Proton receptors regulate synapse-specific reconsolidation in the amygdala

Erin E Koffman[1,2 #], Charles M Kruse[1,2 #], Kritika Singh[2], FarzanehSadat Naghavi[2], Jennifer Egbo[2], Sandra Boateng[2], Mark Houdi BA[2], Boren Lin[2], Jacek Debiec[3], Jianyang Du[1, 4 *]

[1] Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, TN 38163, USA.

[2] Department of Biological Sciences, University of Toledo, Toledo, OH 43606, USA.

[3] Molecular & Behavioral Neuroscience Institute and Department of Psychiatry, University of Michigan, Ann Arbor, MI, USA.

[4] Neuroscience Institute, University of Tennessee Health Science Center, Memphis, TN, United States.

[#] Equal contribution

*** Correspondence:**

Jianyang Du

Department of Anatomy and Neurobiology,

University of Tennessee Health Science Center,

Memphis, TN 38163, USA

TEL: 901-448-3463

E-MAIL: jdu15@uthsc.edu

Abstract

During retrieval, aversive memories become labile during a period known as the reconsolidation window. When an extinction procedure is performed within the reconsolidation window, the original aversive memory can be replaced by one that is less traumatic. Our recent studies revealed that acidosis via inhalation of carbon dioxide (CO_2) during retrieval enhances memory lability. However, the effects of CO_2 inhalation on the central nervous system can be extensive, and there is a lack of prior evidence suggesting that the effects of CO_2 are selective to a reactivated memory. The specific effects of CO_2 depend on acid-sensing ion channels (ASICs), proton receptors that are involved in synaptic transmission and plasticity in the amygdala. Our previous patch-clamping data suggests that CO_2 inhalation during retrieval increases activities of neurons in the amygdala that involve in the memory trace. In addition, CO_2 inhalation during retrieval increases exchanges from Ca^{2+}-impermeable to Ca^{2+}-permeable AMPA receptors. Thus, we hypothesize that CO_2 selectively potentiates memory lability in mice when inhaled during retrieval of aversive memory. In addition, CO_2 inhalation alters memory lability via synaptic plasticity at selectively targeted synapses. Alterations in spine morphology after CO_2 and retrieval with a specific stimulus indicates that CO_2 selectively enhances synaptic plasticity. 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: acid-sensing ion channels; carbon dioxide (CO_2); aversive memory; memory retrieval; AMPA receptors; reconsolidation

Introduction

Recently, aversive memory research has focused on a window of time after aversive memory retrieval, in which the memory is labile and subject to intervention (Lee, 2009; Liu et al., 2012; Schiller et al., 2012). This window of time is known as a reconsolidation window, and is thought to last up to six hours after initiation (Duvarci and Nader, 2004; Sandrini et al., 2015; Schiller et al., 2010). Several studies have demonstrated that interrupting the updating process aroused by retrieval prevents memory restorage, generating selective amnesia (Lee, 2009; Nader et al., 2000). 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 (Schiller et al., 2010). Recently, drug-free paradigms have been proposed that effectively prevent the return of aversive memories in both rodents and humans (Huang et al., 2020; Monfils et al., 2009). 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 (Monfils et al., 2009). Among potential mechanisms, this isolated retrieval trial was contributed to aversive memory lability (Clem and Huganir, 2010; Jarome et al., 2015; Monfils et al., 2009; Quirk et al., 2010). These studies suggest elucidating the mechanism by which retrieval changes the lability of memory is requisite for the development of new clinical treatments for fear-related anxiety disorders.

70 Currently, the combination of retrieval and extinction paradigms is controversial due to
71 conflicting findings (Auber et al., 2013; Chan et al., 2010; Costanzi et al., 2011).
72 Because it is difficult to completely erase the original memory (Ishii et al., 2012), a more
73 reliable strategy for triggering memory erasure is necessary. In search of this strategy,
74 we studied the effects that CO_2 inhalation may have on the erasure of a specific
75 memory. As we showed previously, when mice receive a retrieval tone supplemented
76 by CO_2 inhalation, the memory becomes more labile than the presentation of a retrieval
77 tone alone (Du et al., 2017). Within the reconsolidation window, the labile memory
78 becomes more convertible, either weakened by extinction or strengthened by
79 reconditioning (Du et al., 2017). Moreover, the effects of CO_2 inhalation on the memory
80 lability were dependent on acid-sensing ion channels (ASICs), since disrupting ASICs in
81 the amygdala eliminated these effects of CO_2 (Du et al., 2017).
82
83 Recent studies have revealed that protons are neurotransmitters and ASICs serve as
84 postsynaptic proton receptors that play key roles in neurotransmission and synaptic
85 plasticity in the amygdala, a brain region that is critical for the formation of aversive
86 memories (Du et al., 2014). ASICs are members of the Degenerin/ Epithelial sodium
87 channel (DEG/ ENaC) family (Ben-Shahar, 2011). To date, six family members have
88 been identified (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) (Waldmann et
89 al., 1997; Waldmann and Lazdunski, 1998). These proteins assemble as homo- or
90 heterotrimers to form channels that are proton-gated, voltage-insensitive, permeable to
91 both Na^+ and Ca^{2+} and activated by extracellular protons (Rook et al., 2020). ASIC1a is
92 expressed in many areas of the brain, where it contributes to numerous brain functions

93 and disorders, including hippocampal learning and memory, anxiety, depression, stroke,
94 neurodegeneration, seizure, inflammation, and nerve injury (Chu and Xiong, 2012; Gao
95 et al., 2005; Ortega-Ramírez et al., 2017; Wang et al., 2018; Wemmie et al., 2002;
96 Wemmie et al., 2006). ASIC1a is expressed widely in the amygdala and its ion channel
97 activity is evoked by a reduction in extracellular pH (Taugher et al., 2017; Wemmie et
98 al., 2003). Presynaptic stimulation induces a transient synaptic drop in pH and activates
99 ASIC-like excitable postsynaptic currents (EPSCs) in pyramidal neurons; these EPSC
100 currents are lacking in ASIC1a$^{-/-}$ mice (Mango and Nisticò, 2019; Soto et al., 2018;
101 Wemmie et al., 2003; Wu et al., 2004). Loss of ASIC1a function also leads to impaired
102 high-frequency electrical stimulation-induced long-term potentiation (LTP) (Chiang et al.,
103 2015; Du et al., 2014; Liu et al., 2016; Wemmie et al., 2002). In mice, the disruption of
104 ASIC1a activity reduces conditioned aversive memory (Coryell et al., 2007; Wemmie et
105 al., 2004), whereas its overexpression has the opposite effect (Wemmie et al., 2004).
106 Also, reducing pH by CO_2 inhalation or the injection of acid into the amygdala induced
107 fear-like behavior that was dependent on ASIC1a (Ziemann et al., 2009).
108
109 Evidence suggests that CO_2 inhalation affects the memory trace associated with the
110 retrieval cue(Du et al., 2017). Additionally, inhalation of CO_2 may reduce pH throughout
111 the brain (Dulla et al., 2005; Zandbergen et al., 1989) and can have a profound effect on
112 neural tissue(Ziemann et al., 2009). Despite these discoveries, the mechanism and
113 level of specificity by which CO_2 and ASICs may specifically regulate memory trace in
114 the retrieval period is still unknown. In this study, we found that inhalation of CO_2 during
115 memory retrieval selectively potentiated memory lability in mice. Furthermore,

116 electrorheological and imaging studies in brain slices support the conclusion that the
117 effects of CO_2 on memory retrieval are specific to a given memory. Our study proposes
118 that inhaling CO_2 within the reconsolidation window regulates aversive memory with
119 specificity, providing a unique angle to further study the mechanism by which memory is
120 modulated.
121

122 **Materials and Methods**

123 **Mice**

124 For our experiment, we used both male and female mice between 10-14 months of age.
125 Mice were derived from a congenic C57BL/6 background including wild-type, ASIC1a$^{-/-}$
126 and TetTag-c-fos-tTA mice. TetTag-cFos-tTA mice were obtained from Jackson
127 Laboratory and crossed with C57BL/6J mice. Mice carrying the Fos-tTA transgene were
128 selected; Fos-tTA mice have a Fos promoter driving expression of nuclear-localized,
129 two-hour half-life EGFP (shEGFP) (Du et al., 2017; Koffman and Du, 2017; Ramirez et
130 al., 2013). The Fos promoter also drives the expression of tetracycline transactivator
131 (tTA), which bind to the tetracycline-responsive element (TRE) site on an injected
132 recombinant adeno-associated virus, AAV_9-TRE-mCherry virus, resulting in the
133 expression of mCherry (Du et al., 2017; Koffman and Du, 2017). Binding of the tTA to
134 the TRE site is inhibited by doxycycline (DOX). Inhibition of tTA binding prevents target
135 gene expression (Das et al., 2016; Liu et al., 2012; Ramirez et al., 2013).
136
137

138 Two transgenic lines were crossed to generate Fos-tTA; ASIC1a$^{-/-}$ double knockout
139 transgenic mice. Both male and female mice age10-14 weeks were randomly selected
140 for the experiment groups. Experimental mice were maintained on a standard 12-hour
141 light-dark cycle and received standard chow and water ad libitum. Animal care and
142 procedures met the National Institutes of Health standards. The University of
143 Tennessee Health Science Center Laboratory Animal Care Unit (Protocol #19-0112)
144 and University of Toledo Institutional Animal Care and Use Committee (Protocol
145 #108791) approved all procedures.
146

147 **Aversive conditioning, retrieval, extinction, and memory test**

148 The protocols for each experiment are detailed in the schematics of each figure. All
149 mice were handled by experimenters for 30 minutes on each of 3 days before aversive
150 conditioning. On day 1, mice were habituated to the aversive conditioning chamber
151 (Med Associates Inc.) for 7 minutes. Mice were then exposed to varying conditioning
152 protocols, as described below.
153

154 ***Experiment 1: Standard one-conditioned stimulus (CS) auditory aversive***
155 ***conditioning, retrieval, extinction, and memory tests.***
156 On day 1 in a curated environment (context A), the experimental mice were presented
157 with six pure tones (80 dB, 2 kHz, 20 seconds each) paired with 6 foot shocks - one
158 shock at the end of each tone (0.7 mA, 2 seconds). The interval between each tone was
159 100 seconds. On day 2, the mice were placed into a new environment (context B) and
160 habituated for 4 minutes. Mice then inhaled either unaltered air or air containing 10%

161 CO_2 for 7 minutes. Five minutes after inhalation of CO_2 or air began, mice were
162 presented with one 20 second pure tone to retrieve the memory. The mice were then
163 returned to their home cages. 30 minutes later, the mice returned to the retrieval
164 chamber (context B) and underwent two rounds of extinctions. In the first round of
165 extinction, mice were exposed to 20 pure tones with an interval between tones of 100
166 seconds. Mice were then returned to their home cage. 30 minutes later, the mice went
167 through the extinction protocol again with 20 pure tones. On day 7, the mice were tested
168 to see if their aversive response would recur via spontaneous recovery in context B with
169 4 pure tones. Thirty minutes after spontaneous recovery, the mice were returned to the
170 original context of the aversive memory, context A, in a recovery protocol with 4 pure
171 tones.
172
173 ***Experiment 2: Two distinct CSs aversive conditioning, retrieval, extinction, and***
174 ***memory tests.***
175 This procedure was used to test the specificity of the effects of CO_2 on memory
176 retrieval. The context settings and parameters are similar to the above standard one CS
177 auditory aversive conditioning. In contrast to experiment 1, the mice were presented
178 with three pure tones (80 dB, 2 kHz, 20 seconds each) that alternated with three white
179 noises (60 dB, 2 kHz, 20 seconds each); all six stimuli were paired with foot shocks. On
180 day 2, the mice inhaled either unaltered air or air containing 10% CO_2 for 7 minutes.
181 Five minutes after inhalation of CO_2 or air began, the mice underwent retrieval with one
182 single pure tone followed by one white noise with or without CO_2. This was followed
183 thirty minutes later by two sections of extinctions with either pure tones or white noises.

184 On day 7, the mice were tested via spontaneous recovery and renewal protocols with 4
185 pure tones and 4 white noises respectively.
186
187 ***Experiment 3: Two distinct CSs aversive conditioning, retrieval, anisomycin, and***
188 ***memory tests.***
189 In a series of experiments, the standard extinction procedure was replaced with
190 amygdala infusion of anisomycin (detailed in the surgery procedure below). In brief, the
191 cannula was implanted on the amygdala 4-7 days before the behavioral experiments.
192 Starting on day 1, the mice were subjected to the aversive conditioning described in
193 experiment 2. On day 2, 30 minutes after retrieval, the mice were infused with 62.5
194 μg/μl anisomycin via the cannula in the lateral nuclei of the amygdala (LA) bilaterally
195 and returned to their home cage (Debiec et al., 2010). On day 7, the mice were tested
196 via spontaneous recovery and renewal as described in experiment 2.
197
198 Freezing behavior in mice (the absence of movement beyond respiration) is used as a
199 measure of fear. To evaluate the outcomes of freezing behavior in mice, the percentage
200 of time during CS presentation spent in freezing was scored automatically using
201 VideoFreeze software (Med Associates Inc.). In the spontaneous recovery and renewal
202 tests, outcomes of the percentage of time freezing were averaged from each of the 4
203 CSs.
204
205 **Surgery and chemical infusion**

206 For the cannula placement procedure, mice were anesthetized with isoflurane through
207 an anesthetic vaporizer, secured to the stereotaxic instrument and a cannula made from
208 a 25-gauge needle was inserted bilaterally into LA (relative to bregma: -1.2 mm
209 anterioposterior; ±3.5 mm mediolateral; -4.3 mm dorsoventral) (Du et al., 2017; Koffman
210 and Du, 2017). Dental cement secured the cannula and bone anchor screw in place.
211 Mice recovered for 4-5 days before any subsequent testing was carried out. A 10 μL
212 Hamilton syringe connected to a 30-gauge injector was inserted 1 mm past the cannula
213 tip to inject anisomycin (diluted in 1 μl artificial cerebrospinal fluid (ACSF), pH 7.3) over
214 5 sec. The injection sites were mapped post-mortem by sectioning the brain (10 μm
215 coronal) and performing cresyl violet staining.
216
217 **Brain slice preparation and patch-clamp recording of amygdala neurons**
218 Ten minutes after the memory retrieval experiment ended, mice were euthanized with
219 overdosed isoflurane and whole brains were dissected into pre-oxygenated (5% CO_2
220 and 95% O_2) ice-cold high sucrose dissection solution containing (in mM): 205 sucrose,
221 5 KCl, 1.25 NaH_2PO_4, 5 $MgSO_4$, 26 $NaHCO_3$, 1 $CaCl_2$, and 25 glucose (Du et al., 2017).
222 A vibratome sliced brains coronally into 300 μm sections that were maintained in normal
223 ACSF containing (in mM): 115 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 11
224 glucose, 25 $NaHCO_3$ bubbled with 95% O_2/5% CO_2, pH 7.35 at 20°C-22°C. Slices were
225 incubated in the ACSF at least 1 hour before recording. For experiments, individual
226 slices were transferred to a submersion-recording chamber and were continuously
227 perfused with the 5% CO_2/95% O_2 solution (~3.0 ml/min) at room temperature (20°C -
228 22°C).
229

