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# Abnormal Fear Response and Aggressive Behavior in Mutant Mice Deficient for α-Calcium-Calmodulin Kinase II

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Mice deficient for the gene encoding α-calcium-calmodulin–dependent kinase II (α-CaMKII knockout mice) provide a promising tool to link behavioral and cellular abnormalities with a specific molecular lesion. The heterozygous mouse exhibited a well-circumscribed syndrome of behavioral abnormalities, consisting primarily of a decreased fear response and an increase in defensive aggression, in the absence of any measured cognitive deficits. Unlike the heterozygote, the homozygote displayed abnormal behavior in all paradigms tested. At the cellular level, both extracellular and whole-cell patch clamp recordings indicated that serotonin release in putative serotonergic neurons of the dorsal raphe was reduced. Thus, α-CaMKII knockout mice, in particular the heterozygote, may provide a model for studying the molecular and cellular basis underlying emotional disorders involving fear and aggression.

The recently developed mouse gene knockout technology has allowed us to use a multidisciplinary approach to analyze neurobiological abnormalities at the behavioral and cellular levels. Here we tested mutant mice, in which the α-CaMKII gene was disrupted (*1*), for abnormal behaviors associated with aggression and fear.

We first evaluated in CaMKII mutant and wild-type mice the fear response of freezing (*2*), as characterized by an immobile, crouching posture after footshock, which is an indicator of activation of the fear system (*3*). During training, wild-type animals exhibited a high frequency of freezing after footshock, whereas the heterozygous (*t* test, $P < 0.005$) and homozygous ($P < 0.007$) mutants froze significantly less often (Fig. 1A). On the following day, the animals were returned to the shock chamber (contextual conditioning). In the absence of footshock, the wild-type mice displayed freezing behavior, indicating retention of the fear response (Fig. 1B). In contrast, the heterozygotes showed a low percentage of freezing that rapidly decayed ($P < 0.0001$). The homozygotes showed no freezing at all, suggesting a deficiency in the fear response.

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To establish whether the lower rate of freezing in mutants is caused by a fear-specific abnormality or simply by modifications in freezing-related sensory or motor processing capacities, we examined (i) fear conditioning elicited by a different sensory cue, (ii) pain sensitivity to footshocks, (iii) an innate fear behavior, and (iv) a fear-associated autonomic response. The mice were subjected to another task in which simple tones were paired with footshocks (*2*). The heterozygotes ($P < 0.0001$) and homozygotes ($P < 0.0001$) displayed much less freezing than the wild-type mice during training (Fig. 1C), similar to the contextual conditioning (Fig. 1A). Both groups of mutant mice exhibited partial retention of the fear response to tones (compared with the wild-type mice, $P < 0.0001$; between two mutant groups, $P < 0.0002$) (Fig. 1D). Therefore, both groups of mutant mice display reduced freezing to contextual as well as tonal cues.

Because reduced pain would result in less freezing (*4*), we measured current thresholds for three reactions to nociceptive shock, namely, flinch, jump, and vocalization (*5*). For all responses, the heterozygotes exhibited similar pain thresholds to the wild-type (*t* test, $P < 0.18$), whereas the homozygotes had significantly lower pain thresholds and were thus more sensitive to all the nociceptive stimuli (flinch, $P < 0.005$; jump, $P < 0.001$; vocalization, $P < 0.002$) (Fig. 2A). Therefore, it is unlikely that the attenuated freezing response is caused by modified pain sensitivity.

Another measure of fear was based on the behavior of thigmotaxis in an open field (6). Rodents are known to avoid exposure in the center of an open field (innate fear), and mice tend to move to the perimeter (thigmotaxis), possibly to reduce the risk of being attacked by predators (7). Heterozygous ($P < 0.008$) and homozygous ($P < 0.015$) mice spent more time in the center of the field, and they took longer to enter the outermost track than the wild-type mice (Fig. 2B). All mice began their exploration immediately after being placed in the open field, thereby eliminating freezing behavior as a factor.

