Intrinsic neuronal excitability is reversibly altered by a single experience in fear conditioning

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Running Head (55 characters+spaces): Intrinsic excitability in fear learning and extinction

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Abstract
Learning is known to cause alterations in intrinsic cellular excitability, but to date, these changes have only been seen after multiple training trials. A powerful learning task which can be quickly acquired and extinguished with a single trial is fear conditioning. Rats were trained and extinguished on a hippocampus-dependent form of fear conditioning in order to determine whether learning related changes in intrinsic excitability could be observed after a few training trials and a single extinction trial. Following fear training, hippocampal slices were made and intrinsic excitability was assayed via whole cell recordings from CA1 neurons. Alterations in intrinsic excitability, assayed by the post-burst AHP and firing frequency accommodation, were observed after only three trials of contextual or trace-cued fear conditioning. Animals which had been trained in contextual and trace-cued fear were then extinguished. Context fear conditioned animals extinguished in a single trial, and the changes in intrinsic excitability were reversed. Trace cue conditioned animals only partially extinguished in a single trial and reductions in excitability remained. Thus, a single learning experience is sufficient to alter intrinsic excitability. This dramatically extends observations of learning-specific changes in intrinsic neuronal excitability previously observed in paradigms requiring many training trials, suggesting the excitability changes have a basic role in acquiring new information.
Introduction

An important regulator of intrinsic excitability is the post-burst afterhyperpolarization (AHP), a calcium-dependent potassium current which hyperpolarizes the membrane following a burst of action potentials (Alger and Nicoll 1980; Hotson and Prince 1980; Schwartzkroin and Stafstrom 1980). Learning-related increases in cellular excitability have been demonstrated across a variety of species, tasks, and brain regions (for review, see Disterhoft and Oh 2006). In these studies, an animal is trained in a task, and cellular excitability is assessed by measuring the AHP and accommodation. Learning-related increases in excitability have been demonstrated following trace eyeblink conditioning in hippocampus CA1 (Moyer et al. 1996) and CA3 (Thompson et al. 1996) in rabbits and CA1 of rats (Kuo et al. 2008). Spatial learning is correlated with reductions in the AHP, as measured in rats following watermaze learning (Oh et al. 2003; Tombaugh et al. 2005). Increases in excitability are also observed in piriform cortex following odor discrimination learning in rats (Saar et al. 1998). The tasks named above all require numerous repeated trials before intrinsic plasticity is seen, but rapid changes in intrinsic excitability can be elicited using in vitro preparations (Kaczorowski et al. 2007; Zhang and Linden 2003). Changes in intrinsic excitability have not yet been demonstrated after a single learning experience.

Fear conditioning takes advantage of an animal’s natural fear response by pairing a neutral stimulus, such as an environment or a tone, with an aversive stimulus, such as a shock. While the amygdala is critical for learning this task (Blanchard and Blanchard 1972; LeDoux 2000), certain forms also require the hippocampus. The hippocampus is
required for learning contextual (Kim and Fanselow 1992) and trace cued (McEchron et al. 1998) fear conditioning, and is important in the extinction of contextual fear conditioning (Corcoran et al. 2005). In neurons of the infralimbic prefrontal cortex, the AHP and accommodation are increased after learning (Santini et al. 2008). A learning-related decrease in excitability in these cells is consistent with the region’s potential role in providing a tonic brake on fear expression in the amygdala (Maren and Holt 2000, Corcoran and Maren 2001). Extinction reversed the increase in the AHP (Santini et al. 2008). Middle-aged mice which learn contextual fear conditioning have reduced AHPs in CA1 pyramidal neurons compared to controls that failed to learn (Kaczorowski and Disterhoft 2009).