230 As we described previously (5), pyramidal neurons in the lateral amygdala were studied
231 using whole-cell patch-clamp recordings. The pipette solution containing (in mM): 135
232 KSO3CH3, 5 NaCl, 10 HEPES, 4 MgATP, 0.3 Na3GTP, 0.5 K-EGTA (mOsm=290,
233 adjusted to pH 7.25 with KOH). The pipette resistance (measured in the bath solution)
234 was 3-5 MΩ. High-resistance (>1 GΩ) seals were formed in voltage-clamp mode.
235 Picrotoxin (100 μM) was added to the ACSF throughout the recordings to yield
236 excitatory responses. In AMPAR current rectification experiments, we applied D-APV
237 (100 μM) to block NMDAR-conducted EPSCs. The peak amplitude of ESPCs was
238 measured to determine current rectification. The amplitude was measured ranging from
239 -80 mV to +60 mV in 20 mV steps. The peak amplitude of EPSCs at -80 mV and +60
240 mV was measured for the rectification index. In EPSC ratio experiments, neurons were
241 measured at -80 mV to record AMPAR-EPSCs and were measured at +60 mV to record
242 NMDAR-EPSCs. To determine the AMPAR-to-NMDAR ratio, we measured the peak
243 amplitude of ESPCs at -80 mV as AMPAR-currents, and peak amplitude of EPSCs at
244 +60 mV at 70 ms as NMDAR-currents after onset. Data were acquired at 10 kHz using
245 Multiclamp 700B and pClamp 10.1 (RRID:SCR_011323). The mEPSCs events (>5pA)
246 were analyzed in Clampfit 10.1. The decay time (τ) of mEPSCs was fitted to an
247 exponential using Clampfit 10.1.
248
249 **Immunohistochemistry and cell counting**
250 The pAAV-TRE-mCherry plasmid was obtained from the laboratory of Dr. Susumu
251 Tonegawa (Liu et al., 2012; Ramirez et al., 2013), and was used to produce $AAV_{2/9}$ by
252 the University of Iowa Gene Transfer Vector Core. For one week leading up to virus
253 microinjection, TetTag Fos-tTA mice were fed with food containing 40 mg/kg DOX. We

254 used a 10 µl Hamilton microsyringe and a WPI microsyringe pump to inject virus (0.5 µl
255 of 1.45E+12 viral genomes/ml of $AAV_{2/9}$-TRE-mCherry) bilaterally into the amygdala
256 (relative to bregma: -1.2 mm anterioposterior; ±3.5 mm mediolateral; -4.3 mm
257 dorsoventral), as described previously (Du et al., 2017; Koffman and Du, 2017). For
258 a two-week window between surgery and behavior training, mice were housed and fed
259 with a DOX-containing diet. The DOX-containing diet was ceased twenty-four hours
260 before aversive conditioning began on day one (replaced by a regular diet), then
261 immediately restarted afterward. Thirty minutes after retrieval on day two, the mice were
262 euthanized according to protocol. We used transcardial perfusion with 4%
263 paraformaldehyde (PFA) to fix whole brains, followed by continued fixation in 4% PFA at
264 4°C for 24 hours (Wright et al., 2020). Following perfusion, we used a vibratome (Leica
265 VT-1000S) to dissect 50 µm amygdala coronal slices, which were collected in ice-cold
266 PBS. In order to complete immunostaining, slices were placed in Superblock solution
267 (ThermoFisher Scientific) plus 0.2% Triton X-100 for 1 hour and incubated with primary
268 antibodies (1:1000 dilution) at 4°C for 24 hours (Du et al., 2017). Primary antibodies we
269 used include: rabbit polyclonal IgG anti-RFP (Rockland Cat# 600-401-379 RRID:AB_
270 2209751); chicken IgY anti-GFP (Thermo Fisher Scientific Cat# A10262 RRID:AB_
271 2534023) and mouse anti-NeuN (Millipore Cat# MAB377X RRID:AB_2149209) (Liu et
272 al., 2012; Ramirez et al., 2013). We then washed and incubated slices for one hour with
273 secondary antibodies (Alexa Fluor 488 goat anti-chicken IgG (H+L) (Molecular Probes
274 Cat# A-11039 also A11039 RRID:AB_142924); Alexa Fluor 568 goat anti-rabbit IgG
275 (H+L) (Molecular Probes Cat# A-21429 also A21429 RRID:AB_141761); Alexa Fluor
276 647 goat anti-mouse IgG (H+L) (Thermo Fisher Scientific Cat# A-21235 RRID:AB_

277 2535804), 1:200 dilution). VectaShield H-1500 (Vector Laboratories Cat# H-1500 RRID:
278 AB_2336788) was used to mount slices, while confocal microscopy was used to view
279 the slices. We used ImageJ software (RRID:SCR_003070) to analyze dendritic spine
280 morphology. Thin, mushroom and stubby spines were categorized based on the
281 following parameters: 1) mushroom spines: head-to-neck diameter ratio >1.1:1 and
282 spine head diameter >0.35 μm; 2); thin spines: head-to-neck diameter ratio >1.1:1 and
283 spine head diameter >0.35 μm or spine head-to-neck diameter ratios <1.1:1 and spine
284 length-to-neck diameter > 2.5 μm; 3); stubby spines: spine head-to-neck diameter ratios
285 <1.1:1 and spine length-to-neck diameter ≤ 2.5 μm (Kreple et al., 2014; Wright et al.,
286 2020).
287
288 **Statistical analysis**
289 One-way ANOVA and Tukey's post-hoc multiple comparison tests were used for
290 statistical comparison of groups. An unpaired Student's t-test was used to compare
291 results between two groups. $P<0.05$ was considered statistically significant, and we did
292 not exclude potential outliers from our data. The graphing and statistical analysis
293 software Graphpad Prism 8 (RRID:SCR_002798) was used to analyze statistical data,
294 which was presented as means ± SEM. Sample sizes (n) are indicated in the figure
295 legends, and data are reported as biological replicates (data from different mice,
296 different brain slices). Each group contained tissues pooled from 4-5 mice. Due
297 to variable behavior within groups, we used sample sizes of 10-16 mice per
298 experimental group as we previously described in earlier experiments (Du et al., 2017).
299 In behavioral studies, we typically studied groups with four randomly assigned animals

300 per group, as our recording equipment allowed us to record four separate animal cages
301 simultaneously. The experiments were repeated with another set of four animals until
302 we reached the target number of experimental mice per group. Experimentation groups
303 were repeated in this manner so that each animal had the same controlled environment-
304 the same time of day and with similar handling, habituation, and processes.
305
306 **Results**
307 Our recent studies suggested that CO_2 inhalation throughout memory retrieval
308 enhances the lability of the memory and boosts the efficiency of the memory erasure
309 (Du et al., 2017). To further test whether the effects of CO_2 on the memory retrieval are
310 synapse-specific, we designed a series of unique experiments that were able to
311 generate two distinct auditory aversive memories and identify the specificity of CO_2
312 effects on each of them.
313
314 **CO_2 selectively potentiates the lability of auditory aversive memory in the**
315 **amygdala**
316 Previous studies have described an aversive conditioning paradigm in which memory
317 can be selectively reactivated and reconsolidated, suggesting synapse-specific
318 reconsolidation of distinct aversive memories in the amygdala (Debiec et al., 2010;
319 Doyere et al., 2007). We followed this paradigm albeit with modifications (Fig.1A). On
320 day 1, we trained the animals with two distinct conditioned stimuli: three pure tones and
321 three white noises paired with one foot-shock per stimuli as aversive unconditioned
322 stimuli (US) (see the detailed description in Materials and Methods). We evaluated the
323 outputs of the aversive conditioning through the percentage of the freezing time within

324 the time of CSs. The freezing was significantly increased after each of the three
325 conditioned stimuli, indicating the mice were trained sufficiently under the designed
326 condition (Fig.1B, F).
327
328 On day 2, the animals were placed into a new context (context B) and presented with
329 one pure tone followed by a single noise (or vice versa) to retrieve the memory (Fig. 1C,
330 Fig. s1C). The animals were then returned to their home cages. 30 minutes later, all
331 mice underwent two blocks of extinctions in context B (extinction 1: 20 pure tones;
332 extinction 2: 20 pure tones) (Fig.1D, Fig. s1D). After the extinction, the freezing drops
333 down to a low level (Fig. 1D, Fig. s1D). Five days later, the mice underwent
334 spontaneous recovery (context B) and renewal (context A) respectively. Four tones and
335 four noises were presented throughout the memory test (Fig.1E, Fig. s1E). The group
336 that underwent extinction with a specific CS showed specificity in which freezing
337 response was lowered after extinction (Fig.1E, Fig. s1E). For example, when the pure
338 tone was presented during extinction, freezing in the pure tone group was lower than
339 freezing in the noise group (Fig.1E), and vice versa (Fig. s1E).
340
341 When retrieval was paired with 10% CO_2 inhalation, memory erasure effects were
342 enhanced (Fig.1 F-I, Fig. s1F-I). To further evaluate the specificity of the effects of
343 reconsolidation on memory modifications, we designed another retrieval protocol in
344 which we present pure tone and noise, either of them paired with 10% CO_2 inhalation,
345 followed by an unrelated extinction of CS, white noise, or pure tone (Fig. s2). Consistent
346 with our expectation, CO_2 did not boost the effects on the retrieved memory in absence

347 of extinction (Fig. s2B-I). The application of 10% CO_2 to the retrieval event failed to
348 enhance the outcome after extinction, indicating a specificity of the CO_2 effects. In all,
349 our data suggest that memory encoded in the amygdala can be distinct, and the effects
350 of CO_2 on memory are specific.
351
352 To focus on testing the specific effects of CO_2 on retrieval, we performed a series of
353 experiments replacing extinction with an injection of a protein synthesis inhibitor,
354 anisomycin, to extinguish the aversive memory. Anisomycin, when injected bilaterally
355 into the amygdala after retrieval, causes memory erasure compared to the saline
356 injection group (Debiec et al., 2010). Our experiments found anisomycin to sufficiently
357 disrupt memory during reconsolidation (Fig. 2B-E), and that memory retrieval is required
358 for memory erasure with anisomycin injection (Fig. s3 B-E). Consistent with the above
359 extinction results, when retrieval was paired with 10% CO_2 inhalation, we found
360 anisomycin groups to show an increased reduction in aversive response - further
361 confirming that CO_2 enhances memory lability (Fig. 2F-I). We then conditioned the mice
362 with pure tone and white noise and carried out memory retrieval with both CSs and
363 found anisomycin distinguished the memory in both tone and noise groups (Fig. s3 F-
364 M). When 10% CO_2 was applied while the CSs were presented, the retrieval group
365 paired with CO_2 showed less freezing, regardless of the type of CSs (pure tone or white
366 noise) (Fig.s4 B-I). As a control, we applied CO_2 for both retrieval events together and
367 anisomycin decreased the freezing level in both groups, suggesting that CO_2 had equal
368 effects on both tone and noise (Fig. s4 J-Q). As another control, the saline injection
369 does not cause a memory erasure, indicating the effect of anisomycin on the memory

370 (Fig. s5). In all, our data has confirmed the effects of CO_2 are specific on a distinct
371 memory that is activated by a specific CS. We also found that CO_2 does not affect
372 another CS activated aversive memory, suggesting specificity of CO_2 effects on the
373 memory trace.
374
375 **The specific effects of CO_2 on memory lability is ASIC-dependent**.
376 We have previously found the effects of CO_2 on memory retrieval to be ASIC dependent
377 (Du et al., 2017). However, it is still unknown if CO_2 application applied to a specific
378 memory trace affects an ASIC-dependent mechanism. To answer this question, we first
379 performed distinct aversive conditioning in ASIC1a$^{-/-}$ mice with three pure tones and
380 white noises on day 1 (Fig. 3A), followed by a pure tone and white noise for retrieval
381 on day 2. 30 minutes post-retrieval, we performed extinctions with pure tones. Five days
382 later, we tested spontaneous recovery and renewals with 4 pure tones and white
383 noises. Similar to the response we saw in WT mice, the freezing level in the pure tone
384 group of ASIC1a$^{-/-}$ mice was less than that in the white noise group (spontaneous
385 recovery, 46% decrease; renewal, 47.5% decrease). When 10% CO_2 inhalation was
386 paired with pure tone in retrieval, we found that CO_2 did not have additional effects
387 on the memory with the specific CS in ASIC1a$^{-/-}$ mice (spontaneous recovery, 43.4%
388 decrease; renewal, 45.6% decrease) (Fig. 3F-I). We had predicted that the effects of
389 CO_2 on memory retrieval to be ASIC dependent and our data support this expectation.
390 We also applied the two distinct CSs (pure tone and white noise) in retrieval, then
391 followed retrieval with anisomycin injection. Anisomycin dramatically reduced the
392 freezing in memory tests that followed, whereas pairing with CO_2 in either CSs did not

393 cause an additional reduction in the ASIC1a$^{-/-}$ mice, suggesting an ASIC dependency
394 (Fig. 4).
395
396 To provide evidence that an acute ASIC1a blockage was able to eliminate the effects of
397 CO_2, we injected 100nM PcTX1 into the lateral amygdala bilaterally 1 hour before the
398 application of CO_2 to the retrieval (Fig. 5A). Our data suggest that compared to the
399 saline injection group (Fig. 5 B-E), inhibiting ASIC1a by PcTX1 reduced the CO_2 effects
400 on the memory retrieval (Fig. 5 F-I), similar to the data in the ASIC1a$^{-/-}$ mice (Fig. 3).
401 This evidence leads us to conclude the effects of CO_2 on specific memory traces to be
402 ASIC dependent.
403

