Fig. 4. Immunoprecipitation of T cell receptors of IEL from germ-free mice. Cell surface proteins of IEL isolated from germ-free mice were ^{125}I-labeled by the iodogen method (*30*). Precipitation by anti-CD3 was carried out essentially as described (*31*) with the use of anti-CD3 coupled to Sepharose 4B. For antibody to γ chain precipitation, the anti-CD3 precipitates were disrupted by boiling in the presence of 1% SDS and 10 m*M* dithiothreitol as the reducing agent. The reduced proteins were then alkylated by incubation with 30 m*M* iodoacetamide. The samples were diluted with buffer containing 10 m*M* triethanolamine, 0.15*M* NaCl (*p*H 7.8), 0.5% NP-40, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* EDTA, and 1 μg of leupeptin per milliliter. Rabbit antiserum against the γ chain (*9*) was added with protein A coupled to Sepharose 4B, and the mixture was incubated for 2 hours at 4°C with mixing. The precipitates were washed six times with the above buffer and analyzed by SDS–12% polyacrylamide gel electrophoresis. Lane 1, anti-CD3; lane 2, antiserum to γ chain; and lane 3, control antibody.

case. The normal mice used in these studies were free of antibody to virus but we cannot rule out low levels of inapparent viral infection. This is not to imply that IEL are not capable of responding to viral antigens, only that their repertoire may be skewed toward recognition of bacterially derived determinants. The primordial immune system would require protection against invasion of a primitive digestive tract prior to developing a surveillance system of the internal milieu, and thus TCR-γδ may have been the earliest TCRs, as has been previously proposed (*26, 27*). CD8$^+$ T cells have been implicated in the immune response to pathogenic intracellular bacteria but the TCR status of these cells has not been analyzed (*28*). Although the TCRs of CD8$^+$ IEL could be specific for bacterially derived antigens, the nature of these antigens and the antigen-processing mechanisms required for recognition by IEL will require further study. The phenotypic and functional characteristics of IEL coupled with their highly restricted anatomical distribution should allow a detailed analysis of the in vivo function of T cells expressing TCR-γδ.

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Defensive Behaviors in Infant Rhesus Monkeys: Environmental Cues and Neurochemical Regulation

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To survive, primates must detect danger in time to activate appropriate defensive behaviors. In this study, the defensive behaviors of infant rhesus monkeys exposed to humans were characterized. It was observed that the direction of the human's gaze is a potent cue for the infant. Infants separated from their mothers were active and emitted frequent distress vocalizations. When a human entered the room but did not look at the infant, it became silent and froze in one position. If the human stared at the infant, it responded with aggressive barking. Alterations of the opiate system affected the frequency of the infant's distress calls without affecting barking and freezing, whereas benzodiazepine administration selectively reduced barking and freezing. This suggests that opiate and benzodiazepine systems regulate specific defensive behaviors in primates and that these systems work together to mediate behavioral responses important for survival.

TO SURVIVE, PRIMATES MUST DETECT potentially dangerous situations and then activate appropriate defensive behaviors. These behaviors appear to originate from genetic programs (*1, 2*) and may be similar in rhesus monkeys and human infants (*3, 4*). For example, rhesus infants begin to respond to fearful visual stimuli at 2 to 4 months of age (*1*). Human infants experience a similar period of fearfulness toward strangers beginning between 7 and 9 months of age (*5*). Through experience (*6*) and maturation, infants acquire a more refined understanding of what is dangerous. Consequently, the circumstances that elicit fear-related behaviors become more specific.

Eyes receive and communicate information important for survival. Staring is frequently associated with aggression and may predict or prevent an attack (*7*). The implications of staring have been exploited by various species through the evolution of protective "eyespot" markings (*8*). Initially, we observed that infant rhesus monkeys (*Macaca mulatta*) briefly separated from their mothers dramatically alter their behavior when they detect a human intruder and that their behavioral responses differ greatly, depending on whether the intruder stares at the infant or averts his gaze. We then found selective regulation of these different behavioral patterns by the infant's opiate and benzodiazepine systems.

