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4 **Patterns of Arc mRNA expression in the rat brain following dual recall of fear- and**
5 **reward-based socially acquired information**
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21 # Abstract

22

23 The ability to learn new information and behaviors is a vital component of survival in most
24 animal species. This learning can occur via direct experience or through observation of another
25 individual (i.e., social learning). While research focused on understanding the neural mechanisms
26 of direct learning is prevalent, less work has aimed at understanding the brain circuitry mediating
27 the acquisition and recall of socially acquired information. We aimed to further elucidate the
28 mechanisms underlying recall of socially acquired information by having rats sequentially recall a
29 socially transmitted food preference (STFP) and a fear association via fear conditioning by-proxy
30 (FCbP). Brain tissue was processed for mRNA expression of the immediate early gene (IEG) *Arc,*
31 which reliably expresses in the cell nucleus following transcription before migrating to the
32 cytoplasm over the next 25 minutes. Given this timeframe, we were able to identify whether *Arc*
33 transcription was triggered by STFP recall, FCbP recall, or following recall of both memories.
34 Surprisingly – and contrary to past research examining expression of other IEGs following STFP
35 or FCbP recall separately – we found no differences in any of the *Arc* expression measures across
36 a number of prefrontal regions and the vCA3 of the hippocampus between controls,
37 demonstrators, and observers, though we did detect an overall effect of sex in a number of
38 regions. We theorize that these results may indicate that relatively little *Arc*-dependent neural
39 restructuring is taking place in the prefrontal cortices following recall of a recently socially acquired
40 information or directly acquired fear associations in these areas.

41

42 *Key Words:* Arc mRNA, social transmission of food preference, fear conditioning by-proxy, social
43 learning, memory recall

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44
Introduction

45
46 An animal's capacity to survive in a new environment is largely contingent on their ability
47 to learn about and adapt to their surroundings by identifying both potential threats and sources
48 for fulfilling essential needs. Humans, perhaps more than any other species, are particularly adept
49 at acquiring new strategies to deal with environmental challenges or exploit avenues for securing
50 resources. One of the primary ways in which we are able to learn such strategies at an individual
51 level is through receiving instructions or observing an experienced individual, i.e., via social
52 learning. As such, it should not be surprising that deficits in the ability to socially learn have the
53 potential to significantly impair functioning. This can be seen in autism spectrum disorders, in
54 which much of the symptomology is thought to arise from impairments in the social
55 attention/reward systems and, by extension, the social learning system [1–3].
56 Conversely, there are also drawbacks if social learning occurs too indiscriminately. While
57 valuable information and adaptive behaviors can be acquired socially, this does not preclude
58 individuals from socially acquiring false information or maladaptive behaviors through the same
59 pathway. Clinically, this is often seen in phobias, which are commonly reported to have been
60 acquired through observation or instruction (e.g., watching a parent react with extreme fear to a
61 spider or receiving dire warnings about the danger of spiders, respectively) rather than by direct
62 experience [4,5]. Socially acquired phobias may also be disruptive in ways directly acquired
63 phobias are not, because the individual has not directly experienced the aversive consequences
64 in relation to the feared stimuli. As such, they are free to imagine an associated outcome that may
65 be more intense than what occurs in reality. In line with this idea, individuals with socially acquired
66 phobias report increased cognitive symptomology [6] and respond more favorably to certain
67 treatment methods [4] than do individuals with directly acquired phobias.
68 To truly understand and subsequently develop optimal treatments for conditions arising
69 from under- or over-performing social learning, a thorough understanding of the brain

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70 mechanisms that underlie the social learning process is an essential first step. One of the primary
71 methods we have for exploring such mechanisms are non-human animal models. In rodent
72 species, fear-based social learning has been demonstrated to occur under multiple conditions,
73 including: (1) context or stimulus associated fear acquired by observation through a barrier of a
74 conspecific experiencing pain in a novel environment or following the presentation of a novel
75 stimulus [7,8], (2) enhanced acquisition of natural behaviors by observation of a conspecific
76 responding to a threatening stimuli [9–11], and (3) by observation of a fear conditioned
77 demonstrator reacting to the fear-associated stimuli post-conditioning in a paradigm known as
78 fear conditioning by-proxy (FCbP) [12–14].