We sought to determine if learning hippocampus-dependent forms of fear conditioning using very few training trials is accompanied by altered excitability of CA1 pyramidal neurons. We found that the AHP was reduced after both trace cued and contextual fear conditioning as compared to control conditions. In addition, the learning-related increase in excitability was reset to naïve levels after a single extinction trial, at a time point when freezing to the learned context was abolished. These data are a compelling demonstration that intrinsic excitability, as assayed by the AHP and firing frequency accommodation, can be modulated by a single experience.
Materials & Methods

Animals

Two to three month old males of the F1 generation of Fisher 344 X Brown Norway rats from Harlan were used. Fifty-nine animals were trained in the study, divided between 3 cohorts, plus 6 naïve animals. Rats were housed in small groups with ad libitum access to food and water on a 14/10 hour light/dark cycle. Rats were handled and housed in accordance with the standards established by the Institutional Animal Care and Use Committee of Northwestern University and NIH.

Behavior

Rats were divided into three cohorts; each cohort was subdivided between the behavioral paradigms. The Trained cohort was trained in the fear task for 3 trials spaced over 2 days, then sacrificed on day 3 with no additional testing. The Tested cohort was trained in the fear task for 3 trials over 2 days, given a single testing trial for both tone and context on the 2nd day, then sacrificed on day 3. The Extinguished cohort were also trained in the fear task for 3 trials over two days, and were tested/extinguished for both the tone and context three times over days 2 and 3. Hippocampal slices were made from the Extinguished cohort 18-24 hours after the final testing trial.

Within the cohorts, animals were randomly assigned to tone alone (Tone), shock alone (Shock), or paired (Paired) training paradigms; the Extinguished cohort did not include a tone alone group. In each training trial, Tone animals were presented with one tone,
Shock animals were presented with one shock, and Paired animals received a tone and shock separated by a 30sec “trace” stimulus free interval. Naïve animals were neither handled nor exposed to the training context.

Training took place in a 40cmx40cmx40cm clear plastic box with a grid floor and an open top in a curtained 3mx3m room with movable cues. The stimuli presented consisted of a 15s, 4 kHz 75dB tone and a 1s, 0.8mA shock delivered through the floor, as appropriate. All trials lasted 226s, with the tone (if used) presented 90s after the rat was placed in the chamber and the shock (if used) presented 136s after the rat was placed in the chamber. Testing was divided into two parts, each also lasting 226s. First, animals were tested for contextual learning, during which time they were placed in the original context, no stimuli were presented, and freezing was measured. To test cue learning, animals were placed in a clear plastic chamber in which a new floor had been placed (wood shavings on a plastic floor), the distal cues were changed, a novel scent (banana extract) was added, and the lighting was altered. The tone stimulus was presented in this novel context and freezing was measured.

Freezing behavior was video recorded for the duration of training & testing sessions and analyzed using FreezeFrame software (Actimetrics, Willmette, IL). FreezeFrame uses a pixel based algorithm to detect motion. Minimum freezing bout length was set at 1 second; the movement threshold was determined based on a histogram analysis of total movement during the entire 226 second trial. This allowed each animal’s freezing threshold to be standardized to its over-all activity level. Freezing behavior during
training and the context test was averaged over the 135s preceding the shock onset. Freezing behavior during the tone test was averaged over the 135s following the tone presentation.

Electrophysiological Recordings

Eighteen to 24 hours after training and testing were complete, rats were anesthetized by isofluorane and decapitated. The brains were quickly removed and immersed in ice cold artificial cerebrospinal solution (aCSF) consisting of (in mM) 124 NaCl, 1.25 NaH2PO4, 2.5 KCl, 26 NaHCO3, 25 glucose, 2.4 CaCl2, and 2.0 MgSO4. These solutions were saturated with 95% O2/5% CO2 to maintain a pH of 7.4 and to oxygenate slices. The dorsal half of the hippocampus was dissected out and sliced into 300μm thick sections using a Leica vibratome. Slices were held at 34˚C for 30 minutes, and then allowed to sit at room temperature (~22˚C) until biophysical recordings were made (at least one hour after slicing). The experimenter was blind to the training status of the animal during recording and analysis.

