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13. Direct repeats of the oligonucleotide PG (5′-CCTGCCTGGACTTGCCTGG-3′) were used for the p53 binding sequences. Each copy of the repeat contained the binding region of plasmid C_{BE}, previously shown to bind p53 in vitro (*8*). For the nonbinding control sequence, repeats of the oligonucleotide MG (5′-CCTTAATGGACTTTAATGG-3′) were used. For the CAT reporters, repeats of PG were ligated into the Eco RV site of pBluescript II SK+ (Stratagene, La Jolla, CA) to form the PG_n and MG_n series. The Bgl II–Bam HI fragment of pPyOlCAT [Y. Murakami, M. Asano, M. Satake, Y. Ito, *Oncogene* **5**, 5 (1990)], containing the polyomavirus early promoter and the CAT gene coding region, was then ligated into the Bam HI site of the PG_n and MG_n series clones to form the PG_n-CAT and MG_n-CAT series. The PG_n-MG_n-CAT series was formed by excising the Hind III–Sal I fragments of PG_n-CAT, blunt-ending, attaching Xba I linkers, and ligating into the Xba I site of the MG_n-CAT series plasmids. The construction of the p53–wild-type (p53-wt) expression construct has been described [identical to pC53-SN3 in (*6*)]; the mutant expression plasmids (Fig. 1) were constructed similarly from previously described clones (*10*). The mutants studied included one which was typical of those found in the germ line of Li-Fraumeni patients (Trp^{248}) [D. Malkin *et al.*, *Science* **250**, 1233 (1990); S. Srivastava, Z. Zou, K. Pirollo, W. Blattner, E. H. Chang, *Nature* **348**, 747 (1990)] and three found commonly in a variety of human tumors (Val^{143}, His^{175}, His^{273}) (*4*).
14. Cultures of HCT 116 cells at 50 to 80% confluence in 25-m^2 flasks were transfected with Lipofectin [Bethesda Research Laboratories (BRL), Gaithersburg, MD] according to the manufacturer's instructions. All flasks within an experiment were transfected with the same total amount of plasmid, with additional pCMVneoBam DNA (*6*) as necessary. Cells were harvested at 20 to 24 hours, and the CAT activity of the lysates was measured by acetylation of ^{14}C-labeled chloramphenicol (ICN, Costa Mesa, CA) as previously described [C. M. Gorman *et al.*, *Mol. Cell. Biol.* **2**, 1044 (1982)]. The Bio-Rad protein assay was used to assure equivalence of lysate protein. Percent conversion to the acetylated form of chloramphenicol was calculated after quantitation of excised chromatographic spots by scintillation counting. Results reported are representative of at least two transfections done on separate days.
15. Exons 5 to 8 of the p53 gene from HCT 116 cells were amplified by polymerase chain reaction (PCR) and sequenced as described in [D. Sidransky *et al.*, *Science* **252**, 706 (1991)]. Previously, these exons had been shown to contain more than 90% of the mutations observed in human tumors (*2–4*). No mutations were observed in HCT 116 cells. Small amounts of apparently wild-type protein could be detected in Western blots of HCT 116 protein, performed as described (*18*). In cells transfected with p53 expression vectors, p53 protein concentrations increased by more than 20-fold.
16. Placing an additional 59 to 333 base pairs between the PG multimer and the promoter (PG_n-MG_n-CAT reporters, Fig. 1) had little effect on transactivation. However, placement of the PG binding sequences downstream of the CAT gene (CAT-PG_n, Fig. 1) did not allow transactivation (S. E. Kern *et al.*, unpublished data).
17. The mutant p53 gene expression construct used in these experiments were not toxic to transfected cells, as shown by (i) continued cell cycle progression after transient expression (*6*); (ii) establishment of stable clones from these transfectants (*6*); and (iii) noninhibition of CMV-driven gene expression, as assessed by measurements of the exogenous p53 protein produced in these cells (*18*).
