0.25 M $NaPO_4$ and 1 mM EDTA containing 1.0% NP-40 and 1 μl of *N*-glycanase (specific activity 0.227 U/μl; Genzyme) were added, and then the mixtures were incubated for 24 hours at 37°C. The treated and untreated immunoprecipitates were resuspended in Laemmli buffer with reducing agents and resolved by SDS-PAGE.
19. The FO-1 cell line, a human β_2M-negative cell line [C. M. D'Urso *et al.*, *J. Clin. Invest.* **87**, 284 (1991)], was transfected with 20 μg of circular human CD1d or murine CD1.1 (*6*) cDNA in the pSRα Neo expression vector [Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988)] by electroporation with a gene pulsar unit (Bethesda Research Lab) at 400 V and a capacitance of 1600 μF. After 48 hours, transfectants were selected at 2×10^5 cells per well in 96-well flat-bottomed plates with Geneticin sulfate (G418; 3.0 mg/ml; specific activity 500 μg/mg; Gibco). Geneticin sulfate–resistant wells were screened by flow cytometry with the 1H1 mAb.
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Induction of an Olfactory Memory by the Activation of a Metabotropic Glutamate Receptor

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Female mice form an olfactory memory of male pheromones at mating; exposure to the pheromones of a strange male after that mating will block pregnancy. The formation of this memory is mediated by the accessory olfactory system, in which an increase in norepinephrine after mating reduces inhibitory transmission of γ-aminobutyric acid from the granule cells to the mitral cells. This study shows that the activation of mGluR2, a metabotropic glutamate receptor that suppresses the γ-aminobutyric acid inhibition of the mitral cells, permits the formation of a specific olfactory memory without the occurrence of mating by infusion of mGluR2 agonists into the female's accessory olfactory bulb. This memory faithfully reflects the memory formed at mating.

Female mice form an olfactory memory of the pheromones of the male with which they mate. Subsequent exposure to the pheromones of a strange male will block a female's pregnancy, but exposure to the pheromones of a male of the same strain as the original male does not block pregnancy (*1*, *2*). The synaptic changes underlying this memory formation occur in the accessory olfactory bulb (AOB) (*2*–*6*). In the AOB, the mitral cells, when activated by vomeronasal nerve inputs, depolarize granule cells by means of glutamate released at dendrodendritic synapses (*2*) (Fig. 1). This depolarization in turn releases γ-aminobutyric acid (GABA) from granule cells and hyperpolarizes mitral cells (*2*).

Norepinephrine, the turnover of which is enhanced after mating, reduces the GABA-mediated feedback inhibition and induces an olfactory memory of pheromones present at mating (*4*). A metabotropic glutamate receptor (mGluR), mGluR2, is expressed predominantly at the dendrites of granule cells and, when activated by its potent agonist, (2S,1′R,2′R,3′R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), suppresses the GABA-mediated inhibition of the mitral cells (*7*, *8*). Because DCG-IV and norepinephrine both reduce GABA transmission from granule cells to mitral cells, blockage of the GABA-mediated feedback inhibition by DCG-IV could, in theory, permit the formation of a specific pheromonal memory without the occurrence of mating.

We investigated the role of mGluR2 in olfactory memory formation by designing the following three-stage behavioral protocol (*3*, *9*) (Fig. 2). Drugs were infused into the AOB of estrous females 0 and 1.5 hours after the beginning of a 6-hour exposure to BALB/c male pheromones without mating. At the next estrus, the females were mated with a male from a CBA strain and then reexposed to the pheromones of the BALB/c male to test for formation of a pheromonal memory. Under this protocol, it can be determined that memory formation occurs at the time of drug infusion if the drug treatment prevents pregnancy block by the test pheromonal exposure. The results of a series of experiments are summarized in Fig. 2.

Because DCG-IV, though less effectively than mGluR2, activates the *N*-methyl-D-aspartate (NMDA) receptor (*8*), we first tested *trans*-1-aminocyclopentane-1,3-di-