Reduced freezing or thigmotaxis may also result from a disruption in motor output systems rather than in the fear state. Therefore, we chose to measure defecation, an autonomic response that may be indicative of fear in rodents and does not require motor performance (8). During the contextual fear conditioning test (see Fig. 1B), defecation was significantly less in both the heterozygotes ($P < 0.04$) and homozygotes ($P < 0.0001$) compared with the wild-type mice (Fig. 2C). A similar defecation response was seen when naïve mice were confronted with an open field ($P < 0.001$ for both mutants) (Fig. 2C). These results further suggest that both mutants are less fearful than their wild-type littermates and that this lack of fear may reflect an abnormality in the central fear state rather than in the peripheral information processing.

We noticed an unusually high frequency of fighting, which often resulted in fatal back injuries, among heterozogotes in the animal colony. Hence, we investigated two types of aggressive behavior in male mice. Mice are known to be territorial, and a "resident" mouse will attack an intruder by biting its rear (9). As a measure of offensive aggression, we quantified residents' attacks toward an intruder (10). In this paradigm, a resident mouse was housed individually for 4 weeks, and a wild-type mouse, housed in a group, was then introduced as an intruder (11). We also used a variation of the resident-intruder confrontation, in which the wild-type residents and the intruders had been housed individually (12). The focus in this procedure was on the defensive aggression exhibited by the intruder in response to the resident's behavior. Although offensive aggression by the resident may be intepreted as a behavioral manifestation of aggressiveness, the defensive aggression by the intruder, after being attacked, may be related to both aggression and fear (9, 13).

In the offensive aggression test, heterozygous resident mice were equally as aggressive as the wild-type residents as measured by the number of attack bites toward an intruder ($P > 0.3$), whereas homozygous residents did not display any aggressive behavior (Fig. 2D, left). When becoming an intruder, however, the heterozygotes exhibited more defensive attacks toward the resident mouse than the wild-type intruder ($P < 0.006$) (Fig. 2D, right). In fact, the heterozygous intruder often counter-attacked vigorously by biting the resident, whereas the wild-type intruder assumed an upright defensive posture or fled. The homozygous intruder still provoked few fights (compared with the wild type, $P < 0.003$, and the heterozygotes, $P < 0.002$) and fled when being attacked by the resident. Furthermore, the homozygous intruder often reapproached the resident (in a nonaggressive manner) even after being attacked, a sign of fearlessness or other impairments, which rarely occured in wild-type intruders. In summary, heterozygotes display attenuated

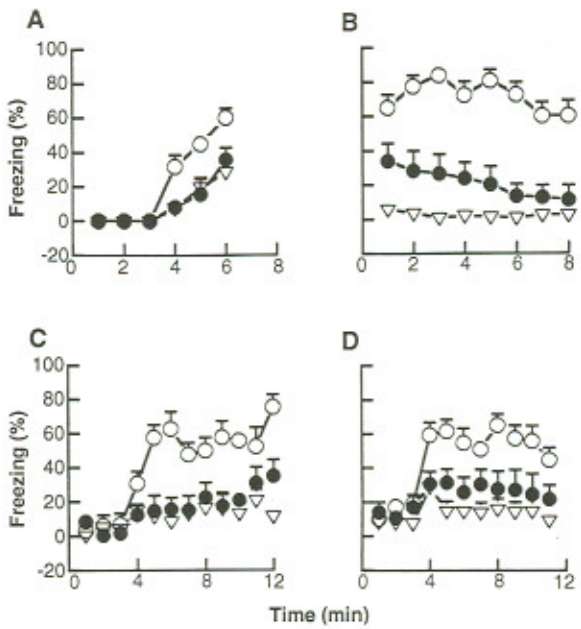


**Fig. 1.** The α-CaMKII mutant mice show reduced freezing responses (percent freezing, mean ± SEM) in fear conditioning (2). Percent freezing was determined by time-sampling in a 2-s interval. (**A**) For contextual training, three footshocks (0.5 mA for 1 s) were given at minutes 3, 4, and 5 ($n$ = 10, wild type; $n$ = 9, mutants). (**B**) The 8-min contextual test 1 day later. No footshocks were applied. (**C**) Three 20-s tones were given at minutes 3, 6, and 9, each terminated with a 1-s shock ($n$ = 8, each group). (**D**) An 8-min tone was given at minute 3 without footshocks. Symbols: open circle, wild type; solid circle, heterozygotes; open triangle, homozygotes.

