The Amygdala Is a Chemosensor that Detects Carbon Dioxide and Acidosis to Elicit Fear Behavior

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SUMMARY

The amygdala processes and directs inputs and outputs that are key to fear behavior. However, whether it directly senses fear-evoking stimuli is unknown. Because the amygdala expresses acid-sensing ion channel-1a (ASIC1a), and ASIC1a is required for normal fear responses, we hypothesized that the amygdala might detect a reduced pH. We found that inhaled CO_2 reduced brain pH and evoked fear behavior in mice. Eliminating or inhibiting ASIC1a markedly impaired this activity, and localized ASIC1a expression in the amygdala rescued the CO_2-induced fear deficit of *ASIC1a* null animals. Buffering pH attenuated fear behavior, whereas directly reducing pH with amygdala microinjections reproduced the effect of CO_2. These data identify the amygdala as an important chemosensor that detects hypercarbia and acidosis and initiates behavioral responses. They also give a molecular explanation for how rising CO_2 concentrations elicit intense fear and provide a foundation for dissecting the bases of anxiety and panic disorders.

INTRODUCTION

Fear is an evolutionarily conserved process that ensures survival by initiating avoidance or escape from predators and harmful situations (Ohman and Mineka, 2001). In humans, abnormal fear and anxiety are hallmarks of many psychiatric diseases; panic disorder and post-traumatic stress disorder are two extreme examples. Previous studies have revealed much about the complex behavioral neurobiology that underlies fear in mammals, including both conditioned (or acquired) and uncondi-tioned (or innate) fear (Gorman et al., 2000; Kim and Jung, 2006; Maren, 2008; Phelps and LeDoux, 2005; Rosen, 2004). Acquired fear involves the learned association of a conditioned stimulus, such as a specific environment, with an unconditioned stimulus such as a footshock. An example is the contextual fear condi-tioning protocol often utilized in mice (Kim and Jung, 2006; Maren, 2008). In contrast, innate fear does not require prior conditioning; examples are a fear of snakes in humans and the innate fear of fox feces odor (trimethylthiazoline) in mice (Ohman and Mineka, 2001; Rosen, 2004).

For both innate and acquired fear behavior, the amygdala, including lateral, basal, and central nuclei, plays a critical role (Adolphs, 2002; Anglada-Figueroa and Quirk, 2005; Bechara et al., 1995; Goosens and Maren, 2001; Kim and Jung, 2006; Maren, 2008; Phelps and LeDoux, 2005; Rabinak and Maren, 2008; Rosen, 2004). The lateral amygdala accepts input arising from sensory systems including sensory cortices and thalamus, from executive cortices, and from the hippocampus. The basal amygdala communicates with both lateral and central nuclei of the amygdala and also contributes to fear behavior. Much of the output from the amygdala flows from the central nucleus of the amygdala, which is critical for signaling areas producing physiological and behavioral manifestations of fear. The amyg-dala is also important in learned fear, forming associations between conditioned and unconditioned stimuli. Thus, the amygdala is central for processing and directing the inputs and outputs that are key to fear behavior. However, the amygdala itself is not known to be a sensor of fear-evoking stimuli.

In previous studies, we discovered that the acid-sensing ion channel-1a (ASIC1a) is particularly abundant in the amygdala and other fear circuit structures (Coryell et al., 2007; Wemmie et al., 2003), and it is required for normal responses in tests of both conditioned and unconditioned fear behavior (Coryell et al., 2007, 2008; Wemmie et al., 2003, 2004). ASIC1a is located in dendrites and on dendritic spines, as well as on neuronal cell bodies (Wemmie et al., 2002; Zha et al., 2006, 2009). ASICs are

subunits that associate as trimers (Jasti et al., 2007) to form channels permeable to Na^+ and Ca^{2+} (Waldmann et al., 1997; Xiong et al., 2004; Yermolaieva et al., 2004). In the central nervous system (CNS), the predominantly expressed ASIC subunits are ASIC1a, -2a, and –2b, which assemble into homo- and heterotrimeric complexes (Benson et al., 2002; Jasti et al., 2007; Lingueglia et al., 1997). ASICs are activated in vitro when extracellular pH falls, and although acidic pH modifies the activity of many receptors and proteins, few besides ASICs are activated by extracellular acidosis, and few are as exquisitely pH sensitive (Diochot et al., 2007; Krishtal, 2003; Wemmie et al., 2006). Disrupting the *ASIC1a* gene (*ACCN2*) in mice eliminates currents evoked by pH as low as 5.0 in central neurons (Askwith et al., 2004; Wemmie et al., 2002). Thus, ASIC1a is critical for acid-evoked currents in CNS neurons.

The contribution of both the amygdala and ASIC1a to fear behavior raised questions of how ASIC1a in the amygdala is activated, especially in vivo. We hypothesized that a reduced pH might induce fear behavior by activating ASIC1a, thereby allowing the amygdala to function as a chemosensor deep within the fear circuit. To explore this issue, we used CO_2 inhalation for three main reasons. First, physiologic effects of CO_2 in mammals typically result from a reduced pH (Zandbergen et al., 1989), which might activate ASIC1a channels. Brain pH rapidly falls when the enzyme carbonic anhydrase catalyzes the conversion of CO_2 and water to carbonic acid (Nattie, 1998). Second, it is well known that rising CO_2 concentrations can elicit intense fear (Papp et al., 1993). Third, the use of CO_2 as a probe has important clinical implications for psychiatric disorders.

Nearly a century ago, it was discovered that CO_2 inhalation can trigger panic attacks, and patients with panic disorder are particularly susceptible (Drury, 1918). Many subsequent clinical studies fostered hope that understanding CO_2 sensitivity would provide unique insight into the neurobiology underlying anxiety disorders and lead to better treatments (Dratcu, 2000; Gorman et al., 2000; Rassovsky and Kushner, 2003; Smoller et al., 1996). Several theories have sought to explain how CO_2 inhalation produces fear and panic (Drury, 1918; Gorman et al., 2000; Klein, 1993; Sanderson et al., 1989). Early on, cardiovascular sensitivity was suspected (Drury, 1918). Others suggested that CO_2 sensitivity in panic might be due to a psychological vulnerability to losing control (Sanderson et al., 1989). A role for brainstem ventilatory chemosensors and hyperventilation has also been speculated (Gorman et al., 2000; Sullivan et al., 2003), and the "false-suffocation-alarm theory" proposes that rising CO_2 heralds impending suffocation and that this alarm is falsely triggered in patients with panic disorder (Klein, 1993). However, the molecular identity and neuroanatomical location of the putative CO_2 alarm have remained uncertain (Dratcu, 2000; Gorman et al., 2000; Griez and Schruers, 1998; Klein, 1993).