18. The transfection and expression efficiency of mutant p53 expression vectors was assessed by Western blot analysis. Protein (100 μg) from each cell lysate was separated on a 10% SDS–polyacrylamide gel, transferred onto a polyvinyl membrane, blocked with 5% nonfat milk, incubated with PAb1801 (1 μg/ml) (Oncogene Sciences, Manhasset, NY) then with ^{125}I-labeled goat antibody to mouse immunoglobulin (IgG) (NEN, Boston, MA), and autoradiographed. Expression of mutant p53 was approximately 0.5-, 1.5-, 2.5-, and 1.5-fold that achieved with the p53-wt vector when p53-143, p53-175, p53-248, and p53-273 were used, respectively. For each expression vector, the concentration of p53 protein increased by two- to fourfold when 2.55 μg instead of 0.85 μg of expression vector was used for transfection.
19. The data on transcriptional activation by wild-type p53, and loss of this activity in p53 mutants, were also observed in transiently transfected HeLa cells (S. E. Kern *et al.*, unpublished data).
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22. When 0.85 μg of p53-wt and 2.55 μg of p53 mutant expression vectors were used for cotransfection, the amount of total p53 protein in the transfected cells was two- to fourfold higher than that achieved with 0.85 μg of p53-wt alone, as assessed by Western blot analysis (*18*).
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27. For the yeast β-gal reporter plasmids, PG and MG sequences in Sal I–Sma I fragments were ligated to the Sal I and filled in Xho I sites of pCZ [A. R. Buchman *et al.*, *Mol. Cell. Biol.* **8**, 5086 (1988)] to create PG_n-*lacZ* and MG_n-*lacZ*. The construction of the yeast p53 expression vectors has been described [J. M. Nigro, R. Sikorski, S. I. Reed, B. Vogelstein, *ibid.* **12**, 1357 (1992)]. The Yp53 expressed wild-type p53, whereas Yp53-143 and Yp53-273 have the same p53 mutation as p53-143 and p53-273, respectively. The reporter and expressor vectors were introduced into *S. cerevisiae*, strain YPH420, and clones were obtained. Overnight cultures grown at 30°C in synthetic liquid medium containing raffinose as the carbon source were diluted to an absorbance at 600 mm of 0.1 in medium containing raffinose and galactose (1:1) and growth continued for another 18 to 24 hours. Cells were harvested and resuspended in breaking buffer [100 mM tris-HCl (pH 8.0), 1 mM dithiothreitol, 0.1 mg/ml of bovine serum albumin, and 20% glycerol]. Cells were disrupted in the presence of 2 mM phenylmethylsulfonyl fluoride by vortexing with glass beads (0.4 mm), clarified by centrifugation; protein concentration was determined by the Bio-Rad assay. The β-gal assays were performed with *o*-nitrophenyl-β-D-galactoside as described [J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972)]. After galactose induction, p53 expression was similar in transformants containing wild-type and mutant expression vectors as assessed by Western blot analysis (*18*).
28. Conversion of an alanine to a valine substitution mutant to a wild-type p53 form at low temperatures has been observed in mammalian cells [D. Michalovitz, O. Halevy, M. Oren, *Cell* **62**, 671 (1990); J. Martinez, I. Georgoff, J. Martinez, A. J. Levine, *Genes Devel.* **5**, 151 (1991)].
29. We thank D. Sidransky for performing the sequence analyses of HCT 116 p53 genes, C. Prives for the recombinant baculoviruses encoding p53, P. Hieter for advice on the yeast experiments, A. Buchman for providing pCZ, M. Brattain for HCT 116 cells, and Pharmagenics, Inc. Allendale, NJ, for synthesis of oligonucleotides. Supported by the Clayton Fund, McAshan Fund, Preuss Foundation, and grants CA06973, CA09243, and CA35494.

