Proton receptors regulate synapse-specific reconsolidation in the amygdala

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SUMMARY

When an extinction procedure is performed within the reconsolidation window, the original aversive memory can be replaced by one that is less traumatic. Recent studies revealed that carbon dioxide (CO_2) inhalation during retrieval enhances memory lability. However, the effects of CO_2 inhalation on the central nervous system can be extensive, and there is lack of evidence suggesting that the effects of CO_2 are selective to a reactivated memory. We discovered that CO_2 inhalation paired with memory retrieval potentiates the specific aversive memory trace, resulting in greater memory lability. The specific effects of CO_2 depend on acid-sensing ion channels (ASICs), the proton receptors that are involved in synaptic transmission and plasticity in the amygdala. In addition, CO_2 inhalation alters memory lability via synaptic plasticity at selectively targeted synapses. Overall, our results suggest that inhaling CO_2 during the retrieval event increases the lability of an aversive memory through a synapse-specific reconsolidation process.

KEYWORDS: carbon dioxide (CO_2); acid-sensing ion channels; proton receptors; aversive conditioning; aversive memory; memory retrieval; AMPA receptors; reconsolidation; synaptic plasticity; spine structure.

INTRODUCTION

Recently, aversive memory research in both rodents and humans has focused on a window of time after aversive memory retrieval, in which the memory is labile and subject to intervention (Kida, 2020; Lee, 2009; Monfils et al., 2009; Nader et al., 2000; Sara, 2000; Schiller et al., 2010; Tronson and Taylor, 2007). This window of time is known as a reconsolidation window, and is thought to last up to six hours after initiation (Clem and Huganir, 2010; Duvarci and Nader, 2004; Monfils et al., 2009; Schiller et al., 2010). Several studies have demonstrated that interrupting the updating process aroused by retrieval prevents memory restorage, generating selective amnesia (Agren et al., 2012; Clem and Huganir, 2010; Mactutus et al., 1979; Monfils et al., 2009; Nader et al., 2000; Schiller et al., 2010). Studies using rodent models have indicated that pharmacological intervention within the reconsolidation window successfully erases reactivated specific aversive memory (Nader et al., 2000; Sara and Hars, 2006; Schiller et al., 2010). Despite the efficacy, ethical and practical concerns may prevent similar pharmacological interventions from being used in humans (Agren et al., 2012; Schiller et al., 2010). Recently, drug-free paradigms have been proposed that effectively prevent the return of aversive memories in both rodents and humans (Bjorkstrand et al., 2016; Clem and Huganir, 2010; Huang et al., 2020; Liu et al., 2014; Monfils et al., 2009; Schiller et al., 2010). Monfils *et al.* developed a novel protocol in which an isolated retrieval trial was followed by an extinction event within a specific time frame. This resulted in the weakening of the original memory trace, thereby preventing reversion of the original aversive memory by spontaneous recovery, renewal, or reinstatement (Clem and Huganir, 2010; Jarome et al., 2015; Monfils et al., 2009; Quirk et al., 2010;

70 Wu et al., 2017). These studies suggest the mechanism by which retrieval changes the
71 lability of memory may become a target for novel clinical treatments for anxiety
72 disorders.
73
74 Currently, the outcomes of the combination of retrieval and extinction paradigms are
75 variable, depends on multiple factors, in both rodents (Auber et al., 2013; Chan et al.,
76 2010; Costanzi et al., 2011; Goode et al., 2017; Ishii et al., 2012) and humans (Golkar
77 et al., 2012; Klucken et al., 2016). Because it is difficult to completely erase the original
78 memory, a more reliable strategy for triggering memory erasure is necessary. In search
79 of this strategy, we studied the effects that CO_2 inhalation may have on the erasure of a
80 specific memory. As we showed previously, when mice receive a retrieval tone
81 supplemented by CO_2 inhalation, the memory becomes more labile than the
82 presentation of a retrieval tone alone (Du et al., 2017). Within the reconsolidation
83 window, the labile memory becomes more convertible, either weakened by extinction or
84 strengthened by reconditioning. Moreover, the effects of CO_2 inhalation on memory
85 lability were dependent on acid-sensing ion channels (ASICs), since disrupting ASICs in
86 the amygdala eliminated these effects of CO_2 (Du et al., 2017).
87
88 Recent studies have revealed that protons are potential neurotransmitters (Du et al.,
89 2014; Highstein et al., 2014; Kawasaki et al., 2009) and ASICs serve as postsynaptic
90 proton receptors that play key roles in neurotransmission and synaptic plasticity in the
91 amygdala, a brain region that is critical for the formation of aversive memories (Cohen
92 et al., 2017; Du et al., 2014; Farley et al., 2018; Ziemann et al., 2009). ASICs are
93 members of the Degenerin/ Epithelial sodium channel (DEG/ ENaC) family (Ben-

94 Shahar, 2011). To date, six family members have been identified (ASIC1a, ASIC1b,
95 ASIC2a, ASIC2b, ASIC3, and ASIC4) (Wemmie et al., 2013). These proteins assemble
96 as homo- or heterotrimers to form channels that are proton-gated, voltage-insensitive,
97 permeable to both Na^+ and Ca^{2+} and activated by extracellular protons (Waldmann et al.,
98 1997; Waldmann and Lazdunski, 1998). ASIC1a is widely expressed in many areas of
99 the brain, where it is associated with numerous brain functions and disorders, including
100 hippocampally-dependent learning and memory, anxiety, depression, stroke,
101 neurodegeneration, seizure, inflammation, and nerve injury (Chu and Xiong, 2012; Gao
102 et al., 2005; Ortega-Ramírez et al., 2017; Wang et al., 2018; Wemmie et al., 2002;
103 Wemmie et al., 2006). ASIC1a is highly expressed in the amygdala and its ion channel
104 activity is evoked by a reduction in extracellular pH within the physiological range
105 (Taugher et al., 2017; Wemmie et al., 2003). Disruption of ASIC1a affects synaptic
106 transmission and plasticity (Mango and Nisticò, 2019; Soto et al., 2018; Wemmie et al.,
107 2003; Wu et al., 2004). Loss of ASIC1a function also leads to impaired high-frequency
108 electrical stimulation-induced long-term potentiation (LTP) (Chiang et al., 2015; Du et
109 al., 2014; Liu et al., 2016; Wemmie et al., 2002). Moreover, we demonstrated that
110 presynaptic stimulation induces a transient synaptic drop in pH and activates ASIC-like
111 excitable postsynaptic currents (EPSCs) in pyramidal neurons (Du et al., 2014; Kreple
112 et al., 2014), suggesting that protons sufficiently activate postsynaptic ASIC1a in
113 synaptic transmission. In mice, disruption of ASIC1a activity reduces innate fear
114 learning and memory and alters neuronal activity in the fear circuit (Coryell et al., 2007),
115 whereas its overexpression has opposite effects (Wemmie et al., 2004). Also, reducing
116 pH by CO_2 inhalation or the injection of acid into the amygdala induced aversive

117 behavior response and enhanced aversive memory, and the CO_2 effects are ASIC1a
118 dependent (Ziemann et al., 2009).
119
120 Although our previous data suggested that CO_2 inhalation affects the aversive memory
121 retrieval and alters the memory lability, we also know that the CO_2 - affected memory
122 trace is associated with the retrieval cue (Du et al., 2017), inhalation of CO_2 may reduce
123 pH outside of the amygdala (Dulla et al., 2005; Zandbergen et al., 1989). Thus, whether
124 CO_2 and ASICs specifically regulate memory trace in retrieval is still unknown and this
125 question is outstanding. In this study, we found that CO_2 inhalation paired with memory
126 retrieval selectively potentiated memory lability in mice. Furthermore, electrorheological
127 and imaging studies in brain slices support the conclusion that the effects of CO_2 on
128 memory retrieval are specifically associated with a given memory. Our study proposes
129 that inhaling CO_2 within the reconsolidation window regulates aversive memory with
130 specificity, providing a unique angle to further study the mechanism by which memory is
131 modulated.
132
133 **EXPERIMENTAL PROCEDURES**
134 **Mice**
135 For our experiment, we used both male and female mice between 10-14 months of age.
136 Mice were derived from a congenic C57BL/6 background including wild-type, ASIC1a
137 knock out (ASIC1a$^{-/-}$), and TetTag-c-fos-tTA mice. TetTag-cFos-tTA mice were
138 obtained from Jackson Laboratory and crossed with C57BL/6J mice. Mice carrying
139 the Fos-tTA transgene were selected; Fos-tTA mice have a Fos promoter driving
140 expression of nuclear-localized, two-hour half-life EGFP (shEGFP) (Du et al., 2017;

141 Koffman and Du, 2017; Ramirez et al., 2013). The Fos promoter also drives the
142 expression of tetracycline transactivator (tTA), which bind to the tetracycline-responsive
143 element (TRE) site on an injected recombinant adeno-associated virus, $AAV_{2/9}$-TRE-
144 mCherry virus, resulting in the expression of mCherry (Du et al., 2017; Koffman and Du,
145 2017). The binding of the tTA to the TRE site is inhibited by doxycycline (DOX).
146 Inhibition of tTA binding prevents target gene expression (Das et al., 2016; Liu et al.,
147 2012; Ramirez et al., 2013).
148
149 Both male and female mice age10-14 weeks were randomly selected for the experiment
150 groups. Experimental mice were maintained on a standard 12-hour light-dark cycle and
151 received standard chow and water ad libitum. Animal care and procedures met the
152 National Institutes of Health standards. The University of Tennessee Health Science
153 Center Laboratory Animal Care Unit (Protocol #19-0112) and University of Toledo
154 Institutional Animal Care and Use Committee (Protocol #108791) approved all
155 procedures.
156
157 **Aversive conditioning, retrieval, extinction, and memory test**

158 The protocols for each experiment are detailed in the schematics of each figure. All
159 mice were handled by experimenters for 30 minutes on each of 3 days before aversive
160 conditioning. On day 1, mice were habituated to the aversive conditioning chamber
161 (Med Associates Inc.) for 7 minutes. Mice were then exposed to varying conditioning
162 protocols, as described below.
163

164 ***Experiment 1: Standard conditioned stimulus (CS) auditory aversive conditioning,***
165 ***retrieval, extinction, and memory tests.***
166 On day 1 in a curated environment (context A), the experimental mice were presented
167 with six pure tones (80 dB, 2 kHz, 20 seconds each) paired with 6 foot shocks - one
168 shock at the end of each tone (0.7 mA, 2 seconds). The interval between each tone was
169 100 seconds. On day 2, the mice were placed into a new environment (context B) and
170 habituated for 4 minutes. Mice then inhaled either unaltered air or air containing 10%
171 CO_2 for 7 minutes. Five minutes after inhalation of CO_2 or air began, mice were
172 presented with one 20 second pure tone to retrieve the memory. The mice were then
173 returned to their home cages. 30 minutes later, the mice returned to the retrieval
174 chamber (context B) and underwent two rounds of extinctions. In the first round of
175 extinction, mice were exposed to 20 pure tones with an interval between tones of 100
176 seconds. Mice were then returned to their home cage. 30 minutes later, the mice went
177 through the extinction protocol again with 20 pure tones. On day 7, the mice were tested
178 to see if their aversive response would recur via spontaneous recovery in context B with
179 4 pure tones. Thirty minutes after spontaneous recovery, the mice were returned to the
180 original context of the aversive memory, context A, in a recovery protocol with 4 pure
181 tones.
182
183 Freezing behavior in mice (the absence of movement beyond respiration) is used as a
184 measure of aversive response. To evaluate the outcomes of freezing behavior in mice,
185 the percentage of time during CS presentation spent in freezing was scored
186 automatically using VideoFreeze software (Med Associates Inc.). In the spontaneous

recovery and renewal tests, outcomes of the percentage of time freezing were averaged from each of the 4 CSs.

Experiment 2: Two distinct CSs aversive conditioning, retrieval, extinction, and memory tests.

This procedure was used to test the specificity of the effects of CO_2 on memory retrieval. The context settings and parameters are similar to the above standard one CS auditory aversive conditioning. In contrast to experiment 1, the mice were presented with three pure tones (80 dB, 2 kHz, 20 seconds each) that alternated with three white noises (60 dB, 2 kHz, 20 seconds each); all six stimuli were paired with foot shocks. On day 2, the mice inhaled either unaltered air or air containing 10% CO_2 for 7 minutes. Five minutes after inhalation of CO_2 or air began, the mice underwent retrieval with one single pure tone followed by one white noise with or without CO_2. This was followed thirty minutes later by two sections of extinctions with either pure tones or white noises. On day 7, the mice were tested via spontaneous recovery and renewal protocols with 4 pure tones and 4 white noises respectively.

Experiment 3: Two distinct CSs aversive conditioning, retrieval, anisomycin, and memory tests.

In a series of experiments, the standard extinction procedure was replaced with amygdala infusion of anisomycin (detailed in the surgery procedure below). In brief, the cannula was implanted on the amygdala 4-7 days before the behavioral experiments. Starting on day 1, the mice were subjected to the aversive conditioning described in experiment 2. On day 2, 30 minutes after retrieval, the mice were infused with 62.5

211 μg/μl anisomycin via the cannula in the lateral nuclei of the amygdala (LA) bilaterally
212 and returned to their home cage (Debiec et al., 2010). On day 7, the mice were tested
213 via spontaneous recovery and renewal as described in experiment 2.
214
215 **Surgery and chemical infusion**
216 For the cannula placement procedure, mice were anesthetized with isoflurane through
217 an anesthetic vaporizer, secured to the stereotaxic instrument and a cannula made from
218 a 25-gauge needle was inserted bilaterally into LA (relative to bregma: -1.2 mm
219 anterioposterior; ±3.5 mm mediolateral; -4.3 mm dorsoventral) (Du et al., 2017; Koffman
220 and Du, 2017). Dental cement secured the cannula and bone anchor screw in place.
221 Mice recovered for 4-5 days before any subsequent testing was carried out. A 10 μL
222 Hamilton syringe connected to a 30-gauge injector was inserted 1 mm past the cannula
223 tip to inject anisomycin (diluted in 1 μl artificial cerebrospinal fluid (ACSF), pH 7.3) over
224 5 sec. The injection sites were mapped post-mortem by sectioning the brain (10 μm
225 coronal) and performing cresyl violet staining.
226
227 **Brain slice preparation and patch-clamp recording of amygdala neurons**
228 Ten minutes after the memory retrieval experiment ended, mice were euthanized with
229 overdosed isoflurane and whole brains were dissected into pre-oxygenated (5% CO_2
230 and 95% O_2) ice-cold high sucrose dissection solution containing (in mM): 205 sucrose,
231 5 KCl, 1.25 NaH_2PO_4, 5 $MgSO_4$, 26 $NaHCO_3$, 1 $CaCl_2$, and 25 glucose (Du et al., 2017).
232 A vibratome sliced brains coronally into 300 μm sections that were maintained in normal
233 ACSF containing (in mM): 115 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 11
234 glucose, 25 $NaHCO_3$ bubbled with 95% O_2/5% CO_2, pH 7.35 at 20°C-22°C. Slices were

