A novel role for acid-sensing ion channels in Pavlovian reward conditioning

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Pavlovian fear conditioning has been shown to depend on acid-sensing ion channel-1A (ASIC1A); however, it is unknown whether conditioning to rewarding stimuli also depends on ASIC1A. Here, we tested the hypothesis that ASIC1A contributes to Pavlovian conditioning to a non-drug reward. We found effects of ASIC1A disruption depended on the relationship between the conditional stimulus (CS) and the unconditional stimulus (US), which was varied between five experiments. In experiment 1, when the CS preceded the US signaling an upcoming reward, Asic1a⁻/⁻ mice exhibited a deficit in conditioning compared to Asic1a+/+ mice. Alternatively, in experiment 2, when the CS coinitiated with the US and signaled immediate reward availability, the Asic1a⁻/⁻ mice exhibited an increase in conditioned responses compared to Asic1a+/+ mice, which contrasted with the deficits in the first experiment. Furthermore, in experiments 3 and 4, when the CS partially overlapped in time with the US, or the CS was shortened and coinitiated with the US, the Asic1a⁻/⁻ mice did not differ from control mice. The contrasting outcomes were likely because of differences in conditioning because in experiment 5 neither the Asic1a⁻/⁻ nor Asic1a+/+ mice acquired conditioned responses when the CS and US were explicitly unpaired. Taken together, these results suggest that the effects of ASIC1A disruption on reward conditioning depend on the temporal relationship between the CS and US. Furthermore, these results suggest that ASIC1A plays a critical, yet nuanced role in Pavlovian conditioning. More research will be needed to deconstruct the roles of ASIC1A in these fundamental forms of learning and memory.

KEYWORDS
ASIC1A, learning and memory, Pavlovian, reward

1 | INTRODUCTION

Classical Pavlovian conditioning is a fundamental form of learning and memory, during which an association is made between at least two previously unrelated stimuli, the conditional stimulus (CS) and unconditional stimulus (US).¹ The CS is neutral and does not evoke a response on its own. The US in contrast, evokes a response, the unconditional response (UR), independent of previous learning and can be either inherently aversive (eg, foot shock) or rewarding (eg, drug or food). With repeated CS-US pairings, a Pavlovian association is learned such that the CS acquires the ability to produce a response on its own, the conditional response (CR), that may resemble the UR.²

Accumulating data suggests that Pavlovian conditioning depends on acid-sensing ion channel-1A (ASIC1A), a member of the degenerin-epithelial Na⁺ channel (DEG-ENaC) family.³ ASIC1A is intrinsically pH-sensitive, permeable to cations including Na⁺ and Ca²⁺, and required for currents evoked by extracellular acidosis in central nervous system neurons.⁴⁻⁵ ASIC1A is expressed throughout the brain, including areas implicated in Pavlovian learning and memory such as the amygdala,⁴⁻⁶⁻⁸ bed nucleus of the stria terminalis,⁹⁻¹⁰ hippocampus,⁵,¹¹ insula¹² and nucleus accumbens (NAc).¹³ In addition,
ASIC1A has been localized to dendritic spines, and implicated in synaptic plasticity and neurotransmission mediated by protons released from neurotransmitter containing vesicles. Effects of ASIC1A disruption on dendritic spine density and glutamate receptor composition and function have been reported as well as effects on long-term potentiation and long-term depression. Thus, through its location and function, ASIC1A may be well positioned to influence Pavlovian conditioning.

Consistent with the above observations, effects of ASIC1A on Pavlovian conditioning to aversive stimuli have been described. Disrupting ASIC1A in mice reduced cued and contextual fear conditioning. These deficits were reproduced with amygda- specific ASIC1A disruption, and a deficit in contextual fear memory was rescued by expressing ASIC1A in the amygdala. In addition, ASIC1A disruption impaired eyelink conditioning and deficits in conditioned place avoidance to carbon dioxide (CO2), a paradigm in which mice learn to avoid a context paired with CO2, an aversive US. In contrast, Asic1a−/− mice acquired conditioned taste aversion normally, although extinction was impaired. Together, these findings suggest an important role for ASIC1A in Pavlovian conditioning to aversive stimuli.