With this background, we hypothesized that inhaling CO_2 would reduce brain pH and that the acidosis would trigger ASIC channels in the amygdala to elicit fear. Whereas CO_2 is known to stimulate chemosensors in the periphery and brainstem, if CO_2 and hence acidosis were to directly activate the amygdala, it would suggest that the amygdala is an important chemosensory structure (Brannan et al., 2001; Gorman et al., 2000).

RESULTS

Loss or Inhibition of ASIC1a Impairs CO_2-Induced Fear Behavior

We developed four paradigms to test CO_2-triggered fear in mice: (1) CO_2-evoked freezing, (2) CO_2 effects in the open-field test, (3) CO_2 aversion, and (4) CO_2-potentiated context fear conditioning.

To assess the response to CO_2, we assayed freezing, which is used as a correlate of fear and panic in mice (Mongeluzi et al., 2003). In humans with panic disorder, breathing 5% CO_2 evokes panic attacks (Rassovsky and Kushner, 2003) but it rarely evokes panic in control subjects. Consistent with this, 5% CO_2 produced little or no freezing in mice (Figure 1A). In humans who do not have panic disorder, breathing higher CO_2 concentrations is more likely to evoke panic (Rassovsky and Kushner, 2003). Likewise, breathing 10% CO_2 triggered significant freezing in control mice (Figure 1A). However, disrupting the *ASIC1a* gene substantially blunted the response. Moreover, acutely inhibiting ASIC1a with either psalmotoxin (PcTx1) (Diochot et al., 2007) or A-317567 (Dube et al., 2005) also reduced CO_2-evoked freezing in wild-type mice (Figure 1B). Neither inhibitor reduced freezing in *ASIC1a*$^{-/-}$ animals, suggesting that their effects were ASIC1a specific. Thus, CO_2 produced fear-like freezing behavior that depended on ASIC1a.

The tendency of mice to avoid the center of an open field is used as a measure of their fear and anxiety of open spaces. In wild-type mice, breathing CO_2 concentrations greater than 5% reduced the amount of center activity in an open field (Figure 1C). However this effect was much attenuated in *ASIC1a*$^{-/-}$ mice.

We examined the aversive effect of CO_2 on behavior by allowing mice to move between two chambers, one with 15% CO_2 and one with <2% CO_2. During a 10 min assay, *ASIC1a*$^{+/+}$ mice only briefly sampled the CO_2 chamber, spending >90% of the time in the air chamber (Figure 1D). In contrast, *ASIC1a*$^{-/-}$ mice spent similar amounts of time in both chambers. Failure to avoid CO_2 did not generalize to other aversive stimuli as both genotypes avoided moving air infused into one of the chambers; *ASIC1a*$^{+/+}$ mice spent 69% ± 7% and *ASIC1a*$^{-/-}$ mice 82% ± 4% of time in the non-air-infused chamber (n = 7 per group; p > 0.15; Wilcoxon rank sum).

Context fear conditioning is often used to measure fear and anxiety, and our earlier work showed that disrupting or inhibiting ASIC1a impaired fear conditioning (Coryell et al., 2007, 2008; Wemmie et al., 2004). During training with a series of footshocks, wild-type mice froze (Figure 1E), as previously described (Coryell et al., 2008). However, when training was conducted in the presence of 10% CO_2, mice began to freeze before receiving footshocks and froze even more once the footshocks were delivered. The following day, we returned the mice to the original context (minus footshocks and minus CO_2) and found that wild-type mice trained in CO_2 showed more freezing than those not exposed to CO_2 (Figure 1F). In contrast, CO_2 had no significant effect on context fear conditioning in *ASIC1a*$^{-/-}$ mice. To learn whether CO_2 itself might have served as an unconditioned stimulus, we used wild-type mice and delivered CO_2 or air but no footshocks on the training day. On the testing day, neither group froze in response to the context (Figure 1F, inset). Thus, CO_2 by
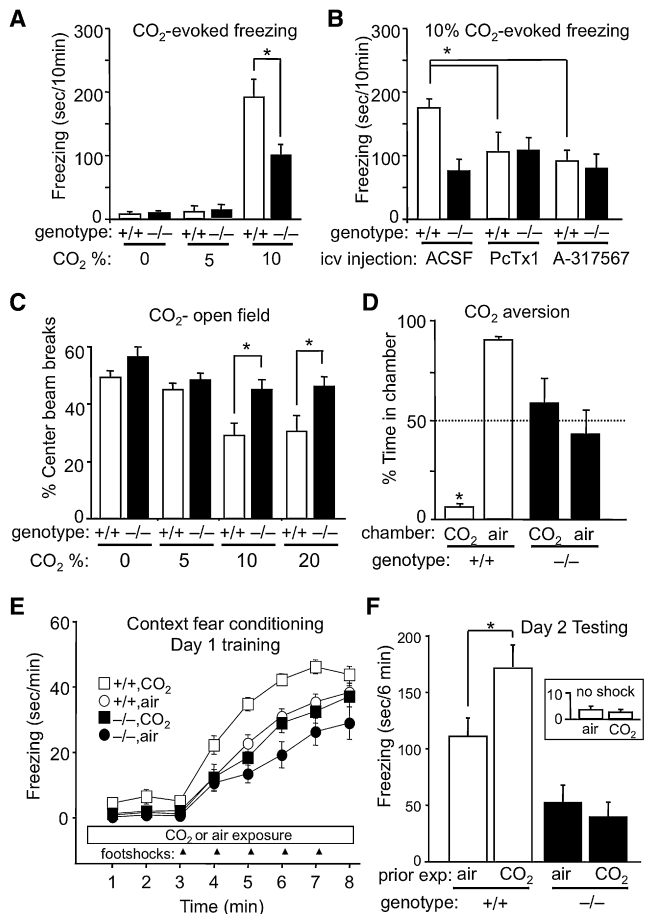
Figure 1. ASIC1a Disruption Attenuates CO_2 Effects in Multiple Behavioral Models of Fear

(A) CO_2 evoked prominent fear-like freezing behavior compared to air. $ASIC1a^{-/-}$ mice were significantly impaired relative to wild-type controls. ANOVA revealed a significant CO_2 by genotype interaction ($F_{(2, 35)} = 4.62$, $p < 0.05$). Planned contrast testing revealed a significant attenuation of freezing in the $ASIC1a^{-/-}$ mice in response to 10% CO_2 (*$p < 0.001$) but not in air or 5% CO_2 ($p > 0.05$). From left to right, groups contained 5, 6, 7, 6, 7, and 10 mice.

(B) ASIC1a antagonists PcTx1 and A-317567 reduced 10% CO_2-evoked freezing in wild-type mice but had no effect on CO_2-evoked freezing in the $ASIC1a^{-/-}$ mice. ANOVA revealed significant effect of group ($F_{(5, 43)} = 3.87$, $p < 0.01$). Significant pairwise comparisons are indicated (*$p < 0.05$; n = 7–10 mice per group).