235 incubated in the ACSF at least 1 hour before recording. For experiments, individual
236 slices were transferred to a submersion-recording chamber and were continuously
237 perfused with the 5% CO_2/95% O_2 solution (~3.0 ml/min) at room temperature (20°C -
238 22°C).
239
240 As we described previously (Du et al., 2017), pyramidal neurons in the lateral amygdala
241 were studied using whole-cell patch-clamp recordings. The pipette solution containing
242 (in mM): 135 KSO_3CH_3, 5 NaCl, 10 HEPES, 4 MgATP, 0.3 Na_3GTP, 0.5 K-EGTA
243 (mOsm=290, adjusted to pH 7.25 with KOH). The pipette resistance (measured in the
244 bath solution) was 3-5 MΩ. High-resistance (>1 GΩ) seals were formed in voltage-
245 clamp mode. Picrotoxin (100 μM) was added to the ACSF throughout the recordings to
246 yield excitatory responses. In AMPAR current rectification experiments, we applied D-
247 APV (100 μM) to block NMDAR-conducted EPSCs. The peak amplitude of ESPCs was
248 measured to determine current rectification. The amplitude was measured ranging from
249 -80 mV to +60 mV in 20 mV steps. The peak amplitude of EPSCs at -80 mV and +60
250 mV was measured for the rectification index. In EPSC ratio experiments, neurons were
251 measured at -80 mV to record AMPAR-EPSCs and were measured at +60 mV to record
252 NMDAR-EPSCs. To determine the AMPAR-to-NMDAR ratio, we measured the peak
253 amplitude of ESPCs at -80 mV as AMPAR-currents, and peak amplitude of EPSCs at
254 +60 mV at 70 ms as NMDAR-currents after onset. Data were acquired at 10 kHz using
255 Multiclamp 700B and pClamp 10.1. The mEPSCs events (>5pA) were analyzed in
256 Clampfit 10.1. The decay time (τ) of mEPSCs was fitted to an exponential using
257 Clampfit 10.1.
258

259 **Immunohistochemistry and cell counting**

260 The AAV-TRE-mCherry plasmid was obtained from the laboratory of Dr. Susumu
261 Tonegawa (Liu et al., 2012; Ramirez et al., 2013), and was used to produce $AAV_{2/9}$ by
262 the University of Iowa Gene Transfer Vector Core. For one week leading up to virus
263 microinjection, TetTag Fos-tTA mice were fed with food containing 40 mg/kg DOX. We
264 used a 10 µl Hamilton microsyringe and a WPI microsyringe pump to inject virus (0.5 µl
265 of 1.45E+12 viral genomes/ml of $AAV_{2/9}$-TRE-mCherry) bilaterally into the amygdala
266 (relative to bregma: -1.2 mm anterioposterior; ±3.5 mm mediolateral; -4.3 mm
267 dorsoventral), as described previously (Du et al., 2017; Koffman and Du, 2017). For
268 a two-week window between surgery and behavior training, mice were housed and fed
269 with a DOX-containing diet. The DOX-containing diet was ceased twenty-four hours
270 before aversive conditioning began on day one (replaced by a regular diet), then
271 immediately restarted afterward. Thirty minutes after retrieval on day two, the mice were
272 euthanized according to protocol. We used transcardial perfusion with 4%
273 paraformaldehyde (PFA) to fix whole brains, followed by continued fixation in 4% PFA at
274 4°C for 24 hours (Wright et al., 2020). Following perfusion, we used a vibratome (Leica
275 VT-1000S) to dissect 50 µm amygdala coronal slices, which were collected in ice-cold
276 PBS. To complete immunostaining, slices were placed in Superblock solution (Thermo
277 Fisher Scientific) plus 0.2% Triton X-100 for 1 hour and incubated with primary
278 antibodies (1:1000 dilution) at 4°C for 24 hours (Du et al., 2017). Primary antibodies we
279 used include: rabbit polyclonal IgG anti-RFP (Rockland Cat# 600-401-379); chicken IgY
280 anti-GFP (Thermo Fisher Scientific Cat# A10262) and mouse anti-NeuN (Millipore Cat#
281 MAB377X) (Liu et al., 2012; Ramirez et al., 2013). We then washed and incubated

282 slices for one hour with secondary antibodies (Alexa Fluor 488 goat anti-chicken IgG
283 (H+L) (Molecular Probes Cat# A-11039); Alexa Fluor 568 goat anti-rabbit IgG (H+L)
284 (Molecular Probes Cat# A-21429); Alexa Fluor 647 goat anti-mouse IgG (H+L) (Thermo
285 Fisher Scientific Cat# A-21235), 1:200 dilution). VectaShield H-1500 (Vector
286 Laboratories Cat# H-1500) was used to mount slices, while confocal microscopy was
287 used to view the slices. We used ImageJ software to analyze dendritic spine
288 morphology. Thin, mushroom and stubby spines were categorized based on the
289 following parameters: 1) mushroom spines: head-to-neck diameter ratio >1.1:1 and
290 spine head diameter >0.35 μm; 2); thin spines: head-to-neck diameter ratio >1.1:1 and
291 spine head diameter >0.35 μm or spine head-to-neck diameter ratios <1.1:1 and spine
292 length-to-neck diameter > 2.5 μm; 3); stubby spines: spine head-to-neck diameter ratios
293 <1.1:1 and spine length-to-neck diameter ≤ 2.5 μm (Kreple et al., 2014; Wright et al.,
294 2020).
295
296 **Statistical analysis**
297 One-way ANOVA and Tukey's post-hoc multiple comparison tests were used for
298 statistical comparison of groups. An unpaired Student's t-test was used to compare
299 results between two groups. $P<0.05$ was considered statistically significant, and we did
300 not exclude potential outliers from our data except the ones did not receive successful
301 aversive conditioning. The graphing and statistical analysis software Graphpad Prism 8
302 was used to analyze statistical data, which was presented as means ± SEM. Sample
303 sizes (n) are indicated in the figure legends, and data are reported as biological
304 replicates (data from different mice, different brain slices). Each group contained tissues

305 pooled from 4-5 mice. Due to variable behavior within groups, we used sample sizes of
306 10-16 mice per experimental group as we previously described in earlier experiments
307 (Du et al., 2017). In behavioral studies, we typically studied groups with four randomly
308 assigned animals per group, as our recording equipment allowed us to record four
309 separate animal cages simultaneously. The experiments were repeated with another set
310 of four animals until we reached the target number of experimental mice per group.
311 Experimentation groups were repeated in this manner so that each animal had the
312 same controlled environment-the same time of day and with similar handling,
313 habituation, and processes.
314

315 **RESULTS**

316 Our recent studies suggested that CO_2 inhalation throughout memory retrieval
317 enhances the lability of the memory and boosts the efficiency of the memory erasure
318 (Du et al., 2017). To further test whether the effects of CO_2 on memory retrieval are
319 synapse-specific, we designed a series of unique experiments by which we were able to
320 generate two distinct auditory aversive memories and identify the specificity of CO_2
321 effects on each of them.
322

323 **CO_2 selectively enhances the lability of auditory aversive memory in the**
324 **amygdala**

325 Previous studies have described an aversive conditioning paradigm in which memory
326 can be selectively reactivated and reconsolidated, suggesting synapse-specific
327 reconsolidation of distinct aversive memories in the amygdala (Debiec et al., 2010;
328 Doyere et al., 2007). We followed this paradigm albeit with modifications **(Fig.1A, Fig.**

329 **s1A)**. On day 1, we trained the animals with two distinct conditioned stimuli: three pure
330 tones and three white noises paired with one foot-shock per stimuli as aversive
331 unconditioned stimuli (US) (see the detailed description in Materials and Methods). We
332 evaluated the outputs of the aversive conditioning through the percentage of the
333 freezing time within the time of CSs. The freezing was significantly increased after each
334 of the three conditioned stimuli, indicating the mice were trained sufficiently under the
335 designed condition **(Fig.1B, Fig. s1B)**.
336
337 On day 2, the animals were placed into a new context (context B) and presented with
338 one pure tone followed by a single noise (or vice versa) to retrieve the memory **(Fig. 1A,**
339 **C, Fig. s1A, C)**. The animals were then returned to their home cages. 30 minutes later,
340 all mice underwent two blocks of extinctions in context B, each extinction contains 20
341 pure tones **(Fig.1D)** or 20 white noise **(Fig. s1D).** At the end of the extinction procedure,
342 the freezing dropped down to a low level **(Fig. 1D, Fig. s1D)**, suggesting that the
343 extinction procedure was sufficient to suppress the memory. Five days later, the mice
344 underwent spontaneous recovery (context B) and renewal (context A) respectively. Four
345 tones and four noises were presented throughout the memory test **(Fig.1A, Fig. s1A)**.
346 The group that underwent extinction with a specific CS showed specificity in which
347 freezing response was lowered after extinction **(Fig.1E, Fig. s1E)**. For example, when
348 the pure tone was presented during extinction, freezing in the pure tone group was
349 lower than freezing in the noise group **(Fig.1E)**, and vice versa **(Fig. s1E)**. When
350 retrieval was paired with 10% CO_2 inhalation, memory erasure effects were enhanced
351 **(Fig.1 F-I, Fig. s1F-I)**, and there are statistical significances between the groups with or

352 without CO_2 in both spontaneous recovery and renewal (green columns, **Fig.1 J-K** and
353 red columns, **Fig. s1J-K**). To further evaluate the specificity of the effects of
354 reconsolidation on memory modifications, we designed another retrieval protocol in
355 which we presented pure tone and white noise, either of them paired with 10% CO_2
356 inhalation, followed by an unrelated extinction of CS, white noise, or pure tone
357 respectively **(Fig. s2)**. Consistent with our expectation, CO_2 did not boost the effects on
358 the retrieved memory in the absence of paired extinction, compared to the data in Fig. 1
359 and Fig. S1 **(Fig. s2B-I)**. When compared to the data in **Fig. 1F-I** and **Fig. s1F-I**, we
360 found the application of 10% CO_2 to the retrieval event failed to enhance the outcome
361 after extinction, indicating a specificity of the CO_2 effects. In all, our data suggest that
362 memory encoded in the amygdala can be distinct, and the effects of CO_2 on memory
363 are specific.
364
365 To focus on testing the specific effects of CO_2 on retrieval, we replaced the extinction
366 procedure with an injection of a protein synthesis inhibitor, anisomycin, to obliterate the
367 aversive memory **(Fig. 2A)**. Anisomycin, when injected bilaterally into the amygdala
368 after retrieval, causes memory erasure compared to the saline injection group (Debiec
369 et al., 2010). We conditioned the mice and retrieve the memory with pure tones
370 **(Fig. 2B-C)**, followed by anisomycin/saline injection **(Fig. 2D)**. Our experiments show
371 that anisomycin disrupts the memory during reconsolidation **(Fig. 2E)**, and that memory
372 retrieval is required for memory erasure with anisomycin injection **(Fig. s3A-E)**.
373 Consistent with the extinction results in Fig. 1, when retrieval was paired with 10%
374 CO_2 inhalation, we found anisomycin reduced more aversive response-further

375 confirming that CO_2 enhances memory lability specifically **(Fig. 2F-I)**. To exclude the
376 possibility that anisomycin associates with one CS other the other, as rigorous controls,
377 we conditioned the mice with both pure tone and white noise and carried out memory
378 retrieval with both CSs and found anisomycin has equal effects on memory in both tone
379 and noise groups **(Fig. s3F-M)**. When 10% CO_2 was applied while the CSs were
380 presented, the retrieval group paired with CO_2 showed less freezing, regardless of the
381 type of CSs (pure tone or white noise) **(Fig.s4 B-I)**. As rigorous controls, we applied
382 CO_2 for both retrieval events together and anisomycin decreased the freezing level in
383 both groups, suggesting that CO_2 had equal effects on both tone and noise **(Fig. s4 J-**
384 **Q)**. As another control, saline injection following retrieval and CO_2 did not cause
385 memory erasure, indicating that anisomycin was necessary to disrupt the reactivated
386 memory **(Fig. s5)**. Talking together, our data demonstrate that the effects of CO_2 are
387 specific to a distinct memory that is activated by a specific CS.
388
389 **The specific effects of CO_2 on memory lability is ASIC-dependent**.
390 We have previously found the effects of CO_2 on memory retrieval to be ASIC dependent
391 (Du et al., 2017). However, it is still unknown if CO_2 application to a specific memory
392 trace affects an ASIC-dependent mechanism. To answer this question, we first
393 performed distinct aversive conditioning in ASIC1a$^{-/-}$ mice with three pure tones and
394 white noises on day 1 **(Fig. 3A)**, followed by a pure tone and white noise for retrieval
395 on day 2. 30 minutes post-retrieval, we performed extinctions with pure tones. Five days
396 later, we tested spontaneous recovery and renewals with 4 pure tones and white
397 noises. Similar to the response we saw in WT mice, the freezing level in the pure tone

398 group of ASIC1a$^{-/-}$ mice were less than that in the white noise group (spontaneous
399 recovery, 46% decrease; renewal, 47.5% decrease) **(Fig. 3B-E)**. When 10% CO_2
400 inhalation was paired with pure tone in retrieval, we found that CO_2 did not have
401 additional effects on the memory with the specific CS in ASIC1a$^{-/-}$ mice (spontaneous
402 recovery, 43.4% decrease; renewal, 45.6% decrease) **(Fig. 3F-I)**, and there are no
403 statistical significances between the groups with or without CO_2 in both spontaneous
404 recovery and renewal (green columns, **Fig. 3J, K**). We had hypothesized that the
405 effects of CO_2 on memory retrieval would be ASIC dependent and our data supported
406 this prediction. We then replaced the extinction procedure with anisomycin, to obliterate
407 the aversive memory **(Fig. 4A)**. The ASIC1a$^{-/-}$ mice were conditioned with pure tones
408 **(Fig. 4B)** and followed by a single tone as retrieval **(Fig. 4C)**. We then apply anisomycin
409 infusions **(Fig. 4D)**and test the memory 5 days later **(Fig. 4E)**. Anisomycin dramatically
410 reduced freezing in memory tests that followed, whereas pairing with CO_2 in the CS did
411 not cause an additional reduction in the ASIC1a$^{-/-}$ mice, suggesting an ASIC
412 dependency **(Fig. 4E)**. We also presented two distinct CSs (pure tone and white noise)
413 during conditioning and then retrieval, with or without 10% CO_2, followed by anisomycin
414 infusions in the ASIC1a$^{-/-}$ mice **(Fig. 4F)**. Anisomycin dramatically reduced freezing in
415 memory tests that followed, whereas pairing with CO_2 in either CSs (pure tone or white
416 noise) did not cause an additional reduction in the ASIC1a$^{-/-}$ mice, suggesting an ASIC
417 dependency **(Fig. 4G-N)**.
418
419 To provide evidence that an acute ASIC1a blockage was able to eliminate the effects of
420 CO_2, we injected 100nM PcTX-1 into the lateral amygdala bilaterally 1 hour before the
421 application of CO_2 to the retrieval **(Fig. 5A)**. Our data suggest that compared to the

422 saline injection group **(Fig. 5 B-E)**, inhibiting ASIC1a by PcTX-1 significantly reduced
423 the CO_2 effects on memory retrieval **(Fig. 5 F-I)**, statistical analysis in the spontaneous
424 recovery and renewal groups supported this conclusion (green columns, **Fig. 5J, K**).
425 This pattern of findings suggests that the effects of CO_2 on specific memory traces are
426 ASIC dependent.
427

428 **Activation of ASICs through CO_2 inhalation alters reconsolidation of distinct**
429 **memory through alteration of AMPARs.**