404 **Activation of ASICs through CO_2 inhalation alters reconsolidation of distinct**
405 **memory through alteration of AMPARs.**

406 AMPARs are glutamatergic receptors that have crucial roles in modulating memory
407 retrieval and destabilization (Auber et al., 2013; Chan et al., 2010; Clem and Huganir,
408 2010; Monfils et al., 2009; Quirk et al., 2010). Previous studies have suggested that an
409 exchange of Ca^{2+}- impermeable AMPARs (CI-AMPARs) for Ca^{2+}- permeable AMPARs
410 (CP-AMPARs) occurs after retrieval (Clem and Huganir, 2010; Hong et al., 2013). 10%
411 CO_2 inhalation during retrieval induced a stronger current rectification of AMPARs (the
412 signature of CP-AMPARs) than in the retrieval alone group, indicating a greater
413 exchange of AMPARs (Du et al., 2017). Interestingly, no further enhancement was
414 observed in the ASIC1a$^{-/-}$ brain slices, indicating that the effect of CO_2 inhalation on
415 AMPAR exchange is ASIC dependent (Du et al., 2017). To further study whether

416 CO_2 specifically alters the AMPARs exchange in retrieval, we designed a unique
417 experiment to separate the aversive conditioning and retrieval and measure the
418 rectification of AMPARs. To study this, we conditioned the mice with 6 pure tones on
419 day 1 (Fig. 6B, left). On day 2, the mice were divided into 4 groups for the retrieval-pure
420 tone only; pure tone plus 10% CO_2 inhalation; white noise only; white noise+10% CO_2
421 inhalation (Fig. 6B, right). Ten minutes after retrieval, we dissected brain slices and
422 AMPAR current was recorded in the pyramidal neurons in the lateral amygdala through
423 stimulation of thalamic inputs (Fig. 6A). Rectification, a signature of CP-AMPARs, was
424 compared among all groups. Consistent with earlier reports, pure tone retrieval
425 increased current rectification (Clem and Huganir, 2010; Hong et al., 2013) and
426 CO_2 paired with pure tone retrieval caused stronger rectification (Fig. 6C). However,
427 when white noise presented as the retrieval event, both white noise and white noise
428 plus CO_2 failed to cause a significant rectification compared to the pure tone group
429 (Fig. 6C). This data supports our prediction that CO_2 associates with a specific memory
430 trace that was reactivated. To avoid artificial effects by conditioned stimuli, we switched
431 over the pure tone and white noise in the aversive conditioning and retrieval. Similar
432 results were observed, confirming the effects of CO_2 were not artificial (Fig. 6D, E).
433
434 In the duration of these experiments, we were interested in determining whether
435 synaptic strength had been changed with the application of an unrelated retrieval CS
436 and CO_2. The ratio of AMPAR-EPSCs to NMDAR-EPSCs might represent the strength
437 of the synapse (Rao and Finkbeiner, 2007). Previous studies reported that the AMPAR/
438 NMDAR-EPSC ratio increased after aversive conditioning whereas retrieval does not

439 potentiate further increase, suggesting that memory retrieval did not alter the synaptic
440 strength (Clem and Huganir, 2010; Hong et al., 2013). Our previous studies also
441 indicated that CO_2 inhalation during memory retrieval did not strengthen the synapse in
442 the amygdala (Du et al., 2017). Here, we further tested whether the pairing of CO_2
443 inhalation with the specific retrieval CS influences the strength of a synapse. Currents
444 were recorded at -80mV for AMPAR-EPSCs and +60mV for NMDAR-EPSCs. Our data
445 suggest that retrieval plus CO_2 inhalation did not change the AMPAR/ NMDAR-EPSCs
446 ratio (Fig. 6F). Moreover, the characteristic of miniature EPSCs (mEPSCs) was not
447 altered (Fig. 6G). In addition, the pairing of CO_2 with another unrelated CS in retrieval
448 did not change the strength of the synapse (Fig. 6F, G). This data suggests that
449 memory retrieval and CO_2 inhalation enhance the destabilization of the synapse without
450 changing synaptic strength.
451
452 **The effects of CO_2 inhalation on distinct memory trace.**
453 Our previous studies indicate that CO_2 enhances memory trace that is associated with
454 aversive conditioning [5]. In this experiment, we examined the mechanism behind the
455 specificity of CO_2 effects on memory traces. Using the TetTag-c-fos driven-GFP mouse
456 model, neurons in the amygdala involved in memory trace after aversive conditioning
457 can be labeled with a long-lasting mCherry fluorescent protein and the neuron in the
458 retrieval trace can be labeled with a short-half life (2 hours) nuclear-localized EGFP
459 (shEGFP) (Fig. 7A, B) (see the details in Materials and Methods) (Du et al., 2017;
460 Koffman and Du, 2017). In this experiment, the mice were first conditioned with pure
461 tone sounds, activating the associated neurons that were labeled with mCherry (Fig.

462 7C). Right after aversive conditioning, the mice were immediately fed with a DOX diet
463 thereby preventing further mCherry labeling. On day 2, the mice were divided into two
464 groups - one group of mice were presented with a single pure tone to retrieve the
465 memory, another was presented with a white noise. A temporary, 2-hour half-life,
466 shEGFP was labeled after the retrieval. Thirty minutes after the retrieval event, we
467 sliced the amygdala and imaged shEGFP- and mCherry-positive cells (Fig. 7B, D).
468 Compared to pure tone aversive conditioning/pure tone retrieval group, inhalation of
469 CO_2 in the pure tone aversive conditioning/white noise retrieval group did not result in an
470 increase of neurons positive for expression of both mCherry-positive and shEGFP-
471 positive neurons (overlapped labeling, yellow) (Fig. 7E). Control experiments to identify
472 the efficiency of the aversive conditioning on the expression of mCherry on the cells
473 were performed (Fig. 7F). These findings indicate that CO_2 only enhances the memory
474 trace that has been reactivated; CO_2 paired with unrelated triggers does not affect the
475 original memory trace. These findings suggest a specific effect of CO_2 on the memory
476 trace.
477
478 Dendritic spine morphology after retrieval was also a topic of interest for us to study;
479 while not absolute, spine morphology has been widely indicated in the mechanism of
480 synaptic plasticity (Woolfrey and Srivastava, 2016; Wright et al., 2020; Yang et al.,
481 2009). Dendritic spines are the primary target of neurotransmission input in the central
482 nervous system (Bourne and Harris, 2008), and their density and structure provide the
483 basis for physiological changes in synaptic efficacy that underlie learning and memory
484 (Bailey et al., 2015). Spine formation and plasticity are regulated by many conditions,

485 including exterior stimulation and behavior (Gipson and Olive, 2017). We hypothesized
486 that CO_2 inhalation during retrieval alters both structure and plasticity of dendritic spines.
487 The molecular mechanism by which CO_2 regulates spine plasticity may explain how CO_2
488 converts memory into the labile stage.
489
490 Using the TetTag-c-fos driven-GFP mouse model, we imaged spine structure in
491 overlapping neurons in each group (pure tone aversive conditioning, pure tone retrieval
492 and pure tone aversive conditioning, white noise retrieval) (Fig. 7A, C). We assessed
493 spine density and morphology in the amygdala of the brain slices. Mature spines-most
494 of which display “mushroom-like” morphology - have more stable postsynaptic
495 structures enriched in AMPARs. In contrast, immature spines with a “thin-like”
496 morphology, are unstable postsynaptic structures that have the transitional ability.
497 Immature dendritic spines are thought to be responsible for synaptic plasticity, as they
498 have the potential for strengthening (Berry and Nedivi, 2017). The categories of spines
499 were identified based on the parameters in the previous studies (Fig. 7G) (see the
500 details in the Material and Methods) (Kreple et al., 2014; Wright et al., 2020). The
501 behavior procedure was described in Fig. 7C, the animal was trained by aversive
502 conditioning with a tone as a CS and followed by a retrieval on day 2 with tone or
503 noise. We found increased spine numbers after aversive conditioning (Fig. 7H),
504 indicating aversive conditioning increases synaptic strength. There was no additional
505 increase in the density of spines in all groups, suggesting that retrieval and CO_2
506 inhalation did not change the synaptic strength (Fig. 7H, lower-right).
507

508 We further analyzed spine subtypes as described in the experimental procedures. Thin
509 spines are deemed to represent the immature structure of the synapses (Berry and
510 Nedivi, 2017), thus we examined the percentage of thin spines to total spines.
511 Interestingly, we found there to be more thin spines in the retrieval group; this suggests
512 a greater amount of synaptic plasticity in this group (Fig. 7H, lower-middle). More
513 excitingly, when the retrieval group (tone) was paired with CO_2, we found an additional
514 increase of thin spines compared to the retrieval group alone (Fig. 7H, lower-middle).
515 This finding suggests that CO_2 indeed boosts synaptic plasticity compared to memory
516 retrieval alone. Consistently, the mushroom spine numbers decreased in the tone and
517 CO_2 paired retrieval groups, suggesting a higher turnover rate when the animal
518 underwent memory retrieval. However, in experiments where the mice were trained with
519 pure tone but in the retrieval were presented with white noise (a generated unrelated
520 CS), we found that in the noise-retrieval group with or without CO_2 inhalation, the thin
521 spine number did not increase compared to the pure tone - retrieval group (Fig. 7H,
522 lower-middle). This finding supports the specific effect of CO_2 on the memory trace.
523

524 **Discussion**

525 When a newly formed aversive memory turns into a labile stage before being stored in a
526 long-term stable stage, it can be easily disrupted (Alberini, 2011). The time window in
527 which a memory is labile is known as reconsolidation (Schiller et al., 2012). In previous
528 studies, it has been found that a retrieval event utilizing a single tone can retrieve the
529 memory and turn into the labile stage during the reconsolidation window (Monfils et al.,
530 2009). Within this reconsideration window, memory is sensitive to the updating process

531 that may either enhance or weaken the original memory (Du et al., 2017). As a result,
532 the reconsolidation window is the key to determining the lability of a memory. A recent
533 study of ours found that when mice inhale 10% CO_2 during retrieval, memory lability
534 increases, and later update processes within the reconsolidation window can replace
535 the original memory (Du et al., 2017). Although the effects of CO_2 on memory retrieval is
536 significant, whether CO_2 has direct and specific effects on memory retrieval is still
537 unknown. Also, it is known that two associative memories can be independently
538 reconsolidated even though they share the same aversive outcome (Debiec et al., 2010;
539 Doyere et al., 2007). We thus designed unique behavioral approaches with distinct CS
540 and US to address the specificity of the effects of CO_2. These behavioral protocols
541 provide approaches to study the specific effects of retrieval and reconsolidation.
542 Applications of CO_2 to the one of two (aversely conditioned) reactivated memories
543 enhances its lability, but not the other one.
544
545 How does CO_2 enhance the lability of aversive memory specifically? We have shown
546 that CO_2 activates ASIC1a by decreasing pH in the brain during retrieval. Activation
547 of ASIC1a increases post-synaptic intracellular calcium and increases AMPAR
548 exchange (Du et al., 2017). Previous studies regarding the mechanism of retrieval have
549 revealed the exchange from CI-AMPARs to CP-AMPARs in synapses in the amygdala
550 (Clem and Huganir, 2010; Hong et al., 2013) after presenting the CS. The emphasis of
551 this study is to detect the specificity of CO_2 effects on the exchange of the AMPARs in
552 synapses of the lateral amygdala. We conditioned the mice with a pure tone CS and
553 reactivated the memory with the pure tone with or without CO_2 inhalation. Consistent
554 with our previous data, CO_2 increases AMPAR exchange when it is paired with retrieval.

555 However, when mice were presented with an unrelated CS during the retrieval stage,
556 CO_2 did not change the AMPAR exchange - suggesting the effects of CO_2 on memory
557 trace are specific. In addition, synaptic strength (ratio of AMPAR/NMDAR and amplitude
558 of the mEPSCs) was not altered while applying CO_2 during retrieval, with or without
559 combining with the memory trace. The exchange of CI-AMPARs to CP-AMPARs
560 indicates a synaptic plasticity change. Interestingly, CO_2 did not increase the total
561 number of spines compared to the retrieval alone group, which suggests the strength of
562 the synapse in a memory trace does not change. In addition, thin spine density
563 significantly increased when retrieval was combined with CO_2 inhalation, suggesting that
564 CO_2 application changes plasticity. Using this model, we found when an unrelated CS
565 was presented in retrieval, no additional increase of immature spine density was found.
566 This finding indicates no plasticity change in the memory trace. Thus, we can conclude
567 from our findings - the effects of CO_2 on memory trace are specific.
568
569 Identifying the specificity of how CO_2 affects brain function is not a simple finding.
570 Although we have provided evidence that CO_2 indeed acts with specificity on a memory
571 trace, this study did not completely uncover *why* CO_2 directly regulates a memory; we
572 cannot exclude the possibility that CO_2 might trigger specific effects on memory through
573 other targets. For instance, CO_2 inhalation increases cerebral blood flow and arterial
574 blood pressure and might affect brain functions, such as cognition. Although no direct
575 evidence supports the possibility that increased cerebral blood flow and arterial blood
576 pressure affect learning and memory, further studies still need to be performed in the
577 future to address why CO_2 affects learning and memory with specificity. Moreover, we
578 cannot exclude the probability that synapses in other brain regions and that other

579 behavior (*e.g.* appetitive behaviors and drug additive behaviors) might show similar
580 responses to CO_2. This opens the door for future research to further explore the
581 specificity of CO_2 effects on other behaviors.
582
583 In conclusion, the effects of CO_2 on the lability of aversive memory are found to be
584 specific under certain conditions. Our research tests the novel hypothesis that protons
585 are neurotransmitters that activate the postsynaptic proton receptors, ASICs, to
586 manipulate memory updates. This non-invasive, drug-free methodology is innovative,
587 efficacious, and safe for translation to clinical use. As a result, this research may lead to
588 an effective complementary treatment for many mental health-related disorders for
589 which efficient treatments are lacking. We hope this research will lead to new areas of
590 inquiry via CO_2-related mechanisms that underlie memory modification and lead to the
591 development of novel therapies for fear-related anxiety disorders such as PTSD.
592
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594
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601
602

603 **Figure Legends**

604 **Figure 1: CO_2 inhalation during a selective memory retrieval potentiate the effect**
605 **of the extinction. (A)** Representative schematic of protocol for the aversive
606 conditioning, memory retrieval, extinction, and memory test (spontaneous recovery and
607 renewal). On day1, the mice were subjected to 3 pure tones and 3 white noises, paired
608 with 6-foot shocks in context A. One day after, the mice were placed in context B and
609 were subjected to both tone and noise as retrieval events. 30 mins after retrieval, the
610 mice were treated with 2 blocks of extinctions with a pure tone as the CS. On day 7,
611 spontaneous recovery and renewal were tested individually in context B and then
612 context A. 4 pure tones and 4 white noises were presented as CSs during each memory
613 testing. **(B-E)** Data are presented by the percentage of freezing time during the CSs
614 (tone and noise) in aversive conditioning **(B)**, retrievals (tone and noise) **(C)**, two
615 sections of extinction with tone **(D)**, memory test of spontaneous recovery (Spon Rec)
616 and renewal with tone and noise **(E)**. **(F-I)** Data are presented by the percentage of
617 freezing time during the CSs (tone and noise) in aversive conditioning **(F)**, retrievals
618 (tone plus CO_2 inhalation and noise) **(G)**, two sections of extinction with tone **(H)**,
619 memory test of spontaneous recovery (Spon Rec) and renewal with tone and noise **(I)**.
620 Data are mean ± SEM. n = 10-12 mice in each group. * indicates $p<0.05$ by unpaired
621 Student's t-test.