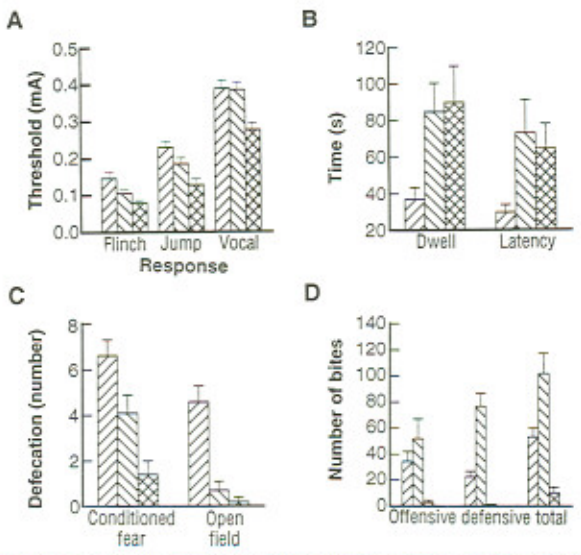


**Fig. 2.** (**A**) Homozygotes have lower pain thresholds (mean ± SEM, $n$ = 6), whereas the heterozygotes are normal (5). (**B**) Both heterozygotes and homozygotes are less fearful of the open field ($n$ = 6) (6). (**C**) Both heterozygotes and homozygotes defecate less in fearful environments. The number of fecal bolluses (mean ± SEM) was counted for 8 min during the contexual fear test or for 5 min during the open field test. (**D**) Heterozygotes show normal offensive but heightened defensive aggression, whereas the homozygotes are permissive for both. The resident attacks (11) were used to demonstrate offensive aggression (left bars, $n$ = 8); defensive aggression (12) was demonstrated by the number of counter-attack bites from three 5-min sessions in a different setting (middle bars). The right bars show combined attacks originating from both the resident and intruder during the defensive aggression test. Symbols: right hatch (/), wild type; left hatch (\), heterozygote; cross hatch, homozygote.

**Table 1.** Summary of behavioral phenotypes in α-CaMKII mutant mice (24).

| Behavioral phenotype | Heterozygote | Homozygote |
|---|---|---|
| Fear-related responses | Decrease | Decrease |
| Offensive aggression | Normal | Decrease |
| Defensive aggression | Increase | Decrease |
| Pain sensitivity | Normal | Increase |
| Startle response | Normal | Increase |
| Vigilance | Normal | Increase |
| Mating | Decrease | Decrease |
| Maze learning | Normal | Decrease |

fear but normal offensive aggression. When being attacked, heterozygotes show heightened defensive aggression, which is consistent with their lack of fear. In contrast, homozygotes have little fear and aggression in addition to many other behavioral abnormalities (Table 1).

Serotonin (5-HT) depletion and neuronal activity in the dorsal raphe correlate with aggressive behaviors (*14*). We thus studied 5-HT transmission: release, receptor response, and reuptake. Single-unit recordings (*15*) were used to monitor the effect of 5-HT and the 5-HT reuptake inhibitor fluoxetine on spontaneous firing of putative serotonergic neurons. In slices from wild-type mice, bath application of 5-HT (100 μM) activated autoreceptors, resulting in an abolition of firing, which recovered as 5-HT was washed out (Fig. 3A). Application of fluoxetine (20 μM) also caused an abolition of spontaneous firing. In slices from mutant mice, application of 5-HT was equally effective in the blockade of spontaneous firing, suggesting that the $5\text{-HT}_{1A}$ autoreceptor response was functional in mutants (*16*). The reuptake system also appeared functional because recovery of the firing rate after the washout of 5-HT was similar to the rate in the wild type. In contrast, the inhibition of neuronal firing by fluoxetine was delayed in onset and attenuated in the heterozygote, and the response was absent in the homozygote (Fig. 3A). However, application of 5-HT subsequent to fluoxetine had a prolonged recovery time after washout (*17*), indicating that the efficacy of the reuptake inhibitor was not reduced and therefore did not cause the observed difference. When the latency to 50% reduction of firing rate by fluoxetine was examined, it was significantly longer in heterozygotes than in the wild-type mice ($P < 0.0001$) (Fig. 3B).