CA1 pyramidal neurons were visually identified for whole cell current clamp recordings. Recordings were made at 32˚C. Patch electrodes contained (in mM): 120 KMeSO4, 10 KCl, 10 HEPES, 10 phosphocreatine sodium salt, 4 ATP magnesium salt, 0.4 GTP sodium salt and 0.5% neurobiotin with pH corrected to 7.4 with KOH and osmolarity of 285 ± 5mOsm. Neurons were included if they had a resting membrane potential of less than -58mV, an input resistance greater than 25MΩ, action potential amplitude of greater than 80mV from rest, and stable series resistance of less than 20MΩ. Electrode
capacitance and series resistance were monitored and compensated throughout recording; cells were held at or near -65mV with injected current. Data was collected using a Dagan BVC-700 amplifier and PClamp 9.2 (Axon), and digitized using a Digidata 1322A A-to-D converter. Data was analyzed using PClamp 9.2 (Axon).

The AHP was elicited by injecting 2ms current pulses of 1.8nA, fifteen times, at 50Hz in order to elicit fifteen action potentials. The amplitude of the AHP was measured relative to baseline at its peak (peak AHP) and 1s following the end of the final action potential (slow AHP). The integrated area of the AHP was measured from the end of the last action potential in the train until the membrane potential returned to baseline levels.

Accommodation was measured by giving a 1000ms current step of sufficient strength to elicit 5 action potentials in the first 100ms. The total number of action potentials in the entire step was counted. A single action potential was elicited to measure the spike threshold, rheobase, and amplitude. I-V relations were studied using an 800ms current injection (-0.3 to +0.1 nA). Sag was measured as the difference between the peak and steady-state hyperpolarization in response to a 800ms, -300pA current step. The input resistance was calculated as the slope of the line of the current plotted against the voltage at the last 100msec of the current step.

Statistics

Statistics were performed using Microsoft Excel and StatView. Differences were evaluated using t-tests, one-way ANOVA, repeated measures ANOVA, and Fischer's PLSD post hoc tests where appropriate. All data are reported as mean ± SEM.
Results
Young adult male rats were divided into Trained, Tested, and Extinguished cohorts. Within cohorts, groups were Naïve, tone-contextual control (Tone), contextually conditioned (Shock), or trace-cue conditioned (Paired). In each group, rats were placed in the training apparatus for three 226 second trials spaced over two days. A trial consisted of a 90s baseline, a 15s tone, 30s trace interval, 1s shock and a 90s post-shock period, as appropriate (Figure 1A, 2A, and 3A). Fear was measured by freezing. In the Trained cohort, Shock and Paired groups showed contextual fear conditioning as measured during each training session in the baseline period prior to the shock (Figure 1B) (repeated measures ANOVA, Paired – $F_{(2,10)}=16.744, p=0.0006$, Shock – $F_{(2,10)}=10.496, p=0.004$). Rats from the Tested cohort also developed contextual fear conditioning during training (Figure 2B) (repeated measures ANOVA, Paired – $F_{(2,14)}=20.504, p<0.0001$, Shock – $F_{(2,10)}=6.551, p=0.02$), as did rats from the Extinguished cohort (Figure 3B) (repeated measures ANOVA, Paired – $F_{(2,18)}=13.955, p=0.0002$, Shock – $F_{(2,16)}=6.412, p=0.009$). Rats from the Tested and Trained cohorts also showed contextual learning in comparison to the tone-alone group during the final trial (ANOVA, Trained – $F_{(2,17)}=4.775, p=0.023$, Tested – $F_{(2,17)}=3.793, p=0.04$). Detailed freezing averages are in the Supplemental Table.

Intrinsic excitability was measured 24 hours after the last training trial from animals in the Trained cohort. The AHP was elicited by fifteen action potentials and was measured at its peak and 1 second later, reflective of the slow AHP. For both the peak and slow AHP, CA1 pyramidal neurons from Shock and Paired animals were
significantly more excitable than those from *Naïve* and *Tone* animals (ANOVA, Peak AHP: $F_{(3,85)}=6.917, p=0.0003$; slow AHP: $F_{(3,85)}=5.039, p=0.0019$) (Figure 1C). The integrated area of the AHP was also significantly smaller in cells from *Paired* and *Shock* animals than in *Tone* and *Naïve* (data not shown, $F_{(3,85)}=3.600, p=0.017$, *Paired* - 7.64±0.65 mV*s, *Shock* -8.06±0.79 mV*s, *Tone* -9.52±0.54 mV*s, *Naïve* -10.67±0.82 mV*s). Distributions of these data can be seen in the Supplemental Figure.