430 AMPARs are glutamatergic receptors that have crucial roles in modulating memory
431 retrieval and destabilization (Auber et al., 2013; Chan et al., 2010; Clem and Huganir,
432 2010; Monfils et al., 2009; Quirk et al., 2010). Previous studies suggest that the
433 exchange of Ca^{2+}-impermeable AMPARs (CI-AMPARs) for Ca^{2+}-permeable AMPARs
434 (CP-AMPARs) occurs after retrieval (Clem and Huganir, 2010; Hong et al., 2013). 10%
435 CO_2 inhalation paired with retrieval induces a stronger current rectification of AMPARs
436 (the signature of CP-AMPARs) than in the retrieval alone group, indicating a greater
437 exchange of AMPARs (Du et al., 2017). Interestingly, no further enhancement was
438 observed in the ASIC1a$^{-/-}$ brain slices, indicating that the effect of CO_2 inhalation on
439 AMPAR exchange is ASIC dependent (Du et al., 2017). To further study whether
440 CO_2 specifically alters the AMPARs exchange in retrieval, we designed a unique
441 experiment to separate the aversive conditioning and retrieval and measure the
442 rectification of AMPARs **(Fig. 6A)**. To study this, we conditioned the mice with 6 pure
443 tones on day 1 **(Fig. 6B, left)**. On day 2, the mice were divided into 4 groups based on
444 retrieval conditions - the first group received pure tone only; the second group-pure tone

445 plus 10% CO_2 inhalation; the third group - white noise only; and the fourth group
446 received white noise+10% CO_2 inhalation **(Fig. 6B, right)**. Ten minutes after retrieval,
447 we dissected brain slices and AMPAR current was recorded in the pyramidal neurons in
448 the lateral amygdala through stimulation of thalamic inputs **(Fig. 6A)**. Rectification, a
449 signature of CP-AMPARs, was compared among all groups. Consistent with earlier
450 reports, pure tone retrieval increased current rectification (Clem and Huganir, 2010;
451 Hong et al., 2013) and CO_2 paired with pure tone retrieval caused stronger rectification
452 **(Fig. 6C)**. However, when white noise was presented as the retrieval event, both white
453 noise and white noise plus CO_2 failed to cause a significant rectification compared to
454 the pure tone group **(Fig. 6C)**. This data supports our prediction that CO_2 was
455 associated with a specific memory trace that was reactivated. To control for the possible
456 effects of the order of presentation of CSs, we switched over the pure tone and white
457 noise in the aversive conditioning and retrieval. Similar results were observed,
458 confirming the effects of CO_2 were not artificial **(Fig. 6D, E)**.
459
460 We then asked whether synaptic strength changed with the application of an unrelated
461 retrieval CS and CO_2. The ratio of AMPAR-EPSCs to NMDAR-EPSCs might represent
462 the strength of the synapse (Rao and Finkbeiner, 2007). Previous studies reported that
463 the AMPAR/ NMDAR-EPSC ratio increased after aversive conditioning whereas
464 retrieval did not potentiate further increase, suggesting that memory retrieval did not
465 alter the synaptic strength (Clem and Huganir, 2010; Hong et al., 2013). Our previous
466 studies also indicated that CO_2 inhalation during memory retrieval did not strengthen the
467 synapse in the amygdala (Du et al., 2017). We further tested whether the pairing of CO_2

468 inhalation with the specific retrieval CS influenced the strength of a synapse. Currents
469 were recorded at -80mV for AMPAR-EPSCs and +60mV for NMDAR-EPSCs. Our data
470 suggest that retrieval plus CO_2 inhalation did not change the AMPAR/ NMDAR-EPSCs
471 ratio **(Fig. 6F)**. Moreover, the characteristics of miniature EPSCs (mEPSCs), including
472 amplitude, frequency, and decay times, were not altered **(Fig. 6G)**. In addition, the
473 pairing of CO_2 with another unrelated CS in retrieval did not change the strength of the
474 synapse **(Fig. 6F, G)**. This data suggests that the effects of CO_2 inhalation on specific
475 memory retrieval enhance the destabilization of the synapse without changing synaptic
476 strength.
477
478 **The effects of CO_2 inhalation on distinct memory trace.**
479 Our previous studies indicate that CO_2 enhances memory trace that is associated with
480 aversive conditioning (Du et al., 2017). In this experiment, we examined the mechanism
481 behind the specificity of CO_2 effects on memory traces. Using the TetTag-c-fos driven-
482 GFP mouse model, neurons in the amygdala involved in memory trace after aversive
483 conditioning can be labeled with a long-lasting mCherry fluorescent protein and the
484 neuron in the retrieval trace can be labeled with a 2-hour short half-life nuclear-localized
485 EGFP (shEGFP) **(Fig. 7A, B)** (see the details in Materials and Methods) (Du et al.,
486 2017; Koffman and Du, 2017). The overlapped labeling (yellow) represents the neurons
487 in the same memory trace of aversive and retrieval (Du et al., 2017; Liu et al., 2012;
488 Ramirez et al., 2013). In this experiment, the mice were first conditioned with pure tone,
489 activating the associated neurons that were labeled with mCherry **(Fig. 7C)**. Right
490 after aversive conditioning, the mice were immediately fed with a DOX diet thereby

491 preventing further mCherry labeling. On day 2, the mice were divided into two groups-
492 one group of mice were presented with a single pure tone to retrieve the memory,
493 another was presented with white noise. A temporary, 2-hour half-life, shEGFP was
494 labeled after the retrieval. Thirty minutes after the retrieval event, we sliced the
495 amygdala and imaged shEGFP- and mCherry-positive cells **(Fig. 7D)**. Compared to
496 pure tone aversive conditioning/pure tone retrieval group (same memory trace of
497 aversive conditioning and retrieval), inhalation of CO_2 in the pure tone aversive
498 conditioning/white noise retrieval group did not result in an increase of neurons positive
499 for expression of both mCherry-positive and shEGFP-positive neurons (overlapped
500 labeling, yellow) **(Fig. 7E)**. Control experiments to identify the efficiency of the aversive
501 conditioning on the expression of mCherry on the cells were performed **(Fig. 7F)**. These
502 findings indicate that CO_2, when paired with retrieval, only enhances the memory trace
503 that has been reactivated; CO_2 paired with unrelated retrieval cue does not affect the
504 original memory trace. These findings suggest a specific effect of CO_2 on the memory
505 trace.
506
507 We then examined the effects of CO_2 on dendritic spine morphology after memory
508 retrieval. Spine morphology has been widely indicated in the mechanism of synaptic
509 plasticity (Woolfrey and Srivastava, 2016; Wright et al., 2020; Yang et al., 2009).
510 Dendritic spines are the primary target of neurotransmission input in the central nervous
511 system (Bourne and Harris, 2008), and their density and structure provide the basis for
512 physiological changes in synaptic efficacy that underlie learning and memory (Bailey et
513 al., 2015). Spine formation and plasticity are regulated by many conditions, including

514 exterior stimulation and behavior (Gipson and Olive, 2017). We hypothesized that CO_2
515 inhalation during retrieval alters both structure and plasticity of dendritic spines. The
516 molecular mechanism by which CO_2 regulates spine plasticity may explain how CO_2
517 converts memory into the labile stage.
518
519 Using the TetTag-c-fos driven-GFP mouse model, we imaged spine structure and
520 assessed spine density and morphology in overlapping neurons of the amygdala in
521 each group (pure tone aversive conditioning, pure tone retrieval and pure tone aversive
522 conditioning, white noise retrieval) **(Fig. 7A, C)**. Mature spines - most of which display
523 “mushroom-like” morphology - have more stable postsynaptic structures enriched in
524 AMPARs. In contrast, immature spines with a “thin-like” morphology, are unstable
525 postsynaptic structures that have the transitional ability. Immature dendritic spines are
526 thought to be responsible for synaptic plasticity, as they have the potential for
527 strengthening (Berry and Nedivi, 2017). The categories of spines were identified based
528 on the parameters in the previous studies **(Fig. 7G)** (see the details in the Material and
529 Methods) (Kreple et al., 2014; Wright et al., 2020). The behavior procedure was
530 described in **Fig. 7C**, the animals underwent aversive conditioning with a tone as a
531 CS and followed by a retrieval on day 2 with tone or noise. We found increased spine
532 numbers after aversive conditioning, indicating that aversive conditioning increases
533 synaptic strength. There was no additional increase in the density of spines in all
534 groups, suggesting that retrieval and CO_2 inhalation did not change the synaptic
535 strength **(Fig. 7H, lower-right)**.
536

537 We further analyzed spine subtypes as described in the experimental procedures. Thin
538 spines are deemed to represent the immature structure of the synapses (Berry and
539 Nedivi, 2017). We examined the ratio of the number of thin spines to the total number of
540 spines. Interestingly, we found that there were more thin spines in the retrieval group,
541 which might suggest a greater potential for synaptic plasticity in this group **(Fig. 7H,**
542 **lower-middle)**. Importantly, when the retrieval group (tone) was paired with CO_2, we
543 found an additional increase of thin spines compared to the retrieval group alone **(Fig.**
544 **7H, lower-middle)**. This finding suggests that CO_2 paired with retrieval might boost
545 synaptic plasticity compared to memory retrieval alone. Consistently, the mushroom
546 spine numbers decreased in the tone and CO_2 paired retrieval groups, suggesting a
547 higher turnover rate after memory retrieval **(Fig. 7H, lower-left)**. However, when the
548 mice were trained with the pure tone and during the retrieval session were presented
549 with white noise (a generated unrelated CS), we found that in the noise group with or
550 without CO_2 inhalation, the thin spine number did not increase compared to the pure
551 tone/retrieval group **(Fig. 7H, lower-middle)**. This finding supports the specific effect of
552 CO_2 on the memory trace.
553

554 **DISCUSSION**

555 A newly acquired aversive memory is labile and can be easily disrupted before it is
556 transformed into a long-term stable state (Alberini, 2011). An existing memory, when
557 reactivated, may become labile again during a short post-reactivation period known are
558 reconsolidation window (Schiller et al., 2012). Previous studies using auditory aversive
559 conditioning found that a retrieval event utilizing a single tone CS renders the memory
560 labile during the reconsolidation window (Monfils et al., 2009). During this

561 reconsolidation window, memory is sensitive to the updating processes that may either
562 enhance or weaken the original memory (Bouton et al., 2020; Du et al., 2017;
563 Fukushima et al., 2014). The reconsolidation window offers an opportunity to determine
564 the mechanisms underlying the lability of an existing memory. We have recently found
565 that when mice inhale 10% CO_2 during retrieval, memory lability increases, and the
566 original memory may be replaced through the update mechanisms (Du et al., 2017).
567 Here, we show that the effects of CO_2 on memory are specific to the reactivated
568 conditioned cue (Debiec et al., 2010; Doyere et al., 2007). To address the specificity of
569 the effects of CO_2 on reactivated memories, we used a unique behavioral approach with
570 distinct CSs and US, which allowed us the creation of distinct memories. An application
571 of CO_2 to the reactivated but not no-reactivated memory enhanced its lability.
572
573 We then asked about the cellular mechanisms through which CO_2 enhances the lability
574 of aversive memory. Memory retrieval-induced lability is glutamate receptor-dependent
575 (Rose and Rankin, 2006; Milton, A.L., Everitt, B.J. 2013). We have shown that CO_2
576 activates ASIC1a by decreasing pH in the brain during retrieval and the activation of
577 ASIC1a increases postsynaptic intracellular calcium and increases AMPAR exchange
578 (Du et al., 2017). Previous research studying the mechanism of retrieval of aversive
579 memories have revealed the rapid and transient exchange from CI-AMPARs to CP-
580 AMPARs in the lateral amygdala synapses (Clem and Huganir, 2010; Hong et al., 2013)
581 after presenting the CS. In this study, we focused on the specificity of CO_2 effects on the
582 exchange of the AMPARs in the lateral amygdala's synapses. We conditioned the mice
583 with a pure tone CS and reactivated the memory with the pure tone with or without
584 CO_2 inhalation. Consistent with our earlier findings, we observed that CO_2 increases

585 AMPAR exchange when it is paired with retrieval. However, when mice were presented
586 with an unrelated CS during the retrieval stage, CO_2 did not alter the AMPAR exchange-
587 suggesting the effects of CO_2 on memory trace are memory specific. In addition,
588 synaptic strength (ratio of AMPAR/NMDAR and amplitude of the mEPSCs) was not
589 altered while applying CO_2 during retrieval, with or without combining with the memory
590 trace. The exchange of CI-AMPARs to CP-AMPARs indicates a synaptic plasticity
591 change. Interestingly, CO_2 did not increase the total number of spines compared to the
592 retrieval alone group, which suggests the strength of the synapse in a memory trace
593 does not change. In addition, thin spine density significantly increased when retrieval
594 was combined with CO_2 inhalation, suggesting that CO_2 application changes plasticity.
595 Using this model, we found when an unrelated CS was presented during the retrieval
596 session, no additional increase of immature spine density occurred. This finding
597 indicates no plasticity change in the memory trace. Thus, we can conclude from our
598 findings that the effects of CO_2 on memory trace are specific.
599
600 Although we have provided evidence that CO_2 indeed acts with specificity on a memory
601 trace, this study did not determine how CO_2 directly regulates a memory. We cannot
602 exclude the possibility that CO_2 might trigger specific effects on memory through other
603 targets. For instance, CO_2 inhalation increases cerebral blood flow and arterial blood
604 pressure and might affect brain functions, such as cognition. Although no direct
605 evidence supports the possibility that increased cerebral blood flow and arterial blood
606 pressure affect learning and memory, future studies will have the mechanisms
607 underlying the specificity of CO_2 on memory. Future studies will also have to examine

608 whether CO_2 has similar effects on synapses in other brain regions and on other learned
609 behaviors (*e.g.* appetitive behaviors and drug addictive behaviors).
610
611 In conclusion, the effects of CO_2 on the lability of aversive memory are found to be
612 specific under certain conditions. Our research tests the novel hypothesis that protons
613 are neurotransmitters that activate the postsynaptic proton receptors, ASICs, to
614 manipulate memory updates. This non-invasive, drug-free methodology is innovative,
615 efficacious, and safe for translation to clinical use. As a result, this research may lead to
616 an effective complementary treatment for many mental health-related disorders for
617 which efficient treatments are lacking. We hope this research will lead to new areas of
618 inquiry into CO_2-related mechanisms that underlie memory modification and lead to the
619 development of novel therapies for anxiety disorders such as PTSD.
620

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627

628 **AUTHOR CONTRIBUTIONS**

629 J.DU., J.D., E.K., C.K. and conceived the project. J.DU., E.K., C.K. and J.D. designed
630 the experiments. E.K., C.K., J.E., S.B., M.H., B.L., performed the behavior experiments.

K.S., performed the spine morphology experiments and data analysis. F.N., and J.DU. performed the patch-clamp experiments and data analysis. E.K., and J.DU., wrote the manuscript. All authors reviewed and edited the manuscript.