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Cholecystokinin Antianalgesia: Safety Cues Abolish Morphine Analgesia

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Environmental stimuli that signal the occurrence of aversive or dangerous events activate endogenous opiate analgesia systems. Signals for safety (the nonoccurrence of aversive events) produce the opposite and inhibit environmentally produced analgesia. Stimuli that signal safety are now shown to abolish the analgesic effect of morphine, even when morphine is applied directly to spinal cord. Further, this antiopiate effect occurs because the environmental stimulus leads to release of the neuropeptide cholecystokinin in the spinal cord. This process may contribute to the regulation of pain and the development of opiate tolerance.

The discovery of endogenous pain inhibitory circuitries in the central nervous system (CNS) led to investigation of environmental events that trigger their activation (*1*). In general, these neural circuits are activated by contact either with noxious stimuli or with cues that signal such noxious events (that is, innate or learned danger signals) (*1*). The CNS may also contain circuitry that can inhibit pain inhibition systems (*2–4*). This evidence rests, to date, on the effects of administering exogenous agonists and antagonists of possible antianalgesia neurotransmitters. In agreement with the concept of antianalgesia systems, agonists block whereas antagonists or anti-

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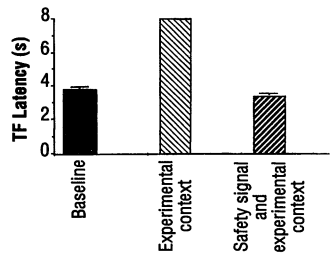

Fig. 1. Safety signal reversal of conditioned analgesia. Baseline pain responsivity was measured before presentation of the experimental context danger signals. All animals then learned to become maximally analgesic in response to the experimental context danger signals (Experimental context). In all animals, conditioned analgesia was abolished by the presentation of the safety signal (light) in the context that produced analgesia (Safety signal and experimental context). TF, tail flick.

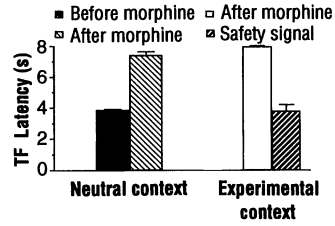

Fig. 2. Safety signal reversal of systemic morphine analgesia, in addition to conditioned analgesia. Animals trained to respond to danger and safety signals were first assessed for baseline pain sensitivity in the colony room, a neutral context where shock had never occurred. All animals then received a subcutaneous injection of 2 mg of morphine sulfate per kilogram body weight. After 15 min, pain sensitivity was again assessed in the neutral context. Animals were then moved to the experimental context where pain sensitivity was again assessed in the presence and absence of the safety signal.

sera potentiate analgesia produced by substances such as morphine (*2–5*).

The coexistence of neural circuitries that produce both analgesia and antianalgesia raises the question of their function in normal organisms. Activation of analgesia systems by signals for impending danger is adaptive, because an analgesic animal, able to ignore the pain of injury, is better equipped to deal with a dangerous situation and more likely to survive the event (*1*).

No similar adaptive or behavioral role has been proposed for antianalgesia systems. These systems have instead been viewed as simple, biochemical homeostatic mechanisms that return an organism to a basal state of pain responsivity after endogenous opiates have produced analgesia (*2–4*). It has been thought that the release of endogenous opiates or the administration of exogenous opiates directly leads to the release of antiopiate substances (*2, 3*).

We believe that antianalgesia systems function as the mirror image of analgesia systems. That is, whereas analgesia systems are activated by danger signals, antianalgesia systems are activated by environmental signals for safety. This too can be viewed as an adaptive response, because once a danger has passed the recognition of safety would once again allow the perception of pain, so that wounds would be attended to promptly. Thus, the activation of antianalgesia by safety signals would provide the animal with more effective recuperative ability.

Signals for safety usually do not produce measurable effects of their own but reverse the behavioral and physiological impact of danger signals. In fact, safety signals reverse the analgesia produced by danger signals (*6*). We gave rats electric shocks in a particular experimental context (*6*). The context (a dimly illuminated room equipped for stimulus presentations) acquired the capacity to elicit a strong analgesic response, a phenomenon called conditioned analgesia. A light cue was established as a safety signal by turning on a 75-s pulse of light after each shock. Thus, the onset of each light presentation was followed by a period free from shock and so signaled the absence of shock (*6*). Neutral stimuli become safety signals under this arrangement and acquire the capacity to inhibit fear responses that are produced by danger (*7*). The light also acquired the ability to block the conditioned analgesia produced by the context (*6*). When the light was turned on, analgesia was completely eliminated (Fig. 1).

However, this demonstration that safety signals inhibit analgesia was only suggestive of the activation of an antianalgesia system. Blockade of analgesia could have been accomplished in a number of ways, because danger and safety signals should both be expected to exert their effects at multiple sites within the brain and spinal cord. For example, danger signals increase arousal, create fear, focus attention, as well as activate analgesia systems. Indeed, attention and fear may activate pain inhibitory circuitry (*1*). Such complex consequences to presentation of danger signals mean that safety signals could inhibit analgesia not through an antianalgesia system but through inhibiting fear, attention, or other consequences of the danger signal.