To measure accommodation, cells were depolarized for 1000ms with a current sufficient to elicit 5 action potentials in the first 100ms. Cells from *Paired* and *Shock* animals fired significantly more action potentials in response to this step than cells from *Tone* or *Naïve* controls (ANOVA: $F_{(3,72)}=8.698, p<0.0001$) (Figure 1D). There was no significant difference between the groups in the amount of current needed to elicit the first five action potentials ($F_{(3,55)}=1.47, p=0.23$). Further, no differences between all cells recorded were observed in the resting membrane potential, the threshold for action potential firing, sag, or the input resistance (see Table 1). The AHP and accommodation measures confirm that intrinsic excitability is increased in those animals which learn either contextual or trace-cued fear.

Learning can be more thoroughly measured by placing the animal in the original context, to assess contextual conditioning, and in a novel context with the cue, to assess cued learning (Figure 2A). The Tested cohort was given these testing trials before hippocampal recordings were made, and both *Paired* and *Shock* rats demonstrated more freezing in the original context than the *Tone* animals (Figure 2C)
(ANOVA, $F_{(2,17)}=5.205$, $p=0.033$). In the novel context, the Paired group froze more in response to the tone than the Shock and Tone groups (ANOVA, $F_{(2,17)}=4.159$, $p=0.034$).

As the testing session presents the training cues in the absence of the shock, it also acts as an extinction trial. We hypothesized that the single testing session was acting to extinguish fear learning in the Shock group. To test this idea, the Extinguished cohort was given repeated testing sessions until freezing to both the context and cue was extinguished in all animals (Figure 2D). During the second test, the Shock animals had significantly reduced freezing to the context, compared to their first testing trial (ANOVA, $F_{(1,8)}=5.89$, $p=0.04$), while the Paired animals still showed fear (ANOVA, $F_{(1,9)}=0.01$, $p=0.97$). Importantly, the Paired group continued to show freezing behavior in the original context after the first extinction trial, although they extinguished to the tone cue after only a single exposure in a new context (ANOVA, $F_{(1,9)}=6.19$, $p=0.03$). By the third trial, both groups had extinguished to both the cue and the original context. (Figure 2F).

Intrinsic excitability measures were made 24 hours following the last testing period from animals which were extinguished for one or three sessions (Tested and Extinguished cohorts). In order to determine the effect of a single extinction trial, comparisons were made between the Tested and Trained cohorts. For the Paired animals, testing had no effect on intrinsic excitability, as measured by comparing the AHP and accommodation of the Tested and Trained cohorts (Figure 3A, B) (ANOVA, Peak: $F_{(1,37)}=0.03$, $p=0.86$; Accommodation: $F_{(1,34)}=1.95$, $p=0.17$). However, neurons from Shock animals from the Tested cohort had significantly larger AHPs and exhibited increased accommodation than those in the Trained cohort (ANOVA, Peak: $F_{(1,29)}=6.73$, $p=0.015$; Accommodation:
After a single testing trial, the excitability of neurons from *Shock* animals was no different than those from *Naïve* animals (ANOVA, Peak: $F_{(1,32)}=0.11$, $p=0.741$; Accommodation: $F_{(1,30)}=1.142$, $p=0.294$). The reversal of the excitability increase in neurons from the *Shock* animals is not due to the exposure to the novel context in the testing session because there was no difference in the size of the AHP from *Tone* animals that were and were not tested (data not shown, *Tone*: $F_{(1,31)}=2.11$, $p=0.16$). The idea that complete extinction was responsible for the difference between the *Shock* animals versus the *Paired* animals was expanded by measuring the excitability from the Extinguished cohort. There was no significant difference in the freezing behavior after extinction between the Paired and Shock groups, nor in the AHP or accommodation measures. Consequently, the electrophysiological data for these groups were pooled. Cells from Extinguished animals had AHPs like those of naïve animals (Figure 3A). Indeed, there was no difference in the size of the AHP between Naïve, Extinguished, and *Shock* animals from the Tested cohort (ANOVA, $F_{(2,56)}=0.267$, $p=0.77$). There was also no difference in accommodation measures (Figure 3B) ($F_{(2,53)}=1.709$, $p=0.19$).