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654 **FIGURE LEGENDS**

655 **Figure 1: CO_2 inhalation during a selective memory retrieval potentiates the effect**
656 **of the extinction. (A)** Representative schematic of protocol for the aversive
657 conditioning (Av. Cond.), memory retrieval (Ret), extinction (Ext), and memory test-
658 spontaneous recovery (Spon Rec) and renewal. On day1, the mice were subjected to 3
659 pure tones and 3 white noises, paired with 6 foot shocks in context A. One day after, the
660 mice were placed in context B and were subjected to both tone and noise as retrieval
661 events. 30 mins after retrieval, the mice were treated with 2 blocks of extinctions with a
662 pure tone as the CS. On day 7, spontaneous recovery and renewal were tested
663 individually in context B and then context A. 4 pure tones and 4 white noises were
664 presented as CSs during each memory testing. **(B-E)** Data are presented by the
665 percentage of freezing time during the CSs (tone and noise) in aversive conditioning
666 **(B)**, retrievals (tone and noise) **(C)**, two sections of extinction with tone **(D)**, memory test
667 of spontaneous recovery (Spon Rec) and renewal with tone and noise **(E)**. **(F-I)** Data
668 are presented by the percentage of freezing time during the CSs (tone and noise) in
669 aversive conditioning **(F)**, retrievals (tone plus CO_2 inhalation and noise) **(G)**, two
670 sections of extinction with tone **(H)**, memory test of spontaneous recovery (Spon Rec)
671 and renewal with tone and noise **(I)**. **(J-K),** comparison data based on spontaneous
672 recovery and renewal respectively from panels **E** and **I**. Data are mean ± SEM. n = 10-
673 12 mice in each group. 'n.s.' indicates not statistically significant. * indicates $p<0.05$, **
674 indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$,by unpaired Student's t-
675 test and one-way ANOVA with Tukey's post-hoc multiple comparisons.
676 See also Figure S1 and Figure S2.
677

678 **Figure 2: CO_2 inhalation during memory retrieval potentiates the effect of the**
679 **anisomycin. (A)** Representative schematic of the protocol for aversive conditioning,
680 memory retrieval, anisomycin injection, and memory test (spontaneous recovery and
681 renewal). On day1, the mice were subjected to 6 pure tones, paired with 6 foot shocks
682 in context A. One day after CS, the mice were placed in context B and were subjected
683 to one single tone as a retrieval event with or without CO_2 inhalation. 30 mins after
684 retrieval, the mice were then infused with 62.5 μg/μl anisomycin or saline and then
685 returned to their home cage. On day 7, spontaneous recovery and renewal were tested
686 individually in context B and then context A. 4 pure tones were presented as CSs during
687 each memory testing. **(B-E)** Data are presented by the percentage of freezing time
688 during the tone presentation in aversive conditioning **(B)**, retrieval (tone) **(C)**, saline or
689 anisomycin infusion in the amygdala **(D)**, spontaneous recovery (Spon Rec), and
690 renewal test with tones **(E)**. **(F-I)** Data are presented by the percentage of freezing time
691 during the tone presentation in aversive conditioning **(F)**, retrieval (tone) with or without
692 CO_2 **(G)**, anisomycin infusion **(H)**, spontaneous recovery (Spon Rec), and renewal test
693 with tones **(I)**. Data are mean ± SEM. n = 8-10 mice in each group. * indicates $p<0.05$,
694 *** indicates $p<0.001$, by unpaired Student's t-test. See also Fig. s3, s4, s5
695
696 **Figure 3: The effect of CO_2 inhalation on selective memory retrieval is ASIC1a-**
697 **dependent. (A)** Representative schematic of protocol for the aversive conditioning,
698 memory retrieval, extinction, and memory test (spontaneous recovery and renewal) in
699 ASIC1a$^{-/-}$ mice. On day1, the mice were subjected to 3 pure tones and 3 white noises,
700 paired with 6 foot shocks in context A. One day later, the mice were placed in context B
701 and subjected to both tone and noise as retrieval events. 30 mins after retrieval, the

702 mice were treated with 2 blocks of extinctions with a pure tone as the CS. On day 7,
703 spontaneous recovery and renewal were tested individually in context B and then
704 context A. 4 pure tones and 4 white noises were presented as CSs during each memory
705 testing. **(B-E)** Data in ASIC1a$^{-/-}$ mice are presented by the percentage of freezing time
706 during the CSs (tone and noise) in aversive conditioning **(B)**, retrievals (tone and noise)
707 **(C)**, two sections of extinction with tone **(D)**, memory test of spontaneous recovery
708 (Spon Rec) and renewal with tone and noise **(E)**. **(F-I)** Data in ASIC1a$^{-/-}$ mice in
709 aversive conditioning **(F)**, retrievals (pure tone plus 10% CO_2 inhalation and white noise)
710 **(G)**, two sections of extinction with pure tone **(H)**, memory test of spontaneous recovery
711 (Spon Rec) and renewal with tone and noise **(I)**. **(J-K),** comparison data based on
712 spontaneous recovery and renewal respectively from panels **E** and **I**. Data are mean ±
713 SEM. n = 12-16 mice in each group. * indicates $p<0.05$, ** indicates $p<0.01$, ***
714 indicates $p<0.001$, by unpaired Student's t-test and one-way ANOVA with Tukey's post-
715 hoc multiple comparisons.
716
717 **Figure 4: The CO_2 potentiated anisomycin effects are ASIC1a-dependent.**
718 **(A)** Representative schematic of protocol for the aversive conditioning, memory
719 retrieval, anisomycin injection, and memory test (spontaneous recovery and renewal).
720 On day 1, the mice were subjected to 6 pure tones, paired with 6 foot shocks in context
721 A. One day after, the mice were placed in context B and subjected to one single tone as
722 a retrieval event with or without CO_2 inhalation. 30 mins after retrieval, the mice were
723 infused with 62.5 μg/μl anisomycin or saline and then returned to their home cage. On
724 day 7, spontaneous recovery and renewal were tested individually in context B and then

725 context A. 4 pure tones were presented as CSs during each memory testing. **(B-E)** Data
726 in ASIC1a$^{-/-}$ mice are presented by the percentage of freezing time during the tone
727 presentation in aversive conditioning **(B)**, retrieval (tone) with or without CO_2 **(C)**,
728 anisomycin infusion in the amygdala **(D)**, spontaneous recovery (Spon Rec) and
729 renewal test with tones **(E)**. **(F)** Representative schematic of protocol for the aversive
730 conditioning, memory retrieval, extinction, and memory test (spontaneous recovery and
731 renewal). On day 1, ASIC1a$^{-/-}$ mice were subjected to 3 pure tones and 3 white noises,
732 paired with 6 foot shocks in context A. One day later, the mice were placed in context B
733 and subjected to both tone and noise as retrieval events. 30 mins after retrieval, the
734 mice were infused with anisomycin or saline and returned to their home cage. On day 7,
735 spontaneous recovery and renewal were tested individually in context B and then
736 context A. 4 pure tones and 4 white noises were presented as CSs during each memory
737 testing. **(G-J)** Data are presented by the percentage of freezing time during the tone
738 presentation in aversive conditioning **(G)**, retrieval (noise plus CO_2 inhalation, then tone)
739 **(H)**, anisomycin infusion in the amygdala **(I)**, spontaneous recovery (Spon Rec), and
740 renewal test with tones **(J)**. **(K-N)** Data are presented by the percentage of freezing time
741 during the tone presentation in aversive conditioning **(K)**, retrieval (tone) with or without
742 CO_2 **(L)**, anisomycin infusion in the amygdala **(M)**, spontaneous recovery (Spon Rec),
743 and renewal test with tones **(N)**. Data are mean ± SEM. n = 12-16 mice in each group.
744 'n.s.' indicates not statistically significant by unpaired Student's t-test between groups.
745
746 **Figure 5: Blockage of ASIC1a in the amygdala reduces the CO_2 effects on**
747 **selective memory retrieval. (A)** Representative schematic of protocol for the aversive
748 conditioning, PcTX-1 injection, memory retrieval, extinction, and memory test

749 (spontaneous recovery and renewal). On day1, the mice were subjected to 3 pure tones
750 and 3 white noises, paired with 6 foot shocks in context A. One day later, the mice were
751 injected with 100nM PcTX-1 or saline, then the mice were placed in context B and
752 subjected to both tone and noise as retrieval events with or without CO_2. 30 mins after
753 retrieval, the mice were treated with 2 blocks of extinctions with a pure tone as the CS.
754 On day 7, spontaneous recovery and renewal were tested individually in context B and
755 then context A. 4 pure tones and 4 white noises were presented as CSs during each
756 memory testing. **(B-E)** Data are presented by the percentage of freezing time during the
757 CSs (tone and noise) in aversive conditioning **(B)**, retrievals (tone plus CO_2 inhalation
758 and noise) after saline injection in the amygdala **(C)**, two sections of extinction with tone
759 **(D)**, memory test of spontaneous recovery (Spon Rec) and renewal with tone and noise
760 **(E)**. **(F-I)** Data are presented by the percentage of freezing time during the CSs (tone
761 and noise) in aversive conditioning **(F)**, retrievals (tone plus CO_2 inhalation and noise)
762 after PcTx-1 injection in the amygdala **(G)**, two sections of extinction with tone **(H)**,
763 memory test of spontaneous recovery (Spon Rec) and renewal with tone and noise **(I)**.
764 **(J-K),** comparison data based on spontaneous recovery and renewal respectively from
765 panels **E** and **I**. Data are mean ± SEM. n = 12 mice in each group. 'n.s.' indicates not
766 statistically significant. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, ****
767 indicates $p<0.0001$, by unpaired Student's t-test and one-way ANOVA with Tukey's
768 post-hoc multiple comparisons.
769
770 **Figure 6: CO_2 inhalation during a selective memory retrieval enhances the**
771 **retrieval dependent AMPAR current rectification. (A)** Representative schematic of

772 the protocol. On day 1, the animal underwent 6 CSs (tones or noses), paired with 6-foot
773 shocks in context A. On day 2, the mice were divided into 4 groups for the retrieval-pure
774 tone only; pure tone plus 10% CO_2 inhalation; white noise only; white noise+ 10% CO_2
775 inhalation. Ten minutes after retrieval, the brain slices were dissected and AMPAR
776 current was recorded in the pyramidal neurons in the lateral amygdala through
777 stimulation of thalamic input. **(B-E)** Mice underwent 6 pure tones **(B)** or 6 noises **(D)** in
778 aversive conditioning, Data are presented by the percentage of freezing time during the
779 tone presentation in aversive conditioning, retrieval (noise plus CO_2 inhalation, then
780 tone). **(C, E)** Left, AMPARs current-voltage relationships in the recorded neurons. Insets
781 show an example of the AMPAR-EPSCs in -80mV and + 60mV. Right, AMPAR
782 rectification index (I-80 mV / I+60 mV). Data are mean±SEM. n = 20-26 for each group.
783 **(F)** Left, examples of EPSC recordings of AMPAR-EPSCs (-80mV) and NMDAR-
784 EPSCs (+60mV). Right, AMPAR/NMDAR EPSC ratios. Current amplitudes were
785 measured 70 ms after onset. n = 20-26 for each group. **(G)** Miniature EPSCs recordings
786 from the neurons after retrieval. Upper, representative mEPSC traces from different
787 groups. Lower, cumulative distributions of mEPSC amplitudes, inter-event intervals, and
788 decay-times. n = 25-40 for each group. Data are mean ± SEM. 'n.s.' indicates not
789 statistically significant. * indicates $p<0.05$, ** indicates $p<0.01$, by ANOVA with Tukey's
790 post hoc multiple comparisons.
791
792 **Figure 7: CO_2 inhalation during a selective memory retrieval enhances the**
793 **retrieval-related memory trace. (A)** Schematic showing the c-Fos-tTA-GFP mouse
794 system combined with an $AAV_{2/9}$-mCherry to label a specific memory trace. The Fos

795 promoter in transgenic mice was activated by activities, followed by a transient, 2-hour
796 half-life, GFP expression in the cells. When the $AAV_{2/9}$-mCherry virus was injected into
797 the brain, the activation of c-FOS also induces the expression of mCherry. When the
798 mice were fed with DOX, the mCherry expression was interrupted. **(B)** an example
799 image showing the efficiency of the expression of GFP and mCherry in the amygdala.
800 **(C)** The procedure of aversive conditioning, memory retrieval, and memory trace
801 labeling using the system in A. Mice was fed with DOX for at least one week, followed
802 by an injection of $AAV_{2/9}$-mCherry in the amygdala. Two weeks later, DOX was removed
803 and mice were subjected to aversive conditioning with a pure tone as the CS. DOX has
804 added back again immediately after aversive conditioning. 24 hours later, the mice
805 underwent retrieval with pure tone or noise. The brain slices were collected 30 mins
806 after retrieval and then were stained for microscopy. **(D)** Left, representative images of
807 the neurons labeled by mCherry, GFP, and DAPI; Right, the enlarged area from the
808 "merge" image showing the overlapping expression of mCherry and GFP neurons. The
809 overlapping neurons indicate their "consanguinity" in the same memory trace. **(E)**
810 Summarized data are the percentage of the overlapping expression of mCherry and
811 GFP neurons in different behavior groups. All mice underwent aversive conditioning
812 with a tone as the CS. 24 hours later, the mice were separated into 4 groups. Tone
813 group: retrieval CS by tone; Tone+CO_2 group: retrieval CS by tone along with CO_2
814 inhalation; Noise group: retrieval CS by noise; Noise +CO_2 group: retrieval CS by noise
815 along with CO_2 inhalation. **(F)** Control experiment showing the expression of mCherry
816 with or without DOX as well as with or without aversive conditioning. **(G)** Left, a
817 representative image showing the spine morphology in the mCherry and GFP

818 colocalized neurons. The mature spines were categorified as “mushroom” spines and
819 the immature spines were categorified as “thin” spines; Right, an enlarged image
820 showing the details of mushroom and thin spines. **(H)** Upper, representative images of
821 the spine structures in different animal groups showed in E; Lower, summarized data of
822 the spine densities of mushroom spines, thin spines, and total spines in the different
823 groups. Data are mean ± SEM. n =8-12 mice for each group. ‘n.s.’ indicates not
824 statistically significant. * indicates $p<0.05$, *** indicates $p<0.001$, **** indicates
825 $p<0.0001$,by unpaired Student’s t-test and one-way ANOVA with Tukey’s post-hoc
826 multiple comparisons.
827
828 **Figure s1: White noise was an appropriate successful CS to involve use in**
829 **studying the effects of CO_2 effects on aversive conditioning and retrieval.**
830 **(A)** Representative schematic of protocol for the aversive conditioning (pure tone and
831 white noise), memory retrieval (pure tone and white noise), extinction (white noise), and
832 memory test (spontaneous recovery and renewal). **(B-E)** Data are presented by the
833 percentage of freezing time during the CSs (tone and noise) in aversive conditioning
834 **(B)**, retrievals (tone and noise) **(C)**, two sections of extinction with white noise **(D)**,
835 memory test of spontaneous recovery (Spon Rec) and renewal with tone and noise **(E)**.
836 **(F-I)** Data are presented by the percentage of freezing time during the CSs (tone and
837 noise) in aversive conditioning **(F)**, retrievals (pure tone and white noise plus CO_2
838 inhalation) **(G)**, two sections of extinction with white noise **(H)**, memory test of
839 spontaneous recovery (Spon Rec) and renewal with tone and noise **(I)**. **(J-K),**
840 comparison data based on spontaneous recovery and renewal respectively from panels

