of animals to later encounters of BA (Fig. 1C) (table S1). Conversely, imprinting with BA did not affect their response to IA. Moreover, the simultaneous presence of odorants in addition to BA or IA did not affect the ability of BA or IA to leave an imprint. We conclude that olfactory imprints are not generated on the level of the whole receptor neuron, but are generated in an odorant-specific manner.

To investigate the potential physiological relevance of olfactory imprinting, we asked whether an olfactory imprint could be used as a memory device for favorable environmental conditions. One environmental condition that is known to affect a plethora of behavioral paradigms in *C. elegans* is the presence or absence of food [reviewed in (6)]. We found that the absence of food during the critical learning phase disrupted olfactory imprinting (Fig. 1D). Serotonin mimics the presence of food in various sensory paradigms (6), and the addition of exogenous serotonin into the food-free agar plates indeed restored olfactory imprinting (Fig. 1D). In contrast, octopamine, another monoamine present in *C. elegans*, did not compensate for food deprivation. We did not observe any significant enhancement of the responses when olfactory imprinting was carried out in the presence of both food and serotonin (5). If animals were starved and exposed to BA and serotonin at the adult stage, no improvement in the subsequent odorant attraction assay was observed, which indicates that the association of food and serotonin with an odorant occurs only during the critical olfactory imprinting period. Taken together, these results suggest that a potential function of olfactory imprinting is the memorization of favorable growth conditions.

The existence of olfactory imprinting in *C. elegans* afforded us the opportunity to start defining the as yet elusive cellular and molecular mechanisms of olfactory imprinting. In the olfactory imprinting phenomena associated with neonatal attachment in rats, it is thought that several different, though poorly defined, central brain areas play an important role (7). We focused our cellular analysis on the two bilaterally symmetric AIY interneurons, which receive synaptic inputs from several distinct classes of sensory neurons, including the BA- and IA-sensing AWC odorsensory neuron class (Fig. 2A). We genetically disabled the AIY interneurons by reducing the activity of the *ttx-3* homeobox gene, which controls the functional differentiation of AIY (8). Using olfactory cues at a concentration at which *ttx-3* mutants are still capable of responding to the cue, we found that preexposure of *ttx-3* mutants to odorants failed to leave an olfactory imprint (Fig. 3A). Because *ttx-3* is expressed in three other neuron classes besides AIY (8), we tested whether *ttx-3* indeed acts in AIY to affect

olfactory imprinting. Driving expression of a *ttx-3* cDNA under control of a cis-regulatory element that is exclusively active in the AIY interneurons (9) rescued the olfactory imprinting defects of *ttx-3* mutant animals (Fig. 3A).

The *ttx-3* homeobox gene directly regulates the expression of scores of AIY-expressed genes, one of which is the *sra-11* gene (8). *sra-11* encodes an orphan, G protein-coupled seven-transmembrane receptor (7TMR) that belongs to a large family of putative chemoreceptor-encoding genes that were uncovered through genome sequence searches (10). Unlike other chemoreceptor family members, which are expressed in sensory neurons, *sra-11* is exclusively expressed in three interneuron classes, AIY, AIA, and AVB (10). Expression can be observed throughout all larval and adult stages (8). Two mutant alleles of *sra-11*, each likely null alleles that delete most if not all of the *sra-11* locus (Fig. 2B), were made available by the *C. elegans* Gene Knockout Consortium. Several assays that test the functionality of AIY, including reversal assays and thermokinesis assays, indicate that previously known aspects of AIY function are unaffected by the absence of *sra-11* (3, 11). As assayed by odorant attraction assays as well as the odorant-induced egg-laying response, *sra-11* null mutants showed a normal response to several odorants tested, including BA (Fig. 3B) (fig. S3). However, the odorant response of *sra-11* null mutants failed to be positively imprinted by BA or IA (Fig. 3A) (fig. S4). Both *sra-11* null alleles showed similar olfactory imprinting

defects. These olfactory imprinting defects could be measured on both motor output levels, the locomotory output (Fig. 3A) and the egg-laying output (Fig. 3C). Animals that lack another AIY-expressed 7TMR, the tyramine receptor *ser-2* (12), showed intact olfactory imprinting; hence, olfactory imprinting was not affected by the disruption of any AIY-expressed 7TMR.