79 While similar reward-based models of social learning in rodents have proven somewhat
80 more difficult to develop [15], one reliable and well-established model of reward-based socially
81 mediated learning does exist in the social transmission of food preference (STFP) paradigm [16–
82 19]. In the STFP paradigm, rats assigned to the 'demonstrator' condition consume a novel food
83 (generally powdered chow mixed with flavoring, such as cinnamon) before interacting with a naïve
84 rat assigned to the 'observer' condition. When observers are later given the choice to consume
85 either the demonstrated flavor or an entirely novel flavor, they reliably show the tendency to
86 consume more of the demonstrated flavor. This effect has been shown to be mediated by the
87 semiochemical carbon disulfide (CS_2) which is present in the nasal cavity of rats and, when paired
88 with a novel scent, is sufficient to induce a preference for similarly scented foods [17].

89 In rodents, there has been a fair amount of research examining the brain mechanisms
90 mediating the acquisition and recall processes for the social transmission of food preference task
91 [20–24] and, to a lesser extent, socially acquired fears [7,12,13,25–27]. Results from research
92 into the latter topic have also found that there are a number of brain areas that seem to be uniquely
93 activated during social fear learning and not direct fear learning [13,27]. Furthermore, integrative
94 models considering the results from both human and non-human animal research into the brain
95 circuitry underlying the social learning of appetitively and aversively motivated

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96 behaviors/associations posit that, while there does seem to be considerable overlap between the
97 brain areas governing direct learning processes and social learning processes, activity in some
98 unique brain regions is required for social learning to occur [13,28].

99 While the neural mechanisms involved in the social acquisition of tasks and information
100 has received some exploration, research explicitly comparing the storage of memories acquired
101 by social learning to memories acquired by direct learning is, to our knowledge, almost
102 nonexistent. In the experiment described here, we attempted to examine activation in various
103 brain regions following recall of a socially acquired memory from both a reward- and fear-based
104 task. Rats were trained in a reward-based form of social learning, STFP, and a fear-based model
105 of social learning, FCbP, after which we initiated sequential recall of both memories. The tissue
106 from these rats was then processed for mRNA expression of the immediate-early gene (IEG) – a
107 class of genes which are rapidly transcribed following neuronal firing or other cellular stimuli - *Arc*
108 which, when transcribed, produces the mRNA for the activity-regulated cytoskeleton associated
109 (Arc) protein. *Arc* mRNA has a predictable pattern of expression such that in the first 5 minutes
110 following transcription it is expressed in the nucleus of the cell and, after about 25 minutes,
111 migrates to the cytoplasm surrounding the nucleus [29]. As such, cells stained for *Arc* mRNA that
112 are activated at both timepoints show expression in both the cytoplasm and nucleus, allowing for
113 precise localization of cell populations activated in multiple tasks. By analyzing the expression of
114 *Arc* mRNA in rat brains perfused following the sequential recall of FCbP and STFP tasks, we
115 aimed to identify brain regions uniquely involved in retrieval of socially acquired information. The
116 anterior cingulate cortex [7,13,28], orbitofrontal cortices [23,24], and infralimbic and prelimbic
117 cortices [23,30–32] were all of particular interest given past research which has implicated them
118 in fear learning, social fear learning, STFP learning, or some combination of the three.
119

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120 # Methods

121 ## Subjects

122

123 Subjects were male and female Sprague-Dawley rats bred in house in the Animal
124 Resource Center of the University of Texas at Austin. Eight breeding pairs were used to produce
125 the subjects for Cohort 1 of this experiment - with seven successfully breeding - while eight
126 separate breeding pairs were used to produce the subjects for Cohort 2. Female breeding animals
127 were Sprague-Dawley rats (between 215-260g at arrival) obtained from Charles-Rivers
128 (Wilmington, MA, USA) while male breeding animals were Sprague-Dawley rats (most between
129 275-300g at arrival with one at 230g) obtained from Harlan (now Envigo) (Houston, TX, USA) to
130 prevent accidental inbreeding. All rats were paired off with an opposite-sex cage mate following
131 arrival to the colony. Once a female began to show clear signs of pregnancy, her paired male was
132 removed from the cage and rehoused.