841 **E** and **I**. Data are mean ± SEM. n = 12 mice in each group. 'n.s.' indicates not
842 statistically significant. * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, ***
843 indicates p<0.0001, by unpaired Student's t-test and one-way ANOVA with Tukey's
844 post-hoc multiple comparisons.
845
846 **Figure s2: CO_2 inhalation does not boost extinction when pairs with an unrelated**
847 **CS during memory retrieval. (A)** Representative schematic of protocol for the aversive
848 conditioning (pure tone and white noise), memory retrieval (pure tone and white noise),
849 extinction (white noise), and memory test (spontaneous recovery and renewal). **(B-**
850 **E)** Data are presented by the percentage of freezing time during the CSs (tone and
851 noise) in aversive conditioning **(B)**, retrievals (pure tone and white noise plus CO_2
852 inhalation) **(C)**, two sections of extinction with pure tone **(D)**, memory test of
853 spontaneous recovery (Spon Rec) and renewal with tone and noise, Blue arrow and %
854 indicated the difference (decreases) between the tone and noise groups **(E)**. **(F-I)** Data
855 are presented by the percentage of freezing time during the CSs (tone and noise) in
856 aversive conditioning **(F)**, retrievals (pure tone plus CO_2 inhalation and white noise) **(G)**,
857 two sections of extinction with white noise **(H)**, memory test of spontaneous recovery
858 (Spon Rec) and renewal with tone and noise, Blue arrow and % indicated the difference
859 (decreases) between the tone and noise groups. **(I)**. Data are mean ± SEM. n = 12 mice
860 in each group. * indicates p<0.05 by unpaired Student's t-test.
861
862 **Figure s3: Anisomycin disrupts the memory within the reconsolidation window.**
863 **(A)** Representative schematic of protocol for the aversive conditioning (pure tone), with
864 or without memory retrieval (pure tone), anisomycin infusion, and memory test

865 (spontaneous recovery and renewal). **(B-E)** Data are presented by the percentage of
866 freezing time during the tone presentation in aversive conditioning **(B)**, with or
867 without retrieval (tone) **(C)**, anisomycin infusion in the amygdala **(D)**, spontaneous
868 recovery (Spon Rec), and renewal test with tones **(E)**. **(F-I)** Data are presented by the
869 percentage of freezing time during the CSs (tone and noise) in aversive conditioning
870 **(F)**, retrievals (pure tone and white noise) **(G)**, anisomycin injection in the amygdala **(H)**,
871 memory test of spontaneous recovery and renewal with tone and noise **(I)**. **(J-M)** Data
872 are presented by the percentage of freezing time during the CSs (tone and noise) in
873 aversive conditioning **(J)**, retrievals (pure tone plus CO_2 inhalation and white noise) **(K)**,
874 saline injection in the amygdala **(L)**, memory test of spontaneous recovery and renewal
875 with tone and noise, **(M)**. Data are mean ± SEM. n = 11-12 mice in each group. 'n.s.'
876 indicates not statistically significant. * indicates $p<0.05$ by unpaired Student's t-test.
877
878 **Figure s4: CO_2 enhances memory retrieval and potentiates the effects of**
879 **anisomycin on the reconsolidation. (A)** Representative schematic of protocol for the
880 aversive conditioning (pure tone and white noise), memory retrieval (pure tone and
881 white noise) with or without CO_2 inhalation, anisomycin infusion, and memory test
882 (spontaneous recovery and renewal). **(B-E)** Data are presented by the percentage of
883 freezing time during the CSs (tone and noise) presentation in aversive conditioning **(B)**,
884 retrieval (tone plus CO_2 and noise) **(C)**, anisomycin infusion in the amygdala **(D)**,
885 spontaneous recovery (Spon Rec) and renewal test with tone and noise **(E)**. **(F-I)** Data
886 are presented by the percentage of freezing time during the CSs (tone and noise) in
887 aversive conditioning **(F)**, retrieval (noise plus CO_2 and tone) **(G)**, anisomycin infusion in
888 the amygdala **(H)**, memory test of spontaneous recovery (Spon Rec) and renewal with

889 tone and noise **(I)**. **(J-M)** Data are presented by the percentage of freezing time during
890 the CSs (tone and noise) in aversive conditioning **(J)**, retrievals (tone and noise) plus
891 CO_2 **(K)**, saline infusion in the amygdala **(L)**, memory test of spontaneous recovery
892 (Spon Rec) and renewal with tone and noise **(M)**. **(N-Q)** Data are presented by the
893 percentage of freezing time during the CSs (tone and noise) in aversive conditioning
894 **(N)**, retrievals (tone and noise) plus CO_2 **(O)**, anisomycin infusion in the amygdala **(P)**,
895 memory test of spontaneous recovery (Spon Rec) and renewal with tone and noise **(Q)**.
896 Data are mean ± SEM. n = 12 mice in each group. 'n.s.' indicates not statistically
897 significant. * indicate $p<0.05$ by unpaired Student's t-test.
898
899 **Figure s5: CO_2 does not affect memory reconsolidation without anisomycin.**
900 **(A)** Representative schematic of protocol for the aversive conditioning (pure tone and
901 white noise), memory retrieval (pure tone and white noise) with or without CO_2
902 inhalation, saline infusion, and memory test (spontaneous recovery and renewal). **(B-**
903 **E)** Data are presented by the percentage of freezing time during the CSs (tone and
904 noise) presentation in aversive conditioning **(B)**, retrieval (tone plus CO_2 and noise) **(C)**,
905 saline infusion in the amygdala **(D)**, spontaneous recovery (Spon Rec) and renewal test
906 with tone and noise **(E)**. **(F-I)** Data are presented by the percentage of freezing time
907 during the CSs (tone and noise) in aversive conditioning **(F)**, retrieval (noise plus CO_2
908 and tone) **(G)**, saline infusion in the amygdala **(H)**, memory test of spontaneous
909 recovery (Spon Rec) and renewal with tone and noise **(I)**. Data are mean ± SEM. n = 12
910 mice in each group. 'n.s.' indicates not statistically significant by unpaired Student's t-
911 test.
912

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1177

Fig. 1

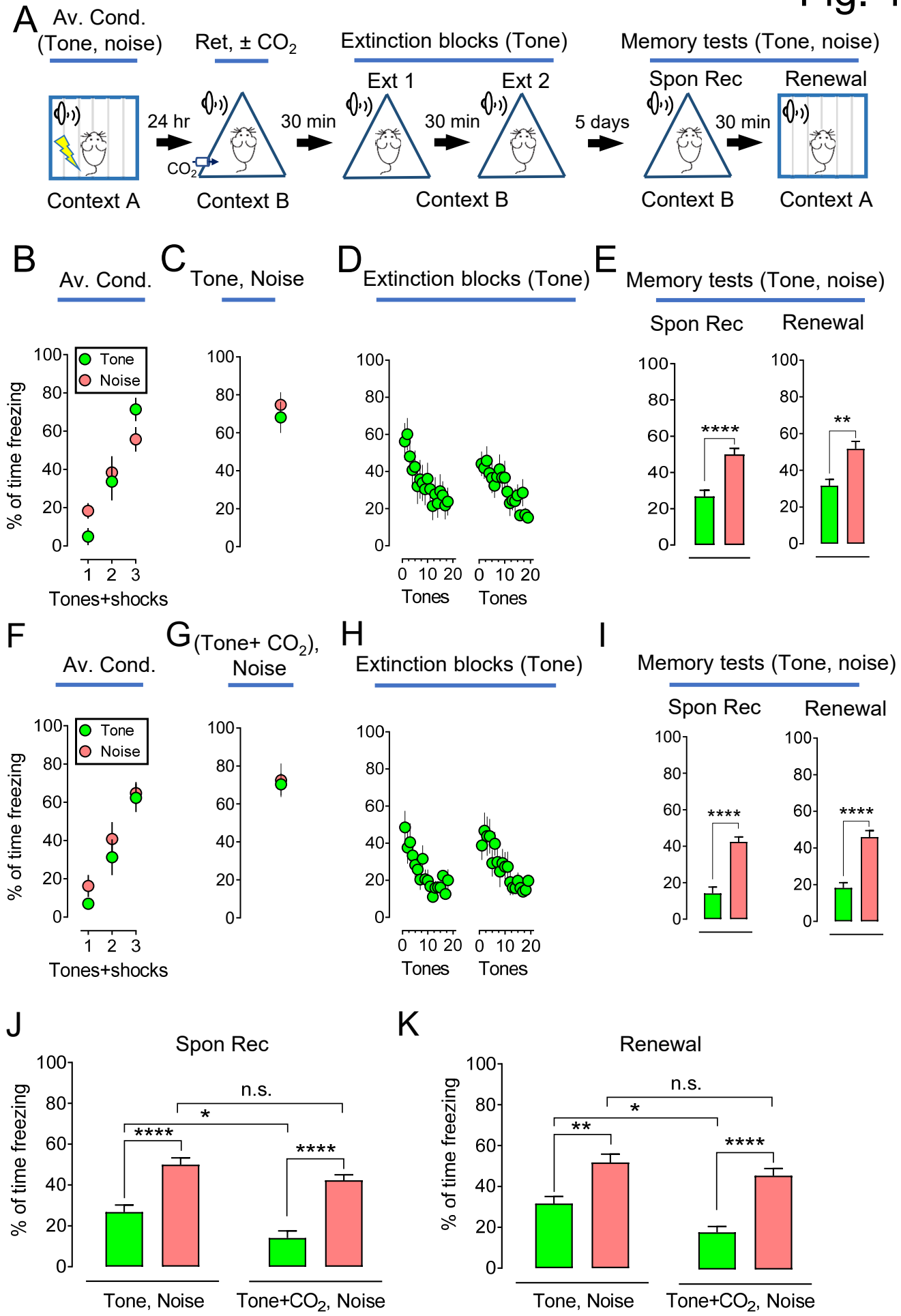

A
Av. Cond. (Tone, noise)
Ret, ± CO2
Extinction blocks (Tone)
Memory tests (Tone, noise)
Ext 1
Ext 2
Spon Rec
Renewal
24 hr
30 min
30 min
5 days
30 min
CO2
Context A
Context B
Context B
Context B
Context A
B
Av. Cond.
C
Tone, Noise
D
Extinction blocks (Tone)
E
Memory tests (Tone, noise)
Spon Rec
Renewal
Tone
Noise
% of time freezing
Tones+shocks
Tones
Tones
****
**
F
Av. Cond.
G
(Tone+ CO2), Noise
H
Extinction blocks (Tone)
I
Memory tests (Tone, noise)
Spon Rec
Renewal
J
Spon Rec
K
Renewal
n.s.
*
****
**
Tone, Noise
Tone+CO2, Noise

Fig. 2

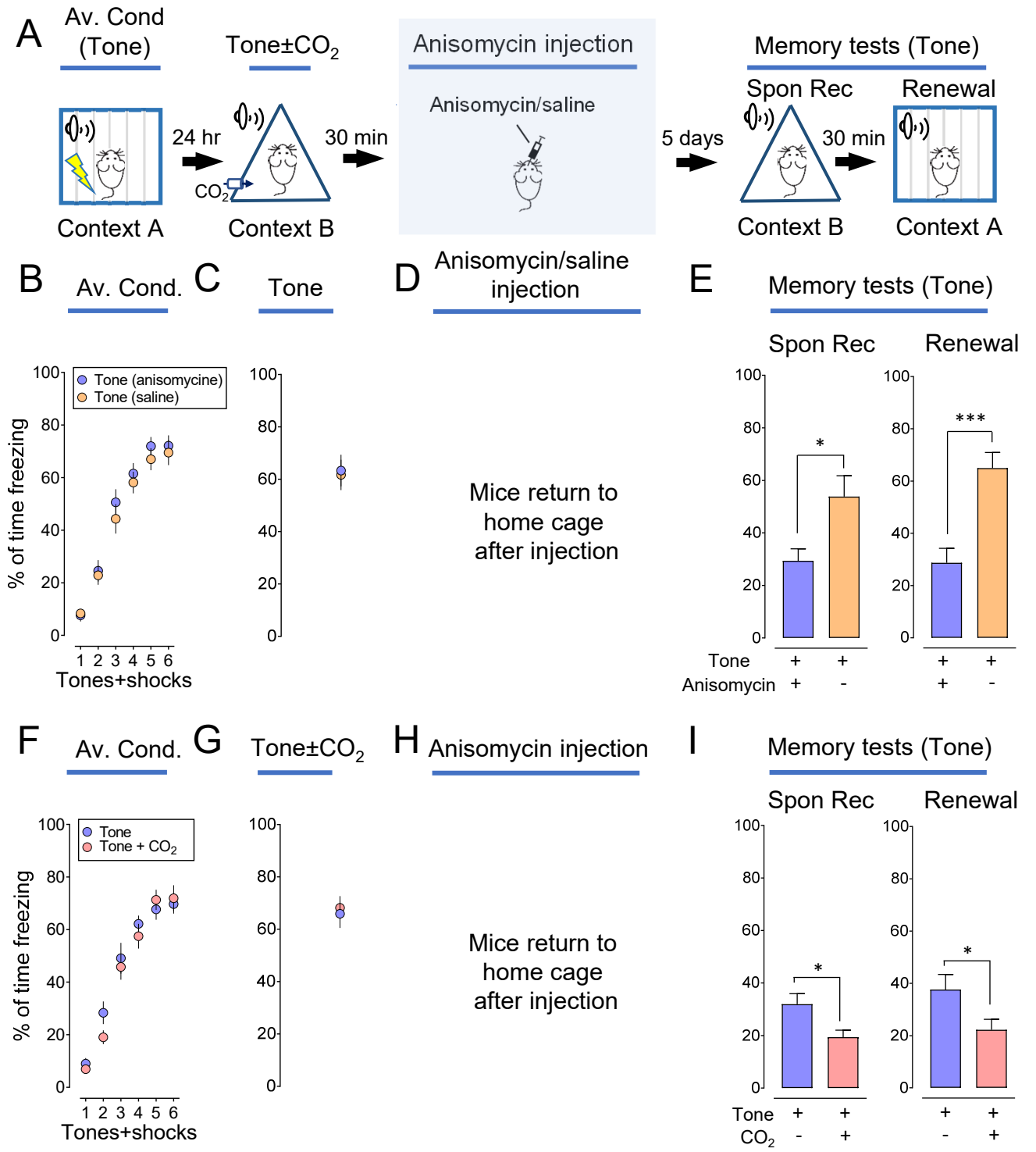

A
Av. Cond (Tone)
Tone±CO2
Anisomycin injection
Memory tests (Tone)
Anisomycin/saline
24 hr
30 min
5 days
30 min
CO2
Spon Rec
Renewal
Context A
Context B
Context B
Context A
B
Av. Cond.
C
Tone
D
Anisomycin/saline injection
E
Memory tests (Tone)
Spon Rec
Renewal
Tone (anisomycine)
Tone (saline)
% of time freezing
Tones+shocks
Mice return to home cage after injection
Tone
Anisomycin
F
Av. Cond.
G
Tone±CO2
H
Anisomycin injection
I
Memory tests (Tone)
Spon Rec
Renewal
Tone
Tone + CO2
% of time freezing
Tones+shocks
Mice return to home cage after injection
Tone
CO2