Because *sra-11* is expressed in two other neuron classes besides AIY (10), we tested whether *sra-11* function is indeed required in AIY by generating transgenic *sra-11* null animals that express *sra-11* under control of an AIY-specific cis-regulatory element (3). As a control, we deleted the G protein-coupled C terminus of *sra-11* and generated animals expressing this construct under control of the same AIY-specific cis-regulatory element. Double-blind scoring of the transgenic lines revealed that only the wild-type construct was able to rescue the *sra-11* null mutant phenotype (Fig. 3, A and C).

Our analysis leads to three main conclusions: (i) *C. elegans* displays a learned olfactory response pattern that can be classified as olfactory imprinting. The imprint is associated with favorable growth conditions (food) and, in analogy to many other olfactory imprinting paradigms, is generated at an early juvenile stage. The imprinted odorant increases the attraction of a mature animal to this odorant and stimulates egg laying, so as to allow the progeny of the animal to exploit the memory of these favorable environmental conditions. (ii) Olfactory imprinting requires a single interneuron pair that is postsynaptic

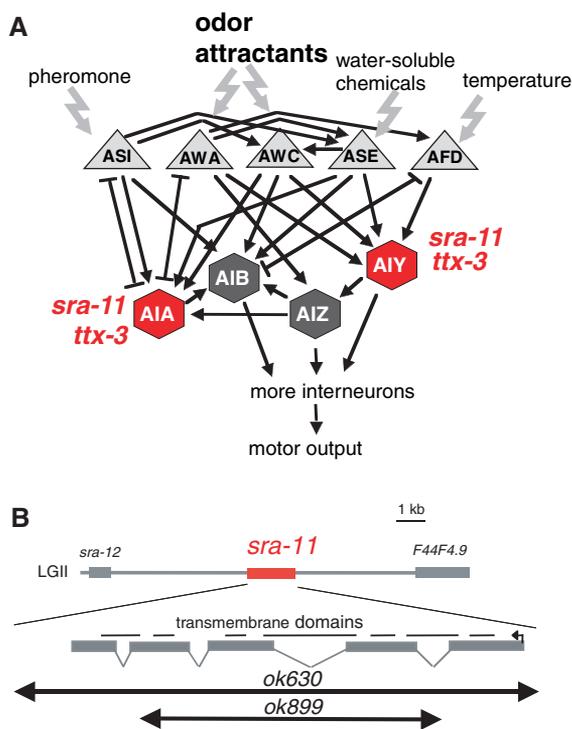


Fig. 2. Cells and genes tested for an effect on olfactory imprinting. (A) Schematic representation of the synaptic connectivity of the AIY interneuron class (14) and the sites of *ttx-3* and *sra-11* expression within this circuit. (B) *sra-11* locus and structure of mutant *sra-11* alleles.