133 Once delivered, pups were weaned into triads of same-sex siblings at post-natal day 21
134 (P21) to help ensure social fear learning [26]. Spare pups were weaned into triads or dyads with
135 unrelated rats and used in other experiments at the University of Texas at Austin. Female pups
136 from our second cohort litter were used in other experiments. The final number of pups used for
137 this experiment were n = 27 for Cohort 1 females, n = 36 Cohort 1 males, and n = 27 Cohort 2
138 males. Pups being utilized in this experiment were allowed to mature with minimal disturbances
139 aside from routine animal husbandry procedures (e.g., cage changes) until habituation
140 procedures (Females triads) or dominance assessment procedures (Male triads) began. All
141 Cohort 1 rats started on habituation procedures between P106-P112 days of age (young
142 adulthood) and all Cohort 2 rats were started between P99-P118 days of age. All subjects were
143 kept on a 3 pm – 3 am lights off light-cycle and all experimental procedures were completed during
144 the subjects' dark cycle. All parts of this experiment were conducted in compliance with the

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145 National Institutes of Health Guide for the Care and Use of Experimental Animals and were
146 approved for use by The University of Texas at Austin Animal Care and Use Committee.
147

148 Apparatus and Stimuli

149 Fear Conditioning

150
151 All fear conditioning and fear conditioning by-proxy procedures were completed in
152 standard conditioning chambers (30.48 cm x 25.4 cm x 30.48 cm) constructed of clear plexiglass
153 walls in the front and back, two steel walls on the side, and a plexiglass ceiling with a hole in the
154 center. The flooring of the chamber was a row of stainless-steel rods connected to a shock
155 generator (Coulbourn Instruments, Allentown, PA). All chambers were enclosed in acoustic
156 isolation boxes (Coulbourn Instruments) and lit with an internal red light. Behavior was recorded
157 by closed-circuit cameras (Panasonic™ WV-BP334) mounted above the conditioning chambers
158 with the lens inserted through the hole in the plexiglass ceiling. Chambers were fully wiped down
159 with 70% alcohol solution between each subject. All stimulus delivery was controlled using the
160 Freeze Frame software (Coulbourn Instruments). The conditioned stimulus (CS) was a 20 second
161 tone (5kHz, 80 dB) and, in procedures with multiple CS presentations, a variable inter-trial interval
162 (ITI) averaging 180 seconds. The unconditioned stimulus (US) was a 1 mA shock that was 500
163 milliseconds in duration and co-terminated with the conditioned stimulus.

164 Social transmission of food preference

165
166 All STFP procedures took place in a room adjacent to the room containing the conditioning
167 chambers. Novel diets were composed by mixing 100g of powdered 5LL2 Purina rodent chow
168 with either 1g of McCormick ground cinnamon (diet Cin) or 2g of Hershey cocoa powder (diet Co).
169 The Plain diet, which was given to all rats during the food restriction period and to Control rats on

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170 the terminal day of experimental procedures, was unadulterated powdered 5LL2 Purina rodent
171 chow. All powdered chows – both during food restriction and experimental procedures – were
172 presented in hanging food cups that were constructed from 4 oz. glass jars and 12-gauge steel
173 utility wire. Food cups were rinsed then wiped down with a 70% ethanol solution before being
174 washed thoroughly with soap and water between every use. All consummatory phases of the
175 STFP experimental procedures took place in standard rat cages (26.7 cm x 48.3 cm x 20.3 cm),
176 with every animal receiving a fresh cage. The interaction phase (STFP acquisition phase) took
177 place in a large plastic bin (50.5 cm × 39.4 cm × 37.5 cm) with wood chip floor bedding that was
178 replaced between every group. Plastic bins were wiped down thoroughly with Windex between
179 each session.

180 Overview of Experimental Design & Social Learning 181 Procedures

182
183 (See Fig 1 for a graphical overview)

184 All rats were food restricted for five days and habituated to handling and the room where
185 STFP procedures would take place for four days immediately prior to day 1 of experimental
186 procedures. While habituation procedures ended prior to day 1 of experimental procedures, food
187 restriction continued through to the end of the experiment. One animal from each triad of rats was
188 assigned to one of three conditions: Demonstrator, Observer, or Control. Cohort 2 male triads
189 had been assessed for dominance and all showed a clear hierarchy and were assigned such that
190 the dominant rat was the Demonstrator and a subordinate was the Observer to enhance social
191 transmission of fear [13]. Individual triads were further randomly subdivided into groups where
192 the Demonstrator and Observer would receive a choice test at STFP recall (Choice) and groups
193 where they would receive only the demonstrated food (Cin).