(C) CO_2 increases anxiety-like behavior in the open field test. ANOVA revealed significant main effects of CO_2 ($F_{(3, 64)} = 8.57$, $p < 0.001$) and genotype ($F_{(1, 64)} = 15.95$, $p < 0.001$), but not a CO_2 by genotype interaction ($F_{(3, 64)} = 1.38$, $p > 0.05$). Planned contrast comparisons revealed significant differences between $ASIC1a^{+/+}$ and $ASIC1a^{-/-}$ mice at the higher CO_2 concentrations (*$p < 0.01$; n = 7–10 mice per group). This difference was not due to differences in general locomotor activity; we previously showed that ASIC1a disruption does not alter total locomotor activity at baseline (Coryell et al., 2007). Similarly, in these studies, during CO_2 exposure we found no effect of ASIC1a disruption on total beam breaks ($F < 1$).

(D) $ASIC1a^{+/+}$ mice avoided the CO_2 chamber, whereas $ASIC1a^{-/-}$ did not. Consequently, the $ASIC1a^{+/+}$ spent significantly less time on the CO_2 side than the $ASIC1a^{-/-}$ mice (*$p = 0.004$, Wilcoxon rank sum test; n = 7 and 9 per group).

(E) CO_2 enhanced freezing in $ASIC1a^{+/+}$ mice during context fear conditioning. Analysis of total freezing during training revealed a significant difference

itself did not act as an unconditioned stimulus, but it enhanced fear memory when coupled with footshocks.

Thus, results in four different models suggest that CO_2 evokes fear-like responses in wild-type mice and that those behaviors depend on ASIC1a.

The Ventilatory Response to CO_2 Is Intact in $ASIC1a^{-/-}$ Mice

It seemed unlikely that genotype-dependent disparities in the arterial pressure of CO_2 (P_aCO_2) would have accounted for differences in fear behavior in $ASIC1a^{+/+}$ and $ASIC1a^{-/-}$ mice. Consistent with that assumption, breathing 10% CO_2 induced a similar elevation of P_aCO_2 in both genotypes (Figure 2A).

Because hypercarbic acidosis is well known to increase ventilation, we also measured the ventilatory response to CO_2 inhalation. Wild-type and $ASIC1a^{-/-}$ mice showed a similar response to hypercarbia (Figure 2B). These data suggest that ASIC1a does not affect the chemosensory system controlling ventilation.

Breathing CO_2 Reduces Amygdala pH

We then turned our attention to the amygdala because of the possible association between the amygdala and panic (Gorman et al., 2000), and because of the robust ASIC1a expression in the basolateral amygdala (Coryell et al., 2007). We measured pH in the basolateral amygdala of anesthetized mice and found that breathing CO_2 reduced pH of both genotypes similarly (Figures 3A and 3B). We recorded similar reductions in the lateral ventricle (Figure 3C). Baseline pH in the mice breathing air was less than the expected range (pH $\sim$7.29–7.39) (Mayhan et al., 1988; Mitchell et al., 1965), likely due to respiratory suppression during anesthesia. Supporting this, $PaCO_2$ in the anesthetized mice was elevated (65 ± 3 mm Hg) (Figure 2A) compared to levels (39–45 mm Hg) reported for awake or mechanically ventilated animals (Mayhan et al., 1988; Mitchell et al., 1965).

To determine if these changes were sufficient to activate ASICs, we measured current in cultured amygdala neurons. In wild-type neurons, lowering pH to 7.2 induced current in 21% of cells, and further reductions generated current in a greater percentage of neurons and evoked larger currents (Figures 4A–4C). The acidosis elicited no current in amygdala neurons from $ASIC1a^{-/-}$ mice, consistent with data from neurons of other brain areas (Askwith et al., 2004; Wemmie et al., 2002; Xiong et al., 2004). We also detected acid-evoked currents in amygdala neurons from $ASIC^{+/+}$, but not $ASIC^{-/-}$ brain slices (Figure 4D). These data are consistent with our studies of cultured neurons and earlier studies in brain slices from other brain regions (Chen et al., 2009; Pidoplichko and Dani, 2006; Zha et al., 2006).

between the groups ($F_{(3, 62)} = 13.94$, $p < 0.001$, ANOVA). Planned contrast comparisons revealed a significant effect of CO_2 in $ASIC1a^{+/+}$ mice ($p < 0.001$) but not in $ASIC1a^{-/-}$ mice ($p > 0.05$).

(F) Previous CO_2 exposure potentiated context-evoked fear memory in $ASIC1a^{+/+}$ mice but not in $ASIC1a^{-/-}$ mice. Significant overall differences were detected between the groups ($F_{(3, 62)} = 7.99$, $p < 0.001$, ANOVA). Planned contrast testing revealed a significant effect of CO_2 in $ASIC1a^{+/+}$ mice (*$p < 0.05$) but not in $ASIC1a^{-/-}$ mice ($p > 0.05$).
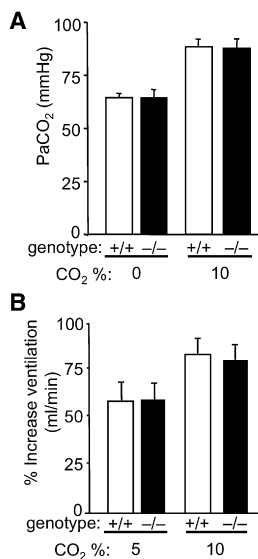
Error bars, SEM.

Figure 2. CO$_2$ Inhalation Increases Arterial CO$_2$ Partial Pressure and Stimulates Ventilation Normally in the _ASIC1a$^{-/-}$_ Mice
(A) PaCO$_2$ did not differ between _ASIC1a$^{+/+}$_ and _ASIC1a$^{-/-}$_ mice. ANOVA revealed significant main effect of CO$_2$ ($F_{(1,8)}$ = 50.2, $p < 0.001$; n = 3 per group) but not genotype ($F < 1$) and no interaction ($F < 1$).
(B) CO$_2$-evoked increases in minute ventilation relative to baseline did not differ between _ASIC1a$^{+/+}$_ and _ASIC1a$^{-/-}$_ mice ($F_{(1, 46)}$ = 0.065, $p = 0.80$, ANOVA; from left to right, groups comprised of 17, 17, 12, and 4 mice).

