

# Protons are a neurotransmitter that regulates synaptic plasticity in the lateral amygdala

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**Stimulating presynaptic terminals can increase the proton concentration in synapses. Potential receptors for protons are acid-sensing ion channels (ASICs), Na<sup>+</sup>- and Ca<sup>2+</sup>-permeable channels that are activated by extracellular acidosis. Those observations suggest that protons might be a neurotransmitter. We found that presynaptic stimulation transiently reduced extracellular pH in the amygdala. The protons activated ASICs in lateral amygdala pyramidal neurons, generating excitatory postsynaptic currents. Moreover, both protons and ASICs were required for synaptic plasticity in lateral amygdala neurons. The results identify protons as a neurotransmitter, and they establish ASICs as the postsynaptic receptor. They also indicate that protons and ASICs are a neurotransmitter/receptor pair critical for amygdala-dependent learning and memory.**

long-term potentiation | PcTX1 | acid sensing ion channel

**A**lthough homeostatic mechanisms generally maintain the brain's extracellular pH within narrow limits, neural activity can induce transient and localized pH fluctuations. For example, acidification may occur when synaptic vesicles, which have a pH of ~5.2–5.7 (1–3), release their contents into the synapse. Studies of mammalian cone photoreceptors showed that synaptic vesicle exocytosis rapidly reduced synaptic cleft pH by an estimated 0.2–0.6 units (4–6). Transient synaptic cleft acidification also occurred with GABAergic transmission (7). Some, but not all, studies also reported that high-frequency stimulation (HFS) transiently acidified hippocampal brain slices, likely as a result of the release of synaptic vesicle contents (8, 9). Neurotransmission also induces a slower, more prolonged alkalization (10, 11). In addition to release of synaptic vesicle protons, neuronal and glial H<sup>+</sup> and HCO<sub>3</sub><sup>−</sup> transporters, channels, H<sup>+</sup>-ATPases, and metabolism might influence extracellular pH (10–12).

ASICs are potential targets of reduced extracellular pH. ASICs are Na<sup>+</sup>-permeable and, to a lesser extent, Ca<sup>2+</sup>-permeable channels that are activated by extracellular acidosis (13–19). In the brain, ASICs consist of homotrimeric and heterotrimeric complexes of ASIC1a, ASIC2a, and ASIC2b. The ASIC1a subunit is required for acid-activation in the physiological range (>pH 5.0) (20, 21). Several observations indicate that ASIC are located postsynaptically. ASICs are located on dendritic spines. Although similar to glutamate receptors, they are also present on dendrites and cell bodies (20, 22–24). ASIC subunits interact with postsynaptic scaffolding proteins, including postsynaptic density protein 95 and protein interacting with C-kinase-1 (20, 24–29). In addition, ASICs are enriched in synaptosome-containing brain fractions (20, 24, 30).

Although these observations raised the possibility that protons might be a neurotransmitter, postsynaptic ASIC currents have not been detected in cultured hippocampal neurons (31, 32), and whether localized pH transients might play a signaling role in neuronal communication remains unclear. In previous studies of hippocampal brain slices, extracellular field potential recordings suggested impaired hippocampal long-term potentiation (LTP)

in *ASIC1a*<sup>−/−</sup> mice (20), although another study did not detect an effect of ASIC1a (33). Another study using microisland cultures of hippocampal neurons suggested that the probability of neurotransmitter release increased in *ASIC1a*<sup>−/−</sup> mice (32).

Here, we tested the hypothesis that protons are a neurotransmitter and that ASICs are the receptor. Criteria to identify substances as neurotransmitters have been proposed (34). Beg and colleagues (35) used these criteria to conclude that protons are a transmitter released from *Caenorhabditis elegans* intestine to cause muscle contraction. Key questions about whether protons meet criteria for a neurotransmitter are: Does presynaptic stimulation increase the extracellular proton concentration? Do protons activate currents in postsynaptic cells? Can exogenously applied protons reproduce effects of endogenous protons? What is the postsynaptic proton receptor? We studied lateral amygdala brain slices because amygdala-dependent fear-related behavior depends on a pH reduction (36). In addition, ASICs are abundantly expressed there, and *ASIC1a*<sup>−/−</sup> mice have impaired fear-like behavior (36–38).