Together, these data suggest that learning cue or contextual fear conditioning increases excitability. Furthermore, at a time point when freezing behavior, and thus learning, were extinguished, the learning-related increase in excitability is rapidly reversed in as little as a single trial.
Discussion

We have shown here that learning either contextual or trace cued fear increases intrinsic excitability in only three trials, and those changes can be reversed by a single experience sufficient to extinguish the learned behavior. The Paired animals learned both contextual and trace cued fear conditioning, as evidenced by their freezing to both the context and the cue during the testing trial. Shock animals learned only the context, as was evident by their lack of freezing in the cue testing trial.

Since hippocampal CA1 neurons in both paired and shock alone animals showed AHPs reduced to similar levels, the conclusion can be drawn that learning contextual fear conditioning is sufficient to reduce the AHP. However, this does not mean that learning trace cued fear conditioning does not alter intrinsic excitability (recall that the Paired animals learned to fear both the original context and the tone in a new context). The degree of AHP reduction we observe here, approximately 30%, is similar to values from previous studies in rats after learning trace eyeblink or Morris water maze (Matthews et al. 2008; Oh et al. 2003). This level of AHP reduction may be optimal to support learning, and further learning may not have an additive effect, possibly to protect the hippocampus from excessive excitation. The fact that excitability is reduced the same degree by learning one association (contextual fear conditioning) or two associations (trace and contextual fear conditioning) suggests that AHP modulation is not additive.

However, a reduction in the AHP caused by learning one task could aid in learning a second one. In fact, learning both Morris water maze and trace eyeblink conditioning reduce the AHP, and simultaneous training in the two tasks facilitates learning trace
eyeblink conditioning (Kuo et al. 2006). Olfactory discrimination also increases intrinsic excitability in CA1 pyramidal neurons during and on the day of learning, but not after the animal has learned the rule. Learning Morris water maze is enhanced if it is started immediately following olfactory discrimination rule learning but not a few days later, and the facilitation correlated with time periods in which olfactory discrimination reduces the AHP (Zelcer et al. 2006). Thus, learning one task and its subsequent increases in intrinsic excitability appears to facilitate the learning of other hippocampus-dependent tasks. The learning-related increase in excitability seen with fear conditioning may facilitate the learning of other hippocampus-dependent tasks.

A testing trial provided the opportunity to more fully assess the degree of learning. But, the act of testing the animals exposed them to an extinction trial, since the tone and context cues were present but the shock was not. In order to determine whether the animals from the Tested cohort did indeed show behavioral extinction after a single trial, the Extinguished cohort was given repeated testing trials until freezing returned to baseline levels. Indeed, by the second extinction trial, at which point AHP recordings were made for the Tested cohort, the Extinguished cohort demonstrated significantly less freezing than during the previous testing trial, but with a differentiation between contextual and cued learning. The Paired animals showed significantly reduced freezing to the cue (while retaining contextual freezing levels), and the Shock animals had significantly reduced freezing to the context as compared to each group’s performance in the first testing trial (Figure 2F). The electrophysiological measures from the Tested cohort showed that a single extinction trial reset the size of the AHP in
neurons from *Shock* animals to that of *Naïve* controls. However, this was not the case for the *Paired* animals, which had reduced AHPs in both the Tested and Trained cohorts (Figure 3A). Note that in the Trained cohort, both the excitability changes and the freezing behavior to the context were similar in the *Paired* and *Shock* groups, so the level of intrinsic excitability after learning was correlated with the behavioral index of learning. The demonstration that cells from animals from the Extinguished cohort had large AHPs further supports this idea. Furthermore, the AHP size after a single extinction trial was correlated with behavioral contextual freezing demonstrated by the *Paired* and *Shock* groups.