Figure 3. CO$_2$ Inhalation Reduces Amygdala pH
(A) Examples of pH recordings in amygdala showing effects of CO$_2$ inhalation.
(B) Mean pH response in basolateral amygdala to CO$_2$ inhalation was normal in _ASIC1a$^{-/-}$_ mice. ANOVA revealed a significant effect of CO$_2$ ($F_{(2, 15)}$ = 7.8, $p < 0.01$) but no effect of genotype ($F_{(1, 15)}$ = 0.2, $p = 0.66$) and no interaction ($F < 1$, n = 3–4 mice per group).
(C) Lateral ventricle pH response to CO$_2$ inhalation. ANOVA revealed a significant effect of CO$_2$ ($F_{(2, 30)}$ = 12.0, $p < 0.001$) but no effect of genotype ($F_{(1, 30)}$ = 1.2, $p = 0.3$) and no interaction ($F < 1$, n = 6 mice per group).
Error bars, SEM.

Administering HCO$_3^-$ Attenuates the CO$_2$-Induced pH Reduction and Fear Behavior

Because reducing amygdala pH was sufficient to trigger fear through ASIC1a, we reasoned that minimizing the pH reduction induced by CO$_2$ inhalation would attenuate the fear response. To counteract hypercarbic acidosis, we administered HCO$_3^-$ systemically and found that it raised amygdala pH (Figure 5A). Subsequent CO$_2$ breathing lowered pH, but not to values as low as observed in control mice (Figure 5B).

We therefore pretreated mice with HCO$_3^-$ and tested CO$_2$-evoked fear behavior. HCO$_3^-$ attenuated freezing in _ASIC1a$^{+/+}$_ mice breathing CO$_2$ (Figure 5C). In contrast, administering HCO$_3^-$ had no effect in _ASIC1a$^{-/-}$_ mice. These data further indicate that a reduced pH initiates the fear-related response to CO$_2$ inhalation.

HCO$_3^-$ Impairs ASIC1a-Dependent Effects on Conditioned and Unconditioned Fear Behavior

In earlier studies, we found that ASIC1a was required for normal responses in tests of acquired and innate fear (Coryell et al., 2007, 2008; Wemmie et al., 2003, 2004). Our current results raised the question of whether HCO$_3^-$ administration might also interfere with those fear-related behaviors. We injected mice with HCO$_3^-$ and within 15 min, while brain pH was still elevated, we trained them in a contextual fear conditioning task. The following day, after brain pH had normalized, we tested conditioned fear memory. HCO$_3^-$ reduced conditioned freezing in ASIC1a$^{+/+}$ mice but not in ASIC1a$^{-/-}$ mice (Figure 5D). These results indicate that elevating brain pH interferes with the ability of ASIC1a to promote fear learning.
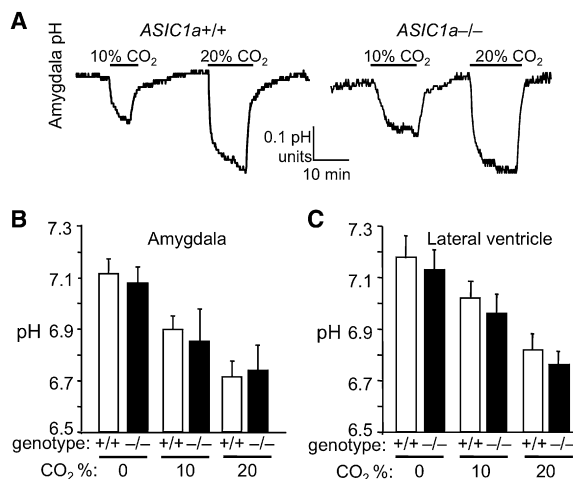
We also tested the effects of HCO$_3^-$ on unconditioned fear. Alkalinizing brain pH by administering HCO$_3^-$ reduced freezing evoked by the predator odor trimethylthiazoline (TMT) in ASIC1a$^{+/+}$ but not ASIC1a$^{-/-}$ mice (Figure 5E). The ability of HCO$_3^-$ to reduce conditioned and unconditioned fear in wild-type mice and the absence of this effect in ASIC1a null mice suggest that ASIC1a activation in vivo is pH dependent.

Reducing pH in the Amygdala Elicits Fear Behavior

Our data indicate that CO$_2$ reduces amygdala pH, that the reduction is sufficient to activate amygdala ASIC currents, and that ASIC1a is required for CO$_2$-induced fear behavior. However, breathing CO$_2$ likely reduces pH throughout the brain, as indicated by the reduced pH in the ventricles (Figure 3C). Moreover, although ASIC1a expression is abundant in the amygdala, it is also widely distributed in the CNS. Therefore, we asked if pH reductions limited to the amygdala would produce fear behavior.

We microinjected acidified ACSF into the amygdala and, with a pH sensor positioned <1 mm from the injection site, showed that pH fell to ~6.8 (Figures 6A and 6B). We detected no acidosis with the sensor ~3 mm from the injector tip, indicating a localized pH reduction. Microinjections of acidic ACSF into the amygdala produced robust freezing in wild-type mice (Figures 6C and 6D). There was no freezing when we microinjected neutral pH ACSF, and injections that missed the amygdala produced much less freezing (Figures 6C and 6D). In contrast, acidic microinjections induced very little freezing in ASIC1a null mice. This freezing deficit did not carry over to other stimuli since both genotypes developed similar freezing when we electrically stimulated the basolateral amygdala (Figure 6E). These data suggest that
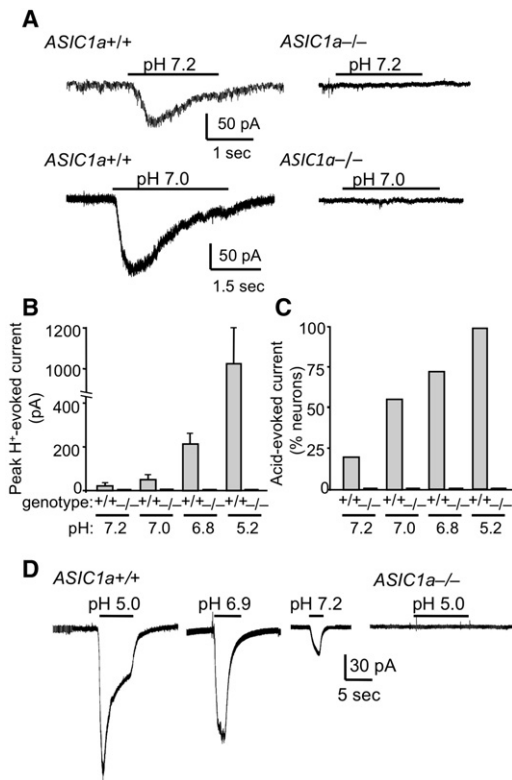
Figure 4. Acid-Evoked Currents in Amygdala Neurons Are Mediated by ASIC1a

(A) Representative examples of responses to pH 7.2 and 7.0 in cultured amygdala neurons from $ASIC1a^{+/+}$ and $ASIC1a^{-/-}$ mice recorded by whole-cell voltage clamp.