## Results and Discussion

### Presynaptic Stimulation Induces ASIC Excitatory Postsynaptic Currents.

We found that an acidic pH stimulated currents in lateral amygdala pyramidal neurons, and *ASIC1a*<sup>−/−</sup> neurons lacked those currents (Fig. 1 *A–C*). We also stimulated cortical inputs and recorded excitatory postsynaptic currents (EPSCs). Under basal conditions, wild-type (WT) and *ASIC1a*<sup>−/−</sup> EPSCs had similar amplitudes (Fig. 1 *E–G*), and previous studies showed similar NMDA and AMPA receptor currents in cultured hippocampal neurons of both genotypes (20). After glutamate receptor (GluR) blockade with AMPA and NMDA receptor blockers, a small component of the EPSC remained (Fig. 1 *E–G*). HFS of cortical inputs also generated postsynaptic currents after GluR blockade (Fig. S1).

*ASIC1a*<sup>−/−</sup> neurons lacked the current that was revealed in the presence of GluR blockers (Fig. 1 *F* and *G* and Fig. S1). Amiloride, which blocks ASICs (albeit a nonselective blocker) (39), inhibited

## Significance

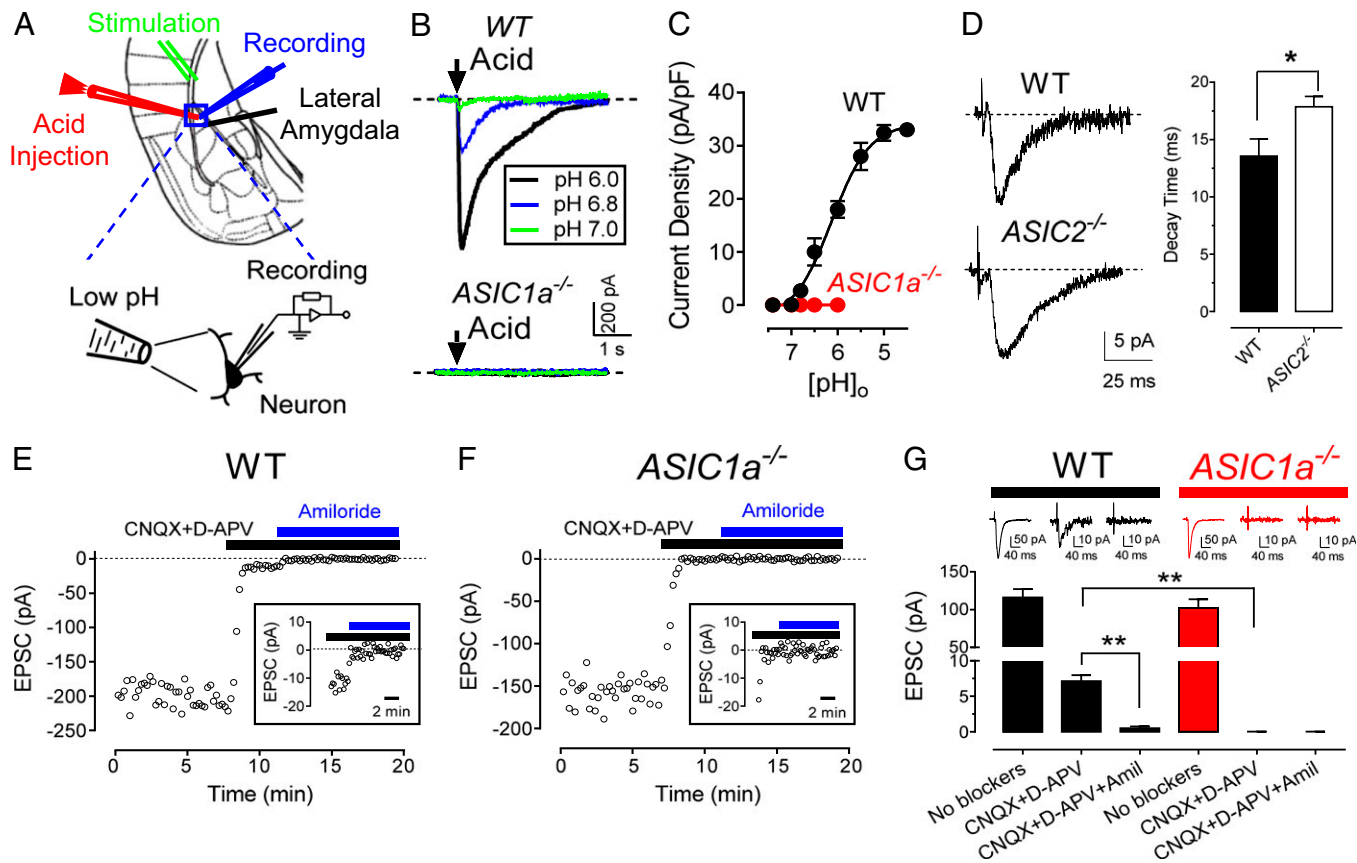
Synaptic transmission involves the release of neurotransmitters that activate receptors on postsynaptic cells. The results reveal that protons fulfill the criteria for a neurotransmitter and that they activate postsynaptic acid-sensing ion channels. This activity facilitates synaptic plasticity, a requirement for learning and memory in the amygdala.