The slower extinction of freezing to the context in the *Paired* group than the *Shock* group may have occurred because the *Paired* group made a compound association during training (context + cue), and therefore the testing sessions exposed the *Paired* animals to two separate partial extinction sessions (context only or cue only), while the *Shock* group received a complete extinction session with each test (Rescorla and Wagner 1972). Another hypothesis is that the slower rate of contextual extinction for the *Paired* group may be indicative of the richer informational content created by the addition of a cue to the training paradigm, i.e. the *Paired* animals learned a more complex stimulus and thus extinguished more slowly. Regardless of why the extinction rates differed, the AHP was predictive of the freezing behavior of the animals in the context.
The difference between the reversal in AHP reduction between the *Paired* and the *Shock* animals with extinction may also be due to the involvement of different neuronal circuits for the two tasks. The extinction of contextual fear conditioning is hippocampus dependent (Corcoran et al. 2005), but the involvement of the hippocampus in extinction of trace cued fear is unknown. Other brain regions are required for cued fear conditioning and extinction. For example, the prefrontal cortex is important for extinction of cued fear, but not of contextual fear conditioning (Morgan and LeDoux 1999; 1995; Quirk et al. 2006). The reversal of the AHP reduction observed in the *Shock* animals after a single extinction trial is qualitatively similar to that observed in the infralimbic prefrontal cortex, which inhibits fear expression. Learning cued fear conditioning is correlated with a reduction in intrinsic excitability in this region while its extinction increases it (Santini et al. 2008). Thus, while the hippocampus is involved in extinction of contextual conditioning, the involvement of other brain regions may mean that other sites of plasticity are involved in extinguishing cued fear.

The central observation in this study is that learning-related changes in intrinsic excitability were evident after just three training trials or only a single extinction trial. Utilization of this training paradigm in future experiments could help elucidate the mechanism(s) of the learning-related reduction in the post-burst AHP, which are not yet fully understood. It has been shown that PKA mediates this reduction (Oh et al. 2009), but the time course of the changes relative to the training time course is unknown. Previous demonstrations of learning-related alterations in the AHP utilized learning paradigms which required many training trials over several days. But the data reported
here indicate that the cellular changes underlying excitability modulation can be rapidly
induced. It is well documented that a single experience, requiring learning or merely
novel, is sufficient to induce changes in gene expression of proteins known to be
important for learning and memory (Sweatt 2004; Tzingounis and Nicoll 2006). For
example, mice that were exposed to a novel context for 3 minutes with or without a
shock and a tone show high levels of FOS production in the hippocampus (Radulovic et
al. 1998). A single lap around a rectangular track is sufficient to trigger immediate early
gene Arc transcription in both CA3 and CA1 (Miyashita et al. 2009). Further, a single
contextual fear conditioning trial with a single shock results in up-regulation of Erk1/2
and Elk1 signaling in CA3 and the dentate gyrus (Sananbenesi et al. 2002). Erk
activation is often preceded or paralleled by PKA activation (Ferguson and Storm 2004;
Winder and Sweatt 2001) providing a possible mechanism for the learning-related
changes in excitability reported here. Also, Erk signaling can lead to CREB activation,
which has also been shown to correlate with AHP modulation (Lopez de Armentia et al.
2007). These changes in gene expression after a single trial could activate signaling
cascades which result in the alterations in the AHP observed after three training trials or
a single extinction trial.

The finding that intrinsic excitability is increased after learning contextual or trace cued
fear, and is rapidly decreased after a single extinction event sufficient to eliminate the
learned behavior, underscores the importance of intrinsic plasticity as a cellular
substrate for learning. A more complete understanding of the mechanisms by which
excitability is modulated and interacts with synaptic properties that also change during
learning should augment treatments to restore the impaired cognitive ability due to aging or injury.