(B) Mean peak current evoked by increasingly acidic pH levels in cultured amygdala neurons from $ASIC1a^{+/+}$ and $ASIC1a^{-/-}$ mice. From left to right, $ASIC1a^{+/+}$ groups included 14, 17, 19, and 9 neurons. $ASIC1a^{-/-}$ groups each included 7 neurons.

(C) As pH was lowered, a greater percentage of amygdala neurons from $ASIC1a^{+/+}$ mice exhibited acid-evoked current. None of the $ASIC1a^{-/-}$ neurons showed acid-evoked current.

(D) Examples of acid-evoked currents in basolateral amygdala neurons in acute slices from $ASIC1a^{+/+}$ and $ASIC1a^{-/-}$ mice. The number of cells exhibiting acid-evoked currents versus the total number tested at each pH value were as follows: ($ASIC1a^{+/+}$: pH 5 = 16/21, pH 6.9 = 6/9, pH 7.2 = 2/5), ($ASIC1^{-/-}$: pH 5 = 0/9, pH 6.9 = 0/7, pH 7.2 = 0/4).

Error bars, SEM.

amygdala acidosis evokes freezing behavior and that ASIC1a plays a critical role.

ASIC1a in the Amygdala Is Sufficient for CO_2-Evoked Fear Behavior

These findings raised the question of whether ASIC1a expression restricted to the amygdala was sufficient for CO_2-evoked fear behavior. To answer this question, we injected an adeno-associated virus encoding ASIC1a into the amygdala of $ASIC1a^{-/-}$ mice (Figures 7A and 7B), as we have previously described (Coryell et al., 2008). This injection involved lateral and basal nuclei of the amygdala, as did the acid injection described above. We found that expressing ASIC1a in the baso-
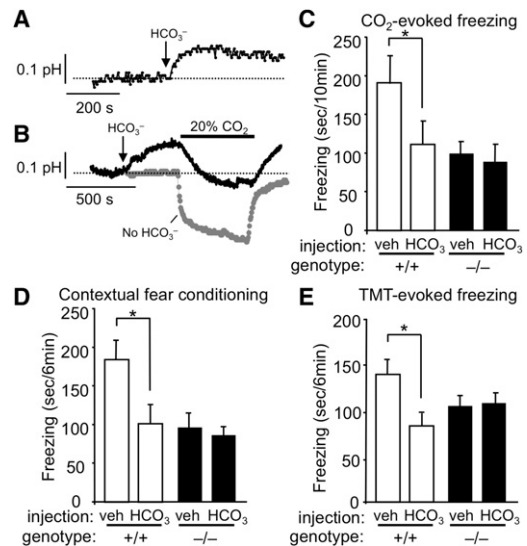

Figure 5. Raising Brain pH with HCO_3^- Blocks CO_2-Evoked Freezing, Fear Conditioning, and Predator Odor-Evoked Fear

(A) Representative tracing of amygdala pH response to HCO_3^- injection (2 mM/kg, intraperitoneally).

(B) Effect of HCO_3^- injection on pH response to CO_2 inhalation (black trace) versus no HCO_3^- injection (gray trace).

(C) HCO_3^- reduced CO_2-evoked freezing in $ASIC1a^{+/+}$ mice but not in $ASIC1a^{-/-}$ mice. ANOVA revealed significant differences between groups (F(3, 30) = 3.92, p < 0.05). Planned contrast testing revealed a significant effect of HCO_3^- in $ASIC1a^{+/+}$ mice (*p < 0.05) but not in $ASIC1a^{-/-}$ mice (p = 0.8). From left to right, group sizes were 8, 7, 10, and 9 mice.

(D) HCO_3^- injection prior to fear conditioning inhibited the contextually conditioned fear response in $ASIC1a^{+/+}$ mice but not in $ASIC1a^{-/-}$ mice. ANOVA revealed significant differences between groups (F(3, 30) = 5.91, p < 0.01). Planned contrast testing found a significant effect of HCO_3^- in $ASIC1a^{+/+}$ mice (*p < 0.05) but not in $ASIC1a^{-/-}$ mice (p = 0.9). From left to right, group sizes were 14, 6, 7, and 7 mice.

(E) HCO_3^- injection prior to predator odor exposure reduced freezing in $ASIC1a^{+/+}$ mice but not in $ASIC1a^{-/-}$ mice. ANOVA revealed significant differences between groups (F(3, 26) = 3.46, p < 0.05). Planned contrast testing found a significant effect of HCO_3^- in $ASIC1a^{+/+}$ mice (*p < 0.01) but not in $ASIC1a^{-/-}$ mice (p = 0.9). From left to right, group sizes were 5, 7, 10, and 8 mice.

Error bars, SEM.

lateral amygdala restored CO_2-evoked freezing to levels comparable to or higher than those in $ASIC1a^{+/+}$ mice (Figure 7B). However, injections that missed the amygdala or expression of GFP had minimal effects on CO_2-evoked freezing.

DISCUSSION

Earlier studies demonstrated the critical contribution of the amygdala to the fear circuit and fear-related behavior (Adolphs, 2002; Kim and Jung, 2006; Maren, 2008; Phelps and LeDoux, 2005; Rosen, 2004). Our results now indicate that the amygdala does more than mediate the fear response; it also has an important chemosensory role. ASIC1a channels in the amygdala detect a reduced pH arising from an increased CO_2 or from direct injection of acid. That CO_2 initiates a fear response is particularly intriguing because rising CO_2 forewarns suffocation, a terrifying
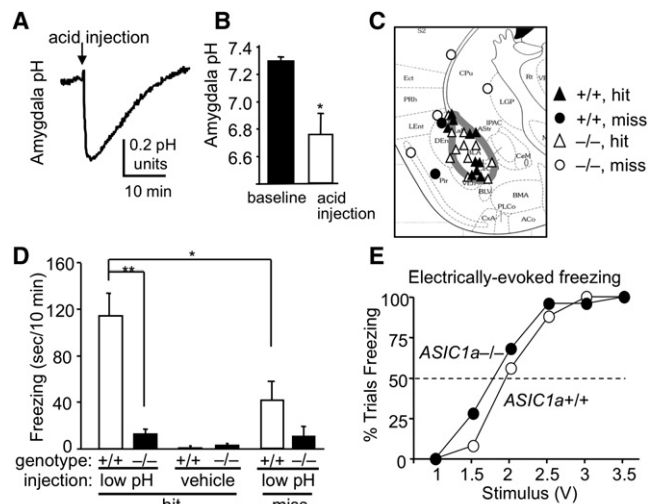
Figure 6. Amygdala Acidosis Evokes ASIC1a-Dependent Freezing Behavior

(A) Injecting acidic ACSF into the amygdala of an anaesthetized mouse lowered amygdala pH by several tenths of a pH unit.