Considering these findings, it is conceivable that ASIC1A may also play an important role in Pavlovian conditioning to rewarding stimuli. Supporting this possibility, ASIC1A is abundant in reward-related brain areas such as the NAc, and recent work indicates differential locomotor sensitivity to the effects of cocaine and enhanced conditioned place preference (CPP) to cocaine and morphine in mice lacking ASIC1A. Similarly, selective ASIC1A deletion in the NAc increases cocaine CPP, and restoring ASIC1A to the NAc of Asic1a−/− mice returns cocaine CPP to wild-type levels. These results suggest that ASIC1A reduces conditioning to drug rewards. Importantly, these effects contrast sharply with the apparent ability of ASIC1A to promote aversive conditioning. Thus, ASIC1A may have differential effects depending on whether the US is rewarding or aversive, although more study is needed to test this possibility.

Here, we used a non-drug reward to test the role of ASIC1A in Pavlovian conditioning. Given the effects of ASIC1A on conditioning to drugs of abuse, we hypothesized that loss of ASIC1A would enhance conditioning to a non-drug reward. However, our results suggest that this is not necessarily the case, as loss of ASIC1A either impaired or enhanced conditioning depending on the timing of the relationship between the CS and US.

2 | MATERIALS AND METHODS

2.1 Mice

Asic1a−/− mice were originally generated as described. Both the Asic1a−/− and Asic1a+/+ mice were generated from homozygous parents which are maintained on a congenic C57BL/6J background. The lines have been backcrossed to standard C57BL/6J mice from The Jackson Laboratory (JA) greater than 10 times, and are routinely backcrossed to this strain to maintain congenicity. Mice were housed in groups of 2 to 5 mice and kept on a 12-hour light-dark cycle, with all experiments occurring during the light cycle. Mice were fed standard chow and water ad libitum. All experimental groups were age- and sex-matched. Sample sizes are presented in figure legends and ranged from 20 to 26 mice per group. Animal care met the standards set by the National Institutes of Health, and all experiments were approved by the University of Iowa Animal Care and Use Committee.

2.2 Pavlovian conditioning apparatus

Pavlovian conditioning experiments were conducted in Med Associates operant chambers (21.6 × 17.8 × 12.7 cm; Med Associates, St. Albans, Vermont) contained within sound attenuating cubicles (Med Associates). Operant chambers were equipped with rod floors, two plexi-glass side walls, front and back metal walls, and a liquid dipper arm that delivered approximately 10 μL of vanilla-flavored Ensure (diluted in water to a 1:1 ratio) from a reservoir. On the back wall, there was a speaker (65 db, 2900 Hz). A house light was placed outside of the left Plexiglas side wall, providing light to the chamber. In the middle of the front wall was a food hole flanked on either side by lever slits, above which cue lights were mounted. For all experiments described here, the CS was a tone and cue light. The CS was always 5 seconds in duration except for experiment 4, when it was 0.5 seconds. The US was the Ensure solution presented for 15 seconds. The food hole was fitted with infrared photobeam sensors that counted the number and time of head entries into the food hole. Twenty-four hours before experiments began, mice were given the Ensure solution for reward habituation and to prevent neophobia during the experiment.

2.3 Chamber acclimatization

On the first day of each experimental paradigm, mice were acclimated to the operant chambers for 10 minutes, with the house light off, before beginning the session. At the 10-minute mark, the Med Associates program was initiated with the illumination of the house light signaling the beginning of the experimental session. The 10-minute acclimation period was excluded during subsequent sessions.

2.4 Pavlovian conditioning

A separate cohort of mice was used for each experiment. Asic1a+/+ and Asic1a−/− mice underwent a 45-minute training session per day consisting of 20 CS-US pairings over the course of eight experimental sessions. The temporal relationship between the CS and US was varied between experiments. The intertrial interval (ITI) between CS-US pairings varied, with an average ITI of 2 minutes. 24 hours after the final training session mice underwent an extinction probe test during which the experimental parameters were the same as training except that the CS was presented in the absence of the US.