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Disclosures
The authors have no conflicts of interest to disclose.
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Table 1. Intrinsic properties of cells included in this study. Resting potential ($V_{\text{rest}}$), action potential threshold ($V_{\text{thresh}}$), and input resistance ($R_{\text{input}}$) were measured for every cell. $V_{\text{rest}}$ was calculated as the potential with 0 pA current injection; $V_{\text{thresh}}$ was calculated as the voltage where the first derivative of the action potential equaled 20mV/ms; $R_{\text{input}}$ was calculated as the slope of the I-V curve generated with an 800ms current injection from (-0.3 to +0.1 nA); Sag was measured as the difference between the peak and steady-state hyperpolarization in response to a 800ms, -300pA current step.

Figure 1. Learning trace and contextual fear increases intrinsic excitability.

A. Training paradigm for Trained Cohort. In each training session, Tone animals were presented with one tone (small white box), Shock animals were presented with one shock (small black dash), and Paired animals received a tone and shock separated by a 30sec “trace” stimulus free interval. Naïve animals were not handled nor exposed to the training context. Testing sessions consisted of 226 seconds in the original context (light grey) with no other stimuli followed 15 minutes later by exposure to the tone in a novel context (dark grey). B. Paired and Shock groups froze more across the training sessions, and also froze significantly more than Tone animals in the last training session, indicating robust contextual learning. All bar graphs show mean ± SEM. C. Cells from Paired and Shock animals had increased intrinsic excitability compared to Tone or Naïve animals as measured by the peak and 1 second amplitude of the AHP. Example traces from a Paired and a Tone animal are shown (Peak AHP: Paired - 4.70±0.36 mV, Shock -4.23±0.38 mV, Tone -5.62±0.21 mV, Naïve -6.07±0.31 mV.
Slow AHP: Paired -2.05±0.19 mV, Shock -2.06±0.24 mV, Tone -2.64±0.15 mV, Naïve -3.07±0.2 mV). D. Cells from Shock and Paired animals showed decreased accommodation by firing more action potentials during a 1s current step sufficient to produce 5 action potentials in the first 100ms, with no difference in the current step (p=0.23). Example traces from a Paired and a Tone animal are shown (Paired 22.4±2.1 APs, Shock 21.7±1.8 APs, Tone 16.3±0.9 APs, Naïve 12.8±1.1 APs).

**Figure 2.** A single testing session confirms learning, but also extinguishes contextual freezing.

A. Training paradigm for Tested Cohort. B. Paired and Shock groups froze more across the training sessions, and also froze significantly more than Tone animals in the last training session, indicating robust contextual learning. C. All animals from the Tested Cohort were tested for contextual and cue learning. Freezing during the testing session of the Tested Cohort revealed robust contextual learning in Shock and Paired groups as compared to the Tone group. Only Paired animals exhibited freezing behavior in response to the cue. D. Training paradigm for Extinguished Cohort. E. Paired and Shock groups froze more across the training sessions, indicating robust contextual learning. F. During the first testing session, freezing was the same as for the Tested cohort, i.e. Shock and Paired groups learned the context while only Paired learned the cue. In the second testing session, Paired animals continued to freeze to the context, but showed extinction to the cue. Shock animals exhibited extinction of contextual learning after only a single testing session; this was the time point at which physiologic recordings were made in the Tested cohort. While it appears that the Shock
animals froze less to the cue in the second testing session, this was not significant
(rMANOVA F(8,2)=1.305, p=0.29). By the third testing session, extinction was complete
for both groups.

Figure 3. Behavioral extinction in single or multiple trials is sufficient to reverse
the learning-related increases in excitability

A. Cells from Paired animals did not show any effect of a single testing trial and their
excitability changes remained (Peak: Trained -4.70±0.36 mV, Tested -4.68±0.34 mV).
In Shock animals, a single testing trial reversed the reduction of the AHP seen after
learning but before testing (Peak: Trained -4.23±0.38 mV, Tested -6.06±0.32 mV). The
AHP from these cells, after a single testing trial, was no different from Naïve cells (Peak:
-6.06±0.31 mV) or than animals from the Extinguished cohort who showed complete
extinction (Peak: -5.77±0.37 mV). B. After a single testing trial, the learning-related
reduction of firing frequency accommodation was also reversed in the Shock group, as
measured by firing fewer action potentials during a 1 second current injection sufficient
to produce 5 action potentials in the first 100 ms. (Trained: 21.7±1.8 APs, Tested:
15.6±1.5 APs ). After a single testing session, firing frequency accommodation in the
Shock group was no different from that of Naïve animals (12.8±1.1 APs) or than animals
from the Extinguished cohort (15.9±1.2 APs). The Paired group showed no effect of a
single testing session on firing frequency accommodation (Trained: 22.4 ±2.1 APs,
Tested: 19.8±2.0 APs).
Table 1 – Intrinsic properties of cells included in this study