Fig. 3

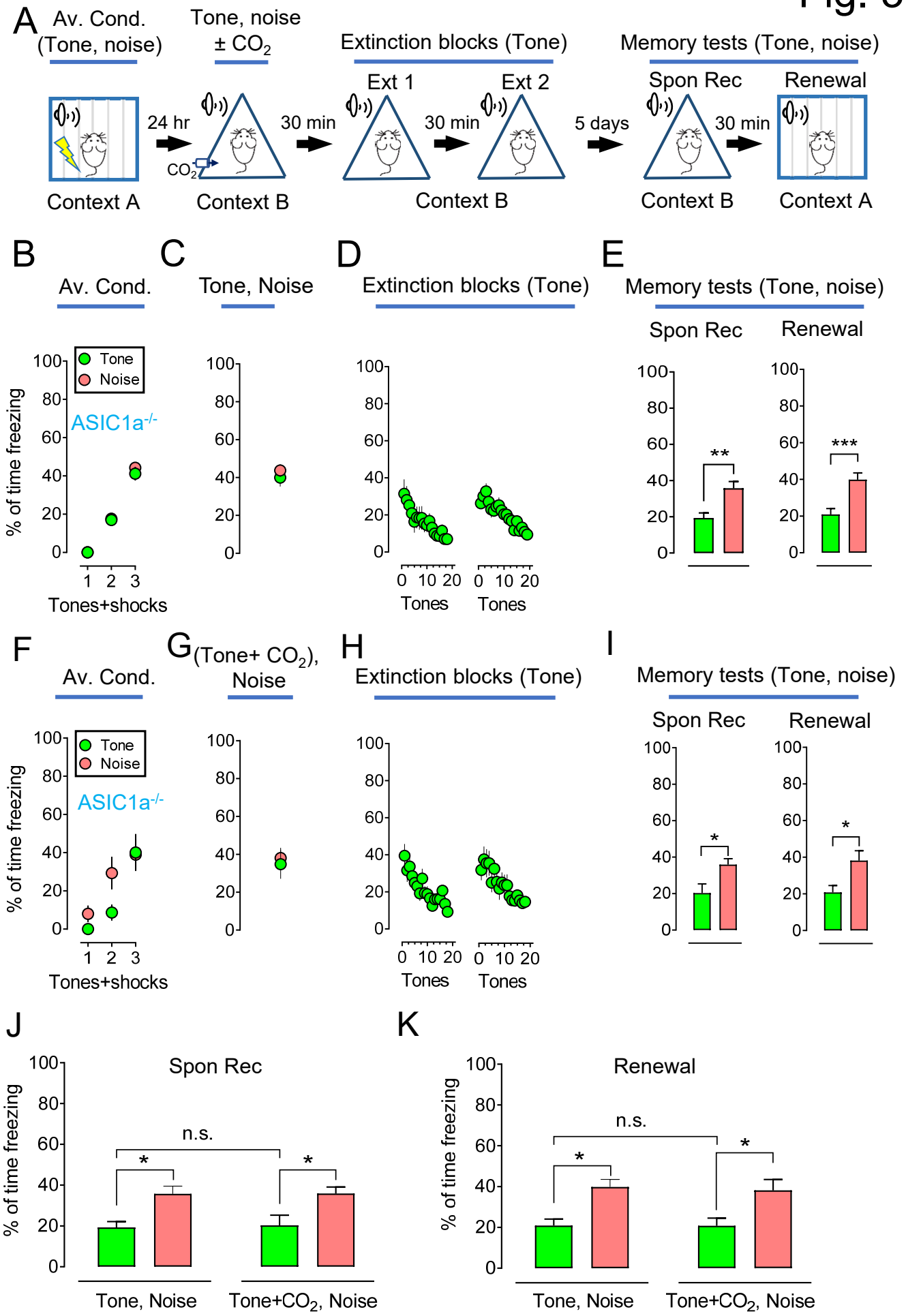

A
Av. Cond. (Tone, noise)
Tone, noise ± CO_2
Extinction blocks (Tone)
Memory tests (Tone, noise)
Ext 1
Ext 2
Spon Rec
Renewal
24 hr
30 min
30 min
5 days
30 min
CO_2
Context A
Context B
Context B
Context B
Context A
B
Av. Cond.
C
Tone, Noise
D
Extinction blocks (Tone)
E
Memory tests (Tone, noise)
Spon Rec
Renewal
Tone
Noise
ASIC1a-/-
% of time freezing
Tones+shocks
Tones
F
Av. Cond.
G
(Tone+ CO_2), Noise
H
Extinction blocks (Tone)
I
Memory tests (Tone, noise)
Spon Rec
Renewal
J
Spon Rec
K
Renewal
n.s.
Tone, Noise
Tone+CO_2, Noise

Fig. 4

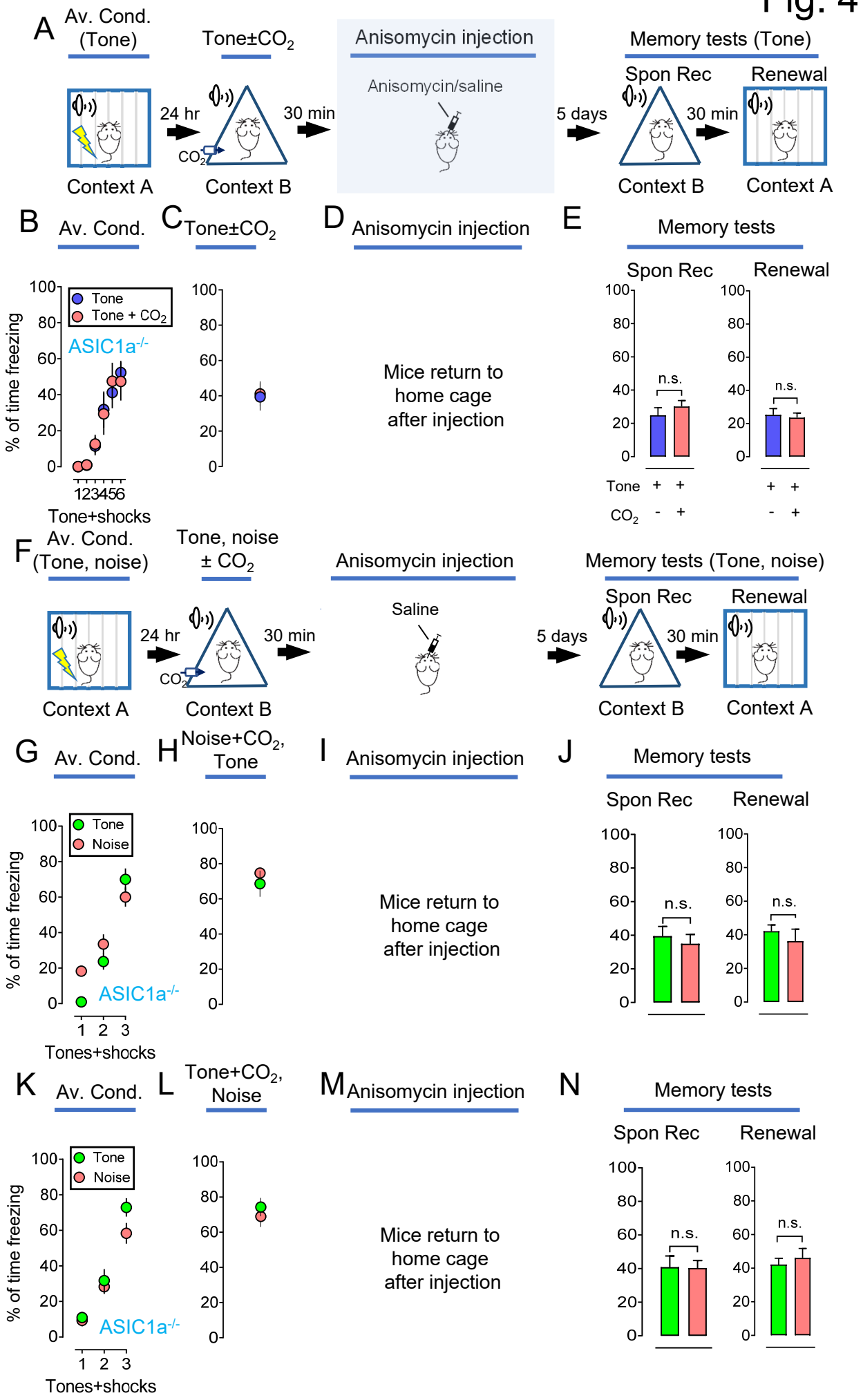

A
Av. Cond. (Tone)
Tone±CO2
Anisomycin injection
Memory tests (Tone)
Spon Rec
Renewal
24 hr
30 min
5 days
30 min
CO2
Anisomycin/saline
Context A
Context B
Context B
Context A
B
Av. Cond.
C
Tone±CO2
D
Anisomycin injection
E
Memory tests
Spon Rec
Renewal
Tone
Tone + CO2
ASIC1a-/-
% of time freezing
Tone+shocks
Mice return to home cage after injection
n.s.
Tone
CO2
F
Av. Cond. (Tone, noise)
Tone, noise ± CO2
Anisomycin injection
Memory tests (Tone, noise)
Saline
Context A
Context B
Context B
Context A
G
Av. Cond.
H
Noise+CO2, Tone
I
Anisomycin injection
J
Memory tests
Tone
Noise
ASIC1a-/-
Tones+shocks
K
Av. Cond.
L
Tone+CO2, Noise
M
Anisomycin injection
N
Memory tests

Fig. 5

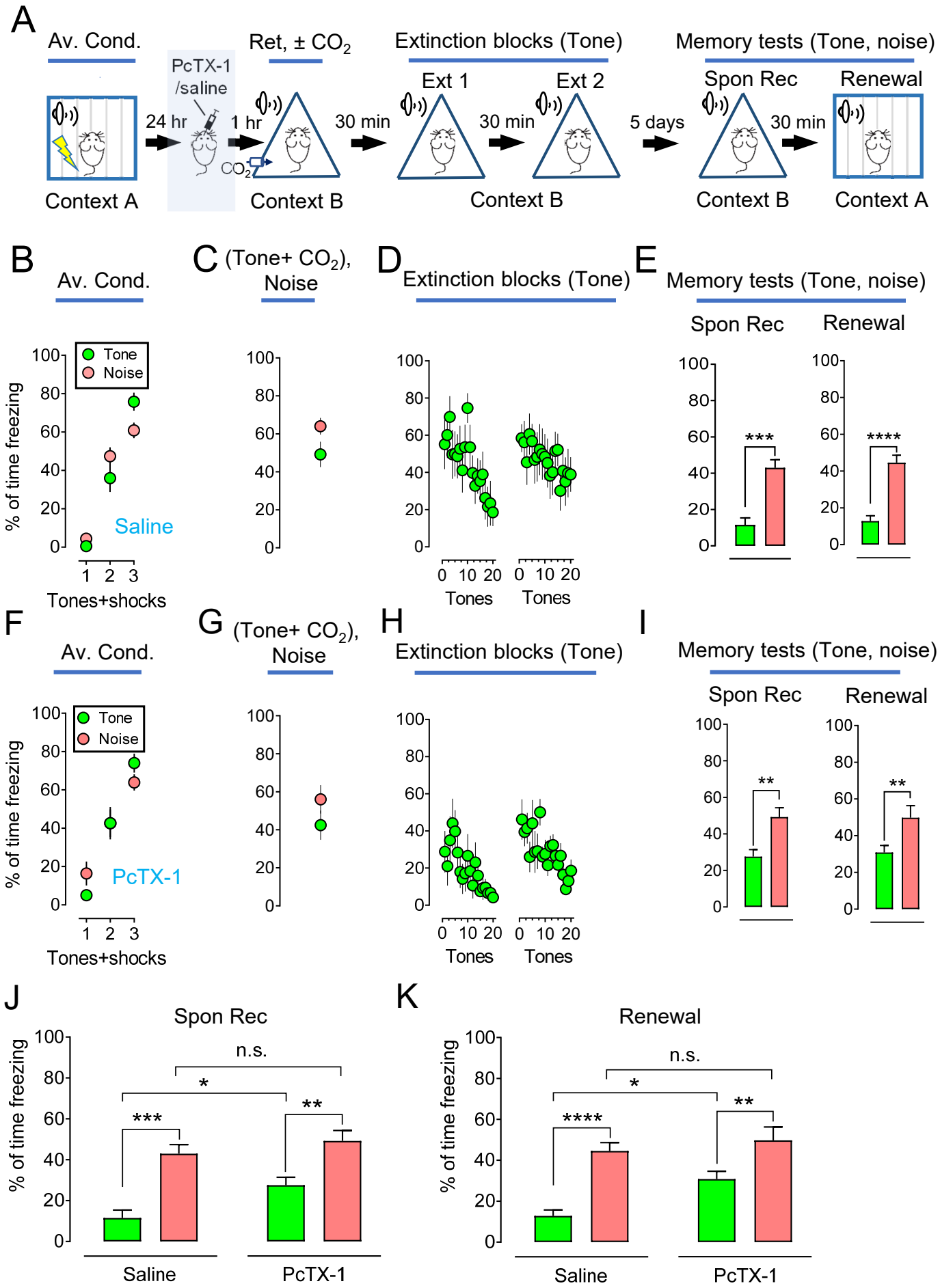

A
Av. Cond.
Ret, ± CO2
Extinction blocks (Tone)
Memory tests (Tone, noise)
PcTX-1 /saline
24 hr
1 hr
30 min
30 min
5 days
30 min
Ext 1
Ext 2
Spon Rec
Renewal
CO2
Context A
Context B
Context B
Context B
Context A
B
Av. Cond.
C (Tone+ CO2), Noise
D Extinction blocks (Tone)
E Memory tests (Tone, noise)
Spon Rec
Renewal
Tone
Noise
% of time freezing
Saline
Tones+shocks
Tones
F
Av. Cond.
G (Tone+ CO2), Noise
H Extinction blocks (Tone)
I Memory tests (Tone, noise)
Spon Rec
Renewal
PcTX-1
J
Spon Rec
K
Renewal
n.s.
Saline
PcTX-1

Fig. 6

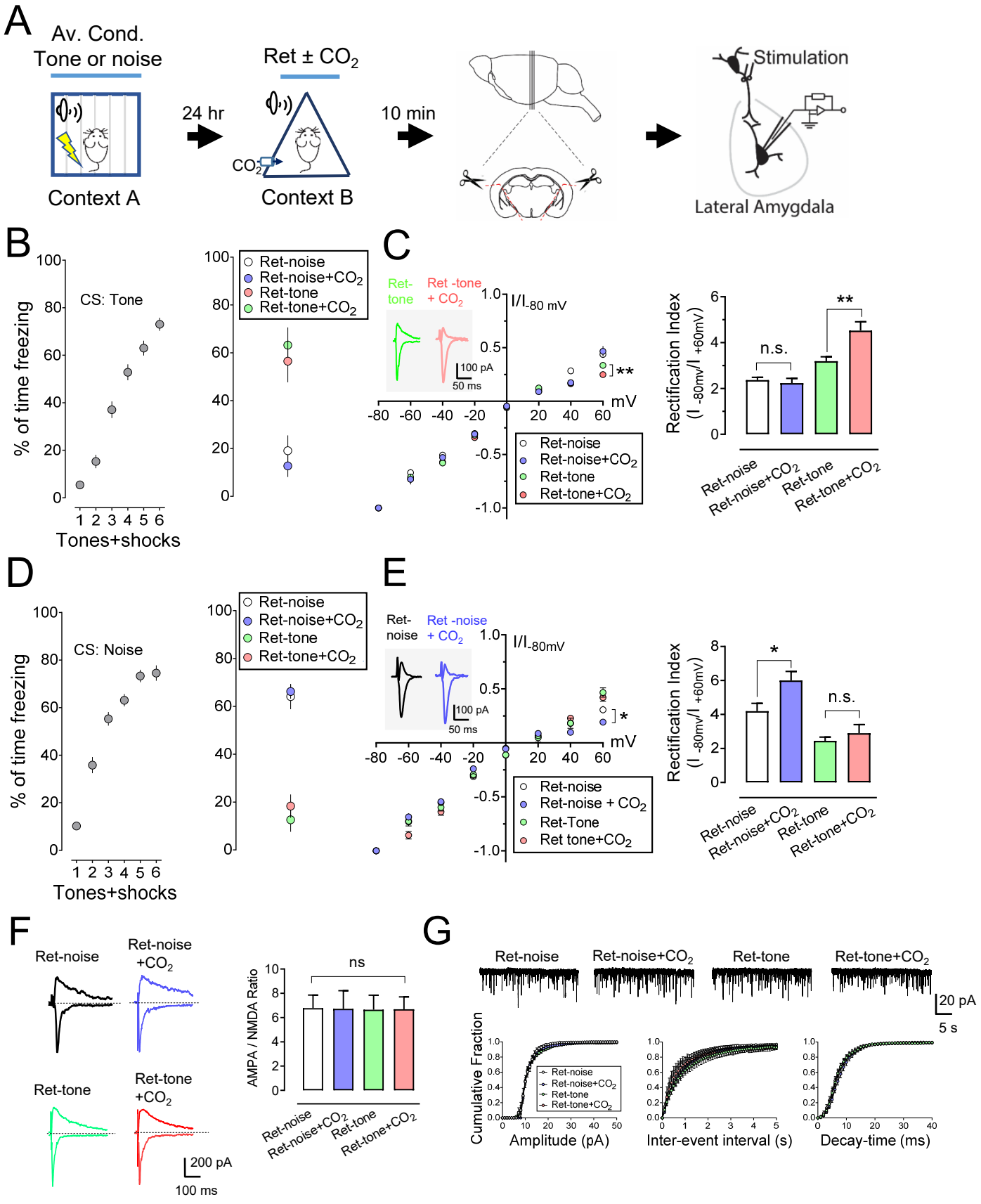

A
Av. Cond.
Tone or noise
Context A
24 hr
Ret ± CO2
CO2
Context B
10 min
Stimulation
Lateral Amygdala
B
CS: Tone
% of time freezing
Tones+shocks
Ret-noise
Ret-noise+CO2
Ret-tone
Ret-tone+CO2
C
Ret-tone
Ret -tone + CO2
100 pA
50 ms
I/I-80 mV
mV
Rectification Index (I -80mV/I +60mV)
n.s.
**
D
CS: Noise
E
Ret-noise
Ret -noise + CO2
I/I-80mV
Ret-Tone
Ret tone+CO2
*
F
Ret-noise
Ret-noise +CO2
Ret-tone
Ret-tone +CO2
200 pA
100 ms
AMPA / NMDA Ratio
ns
G
20 pA
5 s
Cumulative Fraction
Amplitude (pA)
Inter-event interval (s)
Decay-time (ms)