622

623 **Figure 2: CO_2 inhalation during memory retrieval potentiate the effect of the**
624 **anisomycin. (A)** Representative schematic of the protocol for aversive conditioning,
625 memory retrieval, anisomycin injection, and memory test (spontaneous recovery and

626 renewal). On day1, the mice were subjected to 6 pure tones, paired with 6 foot shocks
627 in context A. One day after CS, the mice were placed in context B and were subjected
628 to one single tone as a retrieval event with or without CO_2 inhalation. 30 mins after
629 retrieval, the mice were then infused with 62.5μg/μl anisomycin or saline and then
630 returned to their home cage. On day 7, spontaneous recovery and renewal were tested
631 individually in context B and then context A. 4 pure tones were presented as CSs during
632 each memory testing. **(B-E)** Data are presented by the percentage of freezing time
633 during the tone presentation in aversive conditioning **(B)**, retrieval (tone) **(C)**,
634 anisomycin or saline infusion in the amygdala **(D)**, spontaneous recovery (Spon Rec),
635 and renewal test with tones **(E)**. **(F-I)** Data are presented by the percentage of freezing
636 time during the tone presentation in aversive conditioning **(F)**, retrieval (tone) with or
637 without CO_2 **(G)**, anisomycin infusion **(H)**, spontaneous recovery (Spon Rec), and
638 renewal test with tones **(I)**. Data are mean ± SEM. n = 8-10 mice in each group. *
639 indicates $p<0.05$ by unpaired Student's t-test.
640
641 **Figure 3: The effect of CO_2 inhalation on selective memory retrieval is ASIC-**
642 **dependent. (A)** Representative schematic of protocol for the aversive conditioning,
643 memory retrieval, extinction, and memory test (spontaneous recovery and renewal) in
644 ASIC1a$^{-/-}$ mice. On day1, the mice were subjected to 3 pure tones and 3 white noises,
645 paired with 6 foot shocks in context A. One day later, the mice were placed in context B
646 and subjected to both tone and noise as retrieval events. 30 mins after retrieval, the
647 mice were treated with 2 blocks of extinctions with a pure tone as the CS. On day 7,
648 spontaneous recovery and renewal were tested individually in context B and then
649 context A. 4 pure tones and 4 white noises were presented as CSs during each memory

650 testing. **(B-E)** Data in ASIC1a$^{-/-}$ mice are presented by the percentage of freezing time
651 during the CSs (tone and noise) in aversive conditioning **(B)**, retrievals (tone and noise)
652 **(C)**, two sections of extinction with tone **(D)**, memory test of spontaneous recovery
653 (Spon Rec) and renewal with tone and noise **(E)**. **(F-I)** Data in ASIC1a$^{-/-}$ mice in
654 aversive conditioning **(B)**, retrievals (pure tone plus 10% CO_2 inhalation and white
655 noise) **(C)**, two sections of extinction with tone **(D)**, memory test of spontaneous
656 recovery (Spon Rec) and renewal with tone and noise **(E)**. Data are mean ± SEM. n =
657 12-16 mice in each group. * indicates $p<0.05$ by unpaired Student's t-test.
658
659 **Figure 4: The CO_2 potentiated effect of anisomycin is ASIC1a-dependent.**
660 **(A)** Representative schematic of protocol for the aversive conditioning, memory
661 retrieval, anisomycin injection, and memory test (spontaneous recovery and renewal).
662 On day 1, the mice were subjected to 6 pure tones, paired with 6 foot shocks in context
663 A. One day after, the mice were placed in context B and subjected to one single tone as
664 a retrieval event with or without CO_2 inhalation. 30 mins after retrieval, the mice were
665 infused with 62.5 μg/μl anisomycin or saline and then returned to their home cage. On
666 day 7, spontaneous recovery and renewal were tested individually in context B and then
667 context A. 4 pure tones were presented as CSs during each memory testing. **(B-E)** Data
668 in ASIC1a$^{-/-}$ mice are presented by the percentage of freezing time during the tone
669 presentation in aversive conditioning **(B)**, retrieval (tone) with or without CO_2 **(C)**,
670 anisomycin infusion in the amygdala **(D)**, spontaneous recovery (Spon Rec) and
671 renewal test with tones **(E)**. **(F)** Representative schematic of protocol for the aversive
672 conditioning, memory retrieval, extinction, and memory test (spontaneous recovery and

673 renewal). On day 1, ASIC1a$^{-/-}$ mice were subjected to 3 pure tones and 3 white noises,
674 paired with 6 foot shocks in context A. One day later, the mice were placed in context B
675 and subjected to both tone and noise as retrieval events. 30 mins after retrieval, the
676 mice were infused with anisomycin or saline and returned to their home cage. On day 7,
677 spontaneous recovery and renewal were tested individually in context B and then
678 context A. 4 pure tones and 4 white noises were presented as CSs during each memory
679 testing. **(G-J)** Data are presented by the percentage of freezing time during the tone
680 presentation in aversive conditioning **(G)**, retrieval (noise plus CO_2 inhalation, then tone)
681 **(H)**, anisomycin infusion in the amygdala **(I)**, spontaneous recovery (Spon Rec), and
682 renewal test with tones **(J)**. **(K-N)** Data are presented by the percentage of freezing time
683 during the tone presentation in aversive conditioning **(K)**, retrieval (tone) with or without
684 CO_2 **(L)**, anisomycin infusion in the amygdala **(M)**, spontaneous recovery (Spon Rec),
685 and renewal test with tones **(NI)**. Data are mean ± SEM. n = 12-16 mice in each group.
686 P>0.05 by unpaired Student's t-test between groups.
687
688 **Figure 5: Blockage of ASIC1a in the amygdala reduces the CO_2 effects on**
689 **selective memory retrieval. (A)** Representative schematic of protocol for the aversive
690 conditioning, PcTX-1 injection, memory retrieval, extinction, and memory test
691 (spontaneous recovery and renewal). On day1, the mice were subjected to 3 pure tones
692 and 3 white noises, paired with 6 foot shocks in context A. One day later, the mice were
693 injected with 100nM PcTX-1 or saline, then the mice were placed in context B and
694 subjected to both tone and noise as retrieval events with or without CO_2. 30 mins after
695 retrieval, the mice were treated with 2 blocks of extinctions with a pure tone as the CS.
696 On day 7, spontaneous recovery and renewal were tested individually in context B and

697 then context A. 4 pure tones and 4 white noises were presented as CSs during each
698 memory testing. **(B-E)** Data are presented by the percentage of freezing time during the
699 CSs (tone and noise) in aversive conditioning **(B)**, retrievals (tone plus CO_2 inhalation
700 and noise) after saline injection in the amygdala **(C)**, two sections of extinction with
701 tone **(D)**, memory test of spontaneous recovery (Spon Rec) and renewal with tone and
702 noise **(E)**. **(F-I)** Data are presented by the percentage of freezing time during the CSs
703 (tone and noise) in aversive conditioning **(F)**, retrievals (tone plus CO_2 inhalation and
704 noise) after PcTx-1 injection in the amygdala **(G)**, two sections of extinction with tone
705 **(H)**, memory test of spontaneous recovery (Spon Rec) and renewal with tone and
706 noise **(I)**. Data are mean ± SEM. n = 12 mice in each group. * indicates $p<0.05$
707 by unpaired Student's t-test.
708
709 **Figure 6: CO_2 inhalation during a selective memory retrieval enhances the**
710 **retrieval dependent AMPAR current rectification. (A)** Representative schematic of
711 the protocol. On day 1, the animal underwent 6 CSs (tones or noses), paired with 6-foot
712 shocks in context A. On day 2, the mice were divided into 4 groups for the retrieval-pure
713 tone only; pure tone plus 10% CO_2 inhalation; white noise only; white noise+ 10% CO_2
714 inhalation. Ten minutes after retrieval, the brain slices were dissected and AMPAR
715 current was recorded in the pyramidal neurons in the lateral amygdala through
716 stimulation of thalamic input. **(B-E)** Mice underwent 6 pure tones **(B)** or 6 noises **(D)** in
717 aversive conditioning, Data are presented by the percentage of freezing time during the
718 tone presentation in aversive conditioning, retrieval (noise plus CO_2 inhalation, then
719 tone). **(C, E)** Left, AMPARs current-voltage relationships in the recorded neurons. Insets
720 show an example of the AMPAR-EPSCs in -80mV and + 60mV. Right, AMPAR

721 rectification index (I-80 mV / I+60 mV). Data are mean±SEM. n = 20-26 for each group.
722 * indicates p<0.05 by ANOVA with Tukey's post hoc multiple comparisons. 'ns' indicates
723 not statistically significant. **(F)** Left, examples of EPSC recordings of AMPAR-EPSCs (-
724 80mV) and NMDAR-EPSCs (+60mV). Right, AMPAR/NMDAR EPSC ratios. Current
725 amplitudes were measured 70 ms after onset. n = 20-26 for each group. **(G)** Miniature
726 EPSCs recordings from the neurons after retrieval. Upper, representative mEPSC
727 traces from different groups. Lower, cumulative distributions of mEPSC amplitudes,
728 inter-event intervals, and decay-times. n = 25-40 for each group. Data are mean ± SEM.
729 There were no statistically significant differences among groups by ANOVA with
730 Tukey's post hoc multiple comparisons. 'ns' indicates not statistically significant.
731
732 **Figure 7: CO_2 inhalation during a selective memory retrieval enhances the**
733 **retrieval-related memory trace. (A)** Schematic showing the c-Fos-tTA-GFP mouse
734 system combined with an AAV_9-mCherry to label a specific memory trace. The Fos
735 promoter in transgenic mice was activated by activities, followed by a transient, 2-hour
736 half-life, GFP expression in the cells. When the $AAV_{2/9}$-mCherry virus was injected into
737 the brain, the activation of c-FOS also induces the expression of mCherry. When the
738 mice were fed with DOX, the mCherry expression was interrupted. **(B)** an example
739 image showing the efficiency of the expression of GFP and mCherry in the amygdala.
740 **(C)** The procedure of aversive conditioning, memory retrieval, and memory trace
741 labeling using the system in A. Mice was fed with DOX for at least one week, followed
742 by an injection of $AAV_{2/9}$-mCherry in the amygdala. Two weeks later, DOX was removed
743 and mice were subjected to aversive conditioning with a pure tone as the CS. DOX has

744 added back again immediately after aversive conditioning. 24 hours later, the mice
745 underwent retrieval with pure tone or noise. The brain slices were collected 30 mins
746 after retrieval and then were stained for microscopy. **(D)** Left, representative images of
747 the neurons labeled by mCherry, GFP, and DAPI; Right, the enlarged area from the
748 "merge" image showing the overlapping expression of mCherry and GFP neurons. The
749 overlapping neurons indicate their "consanguinity" in the same memory trace. **(E)**
750 Summarized data are the percentage of the overlapping expression of mCherry and
751 GFP neurons in different behavior groups. All mice underwent aversive conditioning
752 with a tone as the CS. 24 hours later, the mice were separated into 4 groups. Tone
753 group: retrieval CS by tone; Tone+CO_2 group: retrieval CS by tone along with CO_2
754 inhalation; Noise group: retrieval CS by noise; Noise +CO_2 group: retrieval CS by noise
755 along with CO_2 inhalation. **(F)** Control experiment showing the expression of mCherry
756 with or without DOX as well as with or without aversive conditioning. **(G)** Left, a
757 representative image showing the spine morphology in the mCherry and GFP
758 colocalized neurons. The mature spines were categorified as "mushroom" spines and
759 the immature spines were categorified as "thin" spines; Right, an enlarged image
760 showing the details of mushroom and thin spines. **(H)** Upper, representative images of
761 the spine structures in different animal groups showed in E; Lower, summarized data of
762 the spine densities of mushroom spines, thin spines, and total spines in the different
763 groups. Data are mean ± SEM. n =4- 5 mice for each group. * indicates $p<0.05$ by
764 ANOVA with Tukey's post hoc multiple comparisons.
765

766 **Figure s1: White noise was an appropriate successful CS to involve use in**
767 **studying the effects of CO2 effects on the fear conditioning and retrieval.**
768 **(A)** Representative schematic of protocol for the aversive conditioning (pure tone and
769 white noise), memory retrieval (pure tone and white noise), extinction (white noise), and
770 memory test (spontaneous recovery and renewal). **(B-E)** Data are presented by the
771 percentage of freezing time during the CSs (tone and noise) in aversive conditioning
772 **(B)**, retrievals (tone and noise) **(C)**, two sections of extinction with white noise **(D)**,
773 memory test of spontaneous recovery (Spon Rec) and renewal with tone and noise **(E)**.
774 **(F-I)** Data are presented by the percentage of freezing time during the CSs (tone and
775 noise) in aversive conditioning **(F)**, retrievals (pure tone and white noise plus CO_2
776 inhalation) **(G)**, two sections of extinction with white noise **(H)**, memory test of
777 spontaneous recovery (Spon Rec) and renewal with tone and noise **(I)**. Data are
778 mean ± SEM. n = 12 mice in each group. * indicates $p<0.05$ by unpaired Student's t-
779 test.
780
781 **Figure s2: CO_2 inhalation does not boost extinction when pairs with an unrelated**
782 **CS during memory retrieval. (A)** Representative schematic of protocol for the aversive
783 conditioning (pure tone and white noise), memory retrieval (pure tone and white noise),
784 extinction (white noise), and memory test (spontaneous recovery and renewal). **(B-**
785 **E)** Data are presented by the percentage of freezing time during the CSs (tone and
786 noise) in aversive conditioning **(B)**, retrievals (pure tone and white noise plus CO_2
787 inhalation) **(C)**, two sections of extinction with pure tone **(D)**, memory test of
788 spontaneous recovery (Spon Rec) and renewal with tone and noise, Blue arrow and %
789 indicated the difference (decreases) between the tone and noise groups **(E)**. **(F-I)** Data