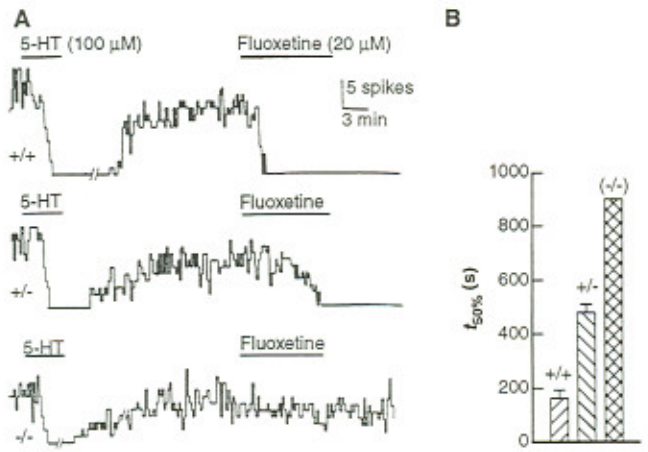


**Fig. 3.** The release of 5-HT is reduced in both heterozygotes and homozygotes. (**A**) Single-unit recordings (*15*) from serotonergic dorsal raphe neurons with bath application of 5-HT and fluoxetine. (**B**) The latency to reach 50% reduction of firing rate ($t_{50\%}$) (mean ± SEM) in response to fluoxetine is prolonged in heterozygotes. The latency for homozygotes could not be quantified because no apparent changes were seen within 15 to 20 min of observation. Symbols: right hatch (/), wild type (+/+; $n = 8$); left hatch (\), heterozygote (+/−; $n = 8$); cross hatch, homozygote (−/−; $n = 5$)

To determine whether the delay or absence of a fluoxetine effect in mutant mice was due to reduced 5-HT release or other network changes, we investigated intracellular responses of serotonergic neurons (*15*). In the neurons of wild-type mice, application of 5-HT (100 μM) caused a membrane hyperpolarization and concomitant decrease in input resistance due to activation of $5\text{-HT}_{1A}$–mediated $K^+$ channels (Fig. 4A) (*16*). After 5-HT was washed out, both the membrane potential and the input resistance recovered. Fluoxetine application (20 μM) induced changes in membrane properties of equal magnitude to those mediated by 5-HT. In both heterozygote and homozygote preparations, the 5-HT effect resembled that of the wild type. However, in contrast to the wild type, the onset of the fluoxetine effect was delayed and the recovery from it on washout was faster in both mutants (Fig. 4B). The latency to 50% change of membrane potential or current, elicited by fluoxetine, in heterozygotes ($P < 0.0005$) and homozygotes ($P < 0.0015$) was significantly longer than that of the wild type (Fig. 4C). This modification suggests that 5-HT release was not absent but was markedly reduced in both mutants. However, no differences in intrinsic electrophysiological properties were detected among mutant and wild-type animals including (i) resting potentials, (ii) input resistance, (iii) number of spikes in response to depolarizing current steps, and (iv) spontaneous firing rates as monitored by single-unit recordings. We also did not see any apparent difference in neuronal morphology and voltage dependence of 5-HT–mediated ionic currents (Fig. 4D) (*16*).