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after vesicle fusion, and/or  $\text{Na}^+/\text{H}^+$  exchange (4, 6, 7). Irrespective of the mechanism, our results are consistent with earlier studies indicating that presynaptic stimulation acutely increases the proton concentration (4–7).

**There is a postsynaptic receptor.** We conclude that ASIC channels are the postsynaptic proton receptors because eliminating ASIC1a eliminated ASIC-dependent EPSCs, GluR EPSCs and ASIC EPSCs manifested different decay times, the biophysical properties of ASIC-dependent EPSCs changed as predicted when ASIC channel subunit composition was altered, PcTX1 and amiloride inhibited ASIC-dependent EPSCs, and ASICs localize to the postsynaptic membrane (20, 23, 24, 26).

**Exogenous application of the chemical mimics the endogenous response.** We found that applying exogenous protons induced action potentials, as well as LTP.

**A mechanism to terminate neurotransmitter action exists.** After acidification, we found that extracellular pH rapidly recovered and alkalinized. Several processes may restore and increase pH, including proton diffusion, pH buffering, and activity of membrane transporters (11). Increased interstitial pH after neurostimulation has also been reported in other preparations (11, 42, 47). Previous studies showed that raising extracellular pH before an acid stimulus reduced steady-state inactivation and increased the amplitude of ASIC currents (43, 44, 48). Thus, alkalinization might prepare postsynaptic ASICs for subsequent stimuli, and thereby maximize their current.

**Blocking the receptor blocks the activity of the neurotransmitter.** Disrupting the *ASIC1* gene eliminated the effect of exogenously applied protons, ASIC-dependent EPSCs, and acid-evoked LTP. Amiloride and PcTX1 also blocked ASIC EPSCs.

**Additional Implications.** First, the results raise the question of whether protons function as a neurotransmitter entirely on their own. Although exogenous acid can activate action potentials, protons might not transmit information in isolation of other transmitters. We say that because when we puffed pH 6.8 solution into amygdala brain slices, LTP induction required delivery of a test pulse to the presynaptic neuron. In addition, compared with glutamate-dependent EPSCs, ASIC EPSCs are small.

Second, every time glutamate is released into a synapse, protons may be coreleased. Co-release has a parallel with other neurotransmitters (49): Co-release implies that proton exocytosis need not have a unique regulatory mechanism; acidification would be initiated by the same  $\text{Ca}^{2+}$ -dependent exocytotic release mechanisms that regulate glutamate release.

Third, both the presence of ASICs in many brain areas (14–17, 22, 38) and a source for proton release at other sites position protons to function as a neurotransmitter in combination with other neurotransmitters and in many brain regions.

Fourth, an acidic pH and ASICs are associated with neuronal injury in models of cerebral ischemia (21, 50), multiple sclerosis (51), and traumatic brain injury (52). Function of protons as a neurotransmitter suggests involvement of excitotoxicity-like mechanisms.