790 are presented by the percentage of freezing time during the CSs (tone and noise) in
791 aversive conditioning **(F)**, retrievals (pure tone plus CO_2 inhalation and white noise) **(G)**,
792 two sections of extinction with white noise **(H)**, memory test of spontaneous recovery
793 (Spon Rec) and renewal with tone and noise, Blue arrow and % indicated the difference
794 (decreases) between the tone and noise groups. **(I)**. Data are mean ± SEM. n = 12 mice
795 in each group. * indicates $p<0.05$ by unpaired Student's t-test.
796
797 **Figure s3: Anisomycin disrupts the reconsolidation. (A)** Representative schematic
798 of protocol for the aversive conditioning (pure tone), with or without memory retrieval
799 (pure tone), anisomycin infusion, and memory test (spontaneous recovery and renewal).
800 **(B-E)** Data are presented by the percentage of freezing time during the tone
801 presentation in aversive conditioning **(B)**, with or without retrieval (tone) **(C)**, anisomycin
802 infusion in the amygdala **(D)**, spontaneous recovery (Spon Rec), and renewal test with
803 tones **(E)**. **(F-I)** Data are presented by the percentage of freezing time during the CSs
804 (tone and noise) in aversive conditioning **(F)**, retrievals (pure tone and white noise) **(G)**,
805 saline injection in the amygdala **(H)**, memory test of spontaneous recovery (Spon Rec)
806 and renewal with tone and noise **(I)**. **(J-M)** Data are presented by the percentage of
807 freezing time during the CSs (tone and noise) in aversive conditioning **(J)**, retrievals
808 (pure tone plus CO_2 inhalation and white noise) **(K)**, anisomycin infusion in the
809 amygdala **(L)**, memory test of spontaneous recovery (Spon Rec) and renewal with tone
810 and noise, **(M)**. Data are mean ± SEM. n = 11-12 mice in each group. * indicates $p<0.05$
811 by unpaired Student's t-test.
812

813 **Figure s4: CO_2 potentiates the effects of anisomycin on the reconsolidation. (A)**
814 Representative schematic of protocol for the aversive conditioning (pure tone and white
815 noise), memory retrieval (pure tone and white noise) with or without CO_2 inhalation,
816 anisomycin infusion, and memory test (spontaneous recovery and renewal). **(B-E)** Data
817 are presented by the percentage of freezing time during the CSs (tone and noise)
818 presentation in aversive conditioning **(B)**, retrieval (tone plus CO_2 and noise) **(C)**,
819 anisomycin infusion in the amygdala **(D)**, spontaneous recovery (Spon Rec) and
820 renewal test with tone and noise **(E)**. **(F-I)** Data are presented by the percentage of
821 freezing time during the CSs (tone and noise) in aversive conditioning **(F)**, retrieval
822 (noise plus CO_2 and tone) **(G)**, anisomycin infusion in the amygdala **(H)**, memory test of
823 spontaneous recovery (Spon Rec) and renewal with tone and noise **(I)**. **(J-M)** Data are
824 presented by the percentage of freezing time during the CSs (tone and noise) in
825 aversive conditioning **(J)**, retrievals (tone and noise) plus CO_2 **(K)**, saline infusion in the
826 amygdala **(L)**, memory test of spontaneous recovery (Spon Rec) and renewal with tone
827 and noise **(M)**. **(N-Q)** Data are presented by the percentage of freezing time during the
828 CSs (tone and noise) in aversive conditioning **(N)**, retrievals (tone and noise) plus
829 CO_2 **(O)**, anisomycin infusion in the amygdala **(P)**, memory test of spontaneous
830 recovery (Spon Rec) and renewal with tone and noise **(Q)**. Data are mean ± SEM. n
831 = 12 mice in each group. * indicate $p<0.05$ by unpaired Student's t-test.
832
833 **Figure s5: CO_2 does not affect memory reconsolidation without anisomycin.**
834 **(A)** Representative schematic of protocol for the aversive conditioning (pure tone and
835 white noise), memory retrieval (pure tone and white noise) with or without CO_2
836 inhalation, saline infusion, and memory test (spontaneous recovery and renewal). **(B-**

837 **E)** Data are presented by the percentage of freezing time during the CSs (tone and
838 noise) presentation in aversive conditioning **(B)**, retrieval (tone plus CO_2 and noise)
839 **(C)**, saline infusion in the amygdala **(D)**, spontaneous recovery (Spon Rec) and renewal
840 test with tone and noise **(E)**. **(F-I)** Data are presented by the percentage of freezing time
841 during the CSs (tone and noise) in aversive conditioning **(F)**, retrieval (noise plus CO_2
842 and tone) **(G)**, saline infusion in the amygdala **(H)**, memory test of spontaneous
843 recovery (Spon Rec) and renewal with tone and noise **(I)**. Data are mean ± SEM. n = 12
844 mice in each group. * indicate $p<0.05$ by unpaired Student's t-test.
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1009

Fig. 1

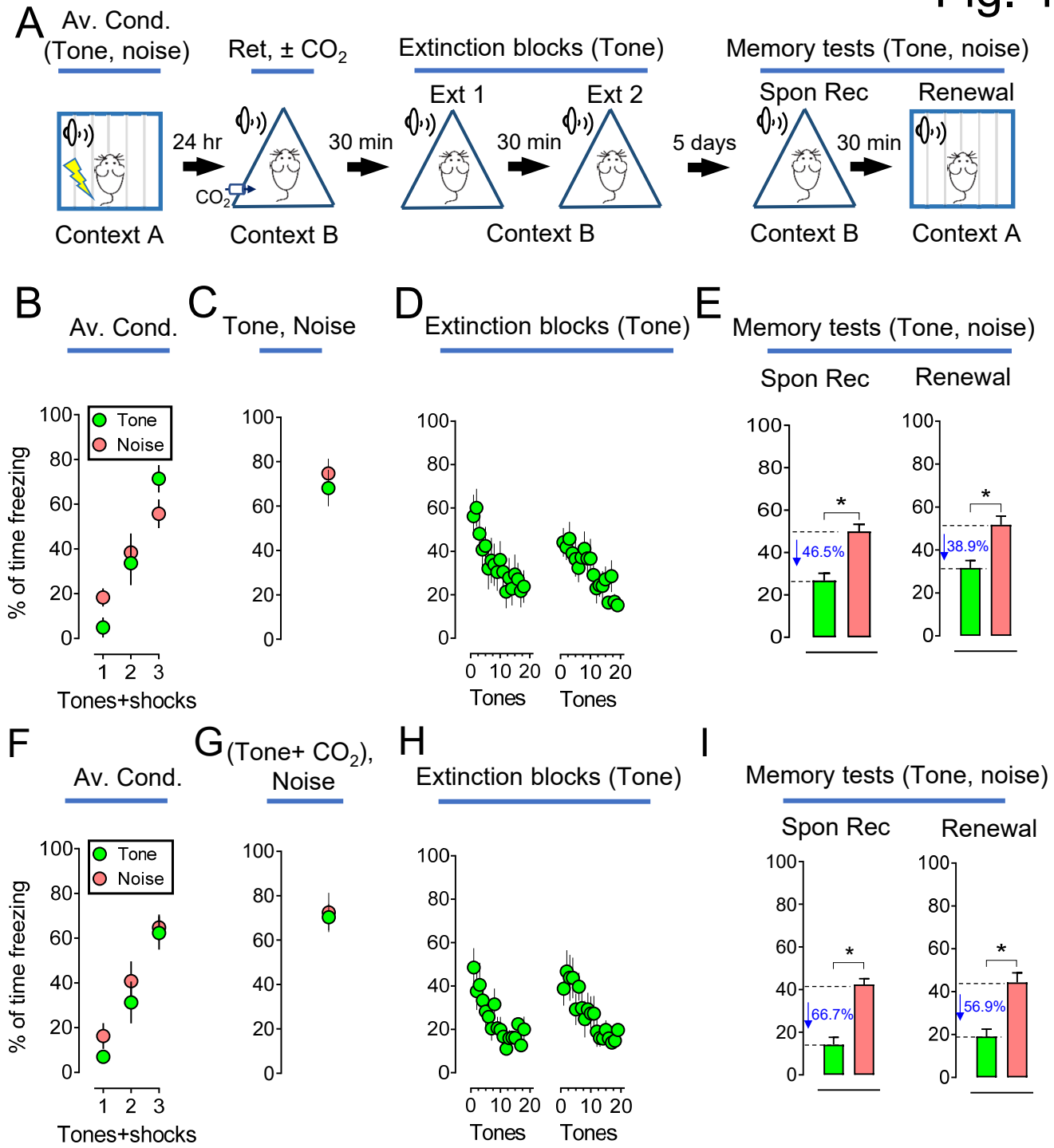

A
Av. Cond. (Tone, noise)
Ret, ± CO2
Extinction blocks (Tone)
Memory tests (Tone, noise)
Ext 1
Ext 2
Spon Rec
Renewal
24 hr
30 min
30 min
5 days
30 min
CO2
Context A
Context B
Context B
Context B
Context A
B
Av. Cond.
C
Tone, Noise
D
Extinction blocks (Tone)
E
Memory tests (Tone, noise)
Spon Rec
Renewal
Tone
Noise
% of time freezing
Tones+shocks
Tones
46.5%
38.9%
F
Av. Cond.
G
(Tone+ CO2), Noise
H
Extinction blocks (Tone)
I
Memory tests (Tone, noise)
Spon Rec
Renewal
66.7%
56.9%

Fig. 2

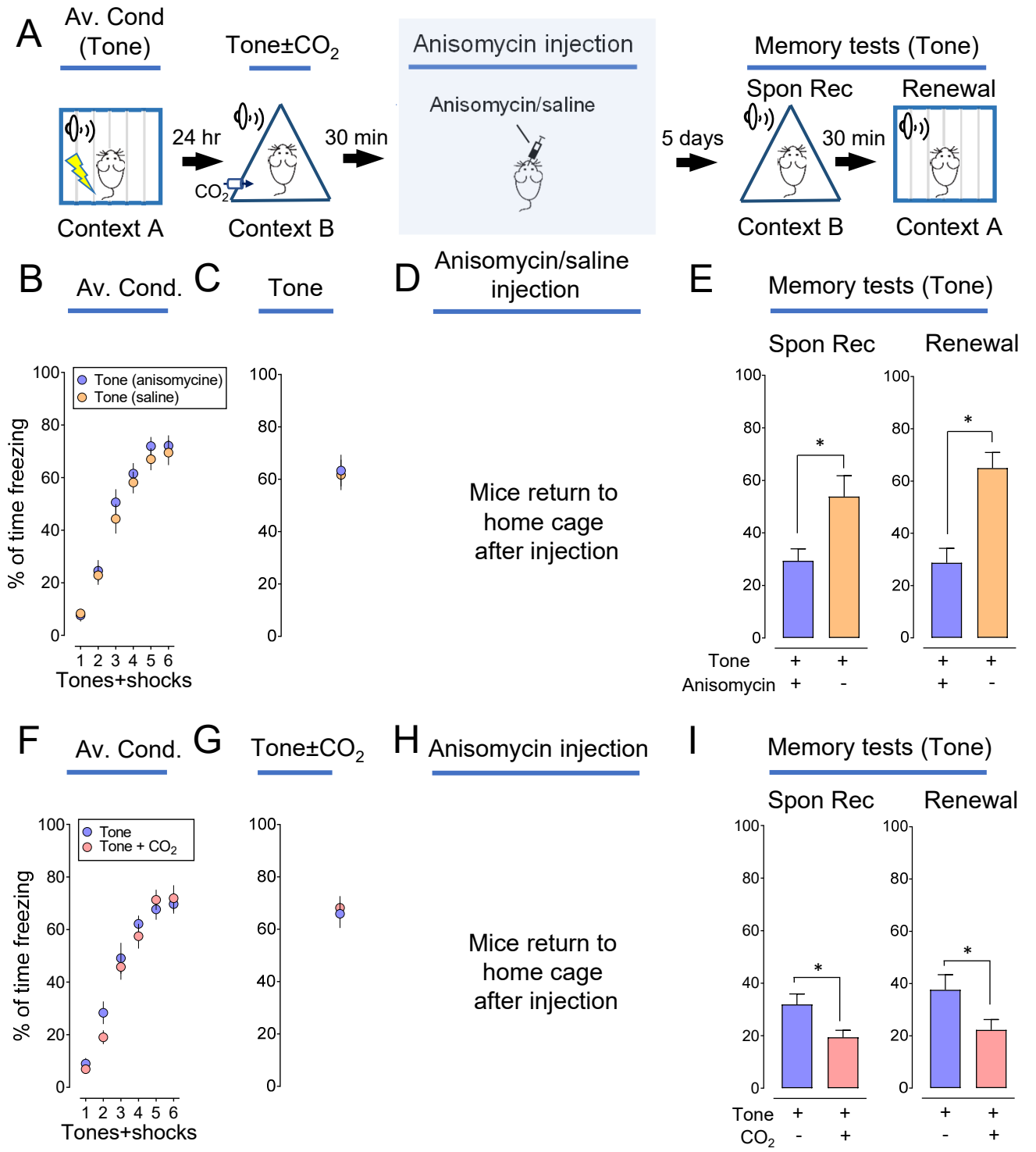

A
Av. Cond (Tone)
Tone±CO2
Anisomycin injection
Memory tests (Tone)
Anisomycin/saline
24 hr
30 min
5 days
30 min
CO2
Spon Rec
Renewal
Context A
Context B
Context B
Context A
B
Av. Cond.
C
Tone
D
Anisomycin/saline injection
E
Memory tests (Tone)
Spon Rec
Renewal
Tone (anisomycine)
Tone (saline)
% of time 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