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194 On day 1 of the experimental procedure, rats assigned to the Demonstrator condition were
195 moved to conditioning chambers and allowed to habituate for 10 minutes before they were

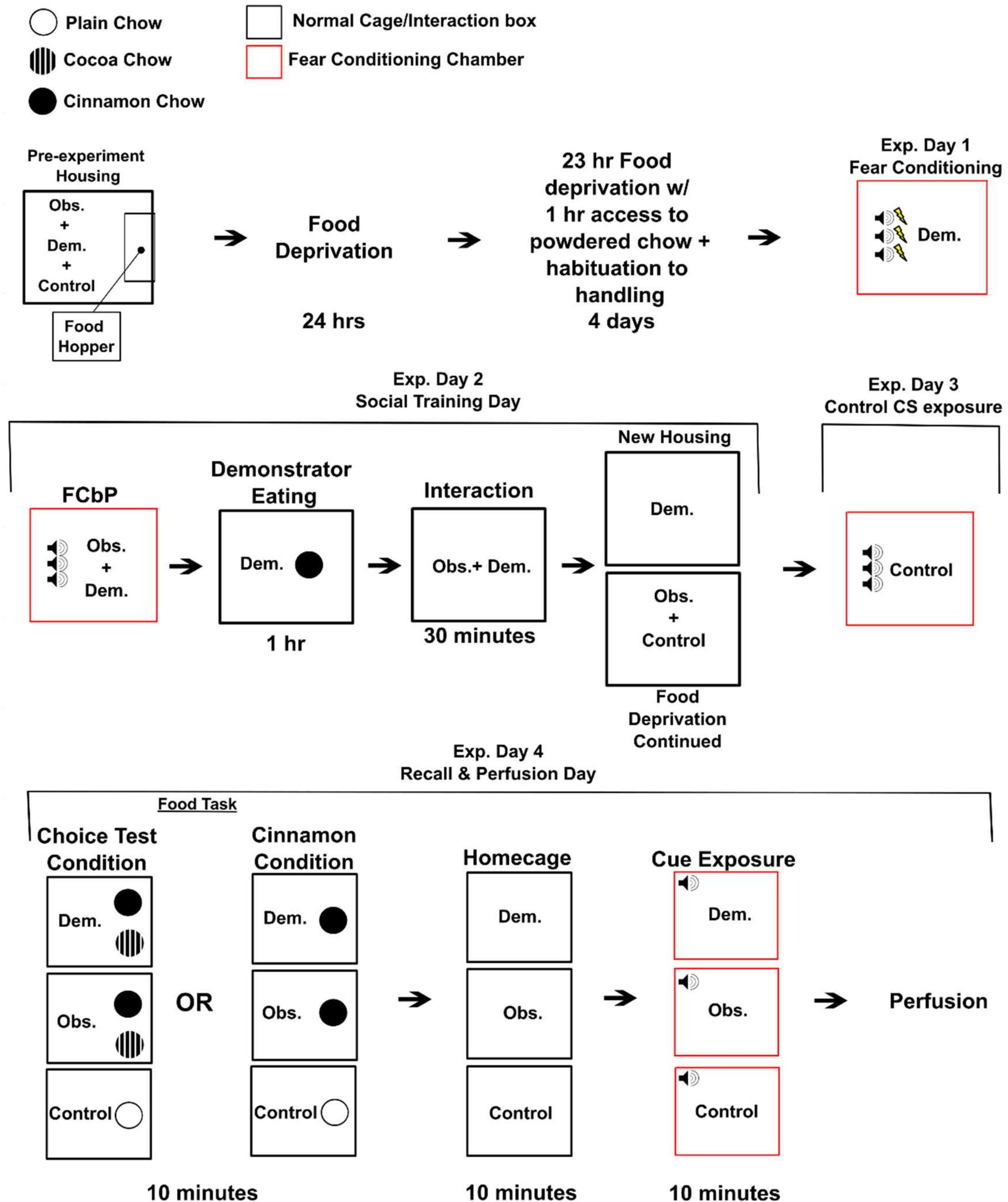

Fig 1. Overview of Experiment Design. This figure outlines the treatment of rats on each day of the experiment from the first day of food restriction on.