To determine whether safety signals block analgesia by activating antianalgesia circuitry, we produced analgesia directly. If safety signals trigger circuits that act directly on pain transmission, then the analgesia produced by agents that act directly on pain transmission should be reversed. We trained rats to respond to both danger and safety signals (*6*) and then gave them an analgesic dose of morphine (*8*) to test whether the safety signal could block morphine analgesia, in addition to again blocking analgesia produced by the danger signal. If the safety signal acts by inhibiting fear or attentional processes, then morphine analgesia will not be affected by the safety signal. If, instead, the safety signal functions through an antianalgesia system, neither morphine nor the danger signal should produce analgesia in the presence of the safety signal. Neither morphine nor the danger signal produced analgesia in the presence of the safety signal (Fig. 2).

Here, morphine analgesia was superimposed on analgesia elicited by the danger signals. To assure that the safety signal was reversing the analgesia produced by the morphine and not the context, we eliminated the ability of the context to produce analgesia. After training rats as before, we then repeatedly exposed them to the shock context, without the shock or light presentations, until all animals exhibited normal pain sensitivity in the presence of the shock-related cues. Thus, the danger signals associated with the context were no longer able to produce analgesia. Now we could test the effect of the safety signal on morphine analgesia without the simultaneous presence of conditioned analgesia. Under these circumstances, the analgesia produced by the morphine injection was again abolished by the safety signal presentation (Fig. 3).

These results establish that animals can learn to activate endogenous antianalgesia systems. The safety signal must activate circuitry that blocks morphine from inhibiting pain. There are multiple sites of morphine action (*9*), but all ultimately act by inhibit-

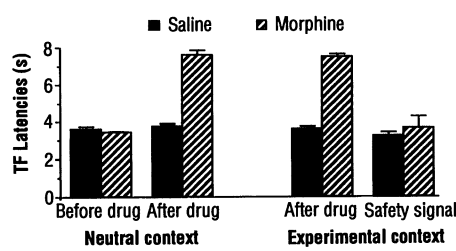

Fig. 3. Safety signal reversal of systemic morphine analgesia, in the absence of conditioned analgesia. Before testing, rats were trained to respond to danger and safety signals, and then the ability of the experimental context to elicit analgesia was eliminated by repeated sessions exposing the animals to this context without presenting the shock or light. During testing, rats were first assessed for baseline pain sensitivity (Neutral context, before drug) in the colony room (where shock never occurred). Rats then received either 2 mg of morphine or saline per kilogram body weight. After 15 min, pain sensitivity was again assessed, revealing potent morphine analgesia (Neutral context, after drug). Rats were then moved to the experimental context and pain sensitivity was again assessed in the absence (Experimental context, after drug) or presence of the light safety signal (Experimental context, safety signal).

ing pain transmission in the spinal dorsal horn. Physiological studies with agonists and antagonists of putative antianalgesia neurotransmitters also suggest that the spinal cord is a critical site for antianalgesia (*2–4*).

There are several candidates for the endogenous spinal cord substances responsible for antianalgesia. Cholecystokinin (CCK) is prominent among these (*3, 10*). Systemic and spinal administrations of exogenous CCK antagonize morphine analgesia, whereas spinal application of CCK antagonists potentiates morphine analgesia.

To examine the possibility that antianalgesia and analgesia systems converge at the spinal cord, we intrathecally administered morphine plus either vehicle or the specific CCK-B receptor antagonist L-365,260 (*11, 12*). This allowed us not only to examine the effect of the safety signal on morphine analgesia induced directly at the spinal cord but also the possible mediation of learned antianalgesia by spinal cord CCK. L-365,260 was chosen because CCK receptors in rat spinal cord are predominantly of the CCK-B subtype (*10*). Rats were trained and responses to the danger signals (shock context) were eliminated as before. Morphine or L-365,260, either alone or in combination, was then microinjected onto the spinal cord. After the onset of morphine analgesia, the safety signal was presented and pain sensitivity reassessed. The safety signal abolished morphine analgesia in animals given morphine alone. Thus the safety signal can inhibit analgesia by initiating processes that act at the spinal cord to reverse the effects of morphine. Moreover, L-365,260 dose dependently prevented the safety signal from abolishing morphine analgesia in rats given morphine plus L-365,260 (Fig. 4A). This effect was due to CCK-B, rather than to CCK-A, receptors because an equimolar dose of the CCK-A antagonist MK-329 (*11*) had no effect (Fig. 4B). These data show that CCK is critically involved in the mediation of antianalgesia and that this effect is mediated through CCK-B receptors at the level of the spinal cord.