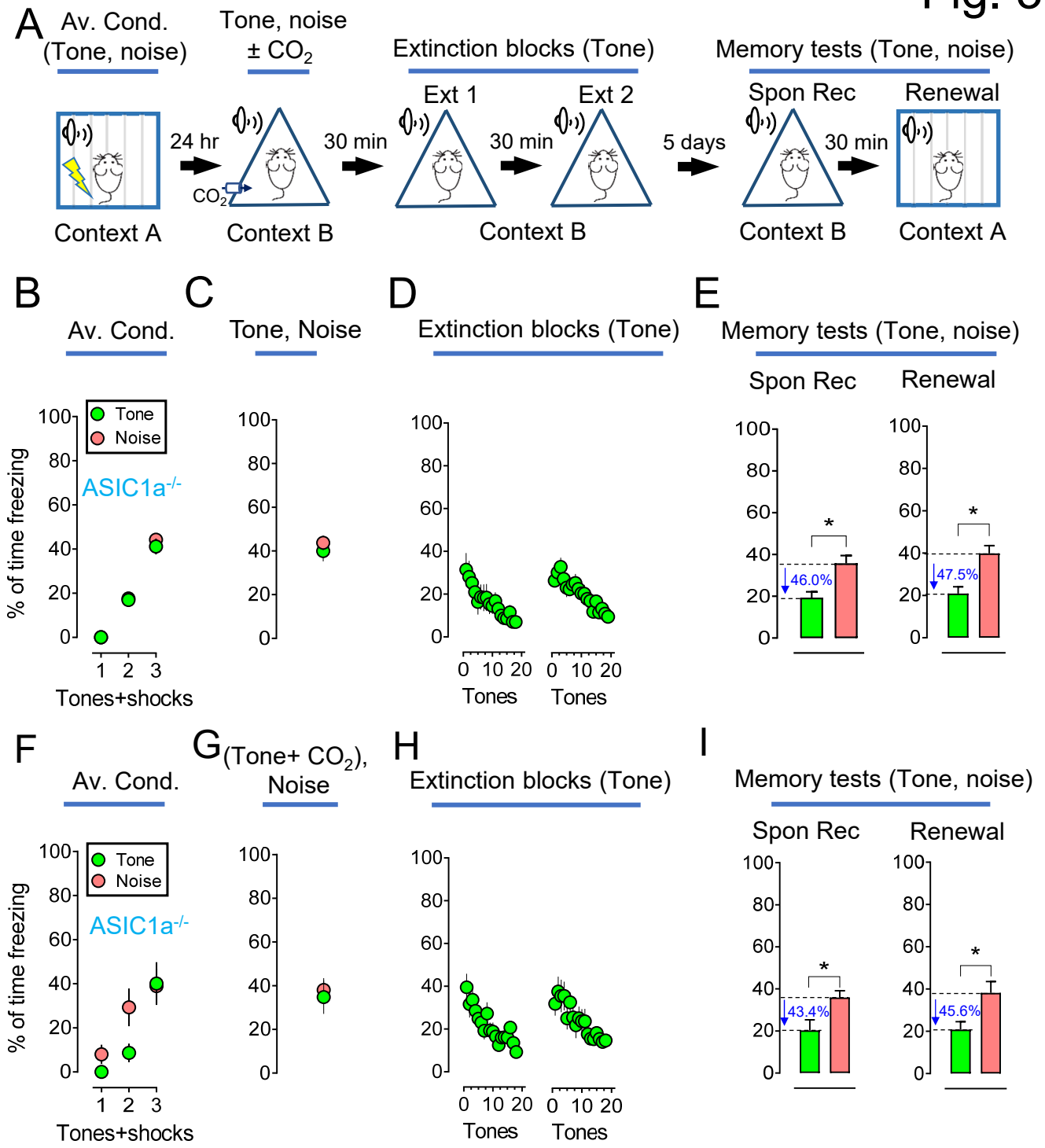

A
Av. Cond. (Tone, noise)
Tone, noise ± CO2
Extinction blocks (Tone)
Memory tests (Tone, noise)
Ext 1
Ext 2
Spon Rec
Renewal
24 hr
30 min
30 min
5 days
30 min
CO2
Context A
Context B
Context B
Context B
Context A
B
Av. Cond.
C
Tone, Noise
D
Extinction blocks (Tone)
E
Memory tests (Tone, noise)
Spon Rec
Renewal
Tone
Noise
ASIC1a-/-
% of time freezing
Tones+shocks
Tones
46.0%
47.5%
F
Av. Cond.
G
(Tone+ CO2), Noise
H
Extinction blocks (Tone)
I
Memory tests (Tone, noise)
Spon Rec
Renewal
43.4%
45.6%

Fig. 4

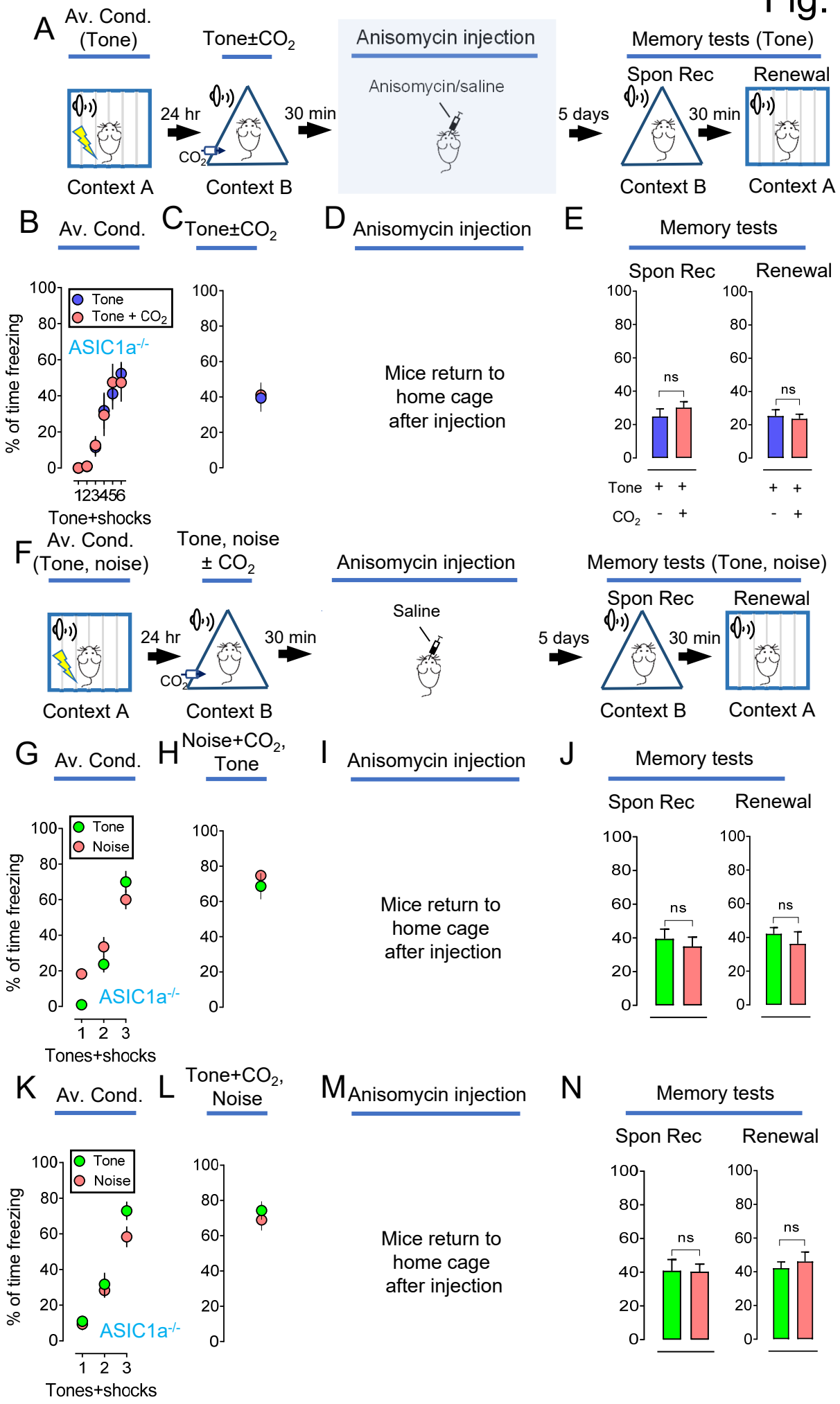

A
Av. Cond. (Tone)
Tone±CO2
Anisomycin injection
Memory tests (Tone)
Spon Rec
Renewal
24 hr
30 min
5 days
30 min
CO2
Anisomycin/saline
Context A
Context B
Context B
Context A
B
Av. Cond.
C
Tone±CO2
D
Anisomycin injection
E
Memory tests
Spon Rec
Renewal
Tone
Tone + CO2
ASIC1a-/-
% of time freezing
Tone+shocks
Mice return to home cage after injection
ns
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

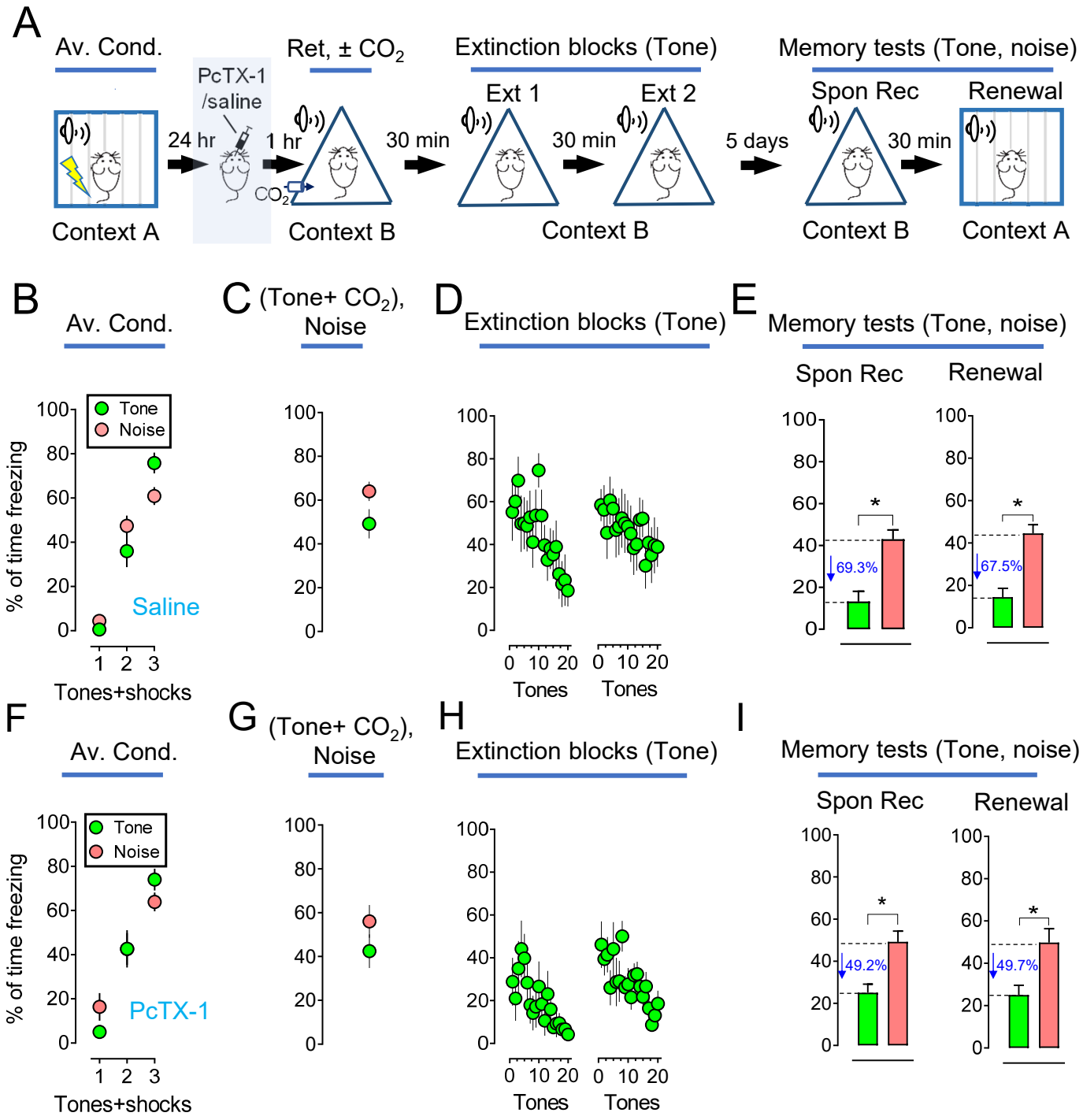

A
Av. Cond.
PcTX-1 /saline
Ret, ± CO2
Extinction blocks (Tone)
Memory tests (Tone, noise)
Ext 1
Ext 2
Spon Rec
Renewal
24 hr
1 hr
30 min
30 min
5 days
30 min
CO2
Context A
Context B
Context B
Context B
Context A
B
Av. Cond.
C (Tone+ CO2), Noise
D Extinction blocks (Tone)
E Memory tests (Tone, noise)
Spon Rec
Renewal
Tone
Noise
% of time freezing
Saline
Tones+shocks
69.3%
67.5%
Tones
F
Av. Cond.
G (Tone+ CO2), Noise
H Extinction blocks (Tone)
I Memory tests (Tone, noise)
Spon Rec
Renewal
PcTX-1
49.2%
49.7%