To characterize these behaviors, we tested 11 infant monkeys (8 females and 3 males, 6 to 11 months old) twice. During the first test, the infant was separated from its mother and placed in a cage in a different room. It remained alone (A_1) for 10 min, while its behavior was recorded on videotape. A human then entered the room and remained motionless 2.5 m from the cage, gazing at the wall and presenting his profile to the infant. At no time did the human engage the

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infant in eye contact (NEC). After 10 min, the human left the room and the infant remained alone (A_2) for another 10 min. It was then returned to its mother.

The second test occurred 1 week later and was identical to the first, except that when the human entered the room, he faced the cage and continuously stared (ST) at the infant. The infant's behavior during each 10-min test condition was recorded in three 3-min periods and scored from the videotapes by expert observers (*9*). As expected, when the infants were separated from their mothers (A_1), they were very active and emitted frequent species-specific distress calls ("coo" vocalizations). The presence of a human and the direction of his gaze made an important difference. During ST, the infants increased their frequency of cooing [$F(2,20) = 9.41$; $P < 0.001$] (Fig. 1). Barking, an aggressive vocalization, was induced by ST but rarely occurred with NEC or when the infant was alone [$F(2,20) = 9.91$; $P < 0.001$]. NEC elicited an insignificant increase in crouching (A_1, 1.0 ± 0.6 s versus NEC, 10.4 ± 7.3 s), but a significant increase in freezing (remaining motionless, except for slow head movements, for at least 3 s) [$F(2,20) = 14.43$; $P < 0.0002$]. These behaviors occurred at extremely low levels during the A_1, A_2, and ST periods, although during ST there was a general reduction in activity.

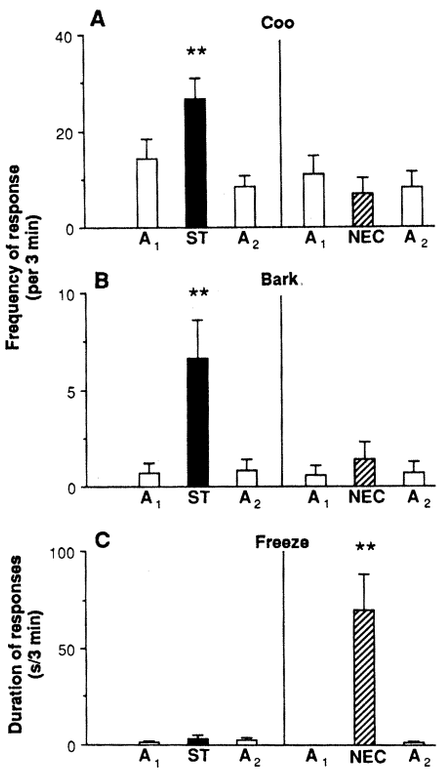

Fig. 1. Behavioral response (mean of three 3-min periods ± SE) of 11 infant rhesus monkeys briefly separated from their mothers (A_1 and A_2), and the effects of a human entering and staring at the infant (ST) or averting his gaze (NEC). $^{**}P < 0.01$.

The quality of the infant's responses during NEC deserves elaboration. When the human entered the room, the infant stopped cooing and froze for a time period characteristic for each animal. Some animals froze almost continuously throughout the 10-min exposure. When freezing ceased, the infant remained crouched in the same position. Eventually it began to locomote—very slowly at first, its face constantly oriented to the intruder; then more rapidly, with less attention to the intruder, until it moved freely around the cage. It then resumed cooing.

An infant's tendency to freeze appears to be a stable characteristic. We tested 12 additional rhesus infants (10 males and 2 females, 5 to 7 months old) twice in the NEC condition with 32 days between tests. There was a strong linear relation between the amount of freezing in the two tests (slope ± SE = 1.06 ± 0.11) (Fig. 2). We followed eight of these animals and found that this relation persisted 5.5 months later, even after eight intervening experiences with the paradigm (slope ± SE = 0.91 ± 0.22).