An external stimulus (light) that occurs in a context in which electric shocks were presented and that is negatively correlated with shock acquires the ability to reverse the analgesic effects of morphine. Moreover, a site of inhibition of morphine analgesia is the spinal cord because the light was able to reverse the effects of morphine applied directly to the cord (*13*). As further support for a spinal site of action for the safety signal, both the specific CCK-B antagonist L-365,260 and the nonselective CCK antagonist proglumide (*14*) applied to the cord reversed the impact of the light. This suggests that the safety signal releases CCK in the spinal cord, with CCK then interacting with opiate mechanisms (*3*). The fact that the effects exerted by L-365,260 or proglumide could not be replicated by the specific CCK-A antagonist MK-329 demonstrates that CCK exerts its antianalgesic effects through spinal cord CCK-B receptors. The results suggest that the safety signal inhibited conditioned analgesia in our prior studies (6) because it activated antianalgesia circuitry in the spinal cord, not just because it reduced fear or diverted attention.

A number of models have been proposed (*15*) that argue that the emotional and physiological reactions to potent events (for example, electric shocks, loss of a loved one, cold, and morphine) elicit subsequent opponent reactions. The interplay between direct and opponent reactions have then been used to explain phenomena such as opiate dependence and tolerance. The details of these models have been somewhat difficult to test, in part because the direct reaction and the opponent have been unspecified in physiological terms and, therefore, measurable only by inference. However, we have demonstrated a potential direct reaction (opiate release in the spinal cord) and a true opponent (CCK release), and both can be measured and their interaction can be assessed.

Spinal cord mechanisms are often thought to be free of the influence of complex psychological variables and to proceed by self-regulatory circuits. This is not the case for spinal antianalgesia mechanisms, just as it is not true of spinal analgesia mechanisms. The influence of psychological events on spinal cord function should perhaps not be surprising given the existence of descending circuits, but demonstrations are rare. Our data suggest that spinal cord antianalgesia mechanisms not only subserve biochemical homeostasis in pain systems but are controlled by more complex psychological and external events. These findings also suggest functions that might be served by antianalgesia. It has been argued that pain promotes recuperative activities (for example, licking a wound or protecting an injury) designed to protect and to promote recovery (*16*). Such activities are incompatible with efficient defensive behavior during danger