Fig. 6

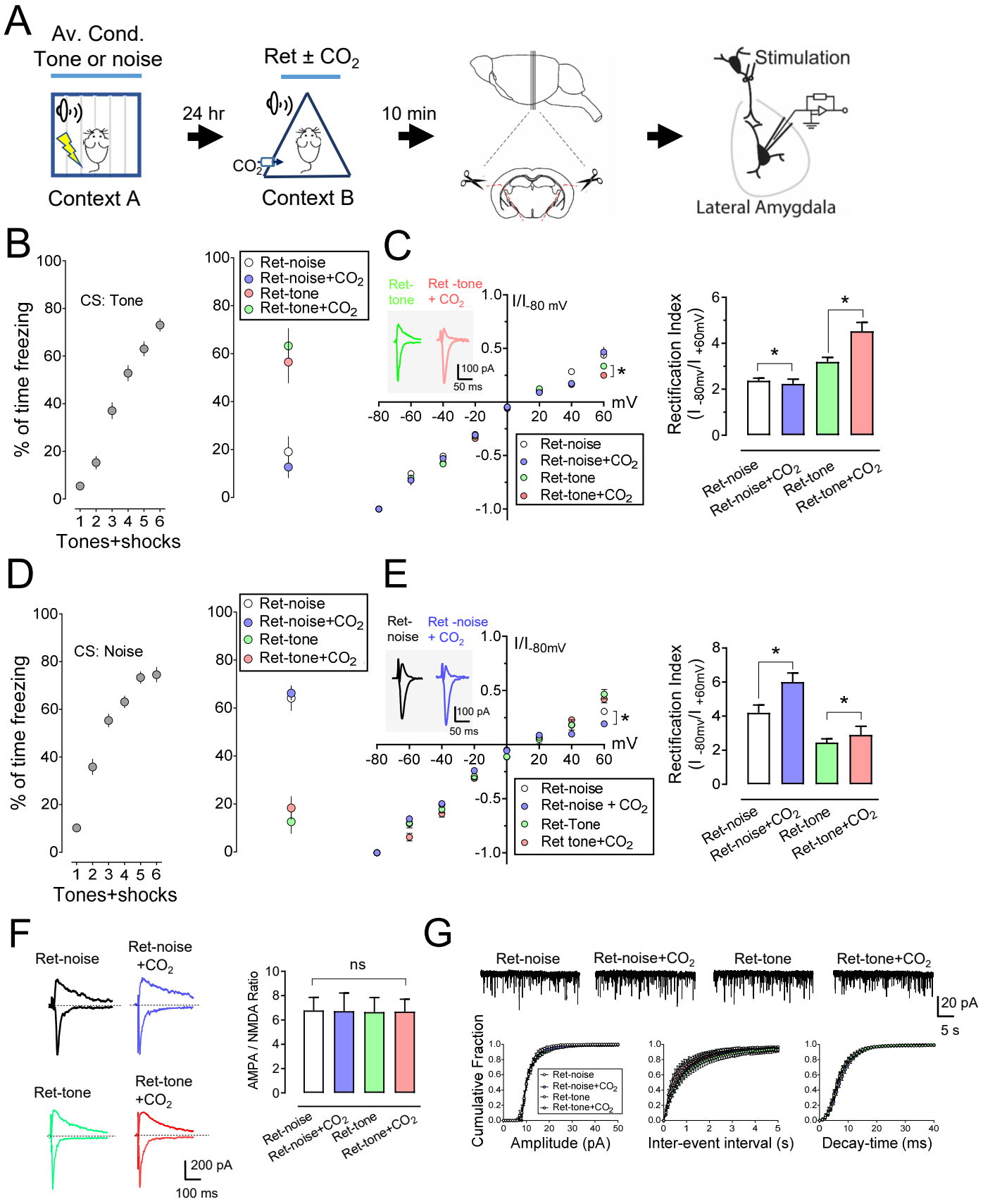

A
Av. Cond.
Tone or noise
Context A
24 hr
Ret ± CO2
CO2
Context B
10 min
Stimulation
Lateral Amygdala
B
CS: Tone
% of time freezing
Tones+shocks
Ret-noise
Ret-noise+CO2
Ret-tone
Ret-tone+CO2
C
Ret-tone
Ret -tone + CO2
100 pA
50 ms
I/I-80 mV
mV
Rectification Index (I -80mV/I +60mV)
D
CS: Noise
E
Ret-noise
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

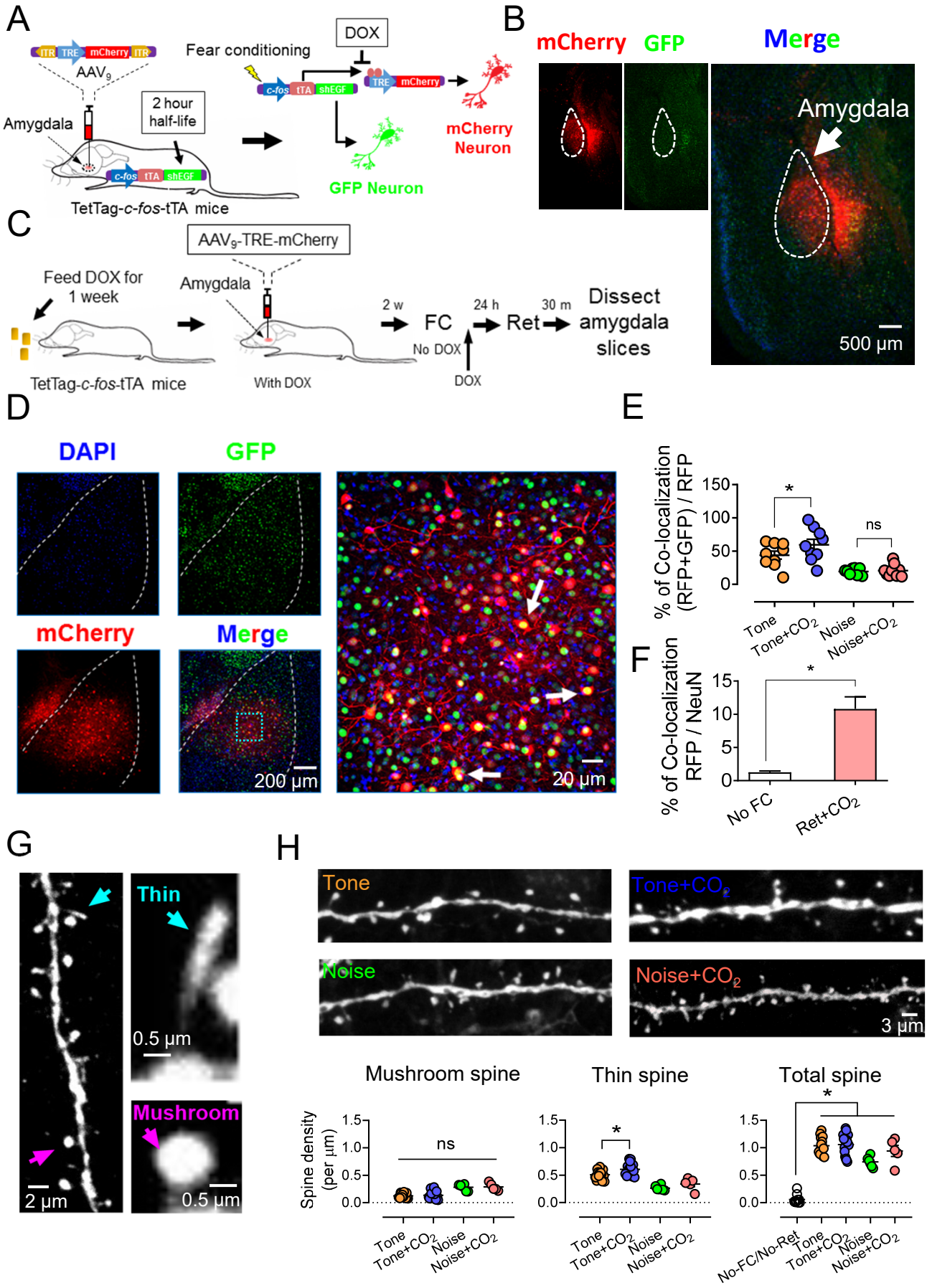

A
ITR TRE mCherry ITR
AAV9
Amygdala
2 hour half-life
c-fos tTA shEGF
TetTag-c-fos-tTA mice
Fear conditioning
DOX
TRE mCherry
mCherry Neuron
GFP Neuron
B
mCherry
GFP
Merge
Amygdala
500 µm
C
AAV9-TRE-mCherry
Feed DOX for 1 week
Amygdala
TetTag-c-fos-tTA mice
With DOX
2 w
FC
No DOX
DOX
24 h
Ret
30 m
Dissect amygdala slices
D
DAPI
GFP
mCherry
Merge
200 µm
20 µm
E
% of Co-localization (RFP+GFP) / RFP
150
100
50
0
*
ns
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
ns
*
*
No-FC/No-Ret
Tone
Tone+CO2
Noise
Noise+CO2

Fig. s1

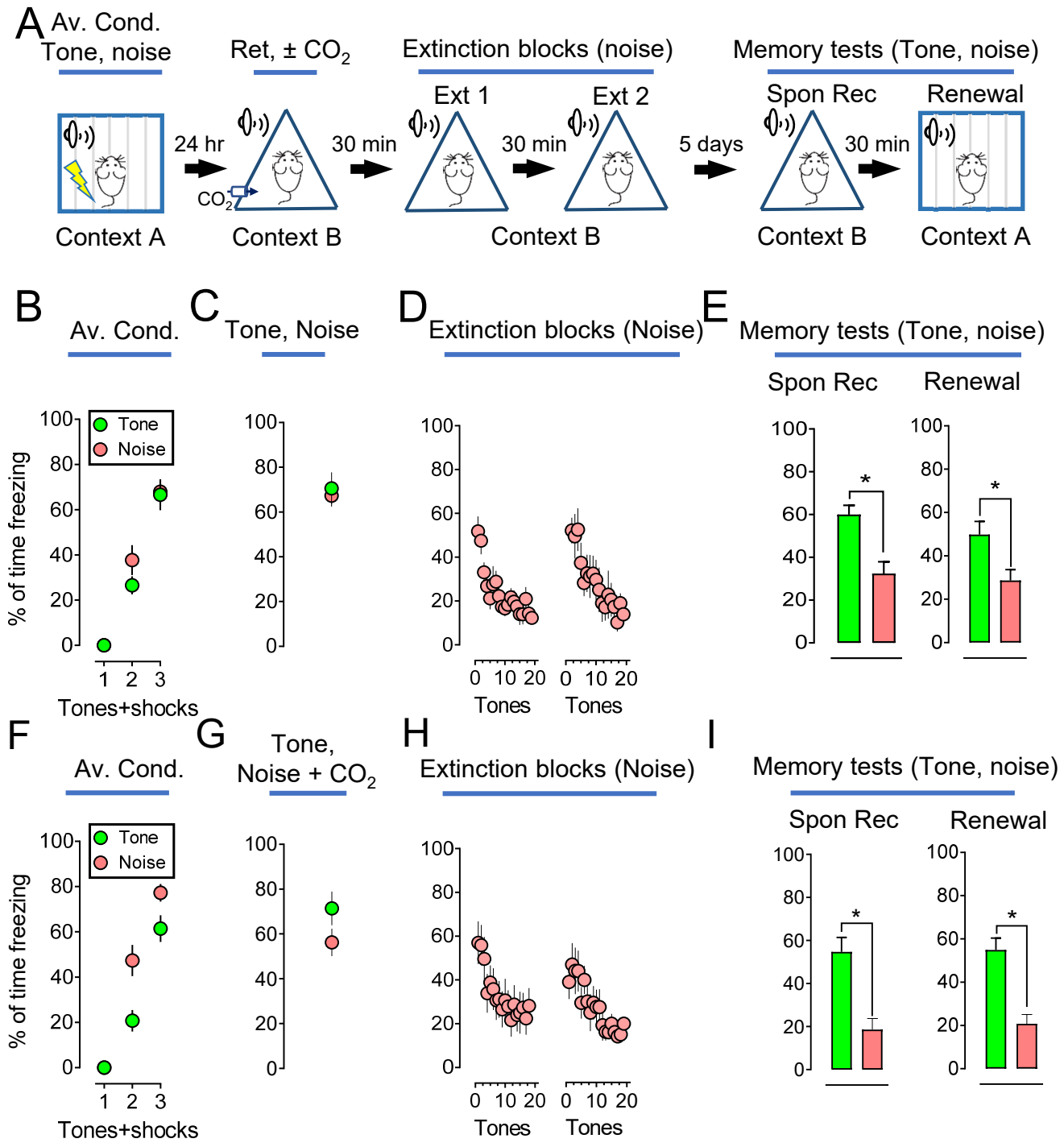

A
Av. Cond.
Tone, noise
Ret, ± CO2
Extinction blocks (noise)
Memory tests (Tone, noise)
Ext 1
Ext 2
Spon Rec
Renewal
24 hr
30 min
30 min
5 days
30 min
CO2
Context A
Context B
Context B
Context B
Context A
B
Av. Cond.
C
Tone, Noise
D
Extinction blocks (Noise)
E
Memory tests (Tone, noise)
Spon Rec
Renewal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

Fig. s2

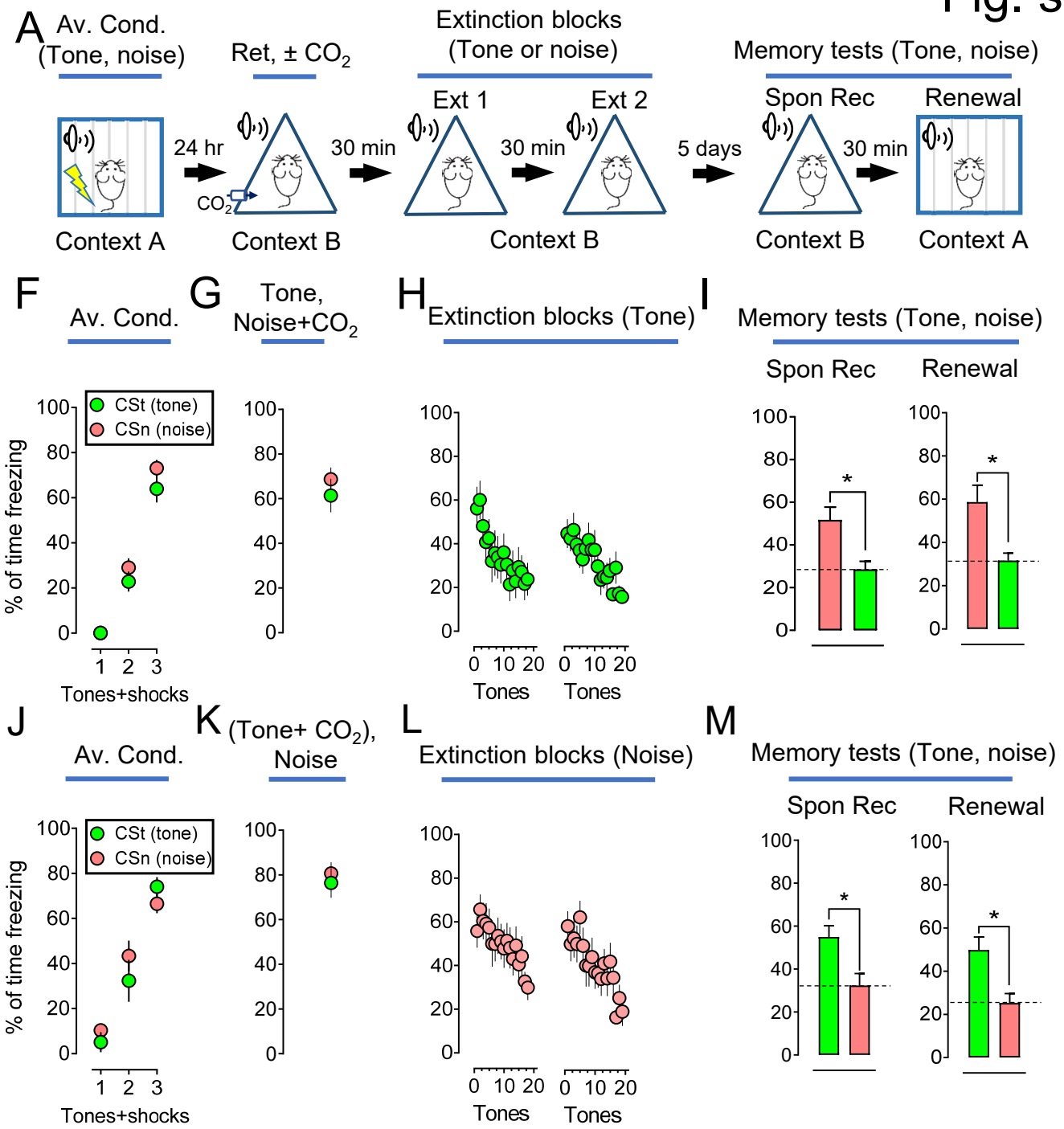

A
Av. Cond.
(Tone, noise)
Ret, ± CO2
Extinction blocks
(Tone or noise)
Memory tests (Tone, noise)
Ext 1
Ext 2
Spon Rec
Renewal
24 hr
30 min
30 min
5 days
30 min
CO2
Context A
Context B
Context B
Context B
Context A
F
Av. Cond.
G
Tone,
Noise+CO2
H
Extinction blocks (Tone)
I
Memory tests (Tone, noise)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