2.5 Behavioral measures

Head entries were recorded by MED-PC software (Med Associates, Med-PC IV Version 4.2). The number of CS trials in which at least one head entry was recorded were quantified for each session of each experiment. Conditioned response latencies were assessed as the time to the first head entry after a CS presentation. Latencies were averaged
across all 20 trials in each session. CS trials in which no head entry occurred were given a default latency of 5 seconds. During training, CR scores were calculated by subtracting the head entry rate during the ITI from the head entry rate during the CS, adapted from.23 For the extinction test, CR scores were calculated by subtracting the head entry rate during 15 seconds of ITI immediately preceding the CS from the head entry rate during the 15 seconds following CS onset (the time period during training when the US would have been present).

2.6 Statistical analysis

All values are plotted as mean ± Standard Error of the Mean (SEM). For all tests, P < 0.05 was considered significant. One-way analysis of variance (ANOVA) was used to assess differences in head entry responses over time for a given genotype. Two-way ANOVA was used to test for interactions between genotype and time, or genotype and sex. When significant effects were observed in the context of the full ANOVA, post hoc Fisher’s Least Significant Difference (LSD) was used to identify differences between genotypes during individual sessions. For the extinction experiment, unpaired t test was used to compare average CR scores between genotypes, and Wilcoxon signed rank test was used to determine if group medians differed significantly from 0. All analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA USA, version 7) software.

3 RESULTS

3.1 ASIC1A disruption impairs conditioning to nondrug reward

To test the hypothesis that ASIC1A plays an important role in Pavlovian reward conditioning, we started with a task similar to that described previously by others23–25 in which the CS preceded the US, a highly palatable liquid food reward (vanilla-flavored Ensure) (Figure 1A). We compared Asic1a+/+ and Asic1a−/− mice across sessions consisting of 20 CS-US pairings per session for eight experimental sessions. We assessed head entries during the CS and found a statistically significant genotype by time interaction (Figure 1B) (F[7,287] = 2.324, P = 0.0255). Furthermore, this effect was driven by the increased head entries in the Asic1a+/+ group (F[1,140] = 3.042, P = 0.0052) but not the Asic1a−/− group, which failed to increase head entries over the eight training sessions (F[1,140] = 1.168, P = 0.3324). To rule out whether the difference between genotypes was because of multiple head entries per trial, we also quantified the number of trials with a head entry (Figure 1C), and continued to observe a statistically significant genotype by time interaction (F[7,287] = 3.274, P = 0.0023). We also quantified conditioned response latency, defined as time from CS onset to the first subsequent head entry, and found conditioned response latencies mirrored the head entry results, such that the Asic1a+/+ mice developed significantly shorter latencies than Asic1a−/− mice across training sessions (Figure 1D) (F[7,287] = 3.644, P = 0.0009).

We next examined whether sex affected the outcome, and found no effect. For example, during the eighth experimental session, there was an effect of genotype (F[1,39] = 4.882, P = 0.0331) but no effect of sex (F[1,39] = 0.1622, P = 0.6893), and no interaction F[1,39] = 0.665, P = 0.4198). Similar analyses of sex were conducted for all subsequent experiments, and no effect was observed (data not shown).

Because the CS signaled upcoming reward availability, we further assessed whether the differences in head entries during the CS translated into differences in head entries when the reward was actually available (Figure 1E). There was an effect of genotype (F[1,41] = 4.829, P = 0.0337) and an effect of time (F[7,287] = 5.918, P < 0.0001), but no
3.2 | ASIC1a<sup>−/−</sup> mice exhibit greater responses when CS and US coinitiate and overlap