(B) Acid injection lowered pH to 6.8 or below.

(C) Acidic injections that hit the amygdala (gray boundary) versus those that missed.

(D) Acidic injections that hit the amygdala in awake and freely moving mice triggered robust freezing in $ASIC1a^{+/+}$ mice and very little freezing in $ASIC1a^{-/-}$ mice. ANOVA revealed a significant pH by genotype interaction ($F(1, 28) = 12.8$, $p < 0.001$). In the low-pH-injected mice, planned contrast testing revealed significantly greater freezing in $ASIC1a^{+/+}$ hit mice compared to $ASIC1a^{-/-}$ hit mice (**$p < 0.001$) and compared to $ASIC1a^{+/+}$ miss mice (*$p < 0.05$) (group sizes from left to right were 11, 10, 4, 7, 2, and 4 mice).

(E) Electrically stimulating the left amygdala with a bipolar electrode evokes similar rate of freezing responses in $ASIC1a^{+/+}$ and $ASIC1a^{-/-}$ mice. Shown are percentages of trials per 5 attempts that produced freezing at each voltage (n = 5 mice per group; $ASIC1a^{+/+}$ $ED_{50} = 1.99$ V, 95% CI = 1.64–2.27; $ASIC1a^{-/-}$ $ED_{50} = 1.81$ V, 95% CI = 1.44–2.09).

Error bars, SEM.

situation that demands sensitive detection and action to ensure survival. Thus, it is interesting that evolution positioned a sensor for hypercarbic acidosis in the amygdala, a structure that stimulates the sympathetic nervous system for fight-or-flight and links to other brain regions involved in the response to threat. Thus, the amygdala both senses a threat, posed by CO_2, and initiates a response.

The amygdala also plays a primary role in forming memories of events that induce strong emotions (Kim and Jung, 2006; Maren, 2008). It is key to learning the association between conditioned and unconditioned, aversive, fear-evoking stimuli (Kim and Jung, 2006; Maren, 2008). Our discovery that CO_2 enhanced context fear conditioning reveals a previously unrecognized relationship between CO_2 and fear memory. Based on our earlier findings that ASIC1a is located postsynaptically (Schnizler et al., 2009; Wemmie et al., 2002, 2003, 2004; Zha et al., 2006, 2009) and that ASIC1a is required for normal synaptic plasticity in the hippocampus (Wemmie et al., 2002; Zha et al., 2006), we speculate that CO_2 may have potentiated synaptic plasticity in the amygdala by activating ASIC1a.
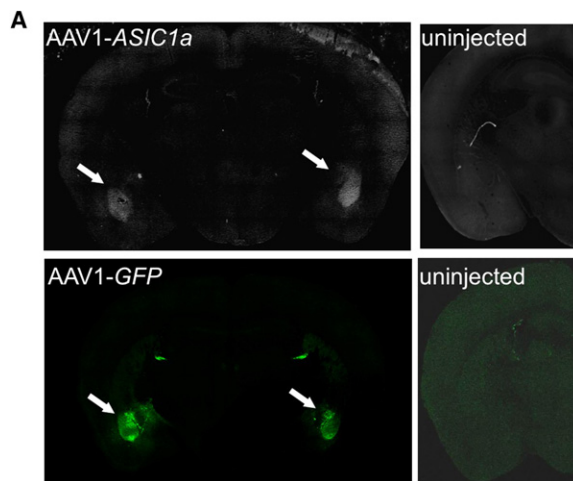

Figure 7. Restoring ASIC1a Expression Bilaterally in the Amygdala of $ASIC1a^{-/-}$ Mice Rescues CO_2-Evoked Freezing

(A) Example of AAV-mediated ASIC1a expression and eGFP expression in bilateral amygdala of $ASIC1a^{-/-}$ mouse.

(B) AAV-$ASIC1a$ injections that hit the amygdala (BLA) bilaterally (ASIC1a-hit) increased CO_2-evoked freezing. One-way ANOVA revealed significant differences between the groups ($F(4, 42) = 4.85$, $p < 0.01$), and planned contrast tests revealed a significant increase in CO_2-evoked freezing in the ASIC1a-hit group relative to other $ASIC1a^{-/-}$ groups (*$p < 0.001$). From left to right, groups sizes were 14, 8, 5, 12, and 8.

ASIC1a loss and inhibition did more than impair CO_2-induced fear behavior; it also attenuated conditioned and unconditioned fear behavior (Figures 1E, 1F, 5D, and 5E) (Coryell et al., 2007, 2008; Wemmie et al., 2003, 2004). In addition, $ASIC1a^{-/-}$ mice tended (not statistically significant) to show less anxiety/fear (enter the center more frequently) than wild-type mice, and the difference between genotypes was accentuated by CO_2 (Figure 1C). These results raised the possibility that ASIC1a action in those assays was also pH dependent. Indeed, supporting that possibility, we found that HCO_3^- administration inhibited contextual fear conditioning and TMT-evoked freezing. Thus, we speculate that when fear-evoking stimuli activate the amygdala, its pH may fall. For example, synaptic vesicles release protons (DeVries, 2001; Palmer et al., 2003), and intense neural activity

is known to lower pH (Kaila and Chesler, 1998). An ability to measure these pH changes in an awake, behaving animal might be informative but also challenging. Supporting an important functional role for pH, studies in *C. elegans* recently demonstrated that protons act as a direct transmitter from intestinal epithelia to muscle cells (Beg et al., 2008). Based on all these considerations, we speculate that local pH fluctuations and ASIC activation might also be important in brain regions outside the amygdala and might explain the requirement of ASIC1a for normal long-term potentiation (Wemmie et al., 2002).

Although our data emphasize the importance of ASIC1a for the amygdala's response to CO_2, they also suggest that there must be additional pH sensors in the brain. For example, the ventilatory response to CO_2 inhalation remained intact in *ASIC1a$^{-/-}$* mice, suggesting that respiratory control centers utilize different molecular mechanisms to detect acidosis. In addition, disrupting the *ASIC1a* gene did not completely eliminate all the behavioral responses to breathing CO_2. It seems unlikely that ASIC2a or -2b are responsible, since they are much less pH sensitive than ASIC1a, and loss of ASIC1a eliminates currents evoked by pH values as low as 5.0 (Wemmie et al., 2002). Other pH-sensitive receptors might include channels such as pH-sensitive TRP channels (Huang et al., 2006), K^+ channels (Trapp et al., 2008), or other pH-sensitive proteins.