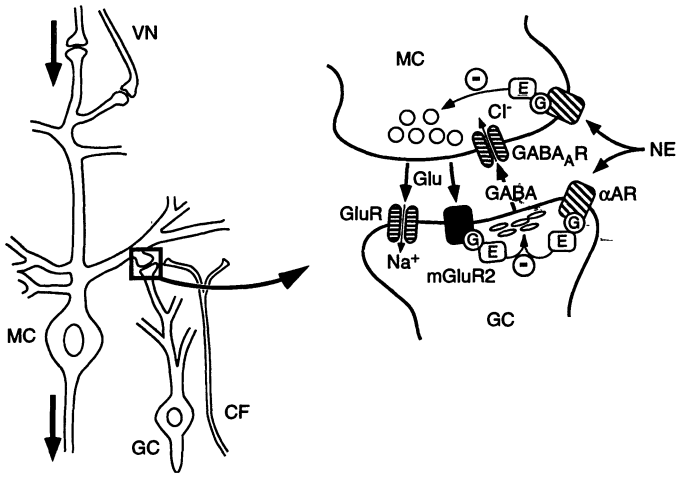

Fig. 1. Synaptic relations in the AOB and a model for the microcircuitry in dendrodendritic synapses between a granule cell and a mitral cell. VN, vomeronasal nerve; MC, mitral cell; GC, granule cell; CF, centrifugal fiber of norepinephrine projection from locus ceruleus; Glu, glutamate; GluR, ionotropic glutamate receptor; $GABA_AR$, $GABA_A$ receptor; αAR, α-adrenergic receptor; G, G protein; E, intracellular effector; NE, norepinephrine. The signaling pathways that inhibit GABA release are marked by minus symbols. As is the case in the synaptic circuitry of the main olfactory bulb (*17*), the reduction of GABA transmission by norepinephrine may be caused by the inhibition of mitral cell excitation. A direct presynaptic action of norepinephrine on the granule cell, however, is also possible; and two pathways for norepinephrine inhibition of GABA transmission are indicated.

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carboxylate (tACPD), a common agonist for all members of the mGluR family that has no effects on either the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-kainate receptors or on the NMDA receptors (*10*). In the control experiment, phosphate-buffered saline infusions during pheromonal exposure caused significant pregnancy block (group 1). In contrast, two infusions of tACPD prevented the pregnancy block that was evoked by a second exposure to the original pheromones (group 2). This protection was reduced by decrease of the infused doses of tACPD (group 3). A single infusion of tACPD was not sufficient to prevent pregnancy block (group 4), which is consistent with the previous observation that the sustained excitation of mitral cells is necessary for memory formation (*4*). These results indicate the involvement of an mGluR in olfactory memory formation.

We then used DCG-IV to define the mGluR subtype that is involved in olfactory memory formation. DCG-IV infused during the females' exposure to male pheromones significantly reduced pregnancy block (group 5). As was consistent with the greater effectiveness of DCG-IV over tACPD as an mGluR2 agonist (*8*), a pheromonal memory was formed by a lower concentration of DCG-IV than of tACPD. DCG-IV itself did not increase spontaneous pregnancy block (group 6). DCG-IV infused without pheromone failed to prevent pregnancy block (group 7), which indicates that memory formation requires the concomitant action of DCG-IV and of pheromonal inputs. Notably, this memory is specific to the pheromones to which the females were exposed during DCG-IV infusions, because a significant pregnancy block was brought about by exposure to pheromones from a different mouse strain (group 8). Thus, this finding shows that DCG-IV produces pheromone-specific memory, and it rules out the possibility that the DCG-IV–mediated reduction in pregnancy block is caused by a nonspecific impairment of the AOB circuitry. The α-adrenergic antagonist phentolamine had no effect on the DCG-IV–mediated memory formation (group 9), which supports the view that this memory formation results from the direct action of DCG-IV on mGluR2 rather than from the enhancement of norepinephrine actions.

We also tested whether an mGluR antagonist, (+)-α-methyl-4-carboxyphenylglycine (three infusions of 10 nmol each), could affect naturally occurring pheromonal memory formation, but we failed to detect the inhibition of this memory. However, because of the relatively weak potency of this compound as an antagonist to mGluR2 (*11*), it remains to be determined if this failure reflects the possible difference in extracellular signaling pathways in which the GABA modulation is used by the naturally occurring and the mGluR2-mediated memory formations (Fig. 1).

Memory formation at mating is blocked by the nonselective ionotropic glutamate receptor antagonist, γ-D-glutamylglycine, but not by the selective NMDA receptor antagonist, D-2-amino-5-phosphonovalerate (D-AP5) (*12*). The AMPA-kainate receptor-selective antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQx), prevented the DCG-IV–mediated memory formation (group 10), whereas the NMDA antagonist D-AP5 was without effect (group 11). Thus, all of the above results indicate that DCG-IV–induced memory formation conforms to the characteristics of the memory formed at mating.