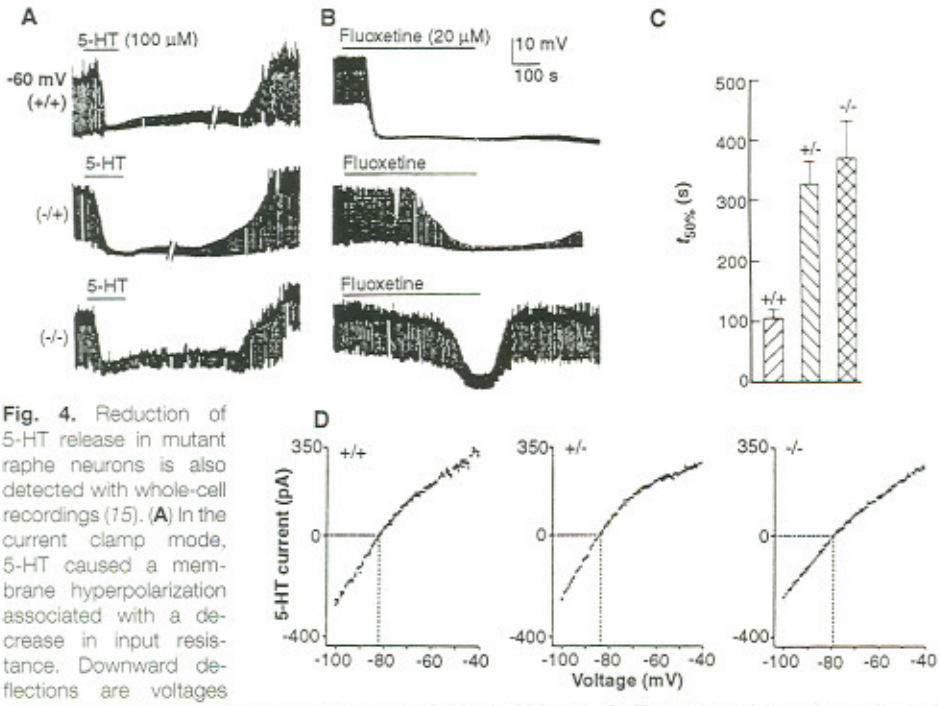


**Fig. 4.** Reduction of 5-HT release in mutant raphe neurons is also detected with whole-cell recordings (*15*). (**A**) In the current clamp mode, 5-HT caused a membrane hyperpolarization associated with a decrease in input resistance. Downward deflections are voltages generated with hyperpolarizing current steps (0.02 nA, 350 ms). (**B**) Fluoxetine mimicked the effect of 5-HT on input resistance, but the latency of membrane hyperpolarization ($t_{50\%}$) increased and its duration decreased in mutant slices. (**C**) The latency to reach 50% reduction of membrane potential in response to fluoxetine is prolonged in heterozygotes and homozygotes (+/+, $n = 6$; +/−, $n = 7$; −/−, $n = 5$). (**D**) The voltage dependence, as shown by representative current voltage curves, of 5-HT-mediated $K^+$ currents is similar in the neurons of wild-type and mutant mice. The pure 5-HT current was obtained in the voltage clamp mode by subtracting ramp currents before and during 5-HT application.

CaMKII facilitates presynaptic transmitter release (*1*, *18*), and CaMK-mediated phosphorylation is required for activation of tryptophan hydroxylase, the rate-limiting enzyme for 5-HT synthesis (*19*). It remains to be determined if either of these factors contributes to the altered 5-HT release seen

in this study. Consistent with our findings, manipulation of 5-HT has been shown to affect fear (or anxiety) (*20*) and aggression (*21*) in animals and humans (*22, 23*).

The distinct behavioral and neuronal deficits in the heterozygote suggest that only those brain regions that have intrinsically low levels of the α-CaMKII gene expression would be most affected by the lower gene dosage. These regions may include the serotonergic nuclei. In contrast, the behavioral abnormalities of the homozygote (in which both copies of the gene are absent) become widespread (Table 1). Such gene dosage effects may exist in human psychiatric diseases, in particular, those involving personality traits associated with increased aggression and decreased fear (consistent with increased risk-taking behaviors).

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15. For the electrophysiology studies, thick brainstem slices (500 μm) were prepared from anesthetized adult mice. Slices were kept in the recording chamber constantly perfused with oxygenated artificial cerebrospinal fluid (ACSF) (1 ml/min at 32°C). Extracellular single units were recorded with a microelectrode, and spike rates (10-s bin) were analyzed with a computer window discriminator. Phenylephrine (20 μM, Sigma) was included in ACSF to increase spontaneous firings (*16*). Whole-cell recordings were obtained in either voltage or current clamp mode with conventional thick-slice techniques. Biocytin (0.25%) was included in electrodes for staining. The 5-HT was obtained from Sigma, fluoxetine from Eli Lilly, and 8-OHDPAC and NAN-190 from Research Biochemicals International.
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