It has long been known that breathing CO_2 triggers panic attacks in patients with panic disorder (Drury, 1918; Klein, 1993; Papp et al., 1993; Sanderson et al., 1989), and that these patients show an increased sensitivity to CO_2 inhalation (Papp et al., 1993; Rassovsky and Kushner, 2003). Dysregulated brain pH has also been implicated in panic disorder (Friedman et al., 2006; Maddock, 2001). In addition, patients with increasing hypercarbia due to respiratory failure become extremely anxious (Smoller et al., 1996). Our studies suggest a molecular and anatomical foundation for further dissecting CO_2-triggered anxiety and panic in humans. They also suggest that new therapeutic strategies for panic and anxiety might target changes in brain pH or ASIC channels.

EXPERIMENTAL PROCEDURES

Mice
ASIC1a$^{-/-}$ mice were described previously (Wemmie et al., 2002). Briefly, the neomycin resistance gene replaced the first translated exon in the ASIC1a-encoding gene (*ACCN2)*, eliminating expression of the ASIC1a protein. *ASIC1a$^{-/-}$* and *ASIC1a$^{+/+}$* mice were maintained in a congenic C57BL/6 background. Experimental groups were matched for age (ranging from 9–18 weeks) and gender. Mice were kept on a standard 12 hr light-dark cycle and received standard chow (LM-485; Teklab, Madison, WI, USA) and water ad libitum. Animal care met National Institutes of Health standards and the University of Iowa Animal Care and Use Committee approved all procedures.

CO$_2$-Evoked Freezing, PcTx1, and A-317567
Freezing behavior was defined as the absence of motion except for respiration and was assessed by placing mice in a plexiglas chamber (20.3 cm wide × 20.3 cm deep × 16.5 cm high), similar to that used previously to test predator odor-evoked freezing (Coryell et al., 2007). CO_2 diffused through a large valve in the ceiling of the chamber to create a steady-state concentration of 5% or 10% CO_2. This setup avoided blowing CO_2 or air, which can be aversive to mice. Behavior was videotaped and scored by a trained observer blinded to genotype and condition. To administer the ASIC1a antagonists, intracerebroventricular (ICV) guide cannulae were implanted into the left lateral ventricle of

anesthetized mice (relative to bregma: anteroposterior −0.3 mm, lateral −1.0 mm, ventral −3.2 mm). Three to five days later, 5 μl of PcTx1-containing venom (9 ng/μl) (SpiderPharm, Yarnell, AZ, USA), A-317567 (0.9 mM from 10 mM stock suspended in 5% DMSO—kindly provided by Drs. Alan Light and Jon Rainier), or ACSF alone (in mM: NaCl 124, KCl 3, NaH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, CaCl$_2$ 2, NaHCO$_3$ 26) were injected over 10 s with a 10 μl Hamilton syringe connected to a 30-gauge injector. CO_2-evoked freezing was assessed 2 hr or 30 min after injecting PcTx1 and A-317567, respectively.

Open Field
Mice were placed in an open field (40.6 cm wide × 40.6 cm deep × 36.8 cm high, 55 lux) (San Diego Instruments). The open field was covered with a clear plexiglas lid and prefilled with compressed air or CO_2 concentrations similar to those that trigger panic in humans, 5% to 20% (in air). Behavior in the open field was assessed for 20 min. Center activity was defined as percent beam breaks in the center (30.5 × 30.5 cm) relative to total beam breaks.

CO$_2$-Aversion Assay
Mice were placed in a clear plexiglas chamber with two sides (20.3 cm wide × 20.3 cm deep × 14.0 cm high) connected by a swinging door (4.3 cm × 4.6 cm) that allowed mice to cross freely but limited gas mixing between the two sides. To further prevent gas mixing the lid of each side was left ajar to provide a low-resistance outflow. CO_2 (20% in 21% O_2, balanced with N_2) was administered to one side (10 l/min), whereas compressed air was administered at the same rate to the opposite side leading to steady-state concentrations of 15% CO_2 and 2% CO_2, respectively. Prior to gas infusion, mice were allowed to explore the chamber until they learned to cross freely between the two sides at least four times. The side receiving CO_2 was randomly alternated between trials. Ten minute trials were videotaped and a trained observer blinded to genotype scored the time each mouse spent in the two sides of the chamber. Aversion to compressed blowing air was similarly assessed by administering compressed air to one side (flow rate 15 l/min) and leaving the other side undisturbed.

Context Fear Conditioning and Predator Odor-Evoked Fear
Mice were placed in a near infrared fear-conditioning chamber (Med Associates, St. Albuns, VT, USA) and allowed to habituate for 3 min. Mice received five footshocks (1 s, 0.75 mA) with an intershock interval of 1 min. Control groups received no footshocks. Ten percent CO_2 or air (9 l/min) was infused into the chamber during conditioning. Mice were then returned to the home cage. Context-evoked freezing was tested 24 hr later by placing the mice back into the conditioning chamber for 5 min (minus CO_2 and minus footshocks). Freezing was defined as an absence of movement other than respiration and was scored with VideoFreeze software (Med Associates), which correlated closely with manual scoring (Coryell et al., 2008). HCO$_3^-$ (2 mM/Kg, ip) was injected 15 min prior to fear conditioning or TMT exposure. Contextual fear conditioning was performed as above except that each footshock was preceded by a 20 s tone. As before, conditioned freezing response to the context was assessed 24 hr after training. TMT-evoked freezing was assessed as previously described (Coryell et al., 2007).

Whole-Body Plethysmography and Arterial Partial Pressure CO$_2$ Measurements
Respiration was assessed in a plethysmograph chamber (Kent Scientific, Litchfield, CT, USA). Mice were habituated to the chamber for 1 hr. Ventilatory patterns were then recorded while perfusing the chamber with compressed air, 5% CO_2, or 10% CO_2 (in air) for 10 min. During stable breathing (no extraneous body movement), the average peak-to-peak ventilatory rate (V_R) was calculated. Tidal volume (V_T) was determined by measuring area under curve (AUC) for 20 expiratory events in at least two epochs of stable breathing. The AUC was then transformed to volume using a calibration injection of a known volume. Minute ventilation was calculated by multiplying V_R by V_T. The CO_2-evoked increase in minute ventilation versus air was then determined. PaCO$_2$ measurements were obtained from 100 μl arterial blood from a right femoral artery line in mice anesthetized with ketamine and xylazine breathing compressed air or 10% CO_2 (21% O_2) for 5 min with an ABL-5 blood gas analyzer (Radiometer Copenhagen).