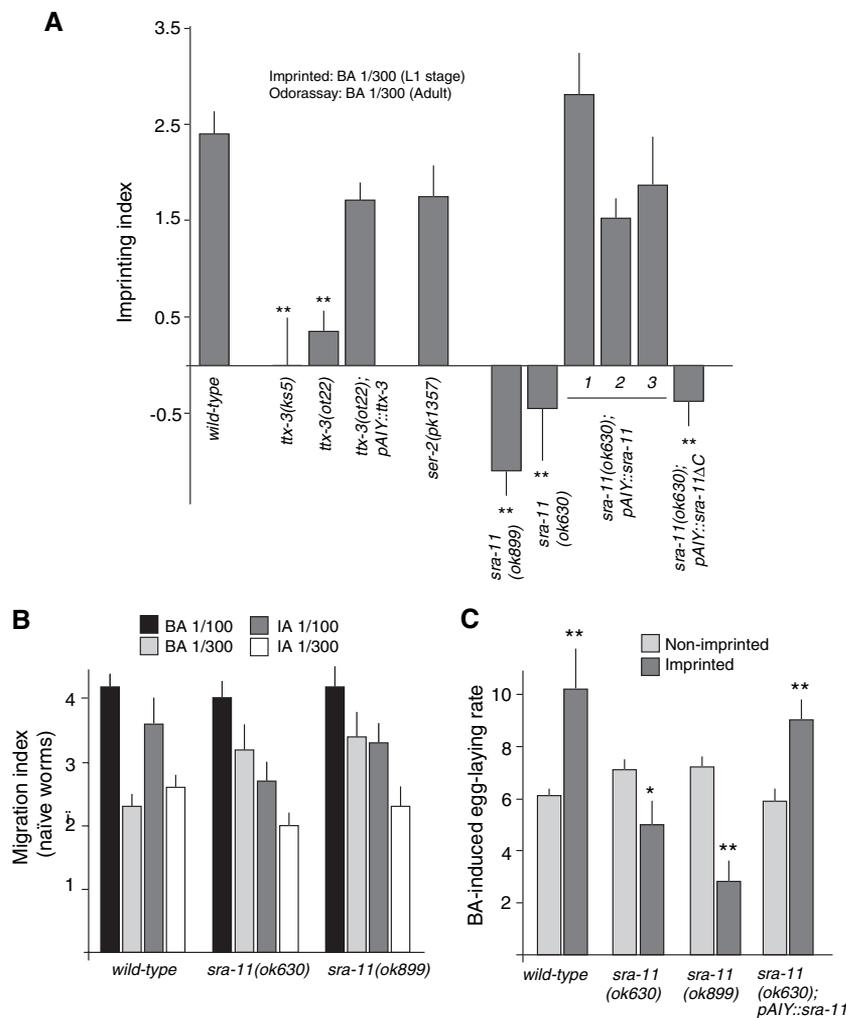


Fig. 3. AIY-expressed *sra-11* is required for olfactory imprinting. (A) *ttx-3* and *sra-11* mutant animals show imprinting defects for BA as measured in an odorant attraction assay. Three independent rescued lines that express *sra-11* exclusively in AIY (“*pAIY::sra-11*”) and one control transgenic line expressing a truncated *sra-11* gene in AIY (“*pAIY::sra-11ΔC*”) are shown. See table S2 for complete data set. $**P < 0.001$ for comparison of wild-type to both *ttx-3* and both *sra-11* alleles and to the control line for rescue and for the comparison of both *ttx-3* mutants to the rescued line and the *sra-11* mutants to the rescued lines. (B) *sra-11(ok630)* null mutants show a normal response to different concentrations of BA and IA. Each assay was done in triplicate with 20 animals each. (C) *sra-11* mutants also show imprinting defects in the egg-laying imprinting assay. One representative rescued line is shown. Values are the means of a total of 80 worms (two independent experiments with 10 worms each on four plates per condition). $**P < 0.01$, $*P < 0.05$, comparing imprinted and nonimprinted animals under each condition. Note that *sra-11* mutants appear to display a negative olfactory imprint, given that imprinted animals are less attracted to BA [as shown in (A)] and lay fewer eggs relative to naïve worms.

to olfactory neurons. (iii) The SRA-11 protein, a member of a large chemosensory receptor family, is specifically required for olfactory imprinting. Surprisingly, SRA-11 does not function in sensory neurons but in interneurons

downstream of the sensory neuron class to control olfactory imprinting. Because the olfactory imprinting process shows odorant selectivity, and because olfactory imprinting to at least two distinct odorants is disrupted in

sra-11 mutants, we infer that *sra-11* is required for a generic rather than odorant-specific aspects of olfactory imprinting. The SRA-11 protein could be a generic subunit of a receptor complex that is activated by an AWC-released ligand upon imprinting by distinct odorants, leaving permanent marks in the AIY interneuron; upon a later encounter of the same odorant by the AWC neuron class, these marks may facilitate signaling through the AIY interneuron. In analogy to glomerular targeting mediated by vertebrate olfactory receptors (13), it is also conceivable that SRA-11 may have a role in determining fine aspects of AWC-AIY connectivity that may be modulated upon olfactory imprinting. Elucidating the nature of the ligand of the SRA-11 protein will provide further insights into the process of olfactory imprinting.

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15. We thank the *C. elegans* Gene Knockout Consortium in Oklahoma for providing the *sra-11* knockout alleles; M. A. Felix (Institut Jacques Monod) and all members of her group; S. Lockery for help with statistics; I. Greenwald, P. Sengupta, and members of the Lockery and Hobert lab for comments on the manuscript; J. Zhu for generating the *sra-11* constructs; and Q. Chen for expert DNA injection assistance. Supported by the Neurobiologie de l’Olfaction et de la Prise Alimentaire laboratory, the Physiologie Animale et Systèmes d’Elevage (PHASE) department of Institut National de la Recherche Agronomique (J.-J.R.), and NIH grant NS039996-05 (O.H.).

Supporting Online Material

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Materials and Methods
Figs. S1 to S4
Tables S1 and S2

28 April 2005; accepted 17 June 2005
10.1126/science.1114209