To test whether the behavioral differences in the ASIC1a<sup>−/−</sup> mice might be sensitive to the temporal relationship between the CS and US, we performed a second experiment with a new cohort of mice, in which we altered the timing of the CS and US presentations. In this paradigm, the CS onset signaled that the reward was immediately available (Figure 2A), and hence minimized the delay between the start of the CS and the ability to sample the US. Otherwise, this second experiment was identical to the first. Over eight training sessions, we found that both genotypes increased head entries during the CS (Figure 2B) (ASIC1a<sup>+/+</sup>, \(F_{7,182} = 15.2, P < 0.0001\); ASIC1a<sup>−/−</sup>, \(F_{7,186} = 30.77, P < 0.0001\)). Moreover, the ASIC1a<sup>−/−</sup> mice exhibited more head entries than the ASIC1a<sup>+/+</sup> mice (genotype by time interaction, \(F_{7,350} = 2.83, P = 0.0070\)). Similar results were observed when we quantified CS trials with a head entry (Figure 2C) (ASIC1a<sup>+/+</sup>, \(F_{7,350} = 4.737, P < 0.0001\)) and CS latency (Figure 2D) (ASIC1a<sup>+/+</sup>, \(F_{7,350} = 3.191, P = 0.0027\)). We also examined head entries after the CS, but while the US was still available (10 seconds) and found that head entries did not differ between genotypes (Figure 2E). There was an effect of time (\(F_{1,50} = 7.068, P < 0.0001\)) but no effect of genotype (\(F_{1,50} = 1.331, P = 0.2541\)) and no interaction (\(F_{7,350} = 0.8918, P = 0.5130\)). The observations above were supported by head entry distributions during CS-US presentations (Figure 2F). Therefore, in contrast to the first experiment, disrupting ASIC1A improved CS-evoked responses in experiment 2. These findings suggest that ASIC1A disruption can have differing effects that depend on the temporal relationship between the CS and US.

3.3 | Sensitivity to CS-US relationship

The opposing effects of ASIC1A disruption between the first and second experiments led us to ask what aspects of the CS-US relationship might be responsible. Two features of the second experiment differed from the first. One difference was that in the first experiment there was no overlap between the CS and US, whereas in the second experiment the CS and US overlapped in time. Another difference was that in the second experiment the CS and US were coinitiated. Therefore, to test whether the overlap or coinitiation of the CS and US mattered, we performed two additional experiments, referred to as experiments 3 and 4. In experiment 3, the CS preceded the US by 2.5 seconds and overlapped with the US by 2.5 seconds (Figure 3A). In experiment 4, the CS was shortened to 0.5 seconds and was coinitiated with the US (Figure 4A). Other aspects of experiments 3 and 4 were identical to experiments 1 and 2, including the duration of the US. We hypothesized that either the overlap or coinitiation would produce an effect similar to experiment 2 and lead to more head entries in the ASIC1a<sup>−/−</sup> mice.

In experiment 3, we found evidence of conditioning in both ASIC1a<sup>+/+</sup> and ASIC1a<sup>−/−</sup> mice, suggested by the increase in head entries during the CS with training (Figure 3B) (ASIC1a<sup>+/+</sup> mice,

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FIGURE 2  Experiment 2. A, co-initiating and overlapping CS and US. B, Comparison of head entries during the CS over eight experimental sessions in ASIC1a<sup>+/+</sup> and ASIC1a<sup>−/−</sup> mice. ASIC1a<sup>−/−</sup> mice exhibit significantly more conditioned head entries than ASIC1a<sup>+/+</sup> mice. C, Comparison of CS trials with a head entry. ASIC1a<sup>−/−</sup> mice exhibit significantly more CS trials with a head entry than ASIC1a<sup>+/+</sup> mice. D, Comparison of conditioned response latency. ASIC1a<sup>−/−</sup> mice exhibit a significant decrease in conditioned response latency than ASIC1a<sup>+/+</sup> mice. E, Comparison of head entries during the last 10 seconds of the US. ASIC1a<sup>−/−</sup> and ASIC1a<sup>−/−</sup> mice exhibit a similar increase in head entries. F, Head entry distributions during the CS and US in sessions 1 and 8. (Fisher’s LSD, *P < 0.05, **P < 0.001; n = 26 ASIC1a<sup>+/+</sup> mice, 26 ASIC1a<sup>−/−</sup> mice)
Experiment 3. Overlapping CS (A) and US (B) comparison of head entries during the CS over eight experimental sessions in Asic1a+/+ and Asic1a−/− mice. Asic1a+/+ and Asic1a−/− mice exhibit a similar increase in head entries. C, Comparison of CS trials with a head entry. Asic1a+/+ and Asic1a−/− mice exhibit a similar increase in CS trials with a head entry. D, Comparison of conditioned response latency. Asic1a+/+ and Asic1a−/− mice exhibit a similar decrease in conditioned response latency. E, Comparison of head entries during the last 12.5 seconds of the US. Asic1a+/+ and Asic1a−/− mice exhibit a similar increase in head entries across training days. F, Head entry distributions during the CS and US in sessions 1 and 8. (n = 27 Asic1a+/+, 25 Asic1a−/−)