Fig. s3

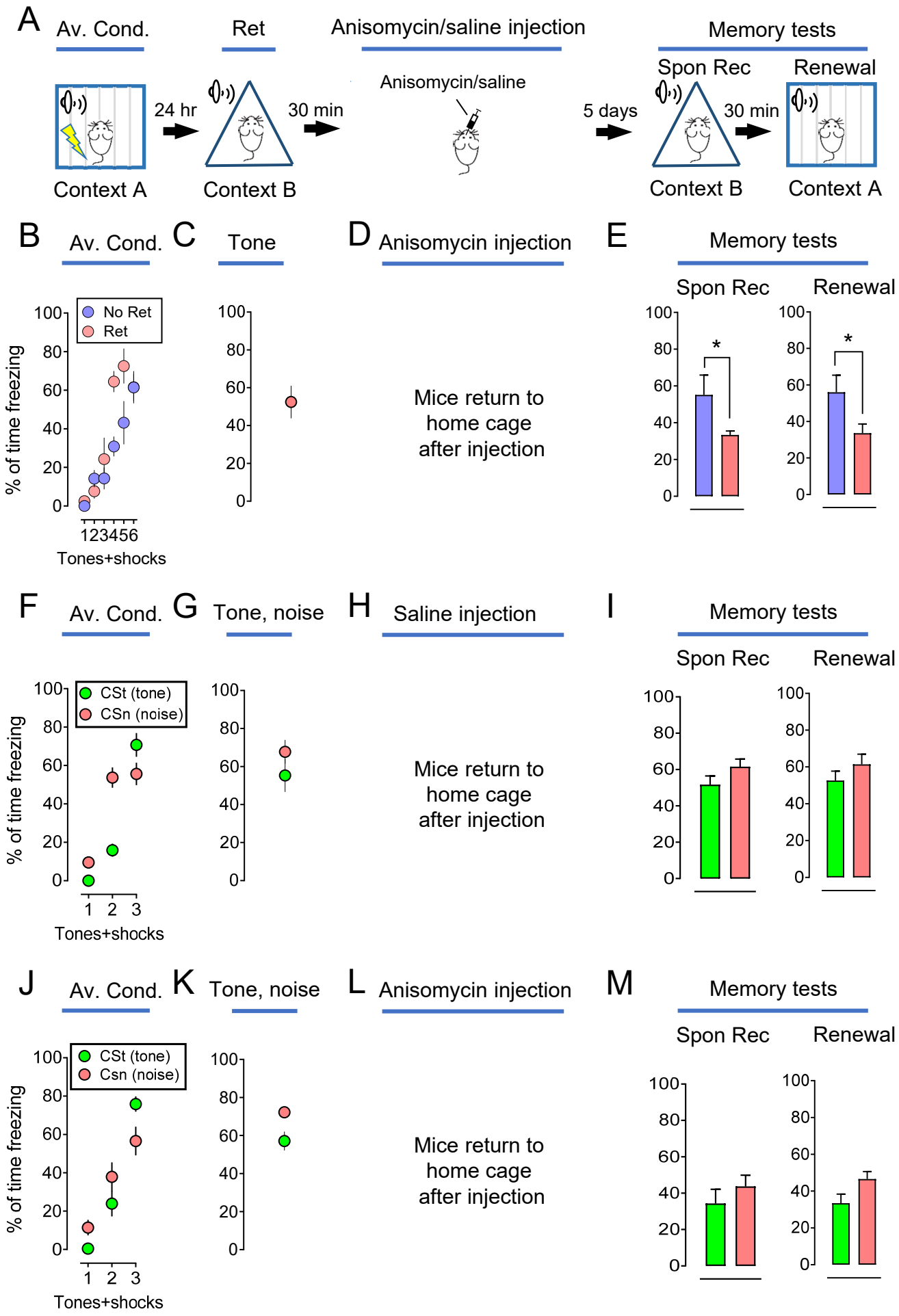

A
Av. Cond.
Ret
Anisomycin/saline injection
Memory tests
Spon Rec
Renewal
24 hr
30 min
Anisomycin/saline
5 days
30 min
Context A
Context B
Context B
Context A
B
Av. Cond.
C
Tone
D
Anisomycin injection
E
Memory tests
Spon Rec
Renewal
No Ret
Ret
% of time freezing
Tones+shocks
Mice return to home cage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

Fig. s4

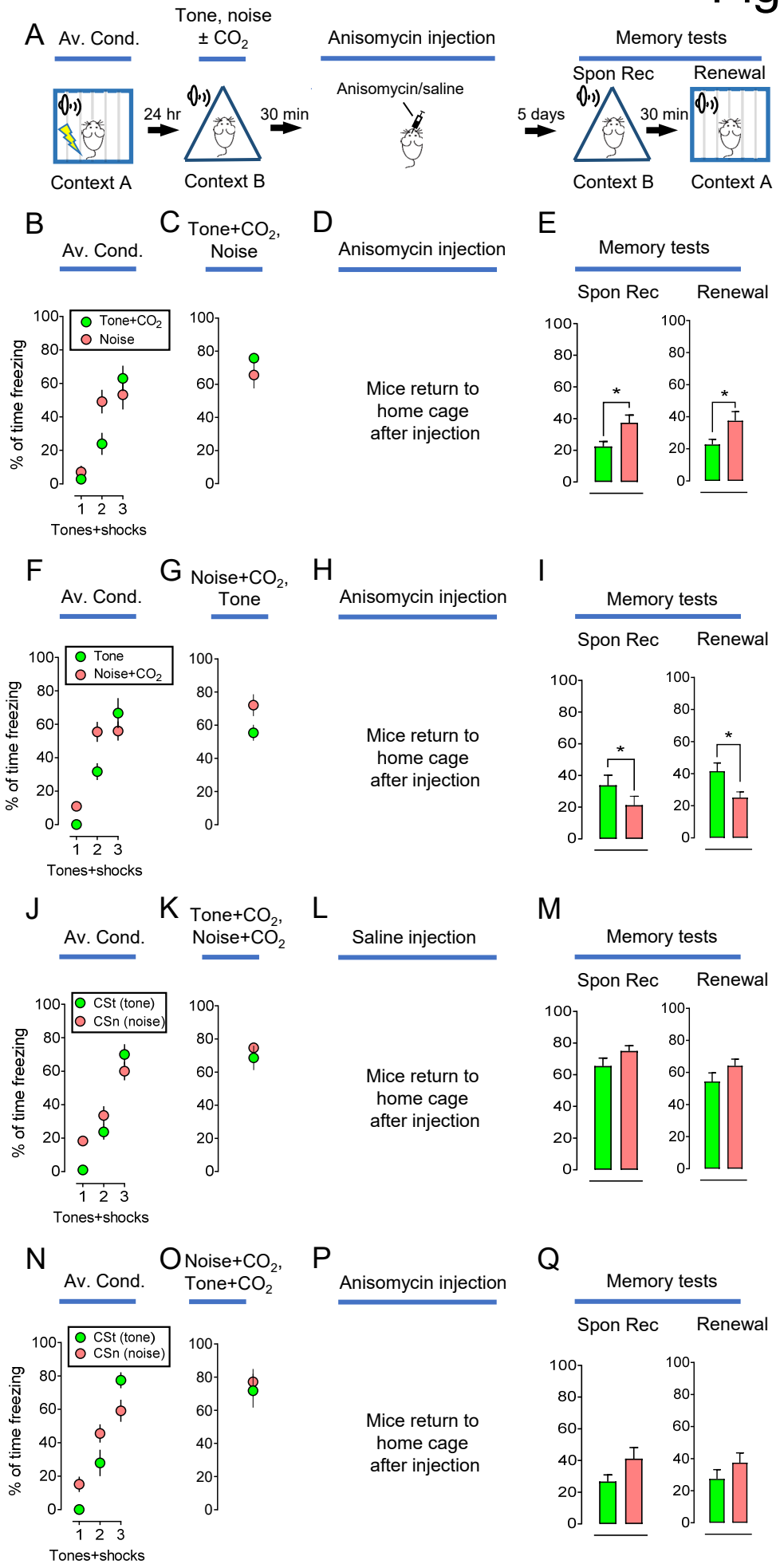
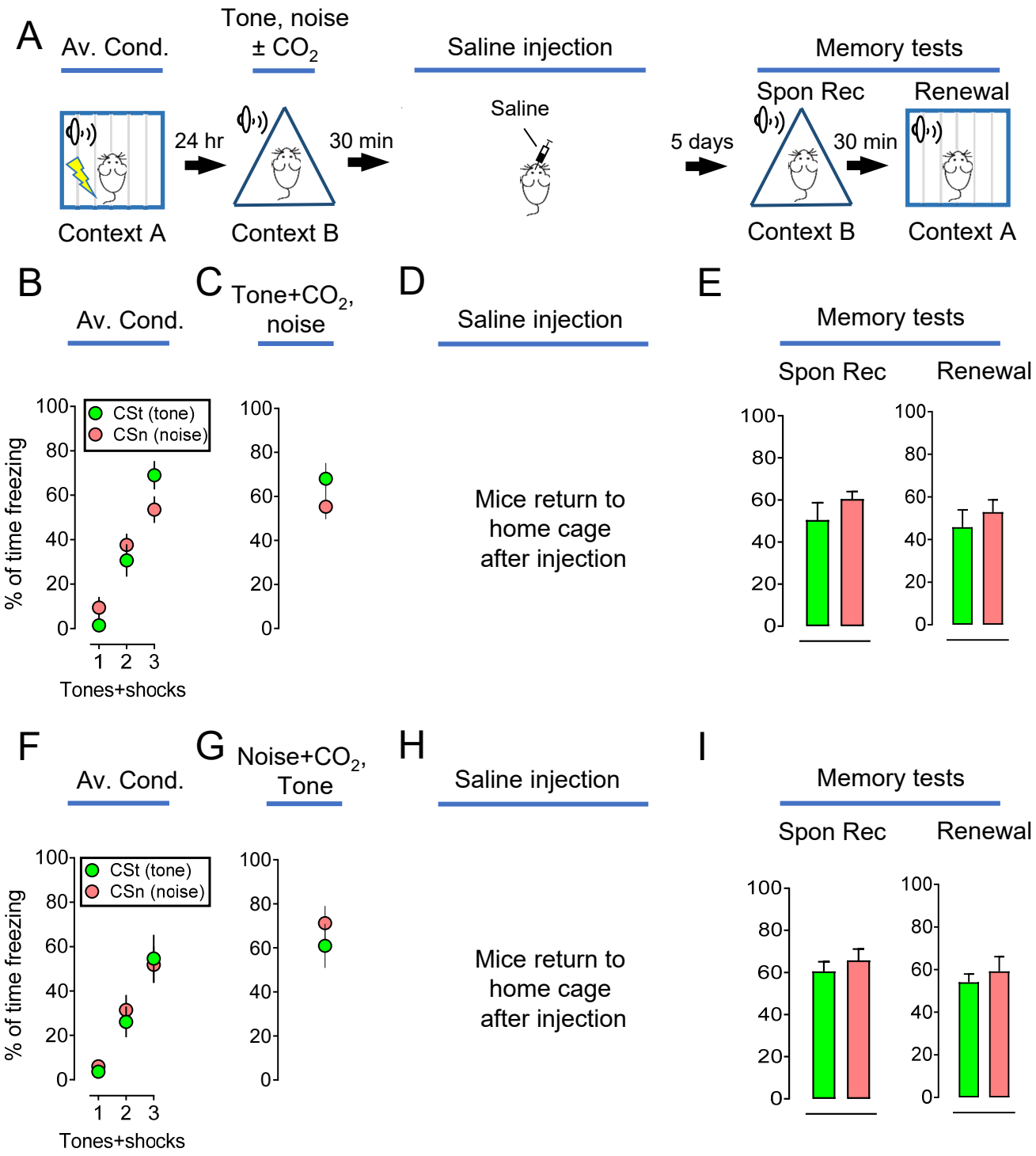

A
Av. Cond.
Tone, noise
± CO2
Anisomycin injection
Memory tests
Spon Rec
Renewal
24 hr
30 min
Anisomycin/saline
5 days
30 min
Context A
Context B
Context B
Context A
B
Av. Cond.
C
Tone+CO2,
Noise
D
Anisomycin injection
E
Memory tests
Spon Rec
Renewal
Tone+CO2
Noise
% of time freezing
Tones+shocks
Mice 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
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

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
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