These findings are important because they characterize different patterns of defensive behavior in the rhesus infant and establish features of the intruder to which the animal selectively responds. Cooing is activated by attachment bond disruption, serving as a signal to the mother to help her locate her lost infant (*4, 10*). Barking connotes aggression and is specific to the ST condition (*11*). Aggressive displays in the face of inescapable danger occur in many species and sometimes discourage the attacker (*12*). The characteristic behavior elicited by NEC is freezing. This is a common response to threatening conditions and reduces the likelihood of attack in the animal's natural environment. This is partially because movement is a powerful stimulus for predatory attack (*12, 13*). Likewise, behavioral inhibition is a typical response of human children confronted by a stranger. This appears to be a stable trait in both species (*14*). Activation of these behaviors in the appropriate context is adaptive. However, fearfulness and defensive responses in the absence of real threat is maladaptive and may result in behavioral,

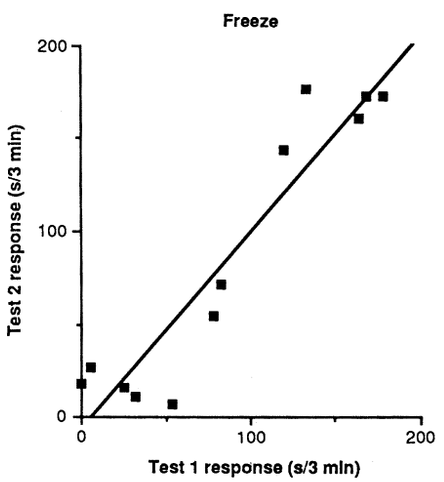

Fig. 2. Relation between amount of freezing elicited by identical conditions in two tests performed 1 month apart ($y = -5.7 + 1.05x$; SE of the slope = 0.11). Twelve rhesus monkeys 5 to 7 months old were exposed to a human who maintained a constant distance from them, keeping his gaze averted to avoid eye contact (NEC).

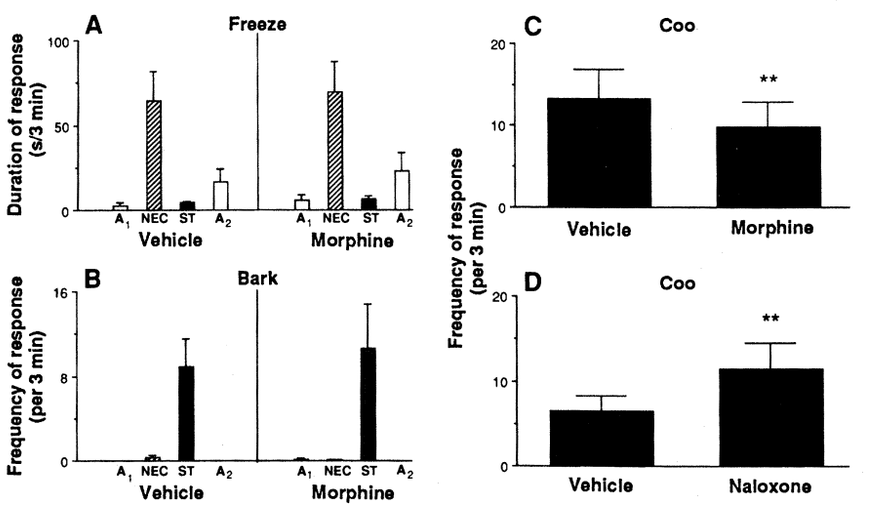

Fig. 3. Effects of opiate system alterations on defensive behaviors in 12 infant rhesus monkeys. (**A**) and (**B**) demonstrate the effects of the NEC condition on freezing (mean ± SE) and the ST condition on barking (mean ± SE). Neither morphine (0.1 mg/kg) nor naloxone (1.0 mg/kg) significantly affected these behaviors. (**C**) and (**D**) show main effects of one drug or the other on the frequency of coos (mean ± SE) emitted across all test conditions. Morphine reduced the frequency of coos, whereas naloxone increased their frequency. $^{**}P < 0.01$.

social, and physiological dysfunction in humans. This may begin early in life, since children who exhibit extreme behavioral inhibition at 2 years of age are at risk to develop fear-related psychopathology (*14*).