\[ F_{(7,154)} = 8.573, \ P < 0.0001; \text{ Asic1a}^{-/-} \text{ mice, } F_{(7,133)} = 6.321, \ P < 0.0001. \] In addition, while there was an effect of time \( (F_{(1,287)} = 13.21, \ P < 0.0001) \), there was no effect of genotype \( (F_{(1,287)} = 2.526, \ P = 0.1196) \) and no interaction \( (F_{(1,287)} = 1.583, \ P = 0.1400) \). Similar outcomes were observed upon quantification of CS trials with a head entry (Figure 3C) (time \( F_{(7,287)} = 25.6, \ P < 0.0001; \text{ genotype } F_{(1,41)} = 0.1108, \ P = 0.7409; \text{ interaction } F_{(7,287)} = 0.8823, \ P = 0.5207) \) and CS latency (Figure 3D) (time \( F_{(7,287)} = 19.38, \ P < 0.0001; \text{ genotype } F_{(1,41)} = 1.542, \ P = 0.2214; \text{ interaction } F_{(7,287)} = 1.24, \ P = 0.2809). \) As for head entries during the US (Figure 3E), there was an effect of time \( (F_{(7,287)} = 14.32, \ P < 0.0001) \) but no effect of genotype \( (F_{(1,41)} = 0.2271, \ P = 0.6362) \) and no interaction \( (F_{(7,287)} = 1.249, \ P = 0.2758) \). Head entry distribution across CS-US presentations (Figure 3F) paralleled the results in Figures 3B, C, and E and suggested similar learning in both genotypes. Together, these data suggest that overlapping the CS and US resulted...

Experiment 4. A, Short CS coinitiating with US. B, Comparison of head entries during the CS and the next 4.5 seconds across experimental sessions in Asic1a+/+ and Asic1a−/− mice. Asic1a+/+ and Asic1a−/− mice exhibit a similar increase in head entries. C, Comparison of CS trials with a head entry. Asic1a+/+ and Asic1a−/− mice exhibit an increase in CS trials with a head entry. D, Comparison of conditioned response latency. Asic1a+/+ and Asic1a−/− mice exhibit a similar decrease in conditioned response latency. E, Comparison of head entries during the last 10 seconds of the US. Both Asic1a+/+ and Asic1a−/− mice exhibit an increase in head entries across training days. F, Head entry distributions during the CS and US in sessions 1 and 8 (Fisher’s LSD, **\( P < 0.001; \ n = 23 \text{ Asic1a}^{+/+}, 20 \text{ Asic1a}^{-/-} \))
in at least some conditioning in the Asic1α−/− mice. However, overlapping the CS and US was not sufficient to reproduce the effects in experiment 2, in which the Asic1α−/− mice exhibited significantly more head entries than wild-types during the CS.

In experiment 4, in which a short CS was co-initiated with the US, we assessed head entries during the 0.5 seconds CS plus the following 4.5 seconds, and also found evidence of conditioning to the CS in both groups (Figure 4B) (Asic1α+/+ mice, \( F_{[7,119]} = 4.737, P < 0.0001 \); Asic1α−/− mice, \( F_{[7,133]} = 4.712, P < 0.0001 \)). However, while there was an effect of time (\( F_{[7,252]} = 8.819, P < 0.0001 \)), there was no effect of genotype (\( F_{[1,36]} = 0.2541, P = 0.6173 \)) and no interaction (\( F_{[7,252]} = 0.5431, P = 0.8013 \)). Similar outcomes were observed upon quantification of CS trials with a head entry (Figure 4C) (time \( F_{[7,252]} = 12.16, P < 0.0001 \); genotype \( F_{[1,36]} = 0.2039, P = 0.6543 \); interaction \( F_{[7,252]} = 0.3724, P = 0.9179 \)) and CS latency (Figure 4D) (time \( F_{[7,252]} = 10.45, P < 0.0001 \); genotype \( F_{[1,36]} = 0.3303, P = 0.5691 \); interaction \( F_{[7,252]} = 0.358, P = 0.9257 \)). When we assessed head entries during the US, we found a significant genotype by time interaction (\( F_{[7,252]} = 2.985, P = 0.0050 \)) (Figure 4E). The timing of head entry distribution during CS-US presentations for sessions 1 and 8 is also shown (Figure 4F). Together, the results of experiment 4 suggest that co-initiating the CS and US by itself was also insufficient to reproduce the elevated CS responses in the Asic1α−/− mice observed in experiment 2. Thus, in experiment 2 both co-initiation and overlap of the CS and US may have combined to produce the exaggerated CS-associated head entries in the Asic1α−/− mice.