Fifth, what is the physiological role of proton–ASIC signaling in neurotransmission? Compared with GluR currents, ASIC

currents make a very small contribution to EPSCs induced by a single test pulse. Thus, we suspect that proton–ASIC signaling has little effect on neurotransmission under basal conditions. That conclusion is consistent with studies of *ASIC1a*<sup>−/−</sup> mice, which show little behavioral difference from WT mice in the absence of stress. However, we speculate that proton–ASIC signaling may be particularly important during intense presynaptic stimulation. Extracellular pH reductions are the greatest during HFS and could have at least two effects: The lower pH would generate larger ASIC currents, and in addition, the reduced pH may inhibit NMDA receptors (53), and thus concurrent activation of ASICs could sustain and enhance synaptic transmission. These effects could explain the involvement of protons and the requirement of ASICs for LTP induced by HFS. Consistent with these conclusions, both ASICs and GluRs are required for normal behavioral responses to stresses such as amygdala-dependent fear-related learning and memory.

## Materials and Methods

Also see *SI Materials and Methods*.

**Mice.** *ASIC1a*<sup>−/−</sup>, *ASIC2*<sup>−/−</sup>, and WT mice were maintained on a congenic C57BL/6 background. The University of Iowa Animal Care and Use Committee approved all procedures.

**Brain Slices.** We used standard procedures to generate brain slices from 4–6-wk-old mice. For experiments with a varying  $\text{HCO}_3^-$  concentration, changes in  $\text{HCO}_3^-$  were balanced with gluconate, and  $\text{Cl}^-$  concentration was maintained constant. Acidic solutions were delivered by direct injection into slices, using a microinjection system, or to the entire slice through the perfusion system.

**Patch-Clamp Recording.** Standard procedures were used to patch-clamp lateral amygdala pyramidal neurons.

**Measurement of Extracellular pH in Brain Slice Cultures.** Amygdala slice cultures were prepared from 3–4-d-old mice with procedures modified from our earlier work (23). A syndecan 2-pHluorin fusion was generated by inserting pHluorin sequence into mouse syndecan 2 cDNA immediately after the signal peptide sequence to generate a fusion with extracellular pHluorin. Biolistic transfection of slices was after 1–3 wk in culture, and slices were studied 48 h later. pHluorin signal was detected with a high-speed confocal microscope. Fluorescence of HeLa cells was measured 24–48 h after transfection.

**Analysis.** Data are presented as means  $\pm$  SEM. To statistically assess LTP, we analyzed the last 5 min of EPSPs of each LTP recording (total 30 EPSP points), averaged those 30 points, and then compared those data with average data before HFS. Statistical comparison of groups used one-way ANOVA and Tukey's post hoc multiple comparison test. A Student *t* test was used to compare two groups.

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# Supporting Information

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## SI Materials and Methods

**Mice.** *ASIC1a*<sup>-/-</sup> (acid-sensing ion channel) and *ASIC2*<sup>-/-</sup> mice were described previously (1,2). *ASIC1a*<sup>-/-</sup>, *ASIC2*<sup>-/-</sup>, and wild-type (WT) mice were maintained on a congenic C57BL/6 background. Experimental groups were matched for age (4–6 wk for studies of acute brain slices and 3–4 d for studies of slice cultures). Mice were kept on a standard 12-h light-dark cycle and received standard chow (LM-485; Teklab) and water ad libitum.

**Brain Slice Preparation.** Mice were anesthetized with isoflurane, and brains were dissected into preoxygenated (5% CO<sub>2</sub> and 95% O<sub>2</sub>) ice-cold high-sucrose dissection solution containing (in mM) 205 sucrose, 5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 5 MgSO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 1 CaCl<sub>2</sub>, and 25 glucose. A vibratome was used to slice brains coronally into 300-μm sections that were maintained in normal artificial cerebrospinal fluid (ACSF) containing (in mM) 115 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 11 glucose, and 25 NaHCO<sub>3</sub> bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> at pH 7.4 at 22–25 °C. Slices were incubated in the solution at least 2 h before recording. For experiments, individual slices were transferred to a submersion-recording chamber and were continuously perfused with the 5% CO<sub>2</sub>/95% O<sub>2</sub> solution (~3.0 mL/min) at 22–25 °C.