We next performed neuropharmacological experiments to assess which neurobiological systems mediate the defensive behaviors induced by the A, NEC, and ST conditions. Since previous studies suggested that opiates play a role in regulating distress vocalizations (*15, 16*), we examined the effects of morphine. Either saline (0.9%) or morphine sulfate (0.1 mg/kg) was administered intramuscularly to 12 rhesus infants (7 male, 5 female) immediately after maternal separation (*17*). The infants were tested twice with 1 week between tests, counterbalancing the order of drug administration. The design was modified by including NEC and ST conditions in one test. Therefore, each infant experienced a 10-min isolation period (A_1) followed by successive 10-min periods of NEC, ST, and A_2.

The effects of this paradigm were similar to those in the first experiment: NEC elicited freezing, whereas ST induced barking and increased the frequency of coos. However, we were now able to observe the infants' ability to rapidly change behavioral patterns. The effects of morphine were highly selective, since it reduced cooing [$F(1,11) = 9.91$; $P < 0.009$] without significantly affecting barking, freezing, or locomotion (Fig. 3).

We then administered the opiate antagonist naloxone (1 mg/kg) and observed increased cooing [$F(1,11) = 8.77$; $P < 0.01$] without significant change in the other defensive behaviors (Fig. 3).

We next assessed the effects of the benzodiazepine diazepam (1.0 mg/kg) on the defensive behaviors (*18, 19*). Unlike morphine and naloxone, diazepam did not significantly alter coos. However, it decreased the duration of freezing [$F(3,33) = 4.10$; $P < 0.01$] and crouching [$F(3,33) = 3.01$; $P < 0.04$] during NEC and reduced the amount of slow locomotion that occurred in all conditions [$F(1,11) = 8.07$; $P < 0.02$]. Barking induced by ST was also decreased by diazepam [$F(3,33) = 5.17$; $P < 0.005$] (Fig. 4).

These studies demonstrate that infant rhesus monkeys dramatically change their behavior when a human intrudes into their environment. When the human averts his gaze, the infant freezes. In contrast, when the human stares at the infant, it engages in aggressive gestures and vocalizations. Such behaviors are associated with fear in other species; thus, it is likely that these actions by infant rhesus monkeys are defensive and represent attempts by the infant to protect itself in a threatening situation. It is noteworthy that the infant monitors the direction of the intruder's gaze, which it takes as a cue to direct its response.

Interestingly, these different behaviors appear to be controlled by different neurotransmitter systems. Thus, manipulations of the opiate system affected coo frequency without affecting barking induced by ST or freezing induced by NEC. If the effects of altering the opiate system were mediated simply by changes in the infant monkey's level of arousal, then barking and freezing would decrease with morphine and increase with naloxone. This was not the case. Conversely, diazepam reduced barking and freezing without significantly affecting cooing.

During a period of threat, an animal must rapidly alter its defensive strategy as the parameters of the threat change (*20*). Our findings suggest selective involvement of opiate and benzodiazepine systems in mediating different defensive responses. When a primate is threatened, the opiate and benzodiazepine systems may work together to orchestrate patterns of defensive behavior necessary for survival. Opiate and benzodiazepine systems may also mediate the development of human psychopathology characterized by excessive or inappropriate fear responses.