### 3.4 Dependence on CS-US pairing

The results thus far led us to ask to what degree the previous outcomes depended on learning the association between the CS and US. Therefore, to further assess the role of ASIC1A in Pavlovian reward conditioning, we performed a final experiment in which the CS and US were explicitly unpaired (Figure 5A). Here, the CS never immediately preceded or overlapped with reward delivery, and thus did not predict that a reward was available. We hypothesized that if the results of the previous experiments reflected conditioning, then responses to the CS should not increase over time when the CS and US are explicitly unpaired. Over eight experimental sessions, we assessed head entries during the CS (Figure 5B) and found that neither genotype increased head entry responses over time (Asic1α+/+ mice, \( F_{[7,147]} = 1.69, P = 0.1156 \); Asic1α−/− mice, \( F_{[7,168]} = 0.5737, P = 0.7766 \)). Similarly, no improvement with training was observed in the Asic1α+/+ or Asic1α−/− mice in CS trials with a head entry (Figure 5C) (\( F_{[7,315]} = 1.542, P = 0.1524 \)) or CS latency (Figure 5D) (\( F_{[7,315]} = 1.646, P = 0.1220 \)). Head entry distribution during the CS presentations also suggested no conditioning to the unreinforced CS (Figure 5E). Together these observations suggest that in the previous experiments, the behavioral responses during the CS did indeed reflect Pavlovian conditioning.

Interestingly, when we analyzed head entries during the US (Figure 5F-H), there was an effect of time in both genotypes (Figure 5F: Asic1α+/+ mice \( F_{[7,147]} = 13.31, P < 0.0001 \); Asic1α−/− mice \( F_{[7,168]} = 13.79, P < 0.0001 \)) (Figure 5G: Asic1α+/+ mice \( F_{[7,147]} = 7.186, P < 0.0001 \); Asic1α−/− mice \( F_{[7,168]} = 11.31, P < 0.0001 \)). Thus, despite the absence of conditioning to the CS, both groups of mice still acquired an improved ability to locate the reward across training sessions, suggesting the animals adopted an alternative learning strategy, independent of the explicit cues. Of note, there was a genotype by time interaction when we quantified the responses during the first 5 seconds of the US (Figure 5F) (\( F_{[7,315]} = 2.526, P = 0.0153 \)) and when we quantified responses during the entire 15 seconds of the US (Figure 5G) (\( F_{[7,315]} = 2.505, P = 0.0161 \)), which was largely because of increased head entries in the Asic1α−/− mice during sessions 4 through 6. This observation might suggest US-associated learning also depended on ASIC1A, although by the end of training (session 8) the two genotypes did not differ.

### 3.5 Conditioned response scores

To account for nonspecific head entries during the ITI, for each experiment, we also assessed CR score, adapted from. Using this score (Figures 6A,B), the results closely paralleled CS-associated head entries (Figures 1–5). Figure 6 further illustrates the uniqueness of experiment 2, in which the CS and US were co-initiated and overlapped for 5 seconds; this paradigm produced the greatest increase in CR score in both genotypes, as observed by comparing CR scores for experiment 2 to the experiment with next highest CR score (Figure 6A, B) (Asic1α+/+ mice, experiment 2 vs experiment 1, \( F_{[1,46]} = 12.1, P = 0.0011 \); Asic1α−/− mice, experiment 2 vs experiment 3, \( F_{[1,43]} = 61.67, P < 0.0001 \)). Moreover, these data further illustrate the difference in experiment 2 between the Asic1α−/− mice and their Asic1α+/+ counterparts (\( F_{[7,350]} = 3.245, P = 0.0024 \)).