**Patch-Clamp Recording.** Slices were visualized with infrared optics, using an upright microscope equipped with differential interference contrast optics (Nikon ECLIPSE FN1). Whole-cell patch-clamp recordings were made from pyramidal neurons in the lateral amygdala. The pipette solution contained (in mM) 135 KSO<sub>3</sub>CH<sub>3</sub>, 5 NaCl, 10 Hepes, 4 MgATP, 0.3 Na<sub>3</sub>GTP, and 0.5 K-EGTA (mOsm = 290, adjusted to pH 7.25 with KOH). Picrotoxin (100 μM) was added to the ACSF throughout the recordings to yield excitatory responses.

Constant current pulses (20–80 μA, 100 μs, 0.05 or 0.1 Hz) were applied through extracellular bipolar electrodes placed at cortical inputs to induce excitatory postsynaptic currents (EPSCs). For whole-cell long-term potentiation (LTP) recordings, high-frequency stimulation (HFS; 100 Hz, 1 s) was used to induce LTP. For injections of acidic ACSF into the slice, the ACSF was buffered with Hepes when pH was >6.0 and was buffered by 10 mM Mes when pH was ≤6.0. Five-second puffs of acidic solution were delivered by a patch-clamp pipette and a Femtojet microinjection system (Eppendorf Inc). For the acid-induced LTP experiments, the test pulse was delivered 50 ms before the end of a 3-s acid injection. The paired acid injection and test pulse were repeated 3 times with a 20-s interval. In other experiments, the pH change in the recording chamber was controlled by the perfusion system. The decay times of EPSCs were fitted with a single exponential.

**Brain Slice Culture and Transfection.** Mouse amygdala organotypic slice cultures were modified from our earlier hippocampal slice culture study (3). Briefly, whole brain from postnatal 3–4-d-old mice were dissected and cut into 300-μm-thick coronal slices with a Vibratome 1000 plus. Amygdala slices were then dissected and

placed in Falcon polyethylene terephthalate-etched membrane culture inserts containing 1-μm pores. Slices were maintained in filter culture medium composed of 25% horse serum, 25% Hanks Balanced Salt Solution, 50% Minimum Essential Medium, 2 mM L-glutamine, 1 mg/mL glucose, 44 mg/mL NaHCO<sub>3</sub>, and 10 units/mL penicillin/streptomycin. Slices were maintained in a 5% CO<sub>2</sub> humidified incubator. The medium was changed every 3 d.

**Synaptic pH Measurement.** A syndecan 2-pHluorin fusion was generated by inserting pHluorin sequence into mouse syndecan 2 cDNA (Origene) immediately after the signal peptide sequence to generate a fusion with the pHluorin on the extracellular surface. Briefly, an AfeI site was created in syndecan 2, using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) with the following primers: forward, 5'-CTGACATCCGATAGCGCTATGTACCTTGAC-3'; reverse, 5'-GTCAAGGTACATAGCGCTATCGGATGTCAG-3'. Cycling conditions were performed according to the manufacturer's protocol. Superecliptic pHluorin was then mutated to introduce 5' and 3' flanking AfeI sites, using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) with the following primers: forward, 5'-AGCGCTATGAGTAAAGGAGAAGAAGCTTTTCACTGG-3'; reverse, 5'-AGCGCTTGTATAGTTTCATCCATGCCATGTGTAAT-3'. The pHluorin containing the flanking AfeI sites and the parent syndecan 2 construct containing the AfeI site were treated with AfeI (NEB) for 2 h at 37 °C. The products were then run on an agarose gel and purified using Qiaquick Gel Extraction (Qiagen). Purified products were ligated together, using Rapid DNA Ligation Kit (Roche), and transformed into MAX Efficiency DH5 Alpha T1 Phage-Resistant Competent Cells (Invitrogen). Colonies were selected and grown overnight in LB Agar containing kanamycin. The next day, DNA was isolated using Wizard Plus SV Minipreps DNA Purification Kit (Promega), and sequence was confirmed by sequencing.