Fig. 7

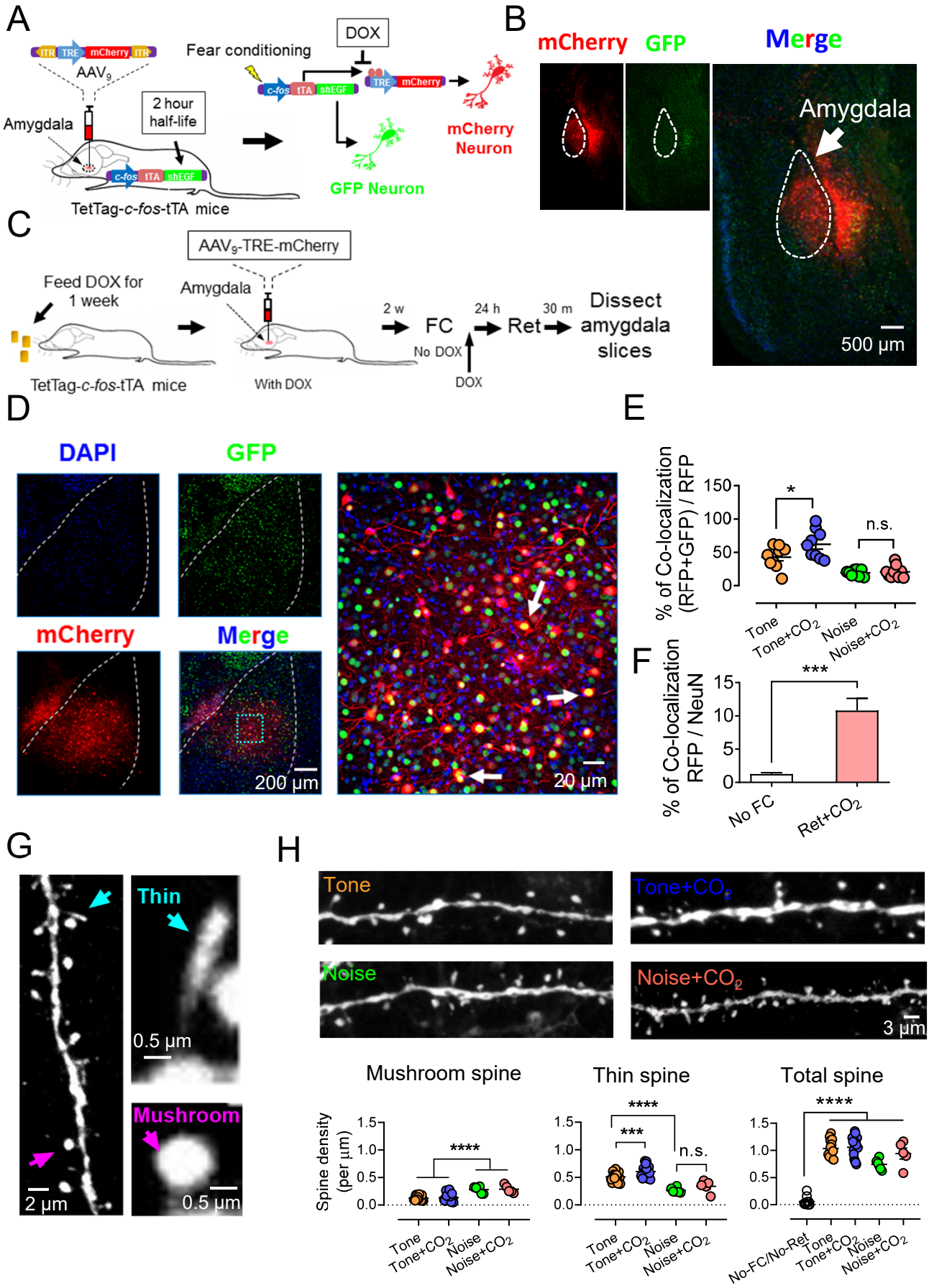

A
ITR TRE mCherry ITR
AAV9
Amygdala
2 hour half-life
c-fos tTA shEGF
TetTag-c-fos-tTA mice
Fear conditioning
DOX
TRE mCherry
mCherry Neuron
GFP Neuron
B
mCherry
GFP
Merge
Amygdala
500 μm
C
AAV9-TRE-mCherry
Feed DOX for 1 week
Amygdala
TetTag-c-fos-tTA mice
With DOX
2 w
FC
No DOX
DOX
24 h
Ret
30 m
Dissect amygdala slices
D
DAPI
GFP
mCherry
Merge
200 μm
20 μm
E
% of Co-localization (RFP+GFP) / RFP
150
100
50
0
*
n.s.
Tone
Tone+CO2
Noise
Noise+CO2
F
% of Co-localization RFP / NeuN
15
10
5
0
***
No FC
Ret+CO2
G
Thin
0.5 μm
Mushroom
0.5 μm
2 μm
H
Tone
Tone+CO2
Noise
Noise+CO2
3 μm
Mushroom spine
Thin spine
Total spine
Spine density (per μm)
1.5
1.0
0.5
0.0
****
***
n.s.
No-FC/No-Ret
Tone
Tone+CO2
Noise
Noise+CO2

Fig. s1

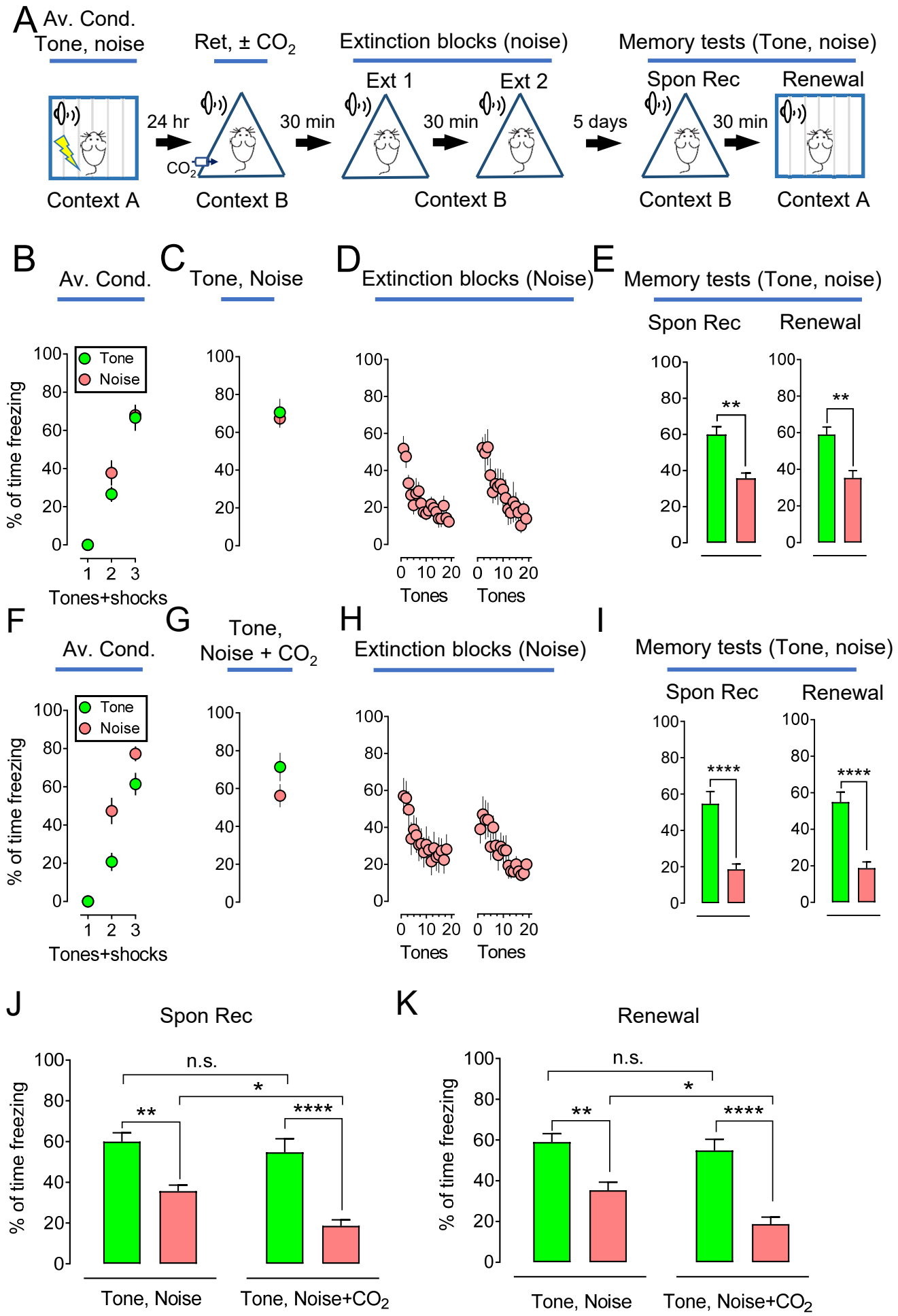

A
Av. Cond.
Tone, noise
Ret, ± CO2
Extinction blocks (noise)
Memory tests (Tone, noise)
Ext 1
Ext 2
Spon Rec
Renewal
24 hr
30 min
30 min
5 days
30 min
CO2
Context A
Context B
Context B
Context B
Context A
B
Av. Cond.
C
Tone, Noise
D
Extinction blocks (Noise)
E
Memory tests (Tone, noise)
Spon Rec
Renewal
Tone
Noise
% of time freezing
Tones+shocks
Tones
Tones
F
Av. Cond.
G
Tone, Noise + CO2
H
Extinction blocks (Noise)
I
Memory tests (Tone, noise)
Spon Rec
Renewal
J
Spon Rec
K
Renewal
n.s.
*
**
****
Tone, Noise
Tone, Noise+CO2

Fig. s2

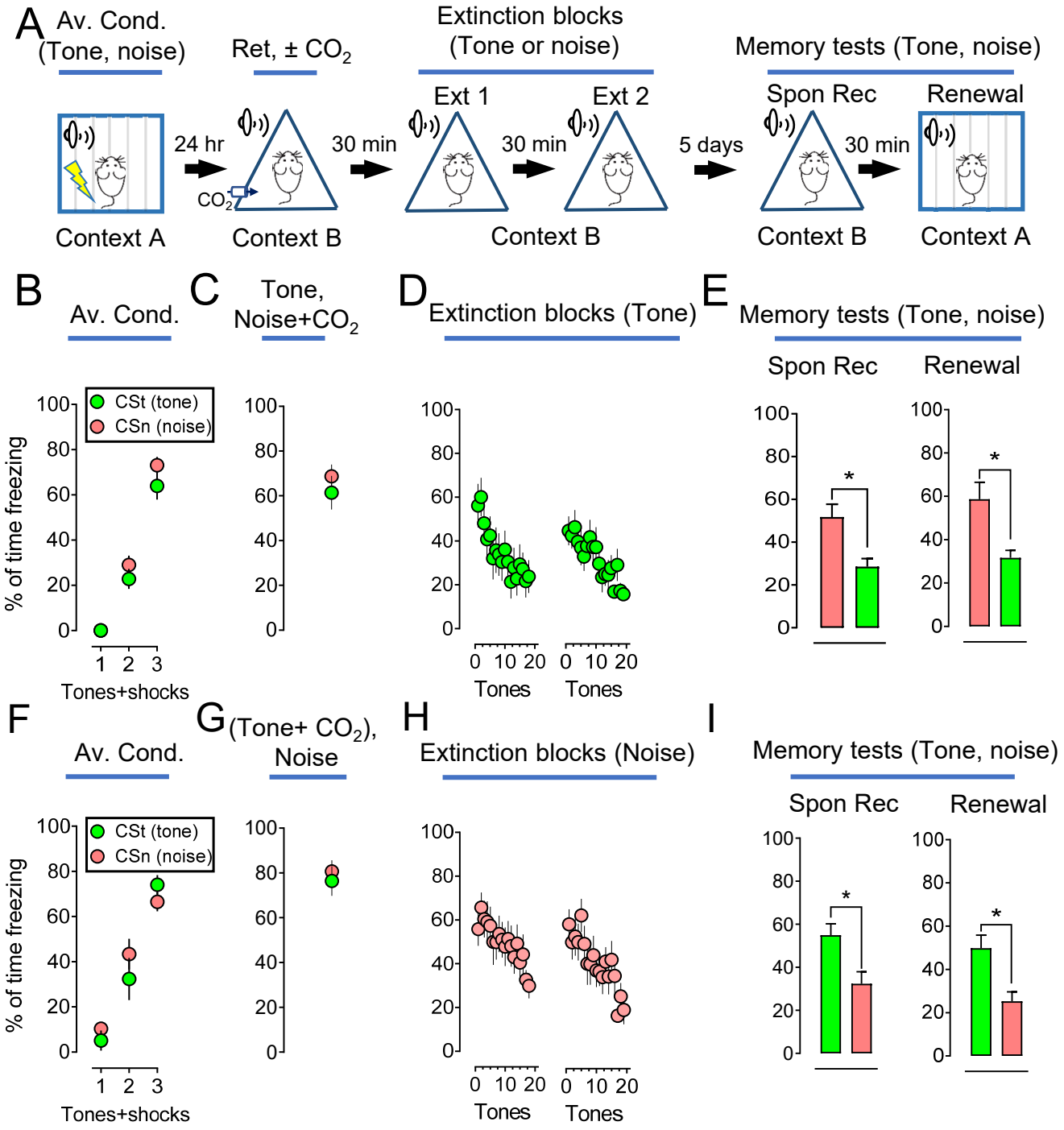

A
Av. Cond.
(Tone, noise)
Ret, ± CO2
Extinction blocks
(Tone or noise)
Memory tests (Tone, noise)
Ext 1
Ext 2
Spon Rec
Renewal
24 hr
30 min
30 min
5 days
30 min
CO2
Context A
Context B
Context B
Context B
Context A
B
Av. Cond.
C
Tone,
Noise+CO2
D
Extinction blocks (Tone)
E
Memory tests (Tone, noise)
Spon Rec
Renewal
CSt (tone)
CSn (noise)
% of time freezing
Tones+shocks
Tones
Tones
F
Av. Cond.
G
(Tone+ CO2),
Noise
H
Extinction blocks (Noise)
I
Memory tests (Tone, noise)
Spon Rec
Renewal
CSt (tone)
CSn (noise)
% of time freezing
Tones+shocks
Tones
Tones