### 3.6 Conditioned responses in the absence of reinforcement

To further assess conditioning, we quantified CR scores when CS presentations were not reinforced with a US during a single extinction session 24 hours after the final training session (Figure 7). In the absence of reinforcement, the head entries evoked by the CS provided a measure of conditioning, although concurrent extinction also likely reduced conditioned responses. Evidence of Pavlovian conditioning was regarded as a mean CR score significantly greater than zero. In all five experiments, only three groups met this criterion for conditioning (Figure 7A); these consisted of the Asic1α+/+ mice in experiment 1 (Wilcoxon signed rank test, \( W = 128, P = 0.0244 \)), the Asic1α−/− mice in experiment 2 (\( W = 297, P < 0.0001 \)), and the Asic1α−/− mice in experiment 2 (\( W = 331, P < 0.0001 \)). With the other measures of conditioning presented in the preceding figures, the greatest CR scores were exhibited by the Asic1α−/− mice in experiment 2, which were significantly greater than their Asic1α+/+ counterparts (\( F_{[51]} = 2.235, P = 0.0229 \)). For a more granular perspective, we quantified average distribution of head entries for all 20 unreinforced CS trials for experiments 1, 2 and 5 (Figure 7B) and CS-evoked responses vs trial number (Figure 7C). In both of these figures, experiment 2 stood out for producing the most striking CS-evoked responses and for elevated CS-evoked head entries in the Asic1α−/− mice. Extinction is harder to discern because of variability in head entry responses across trials (Figure 7C).
To our knowledge, these experiments are the first to assess the role of ASIC1A in Pavlovian reward conditioning to a nondrug reward. The results are consistent with the hypothesis that ASIC1A plays a role in reward conditioning. However, the effects of ASIC1A disruption were more nuanced than initially anticipated. Consistent with previous work in which Asic1a−/− mice exhibited increases in conditioning in some paradigms (cocaine CPP), yet decreases in conditioning in others (fear conditioning), here we found evidence of both impairment and enhancement. Interestingly, in these experiments the role played by ASIC1A was sensitive to relatively subtle variations in the temporal relationship between the CS and US. In experiment 1, when the CS preceded the US, Asic1a−/− mice showed reduced head entry...
responses compared to Asic1a+/+ mice, suggesting that the Asic1a−/− mice did not learn the CS-US relationship normally. In contrast, in experiment 2, when the CS coinitiated with the US and signaled that the reward was immediately available, the Asic1a−/− mice had more head entries, suggesting that their conditioning was enhanced. To test whether the timing of the CS and US mattered, we further manipulated the temporal relationship between the CS and US in experiments 3 and 4, and in these paradigms, we observed no apparent differences between the Asic1a+/+ and Asic1a−/− mice. Taken together, these experiments suggest that the loss of ASIC1A may provide advantages in certain reward learning situations but may also confer disadvantages in other situations, such as when there is no overlap between the CS and US.

To confirm that the responses observed during the CS reflected conditioning, experiment 5 tested explicit unpairing of the CS and US, and thus provided an important control for experiments 1 to 4. As expected, in experiment 5 head entries did not increase over time in response to the unpaired CS. However, head entries during the US did increase over time, suggesting that in the absence of explicit cues the mice adopted an alternative learning strategy. Unfortunately, our experimental design did not allow us to identify how the animals acquired the ability to find the reward. We suspect the mice may have used implicit cues, such as the odor of the Ensure reward, which we could not eliminate. A recent paper suggests ASIC1A disruption may impair olfaction (26); however, we have previously found no differences of Asic1a−/− mice compared to matched Asic1a+/+ mice in the ability to locate buried food by its odor, to detect scent from mice of the opposite sex, and to avoid the aversive odor of butyric acid.27 Regardless of these considerations of olfaction, the learning observed in the unpaired paradigm peaked earlier in the Asic1a−/− mice, which by itself is an interesting observation as it suggests that this alternative learning might also be sensitive to ASIC1A-disruption. Importantly, the behavioral responses to the unpaired US in experiment 5 did not reach the high levels observed in experiment 2 when the US overlapped and coinitiated with the CS, suggesting that the results depended on the CS-US pairing.