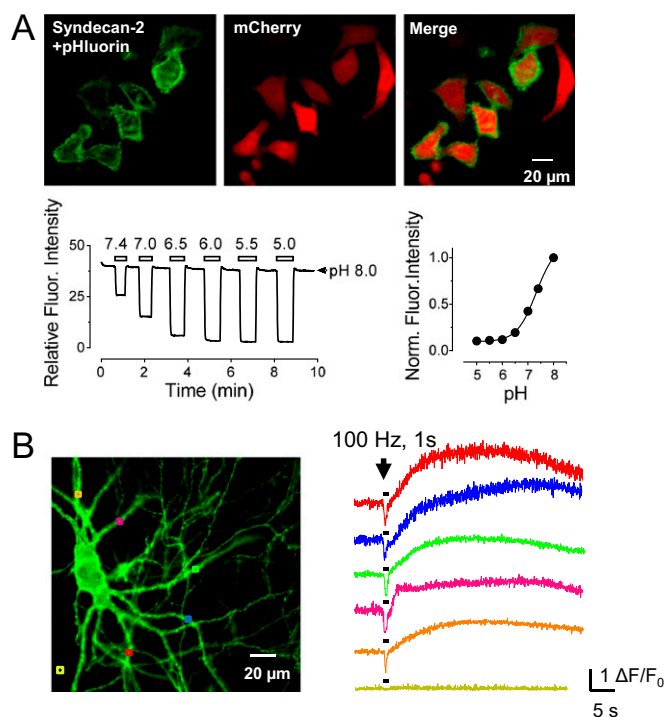
Biolistic transfection of slice cultures was done using a Helios Gene Gun (Bio-Rad) after 1–3 wk culture. Slices were used for synaptic pH measurement 48 h after transfection. In brief, the membrane of the culture insert was cut and transferred to a quick-change imaging chamber (Warner Instruments). Slices were perfused with ACSF at pH 7.4, buffered with 5% CO<sub>2</sub>/95% O<sub>2</sub>. Experiments were controlled and analyzed through Nikon Elements 4.0 software, with off-line background subtraction. Extracellular bipolar electrodes were placed at cortical inputs to generate stimulation. To assess the relationship between stimulus intensity and pH, we applied electrical stimulation at frequencies from 1 to 100 Hz. The pHluorin signal was detected using a NIKON A1R high-speed confocal microscope. For measurements in HeLa cells, the syndecan 2-pHluorin plasmid was transfected into HeLa cells, using Lipofectamine 2000 (Invitrogen), and the cells were studied 24–48 h later with a series of extracellular pH solutions.

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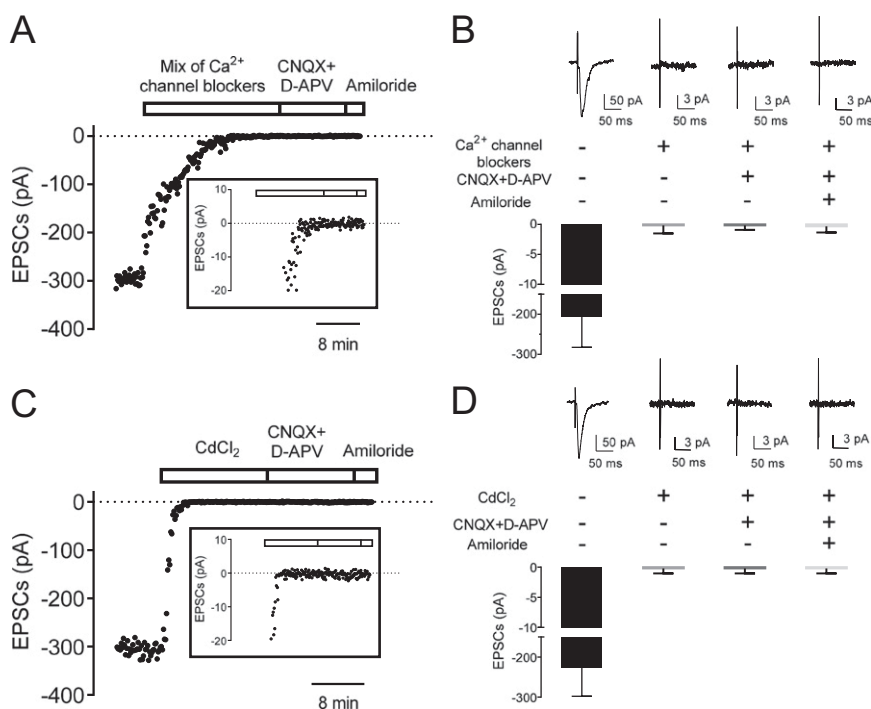
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**Fig. S2.** Comparison of EPSC decay times with and without GluR blockers. (A) Data are mean  $\pm$  SEM of best-fit decay times of EPSCs in pyramidal neurons of lateral amygdala slices under control conditions ( $n = 9$  cells in 4 mice) and in the presence of 25  $\mu$ M CNQX and 50  $\mu$ M D-APV ( $n = 8$  cells in 4 mice).  $*P < 0.05$  (Student *t* test). (B) Best-fitted decay times of HFS-induced EPSCs without and with 25  $\mu$ M CNQX and 50  $\mu$ M D-APV.  $*P < 0.05$  (Student *t* test;  $n = 7$  cells in 4 mice).