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196 exposed to 3 CSs that co-terminated with a painful shock (see **Apparatus and Stimuli** for
197 specifics). Following fear conditioning procedures, Demonstrators were moved back to their
198 original home cage. On day 2 of experimental procedures, 24 hours after fear conditioning,
199 Demonstrators were returned to the conditioning chambers with their cage-mate assigned to the
200 Observer condition and put through the FCbP procedure. Immediately following the FCbP
201 procedure, Observer rats were returned to their home-cage while Demonstrators were moved to
202 an adjacent room and given 1 hour to consume powdered chow flavored with cinnamon. After an
203 hour had passed, Observers were moved to an interaction bin with their Demonstrator and
204 allowed to interact with them for 30 minutes to allow for acquisition of a socially transmitted food
205 preference. Previous research from our lab has validated these timepoints as being sufficient for
206 STFP transmission [33]. Afterwards, Observer rats were returned to their home-cage while
207 Demonstrator rats were moved to single housing to prevent further STFP transmission to the
208 Observer or Control. On day 3 of experimental procedures, Control rats were moved to
209 conditioning chambers alone and, following 10 minutes of habituation to the chamber, were
210 presented with three 20 second CSs with no accompanying shock. This was done on a separate
211 day to minimize the possibility of lingering alarm pheromones – which are known to be released
212 by rats in response to threatening stimuli and effect conspecific learning [34] – still being present
213 in the chamber.

214 On the terminal day of the experiment, day 4, recall was initiated for both the socially
215 transmitted food preference and the fear conditioning/fear conditioning by-proxy memories. All
216 Observers and Demonstrators from triads assigned to the Choice condition were allowed 10
217 minutes *ad libitum* access to both cinnamon and cocoa flavored diets, while Observers and
218 Demonstrators from triads assigned to the Cin condition were given 10 minutes *ad libitum* access
219 to the cinnamon diet only. In all triads, Control rats were given 10 minutes *ad libitum* access to
220 plain powdered chow. Immediately after this, rats were returned to their home-cage and left
221 undisturbed for a 10-minute period before being moved back to the lab space and being placed

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222 in the conditioning chambers. All rats were then given a 3-minute habituation period to the
223 chamber before being presented with a single 20 second CS. 5 minutes after the end of the CS,
224 all rats were euthanized via injection of a pentobarbital and phenytoin solution (Euthasol; Virbac
225 Animal Health) and perfused. Their brains were later processed for *Arc* mRNA expression. Given
226 the time course of our terminal procedure and the known migration timeframe of *Arc* mRNA [28],
227 increases cytoplasmic expression of *Arc* mRNA would be due to STFP recall procedures, while
228 nuclear expression would be due to FC/FCbP recall procedures, with cells showing dual activation
229 having been activated at both timeframes (see Fig 2; also, see **Tissue Analysis** for details on
230 tissue treatment and processing).