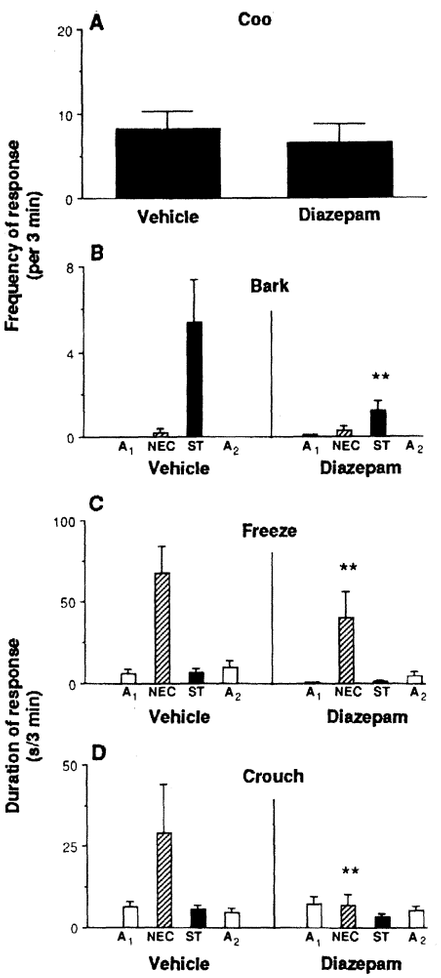

Fig. 4. The effects of diazepam (1 mg/kg) were quite different from those of morphine and naloxone. Diazepam had no statistically significant effect on (**A**) coos (mean ± SE) but significantly reduced the frequency of (**B**) barks (mean ± SE) under ST conditions. Diazepam also significantly reduced behaviors elicited by the NEC condition: (**C**) freezing (mean ± SE) and (**D**) crouching (mean ± SE). $^{**}P < 0.01$.

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9. The care and treatment of all animals were in accordance with institutional guidelines. Infants were reared by their mothers in cages 71.5 cm wide by 84 cm high by 71 cm deep. They remained with their mothers at all times except during the experimental separations. While with their mothers, infants were exposed regularly to the animal caretakers. Animals were maintained on a 12 hour light–12 hour dark cycle (lights on from 0600 to 1800 hours). During the test, infants were separated into a cage 79 cm wide by 76 cm high by 71 cm deep. Throughout all the experiments described, the same man was used as the intruder. In more recent work, we have shown that the animals' responses are not specific to this individual or to males in general, because the infants respond similarly to a woman (N. Kalin and S. Shelton, unpublished data). Videotapes were scored by two expert observers who have had at least 3 years of experience with this system. In the first experiment, each observer scored half of the animals on the two different test days. In the studies with drug administration, all monkeys were scored by the same observer, who was unaware of the drug treatment. Frequently occurring behaviors were coo (vocalization produced by rounding and pursing the lips; characterized by first increase, then decrease in frequency and intensity); bark (threat or warning vocalization, produced with mouth slightly open with lips in normal position; abdomen forces air through vocal cords producing a short, rasping, low-frequency sound); locomotion (ambulation of one or more full steps at any speed, including such behavior as dropping from ceiling to floor or swinging cage shake); freeze (a period of at least 3 s characterized by tense body posture and no movement other than slow movements of the head). Data were analyzed by repeated measures ANOVA. Dun-

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17. In all of the pharmacologic studies reported in this paper, the effects of a single dose of drug were compared with those of vehicle. The dose selected was based on complete dose-response studies in infant monkeys performed earlier (*16, 18*). The same 12 subjects were used over a 2-month period for the morphine, naloxone, and diazepam studies. At the beginning of these studies the infants ranged in age from 9 to 12 months.
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19. Preliminary experiments showed that diazepam decreases vocalizations induced by separation in infant rhesus monkeys. This effect was probably not mediated by diazepam's interaction with benzodiazepine receptors because pretreatment with the benzodiazepine antagonist Ro 15-1788 did not block it. In fact, lower doses of diazepam increased activity levels, an effect that was reversed by pretreatment with the benzodiazepine antagonist.
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Ethanol Inhibits NMDA-Activated Ion Current in Hippocampal Neurons

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The ion current induced by the glutamate receptor agonist *N*-methyl-D-aspartate (NMDA) in voltage-clamped hippocampal neurons was inhibited by ethanol (EtOH). Inhibition increased in a concentration-dependent manner over the range 5 to 50 m*M*, a range that also produces intoxication. The amplitude of the NMDA-activated current was reduced 61 percent by 50 m*M* EtOH; in contrast, this concentration of EtOH reduced the amplitude of current activated by the glutamate receptor agonists kainate and quisqualate by only 18 and 15 percent, respectively. The potency for inhibition of the NMDA-activated current by several alcohols is linearly related to their intoxicating potency, suggesting that alcohol-induced inhibition of responses to NMDA receptor activation may contribute to the neural and cognitive impairments associated with intoxication.