Amygdala Neurons, Culture, Slices, and Whole-Cell Voltage Clamp

Cultured amygdala neurons were obtained from newborn pups as described (Askwith et al., 2004; Wemmie et al., 2003). The amygdala was removed and digested (0.25% trypsin [Sigma] in phosphate-buffered saline) for 10 min at 37°C. Tissue was triturated through Pasteur pipettes of decreasing diameter in MEM medium (GIBCO, USA), with 10% horse serum (Invitrogen, USA), insulin (Sigma, USA), and gentamycin (Invitrogen). Cells were transferred to Petri dishes (35 mm dia.) and adhered to coverslips (12 mm dia.) coated with poly-L-lysine and laminin (Sigma), and incubated in 5% CO_2 (in air) at 37°C. To inhibit glial proliferation, 10 μM cytosine-A-D-arabinofuranoside (Sigma) was added at 72–96 hr. Medium was changed every 24 hr. Neurons were cultured for 14–18 days and studied by whole-cell voltage clamp without selection for neuron size or morphology. Amygdala slices (300 μm) were obtained from 18- to 22-day-old mouse brains sectioned with a Vibratome in ice-cold aCSF, then maintained at room temp for 2 hr in aCSF (in mM: 126 NaCl, 5 KCl, 1.6 $CaCl_2$, 1.8 $MgSO_4$, 10 D-glucose, 27.7 $NaHCO_3$, 1.25 NaH_2PO_4, 95% O_2/5% CO_2) prior to whole-cell voltage-clamp recording. Recordings were obtained at 20°C–23°C using an Axopatch 200B amplifier and Clampex 9.2 (Axon Instruments) sampled at 50 μs interval and filtered at 5 kHz. Membrane voltage was maintained at −70 mV. Recording pipettes (3–7 MΩ) were pulled from capillary glass with a micropipette puller (Sutter instruments), polished (MF830, Narishige, Japan), and contained (in mM): 5 NaCl, 90 Kgluconate, 15 KCl, 1 $MgCl_2$, 10 EGTA, 25 HEPES, and 3 Na_2ATP, adjusted to pH 7.25 with TMAOH. Bath solutions contained for culture (in mM): 145 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 MES, 10 glucose, pH was adjusted with TMA·OH. For slices, the bath solution contained (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 D-glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 95% O_2/5% CO_2, (room temp). Extracellular solutions were changed with a rapid solution changer (SF77B, Warner Instruments).

Measuring Brain pH during CO_2 Inhalation, Acid, and Bicarbonate Injection

A fiber optic pH sensor (pHOptica, WPI; detection range pH 5–9) was placed in the left lateral ventricle (relative to bregma in mm: anteroposterior −0.3, lateral +1.0, ventral −3.2) or the basolateral amygdala (anteroposterior −1.2, lateral +3.6; ventral −5.2 from skull surface) of $ASIC1a^{+/+}$ and $ASIC1a^{−/−}$ mice anesthetized with ketamine and xylazine. Brain pH was measured in response to compressed air or CO_2 (in 21% O_2/69% N_2). To measure the amygdala pH during acid injection, a 30-gauge stainless steel injector was glued to the pH sensor and positioned ~1 mm from the sensor. The pH sensor and injector were inserted into the mouse amygdala. pH was recorded at baseline and in response to injecting 1 μl of ACSF (pH 7.3 and pH 3.0). ACSF pH was lowered to 3 with HCl. Injecting $HCO_3^−$ (2 mM/kg, i.p.) raised brain pH for ~1 hr, as described by others (Schuchmann et al., 2006).

Freezing Response to Amygdala Acidosis

A 26-gauge guide cannula was implanted into the left basolateral amygdala (relative to bregma in mm: anteroposterior −1.2, lateral +3.6, ventral −4.2 from skull surface). Mice recovered from the surgery for at least 3 days. A 10 μl Hamilton syringe connected to a 30-gauge injector was inserted 1 mm past the cannula tip to inject ACSF (1 μl pH 3 or pH 7.3) over 5 s. The behavioral response was videotaped and freezing was scored for 10 min by a trained observer blinded to genotype and condition. The injection sites were mapped post-mortem by sectioning the brain (7 μM coronal) and performing cresyl violet staining (in 100 ml: 0.5 g Cresyl, 20 ml of 100% EtOH, 1.5 ml glacial acetic acid, pH to 3.5–3.7).

Amygdala Stimulation and Electrocorticography Recordings

Custom made bipolar tungsten stimulating electrodes (0.2 mm diameter, 0.5 mm gap, 450 kΩ impedance) were stereotactically implanted into the left amygdala under ketamine/xylazine anesthesia (relative to bregma in mm: anteroposterior −1.2, lateral +3.6, ventral −4.2 from brain surface). Three stainless steel screws (3.2 mm, Stoelting) were implanted above the right frontal lobe, right parietal lobe, and cerebellum. The first two screws served as epidural recording electrodes and the latter as a reference/ground electrode (coordinates from bregma in mm, frontal electrode: anteroposterior +2.0, lateral −1.5; parietal electrode: anteroposterior −2.0 mm, lateral −1.5 mm;

cerebellar electrode: anteroposterior −6.0, lateral 0). The amygdala was stimulated five times at each voltage with a GRASS SD9 stimulator (5 s, 100 Hz, 1–3.5 V in 0.5 V steps). Freezing was scored at the absence of movement except for respiration. Amygdala ECoG recordings were obtained using a TDT MEDUSA preamplifier and base-station (sampling rate 2034.5 Hz, filtered at 1 Hz and 3000 Hz) and recorded with TDT OpenX software and analyzed with MATLAB R2007b. ECoG tracings were examined for evidence of epileptic spike activity and after discharges; none were observed during this experiment.

Adeno-Associated Virus Vectors

Vectors were produced by the University of Iowa Gene Transfer Vector Core and injected as described previously (Coryell et al., 2008). The vectors were AAV2/1 chimeric viruses with AAV1 capsids and AAV2 ITRs and contained a CMV promoter driving an *ASIC1a* or an enhanced green fluorescent protein (*eGFP*) cDNA. To facilitate the screening of AAV-*ASIC1a* transduced brain regions we inserted an internal ribosomal re-entry site (IRES) followed by eGFP, downstream from *ASIC1a* in the AAV-*ASIC1a* vector; however, this vector produced no detectable *eGFP* expression (not shown). Therefore, in the AAV-*ASIC1a* group, we coinjected AAV-*eGFP* as described (Coryell et al., 2008). Mice recovered for 14 days before behavioral analysis. Targeting was confirmed post-mortem as described (Coryell et al., 2008). Correctly targeted injections (hits) were defined as having fluorescence above background within bilateral BLA. Off-target injections (misses) were defined as having fluorescence above background in the temporal lobe, but not in both BLA.

Statistical Analysis

Values are expressed as mean ± standard error of the mean (SEM). Two-way analysis of variance (ANOVA) was used to test for interaction effects. One-way ANOVA was also used to assess overall differences in experiments involving more than two groups. Planned contrast testing within the context of the full ANOVA was used to test relationships hypothesized a priori between groups. Wilcoxon rank sum test was used to assess for significant differences in CO_2 aversion. ED_{50} values were determined by Probit analysis. p values (two-tailed) less than 0.05 were considered significant.

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