One advantage of our approach was that the vanilla-flavored Ensure produced sufficient motivation to support Pavlovian conditioning without food or water restriction, which would have altered appetitive drive. Because the CS-associated responses in the Asic1a−/− mice were reduced, increased, or normal depending on the paradigm, it is unlikely that the differences seen here were because of

![Graphs showing CR scores and head entries](image-url)
abnormalities in motivation. Supporting comparable appetitive drive for food and water, the average weight of Asic1a−/− mice does not differ from Asic1a+/− mice. In addition, because the CS and US remained the same, and the experiments differed primarily in the timing of the CS and US presentation, it is unlikely that observed behavioral differences were because of sensory dysfunction, inability to detect the CS, or an inability to express head entries. Furthermore, we have previously found hearing to be grossly normal in the Asic1a−/− mice, whereas others have not observed vision-based abnormalities that would disrupt detection of visual cues in our experiments.

It is interesting to consider how the present results might relate to our previous findings with Asic1a−/− mice in Pavlovian fear conditioning and CPP. The results here suggest that altering the timing of the CS-US relationship might influence the outcomes in those other paradigms. However in those conditioning paradigms, the nature of the CS and US was very different than those studied here. For example, in fear conditioning, the CS was 20 seconds (auditory tone) or continuous (context), and the footshock (US) was only 1 second. Also, with CPP, the CS (context) was continuous and overlapped with the US (drug reward), although the temporal relationship between the CS and US was not as precise as the relationship here. Nevertheless the results in this manuscript suggest it may be informative to vary the CS-US relationships in those other aversive and appetitive conditioning paradigms.

Although we cannot presently rule out altered brain development as the cause of the behavioral differences observed here, we have previously found that brain morphology and structure are grossly normal in Asic1a−/− mice. Moreover, restoring ASIC1A expression to specific brain sites in adult Asic1a−/− mice normalizes several behaviors and physiological measures, including cocaine CPP and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-to-N-methyl-D-aspartate (NMDA) receptor ratio. Similarily, disrupting or inhibiting ASIC1A in adult mice produces effects resembling those observed in the Asic1a−/− mice in multiple behaviors. Thus, ASIC1A has important effects on behavior and brain function that are independent of brain development.

It is not yet clear where in the brain ASIC1A acts to affect the behaviors studied here. Candidate sites include the amygdala and NAc, which are implicated in various features of Pavlovian learning and where ASIC1A plays a role in Pavlovian conditioning. Additional candidate sites include the lateral hypothalamus and insula, as these sites express ASIC1A and are implicated in the acquisition and expression of conditioned head entry behaviors. Additional work will be needed to determine precise sites of ASIC1A action in Pavlovian reward conditioning.

As for subcellular sites of action, we suspect that the synapse is the best candidate in the behaviors studied here. Recently, a portion of the excitatory postsynaptic current (EPSC) was found to depend on ASIC1A. This cation current is sensitive to ASIC antagonists, pH buffering capacity, and carbonic anhydrase inhibitors, and has been reported in the NAc, lateral amygdala, and Calyx of Held. Protons released from synaptic vesicles during neurotransmission provide the most-likely mechanism for activating ASIC1A during the EPSC. Perhaps related to the ASIC1A-dependent synaptic current, we found a number of synaptic abnormalities in NAc medium spiny neurons following ASIC1A disruption including increases in: AMPA-to-NMDA receptor ratio, AMPA receptor rectification index, miniature EPSC frequency, and dendritic spine density. Also consistent with a synaptic role for ASIC1A, loss or inhibition of ASIC1A reduces long-term potentiation in the amygdala and hippocampus, (but see )

Loss of ASIC1A is also implicated in long-term depression in the insular cortex. Thus, ASIC1A is implicated in both synaptic potentiation and depression in multiple brain areas, which might help explain the diverse effects of ASIC1A disruption observed in these studies.

In conclusion, more needs to be learned about how ASIC1A contributes to brain function and behavior. It will be valuable to discern how disrupting ASIC1A can reduce some types of learning and memory, yet enhance others. Because the structure and function of these channels are well conserved between species, including humans, we expect that ASIC1A plays an important role in the human brain. Thus far, the human Asic1a gene has been implicated in panic disorder in studies with limited sample sizes, but the importance of Pavlovian conditioning in human disorders and the effects of ASIC1A on Pavlovian conditioning in mice suggests that ASIC1A will ultimately be shown to play roles in a variety of psychiatric illnesses.

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CONFLICTS OF INTEREST
The authors declare no competing financial interests.

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