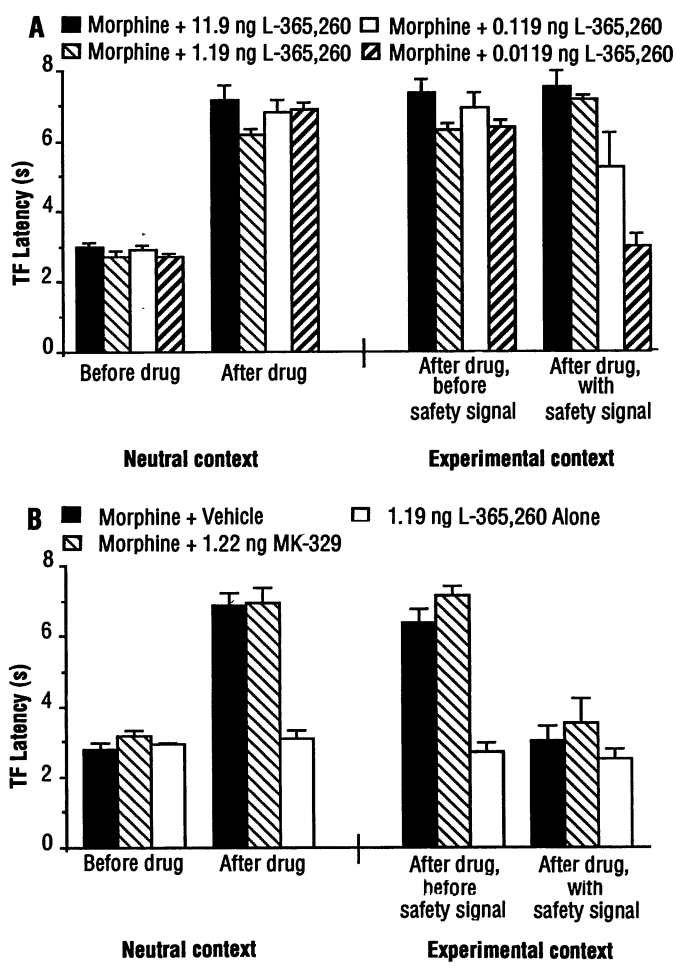

Fig. 4. Safety signal reversal of spinal morphine analgesia and dose-dependent elimination of the safety signal effect by L-365,260, a selective CCK-B antagonist. Rats were first trained to respond to danger and safety signals. Analgesia to the experimental context was then eliminated. (**A**) Animals were first assessed for baseline pain sensitivity (Neutral context, before drug) in the colony room. Rats then received one of seven intrathecal injections: 5 μg of morphine sulfate + 11.9 ng of L-365,260, 5 μg of morphine sulfate + 1.19 ng of L-365,260, 5 μg of morphine sulfate + 0.119 ng of L-365,260, 5 μg of morphine sulfate + 0.0119 ng of L-365,260, 5 μg of morphine sulfate + vehicle, 5 μg of morphine sulfate + 1.22 ng of MK-329 (a selective CCK-A receptor subtype antagonist), or 1.19 ng of L-365,260 alone (without morphine). Pain sensitivity was assessed at 15 min (Neutral context, after drug). Rats were then moved to the experimental context and pain sensitivity was assessed in the absence (Experimental context, after drug, before safety signal) and presence of the safety signal (Experimental context, after drug, with safety signal). (**B**) Rats were treated as in (A) but the effect of morphine + 1.22 ng of MK-329 (equimolar to 1.19 ng of L-365,260) was tested. Injection of 1.19 ng of L-365,260 alone had no effect on pain sensitivity.

and attack, and so the production of analgesia during such times would be adaptive. The same considerations would suggest that the analgesia should be terminated when the situation is safe and danger no longer present, and this is what we have found. This would allow the return of recuperative behaviors. It would seem that pain is a finely tuned sensory system, with an interplay of pain-inhibitory and antipain-inhibitory processes determining the intensity of the sensation.

Antianalgesia mechanisms may be important for the development of opiate tolerance and perhaps dependence, because they involve opioid and antiopioid endogenous substances. The spinal application of proglumide and other CCK antagonists reverses and blocks the development of morphine tolerance (*3*). The implications are that the activation of opiate receptors by morphine triggers CCK release and that each application of morphine with consequent opiate-receptor activation enhances either the output of CCK or the sensitivity of CCK receptors. Thus tolerance develops because the opponent CCK system strengthens with repeated administrations of morphine. In our experiments L-365,260 blocked the effect of the safety signal, which itself interfered with morphine's analgesic action. This suggests that the development of tolerance to morphine might differ, depending on whether morphine is administered in a relatively safe or in a dangerous and fear-arousing environment. Tolerance and perhaps dependence ought to develop more rapidly in a safe environment, where CCK systems are already augmented. Indeed, morphine dependence does not readily develop when morphine is delivered in hospitals, a relatively fear-provoking environment (*17*). Moreover, morphine reactivity is enhanced by mildly fearful and arousing circumstances (*18*). Thus, some of the individual differences in the development of opiate tolerance and dependence might be traceable to the stressfulness of the situation in which the morphine is received, and the interplay between endogenous analgesia and antianalgesia mechanisms may be the mediating link.

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Changes in the Sensory Processing of Olfactory Signals Induced by Birth in Sheep

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After giving birth, sheep and many other species form a selective bond with their offspring based on the sense of smell. Processing of olfactory signals is altered to allow the animals to perform this selective recognition. Lamb odors have little effect on either neurotransmitter release or electrical activity of neurons in the olfactory bulb before birth. However, after birth there is an increase in the number of mitral cells, the principal cells of the olfactory bulb, that respond to lamb odors, which is associated with increased cholinergic and noradrenergic neurotransmitter release. Selective recognition of lambs is accompanied by increased activity of a subset of mitral cells and release of glutamate and γ-aminobutyric acid (GABA) from the dendrodendritic synapses between the mitral and granule cells. The relation between the release of each transmitter after birth also suggests an increased efficacy of glutamate-evoked GABA release.

In sheep, an enduring bond between a mother and her lambs is established very rapidly, usually within 3 hours of parturition (*1*). Before giving birth, pregnant ewes find the smell of amniotic fluid repulsive and are indifferent toward, or violently reject, approaches by lambs. Immediately after the fetal membranes rupture, however, the ewe is attracted to the smell of amniotic fluid (*2*) and, within a few minutes of birth, starts to lick and sniff her newborn lamb and encourages it to adopt a standing posture and suckle. The ewe's interest in lambs and her ability to selec-