ALTHOUGH THE COGNITIVE AND BEHAVIORAL manifestations of EtOH intoxication are well known, the cellular and molecular mechanisms through which EtOH produces its actions are poorly understood. Electrophysiological experiments have shown that EtOH can alter the firing rate or excitability of several types of central nervous system (CNS) neurons (*1*); however, voltage-clamp experiments on mammalian neurons have not revealed a specific membrane ion current that is affected by intoxicating concentrations of EtOH.

Ethanol could produce its effects by altering neural excitation. Glutamate appears to be the major excitatory neurotransmitter in the mammalian CNS (*2*). Glutamate produces its excitatory action through the activation of at least three receptor subtypes distinguished on the basis of their response to the agonists kainate, quisqualate, and *N*-methyl-D-aspartate (*2*). The NMDA receptor is thought to be involved in excitatory neural phenomena (*3*), neural plasticity (*4*), cognitive function (*5*), and certain forms of behavior (*6*). Kainate and quisqualate receptors, on the other hand, appear to mediate fast excitatory synaptic transmission (*7*).

We have examined the effect of EtOH on ion currents activated by glutamate receptor agonists in voltage-clamped hippocampal neurons (*8*). The effect of EtOH on the ion currents induced by the application of NMDA, kainate, and quisqualate in voltage-clamped hippocampal neurons (*9*) is illustrated in Fig. 1. The amplitude of the NMDA-activated current was greatly reduced in the presence of 50 m*M* EtOH (Fig. 1A). Over the concentration range 5 to 100 m*M*, EtOH inhibited the response to NMDA. The average inhibition by 50 m*M* EtOH was 61 ± 3% (n = 14), and the concentration that produced 50% inhibition (IC_{50}) was ~30 m*M* (*10*). (Reported values are mean ± SEM.) The average inhibition produced by 100 m*M* EtOH was 69 ± 6% (n = 5), which was not significantly greater than the inhibition by 50 m*M* EtOH ($P > 0.10$, unpaired t test). Inhibition of the NMDA-activated current was not observed with 2.5 m*M* EtOH; however, in some neurons this concentration increased current amplitude. The percent reduction of kainate- and quisqualate-activated current amplitude by 50 m*M* EtOH was considerably less than the reduction of NMDA-induced current amplitude at the same EtOH concentration (compare Fig. 1, B and C, to Fig. 1A). The average inhibition by 50 m*M* EtOH of the kainate-activated current was 18 ± 2% (n = 5) and of the quisqualate-activated current was 18 ± 2% (n = 5) and of the quisqualate-activated current was 15 ± 2% (n = 5). EtOH was less potent in inhibiting kainate- and quisqualate-activated currents than in inhibiting the NMDA-induced current over a range of EtOH concentrations (Fig. 1, B and C).

Because different alcohols have different potencies for producing intoxication (*11–13*), we examined the effect of several alcohols on the NMDA-activated current. Methanol (200 m*M*) (Fig. 2A), 1-butanol (10 m*M*) (Fig. 2B), and isopentanol (0.5 m*M*) (Fig. 2C) produced an inhibition of the NMDA-activated current comparable to the inhibition by 50 m*M* EtOH (Fig. 1A). These data suggest that the alcohols differ in their potency for inhibiting the response to NMDA. The potency of the alcohols for inhibiting the NMDA-activated current was further evaluated by examining the effect of different concentrations of each of the three alcohols on the response to NMDA. Inhibition of the NMDA-activated current increased with increasing concentrations of each alcohol, but the threshold for inhibition and the IC_{50} differed among the alcohols (Fig. 2). Methanol (Fig. 2A) was less potent than EtOH in inhibiting the NMDA-activated current (compare to Fig. 1A). The threshold for methanol inhibition was ~25 m*M* and the IC_{50} was ~117 m*M*. Both 1-butanol and isopentanol were more potent than EtOH in their inhibition of the NMDA-activated current. The threshold for 1-butanol (Fig. 2B) inhibition was ~0.01 m*M* and the IC_{50} was ~1.14 m*M*. Isopentanol (Fig. 2C) was the most potent of the four alcohols tested, inhibiting the response to NMDA with a threshold of ~0.001 m*M* and an IC_{50} of ~ 0.32 m*M*.

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