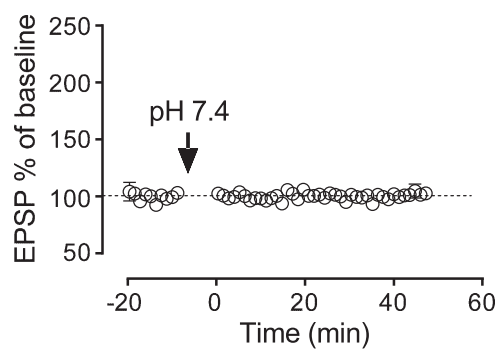


**Fig. S3.** Presynaptic stimulation reduces extracellular pH. (A, Upper) Images of HeLa cells expressing syndecan 2-pHlourin, mCherry (as a control), and the merged image. Syndecan 2-pHlourin preferentially localized to cell membranes. (Bottom, left) Changes in fluorescence intensity elicited by applications of indicated pH. (Bottom, right) Fluorescence intensity at indicated pH values normalized to value at pH 8.0. Best fit of the normalized data yielded  $EC_{50} = 7.33 \pm 0.02$  pH ( $n = 8$ ). (B, Left) Local regions of spines and neighboring dendrites selected for fluorescence measurement. (Right) Fluorescence of regions indicated in left before and after HF5.



**Fig. 5A.** Blocking  $\text{Ca}^{2+}$  channels eliminates ASIC-dependent EPSCs. (A) Example of EPSCs from a WT lateral amygdala pyramidal neuron shown under basal conditions, after addition of a mixture of  $\text{Ca}^{2+}$  channel blockers (10  $\mu\text{M}$  nimodipine, 100 nM Agatoxin, 500 nM  $\omega$ -conotoxin, and 100 nM SNX-842), after addition of GluR blockers (25  $\mu\text{M}$  CNQX and 50  $\mu\text{M}$  D-APV), and then after an ASIC blocker (500  $\mu\text{M}$  amiloride). (Inset) EPSCs with an expanded y axis. (B) Representative traces (Upper) and mean  $\pm$  SEM (Lower) for conditions described for A ( $n = 5$  cells in 3 mice). (C and D) Similar experiments as in A and B, except the nonspecific  $\text{Ca}^{2+}$  channel blocker, 200  $\mu\text{M}$  CdCl<sub>2</sub>, was applied instead of the mixture of  $\text{Ca}^{2+}$  channel blockers ( $n = 6$  cells in 3 mice).

**Fig. S6.** Attenuation of LTP by increased pH buffer was not a result of inhibition of EPSPs or irreversible changes in brain slices. EPSPs recorded from lateral amygdala pyramidal neurons perfused with 90 mM  $\text{HCO}_3^-/15\%$   $\text{CO}_2$  at pH 7.4 ACSF. HFS (arrow at time 0) did not alter EPSPs ( $103 \pm 11\%$  baseline). At time indicated by bars, the solution was changed to 25 mM  $\text{HCO}_3^-/5\%$   $\text{CO}_2$  at pH 7.4. Then HFS was performed a second time ( $141 \pm 9\%$  baseline;  $P < 0.01$ ; Student *t* test). Representative EPSP traces at top were obtained at times indicated. Data are example from one cell; similar results were obtained in 8 cells from 4 mice.



**Fig. S7.** Extracellular application of pH 7.4 solution did not induce LTP. Application of pH 7.4 ACSF to amygdala pyramidal neurons did not induce LTP ( $100 \pm 1\%$  of baseline;  $n = 4$  cells in 3 mice).