DCG-IV is a very potent agonist for both mGluR2 and mGluR3. However, no appreciable expression of mGluR3 mRNA was observed in the adult AOB, nor was any localization of mGluR2 at the mitral dendritic synapses (*8*, *13*). Thus, our data indicate that the activation of mGluR2 at the presynaptic sites of granule cells permits formation of an olfactory memory through reduction of GABA release. As is consistent with this conclusion, the $GABA_A$ receptor antagonist bicuculline also produces an olfactory memory (*3*). However, this memory, unlike DCG-IV–induced memory, lacks the pheromone specificity. This difference may be because mGluR2 is restrictedly expressed in granule cells, as opposed to the wide distribution of the $GABA_A$ receptors in the AOB (*14*). The formation of an olfactory memory depends on the stimulation of both mGluR2 and the AMPA-kainate receptor but seems not to require the NMDA receptor. The mechanism underlying the associative actions of mGluR2 and of AMPA-kainate receptors remains elusive. It has been reported that the expression of immediate-early genes increases in both the mitral cells and the granule cells during the period of memory formation that occurs at mating (*15*). It is thus possible that olfactory memory formation may require the excitation of both the granule cells and the mitral cells by means of the AMPA-kainate receptors and mGluR2.

Because DCG-IV seems not to activate norepinephrine actions that are critical for mating-induced memory formation, mGluR2 may not be directly involved in the olfactory block to pregnancy. However, there are many forms of olfactory memory and learning, which may differ in their synaptic bases (*16*). Our data further indicate that the persistent activation of mGluR2 is necessary for the mGluR2-mediated memory formation. This feature would be important in distinguishing transient olfactory stimuli from those experienced continuously. Thus, mGluR2 could be critical in a different form of odorant-mediated neuronal plasticity in the AOB. In conclusion, this study demonstrates that synthetic chemical agonists for a specific receptor can induce a recognition memory and that a specific metabotropic receptor is involved in evoking neuronal plasticity in the olfactory brain functions.

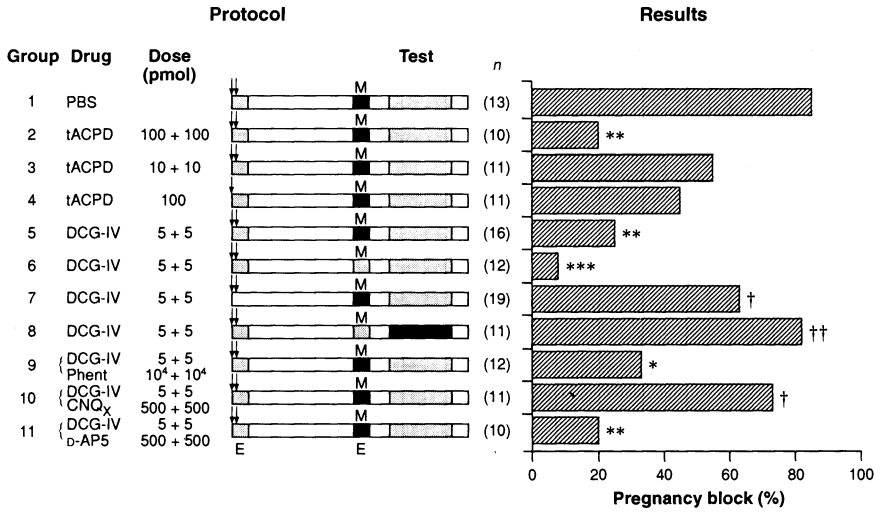

Fig. 2. Olfactory memory formation by mGluR activation in the AOB. Failure of the test exposure to male pheromones to block pregnancy was used to evaluate the formation of an olfactory recognition memory (*9*). M, mating; arrows, drug or phosphate-buffered saline infusions; shaded blocks, exposure to pheromones from the BALB/c male; filled blocks, exposure to pheromones from the CBA male; Phent, phentolamine; and E, estrus. The numbers in parentheses indicate numbers of animals tested. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$, when compared with group 1. †$P < 0.05$, ††$P < 0.01$, when compared with group 5. Data were analyzed with the Fisher exact probability test.

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9. BALB/c female mice received bilateral, stereotaxic implantations of indwelling 25-gauge stainless steel guide cannulae under Avertin anesthesia at least a week before drug infusion. To avoid damage to the AOB and to vomeronasal nerves, these cannulae were implanted immediately anterior to the AOB. Estrous females received one or two 0.5-μl infusions of a drug at 0 and 1.5 hours during a 6-hour exposure to bedding soiled by a BALB/c male or to bedding with no pheromone. At the next estrus, these females were mated either with a male of a CBA strain or with a BALB/c male. The females were left with the stud males for 6 hours after mating and were then removed to a clean cage for the rest of the experiment. Between 24 and 26 hours after mating, the females were exposed to the pheromones of a BALB/c male or of a CBA male for 48 hours to test for pregnancy block. Six days after mating, the females were killed by cervical dislocation and their uteri were examined for implantation sites. In these experiments, no seizures were caused by infusions of either *t*ACPD or DCG-IV. In addition, the seizures that are frequently caused by infusions of ionotropic receptor antagonists (*12*) were also completely prevented by combined infusions with DCG-IV.
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TECHNICAL COMMENTS