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<th>Group</th>
<th>Cells (n)</th>
<th>$V_{\text{rest}}$ (mV)</th>
<th>$V_{\text{thresh}}$ (mV)</th>
<th>Sag (mV)</th>
<th>$R_{\text{input}}$ (MΩ)</th>
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<td>-39.4 ± 2.7</td>
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<td>26</td>
<td>-66.8 ± 0.7</td>
<td>-43.7 ± 2.4</td>
<td>-4.1 ± 0.3</td>
<td>78.0 ± 4.9</td>
</tr>
<tr>
<td>Naïve</td>
<td>19</td>
<td>-67.0 ± 0.6</td>
<td>-45.6 ± 1.5</td>
<td>-3.8 ± 0.2</td>
<td>70.9 ± 4.8</td>
</tr>
<tr>
<td>Extinguished</td>
<td>20</td>
<td>-64.3 ± 0.8</td>
<td>-35.1 ± 4.4**</td>
<td>-3.8 ± 0.3</td>
<td>80.7 ± 5.6</td>
</tr>
<tr>
<td>Tested</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paired</td>
<td>22</td>
<td>-64.3 ± 0.7</td>
<td>-45.8 ± 1.4</td>
<td>-3.5 ± 0.3</td>
<td>71.0 ± 5.0</td>
</tr>
<tr>
<td>Shock Alone</td>
<td>22</td>
<td>-65.0 ± 0.5</td>
<td>-42.6 ± 2.0</td>
<td>-3.5 ± 0.3</td>
<td>67.8 ± 5.0</td>
</tr>
<tr>
<td>Tone Alone</td>
<td>19</td>
<td>-65.2 ± 0.9</td>
<td>-41.3 ± 1.5</td>
<td>-4.3 ± 0.3</td>
<td>78.3 ± 3.3</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

$F_{(7.156)} = 1.98, p = 0.062$
Figure 1: Learning trace & contextual fear increases intrinsic excitability

A) Trained Cohort
- Day 1: AM/Tone, PM/Shock, Paired
- Day 2: AM/Tone, Shock, Paired
- Day 3: Tone, Slice/Shock, Paired

B) % Freezing
- Trial 1, Trial 2, Trial 3
- Paired (7) * p < 0.03
- Shock (6) ** p < 0.001
- Tone (7)

C) Peak AHP, Slow AHP
- AHP (mV)
- Paired (20), Shock (24), Tone (26), Naive (19)
- * p < 0.04

D) # of Action Potentials
- Paired (19), Shock (16), Tone (24), Naive (17)
- * p < 0.01
Figure 2: A single testing trial confirms learning, but also extinguishes contextual freezing

A) Tested Cohort

- Day 1: Tone, Shock, Paired (AM, PM)
- Day 2: Tone, Shock, Paired (AM, PM)
- Day 3: Tone, Shock, Paired (AM, PM)

B) % Freezing

- Paired (8) * p < 0.02
- Shock (6) ** p < 0.001
- Tone (6)

C) % Freezing

- Paired (8)
- Shock (6)
- Tone (6) * p < 0.03

D) Extinguished Cohort

- Day 1: Shock, Paired (AM, PM)
- Day 2: Shock, Paired (AM, PM)
- Day 3: Shock, Paired (AM, PM)

E) % Freezing

- Paired (10) * p < 0.001

F) % Freezing

- Context Tests
  - Paired (10)
  - Shock (9) * p < 0.04

- Cue Tests
  - Paired (10)
  - Shock (9) * p < 0.03
Figure 3: Behavioral extinction in single or multiple trials is sufficient to reverse the learning-related increases in excitability.