Fig. s3

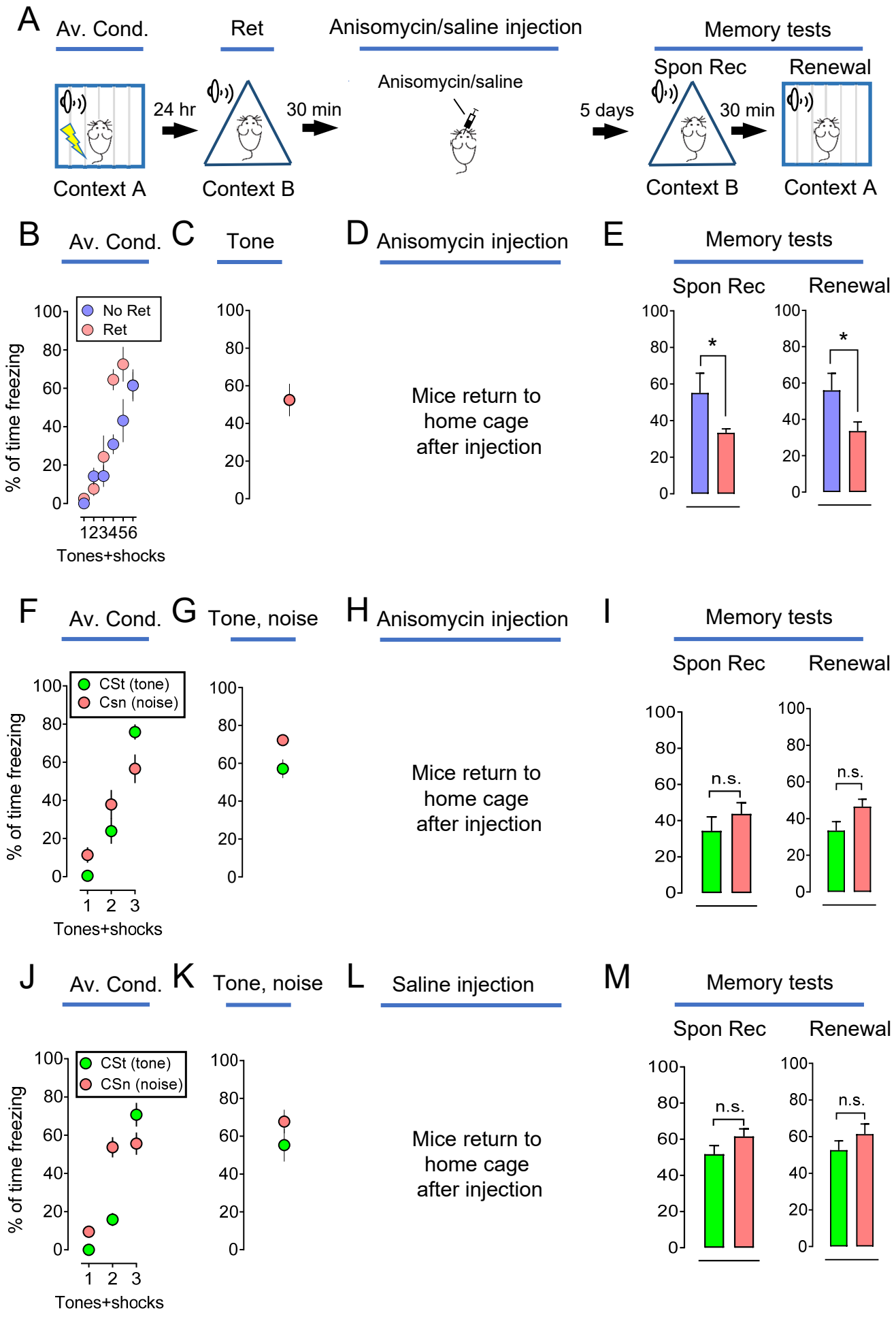

A
Av. Cond.
Ret
Anisomycin/saline injection
Memory tests
Spon Rec
Renewal
24 hr
30 min
Anisomycin/saline
5 days
30 min
Context A
Context B
Context B
Context A
B
Av. Cond.
C
Tone
D
Anisomycin injection
E
Memory tests
Spon Rec
Renewal
No Ret
Ret
% of time freezing
Tones+shocks
Mice return to home cage after injection
F
Av. Cond.
G
Tone, noise
H
Anisomycin injection
I
Memory tests
Spon Rec
Renewal
CSt (tone)
Csn (noise)
% of time freezing
Tones+shocks
Mice return to home cage after injection
n.s.
J
Av. Cond.
K
Tone, noise
L
Saline injection
M
Memory tests
Spon Rec
Renewal
CSt (tone)
CSn (noise)
% of time freezing
Tones+shocks
Mice return to home cage after injection
n.s.

Fig. s4

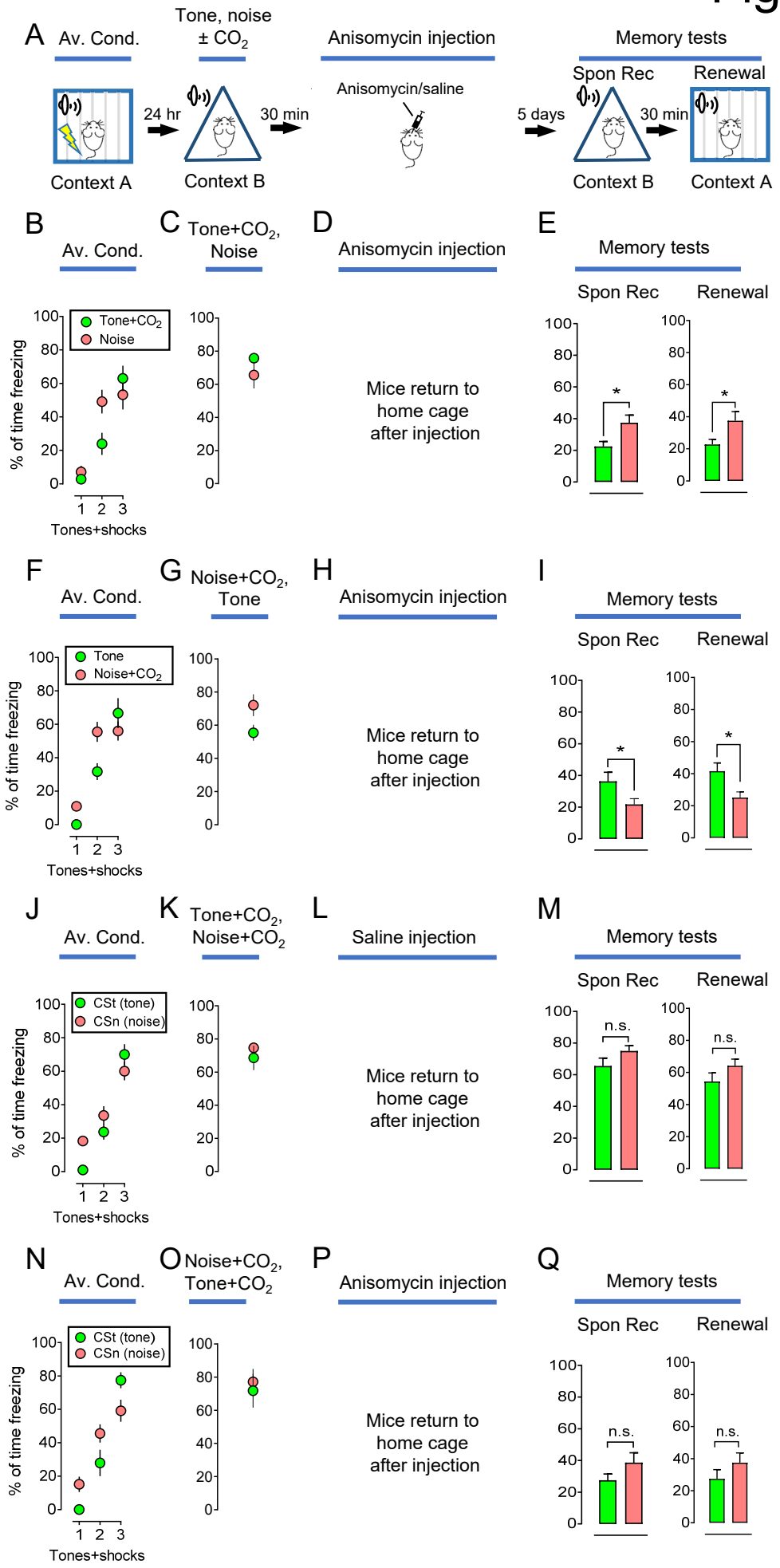
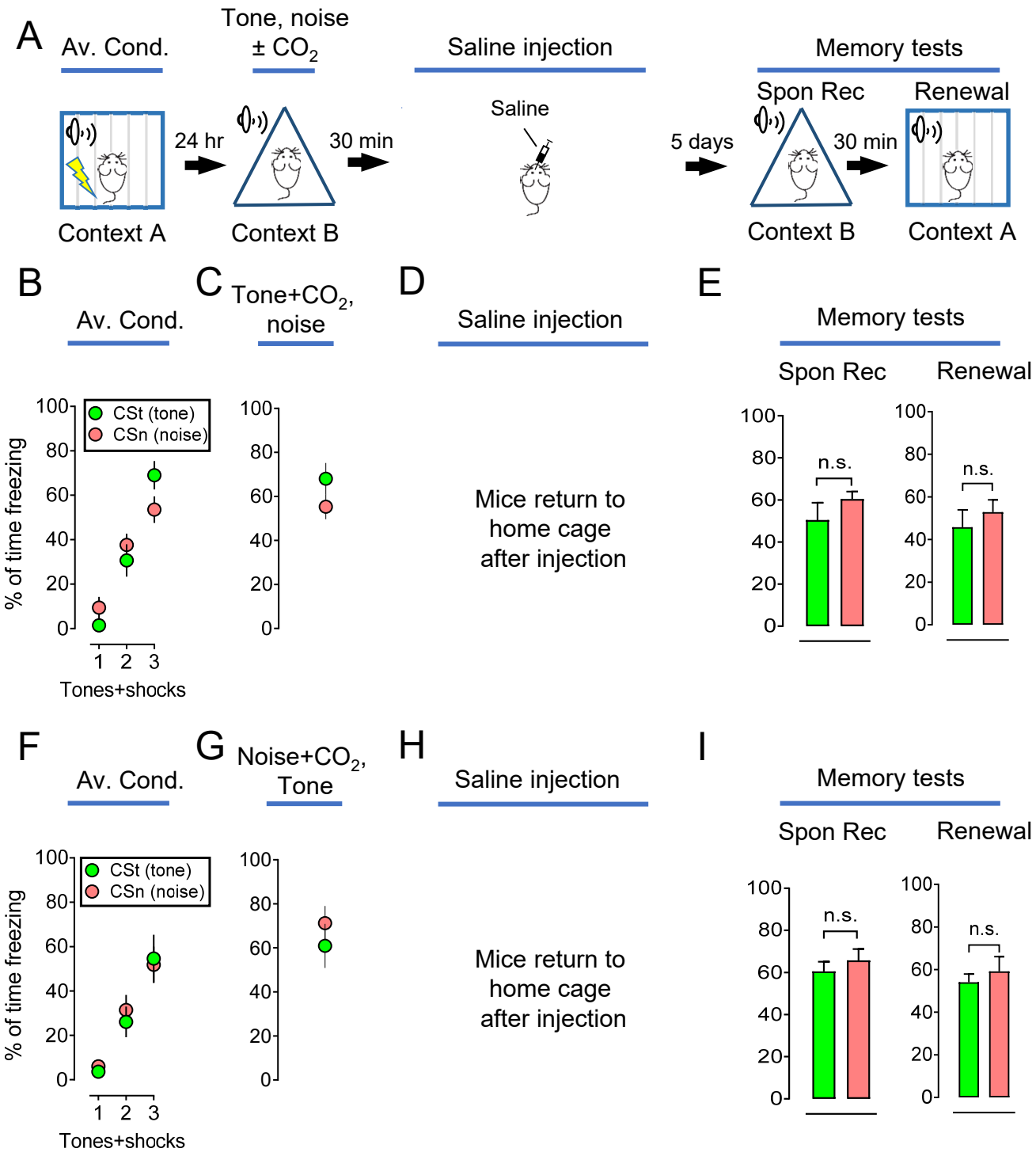

A
Av. Cond.
Tone, noise
± CO2
Anisomycin injection
Memory tests
Spon Rec
Renewal
24 hr
30 min
Anisomycin/saline
5 days
30 min
Context A
Context B
Context B
Context A
B
Av. Cond.
C
Tone+CO2,
Noise
D
Anisomycin injection
E
Memory tests
Spon Rec
Renewal
Tone+CO2
Noise
% of time freezing
Tones+shocks
Mice return to
home cage
after injection
F
Av. Cond.
G
Noise+CO2,
Tone
H
Anisomycin injection
I
Memory tests
Spon Rec
Renewal
Tone
Noise+CO2
% of time freezing
Tones+shocks
Mice return to
home cage
after injection
J
Av. Cond.
K
Tone+CO2,
Noise+CO2
L
Saline injection
M
Memory tests
Spon Rec
Renewal
CSt (tone)
CSn (noise)
% of time freezing
Tones+shocks
Mice return to
home cage
after injection
n.s.
N
Av. Cond.
O
Noise+CO2,
Tone+CO2
P
Anisomycin injection
Q
Memory tests
Spon Rec
Renewal
CSt (tone)
CSn (noise)
% of time freezing
Tones+shocks
Mice return to
home cage
after injection
n.s.

Fig. s5


A
Av. Cond.
Tone, noise
± CO2
Saline injection
Memory tests
Spon Rec
Renewal
24 hr
30 min
Saline
5 days
30 min
Context A
Context B
Context B
Context A
B
Av. Cond.
C
Tone+CO2,
noise
D
Saline injection
E
Memory tests
Spon Rec
Renewal
CSt (tone)
CSn (noise)
% of time freezing
Tones+shocks
Mice return to
home cage
after injection
n.s.
n.s.
F
Av. Cond.
G
Noise+CO2,
Tone
H
Saline injection
I
Memory tests
Spon Rec
Renewal
CSt (tone)
CSn (noise)
% of time freezing
Tones+shocks
Mice return to
home cage
after injection
n.s.
n.s.