Resistance to Murine Acquired Immunodeficiency Syndrome (MAIDS)

I have performed genetic studies on murine acquired immunodeficiency syndrome (MAIDS) susceptibility that suggest an alternative explanation for the results reported by O. Kanagawa *et al.* (*1*). My results raise the possibility that immune suppression caused by the murine retrovirus (LP-BM5 MuLV) may not result from a change in the pattern of cytokine expression of helper T cells from T_H1 to T_H2 phenotype, as Kanagawa *et al.* suggest. The IL-4–deficient mice appear resistant to disease, while their IL-4–sufficient littermates are sensitive. Kanagawa *et al.* conclude that IL-4 deficiency interferes with disease progression by preventing the T_H1 to T_H2 conversion, which implies that the immune response to the virus is pathogenic, but there are genetic factors that could be at work in this system. The embryonic stem (ES) cell line used to generate the "knock-out" mice is derived from a 129 strain mouse (*2*). Therefore, all IL-$4^{-/-}$ progeny must be homozygous for the 129-derived chromosome 11, and conversely, all IL-$4^{+/+}$ progeny must carry both copies of the C57Bl/6-derived homolog.

Inbred mouse strains vary in their sensitivity to LP-BM5 MuLV disease; C57Bl/6 is a sensitive strain, but I have found that 129/J mice are resistant to LP-BM5 disease. There are three documented bases of resistance in mice: MHC ($H2D^d$), Fv-1^n, and R_{mcf}^R, but there is evidence that other loci may be involved (*3*). In F1 crosses between resistant and sensitive strains, *sensitivity* to disease is dominant, rather than resistance. The precise reason for resistance in 129 mice is not known, but IL-$4^{-/-}$ mice may be resistant to MAIDS because they are homozygous for the 129-derived chromosome 11, rather than the defective IL-4 gene.

The 129-derived chromosome 11 could confer resistance by any of a variety of mechanisms. For example, 129 mice carry an endogenous ecotropic retrovirus on chromosome 11 (*4*) that can be expressed as infectious virus. Prior infection of sensitive C57Bl/6 mice with a nonpathogenic ecotropic virus renders them resistant to LP-BM5 disease (*5*). If the 129-derived virus is expressed in the IL-4–deficient mice, then these mice could be resistant as a result of an endogenous infection, not of IL-4 deficiency.

A second possibility is that 129 mice carry another retrovirus resistance locus on chromosome 11. In selecting IL-$4^{-/-}$ mice, Kanagawa *et al.* may have selected mice that were resistant because they were homozygous for a potential resistance locus, not because they were IL-4–deficient.

The absence of resistance in the IL-$4^{+/+}$ littermates should also be addressed. The F1 parents of these mice are Fv-$1^{n/b}$. Fv-1 should segregate independently of IL-4, because these loci are on different chromosomes. One-quarter of the F2 IL-$4^{+/+}$ littermates should be Fv-$1^{n/n}$, and therefore be resistant to LP-BM5 disease, yet 100% (28/28) of the +/+ mice were sensitive.

IL-4 deficiency could indeed be the reason for LP-BM5 MuLV resistance in these mice; in fact, there are many reasons to expect that interfering with immune function may inhibit disease (*6*). However, the genetic factors of viral resistance, or endogenous retroviruses, or both, need to be excluded before making statements as to the effect of cytokine expression, Th phenotype, or both, on the mechanism of retrovirus-induced immune suppression.

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MAIDS is a retrovirus-induced disease of mice with many similarities to HIV-induced disease in humans (*1*, *2*). Studies of both infectious processes have shown that the progression of disease can be associated with a switch from a cytokine profile of a Type 1 T helper cell (T_H1) (high IL-2, low IL-4, and IL-10) to one in which the cytokines of Type 2 T helper cells (T_H2) IL-4 and IL-10 dominate (*3*). The hypothesis that the balance between T_H1 and T_H2 cytokines may be critical to the pathogenesis of these immunodeficiency syndromes (*3*) appears to be supported by several recent findings. First, IL-12, an agent that stimulates T_H1 differentiation and the production of T_H1 cytokines, has been found to inhibit the development of MAIDS (*4*) and to restore in vitro cell-mediated immune responses in individuals that are positive for HIV (*5*). Second, studies of IL-4–deficient (129/Sv × B6)F_2 mice showed them to be resistant to MAIDS (*6*).

Susceptibility and resistance to MAIDS are genetically determined with prominent