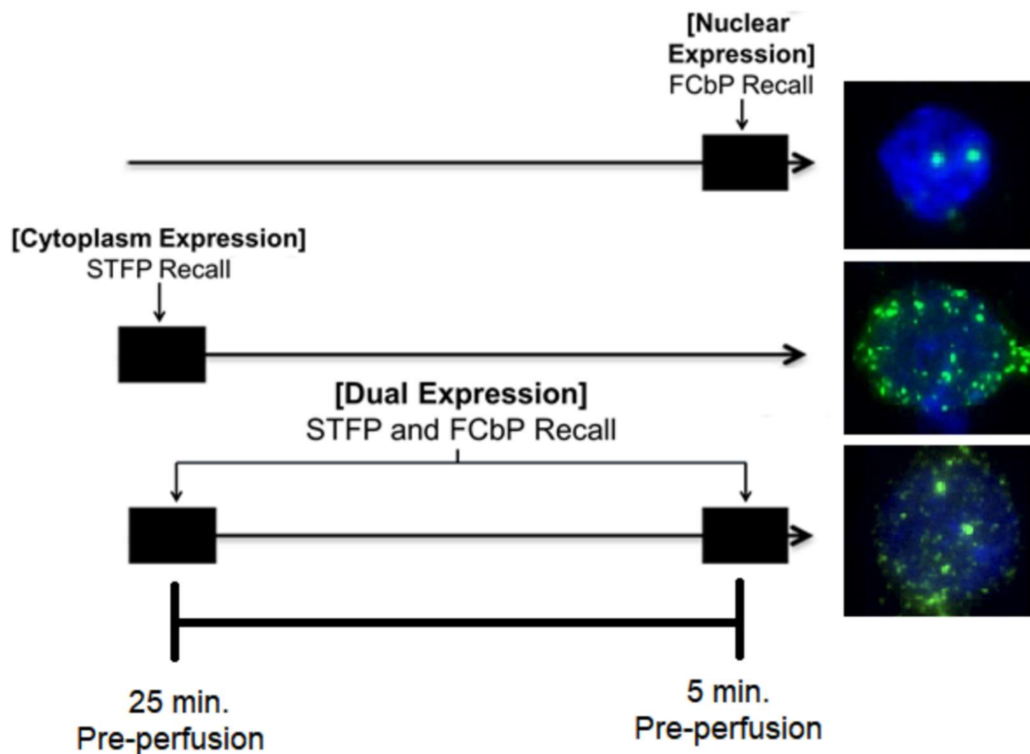

Fig 2. Patterns of *Arc* mRNA Expression. The above figure shows the pattern and area within a cell in which we would see *Arc* mRNA expression triggered by activity at the FCbP recall timepoint, the STFP recall timepoint, or activity that was triggered at both timepoints.

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231 # Procedures

232 ## Habituation and food restriction

233
234 All habituation took place just prior to the first day of experimental procedures. Habituation
235 consisted of each cage of rats being moved into the room in which all STFP experimental
236 procedures would take place and being allowed to habituate to the room for 15 minutes. During
237 this period, each rat was picked up and handled by the experimenter that would be running
238 behavior for 2 minutes to habituate them to handling and that individual. All habituation procedure
239 took place in a dark room under red light, and all rats received 4 days of habituation. Food
240 restriction began the day before habituation began and persisted to the end of the experiment. At
241 the start of food restriction, the food pellets that all subjects had been eating were removed from
242 the cage. Subsequently, all cages were given daily *ad libitum* access to a hanging jar full of plain,
243 powdered Purina 5LL2 diet in their home-cage. Rats were weighed daily starting at the beginning
244 of food restriction until the experiment was over to ensure no unusual loss in weight.

245 ## Play behavior dominance assessment

246
247 A day prior to play dominance assessments, all males were moved to single housing to
248 promote social play behavior. Following a 24-hour isolation period, individuals from each triad
249 were moved to a large plastic bin (50.5 cm × 39.4 cm × 37.5 cm) with woodchip bedding and a
250 camera mounted overhead to record behavior. Rats were allowed to interact for 15 minutes before
251 being removed from the box and returned to single housing. This was repeated for 3 sessions,
252 after which rats were returned to their triads and left undisturbed until the start of the milk
253 competition dominance assessment. Behavior was scored as described below, and rats in Cohort
254 2 were assigned to one of three dominance ranks based on their behavior as following with past
255 research on dominance hierarchies in rats [35]: Dominant, Subordinate 1, or Subordinate 2. Male
256 rats in cohort 1 were randomly assigned condition regardless of dominance rank. As described in

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257 Jones & Monfils [13], dominant rats were the rats that received most nape contact (i.e., play
258 initiations), while subordinate 1 was the rat that initiated the dominant the most, and subordinate
259 2 tended to be avoidant. While all Cohort 1 males were used, male triads in Cohort 2 that did not
260 show dominance hierarchies were removed from the study and used in other experiments.
261 Analysis of play behavior dominance included only Cohort 2 rats.

262 Milk competition dominance assessment

263
264 In order to validate dominance assignments made using the play behavior assessment,
265 we recorded and scored the behavior of male rats allowed access to a desired resource
266 (sweetened milk solution), a dominance assessment that our lab previously found to be effective
267 [13]. The milk solution used in this dominance assessment was a mixture of 2/3 tap water and 1/3
268 sweetened condensed milk (Eagle™) stored in a 2 oz glass jar filled to the top with the solution.
269 Prior to running the dominance assessment, rats from all male triads were moved to single-
270 housing and given access to a full jar of the milk for 5-hours to ensure that each individual rat had
271 the opportunity to overcome their neophobia of the milk solution. Following this, rats were returned
272 to their triads and allowed access to a full jar of the milk solution as a group daily for four days. In
273 order to assure that rats would be motivated to drink, food hoppers were removed from all triads
274 12 hours before the milk was introduced. Following the 3-hour milk access period, hoppers were
275 returned until removal time for the next day of habituation.

276 Once habituation to the milk solution had been completed, triads were run through the
277 formal dominance assessment. As during habituation, food hoppers were removed 12 hours
278 before the start of assessment to promote competition. 2 oz glasses were filled with to the top
279 with the milk solution and secured with adhesive strips to the bottom of a large plastic bin (50.5
280 cm × 39.4 cm × 37.5 cm) with woodchip bedding and portable cameras were mounted above the
281 box for an over-the-head view of all behavior. Rats were placed in the bin and allowed access for
282 either 12 minutes (Cohort 1) or 10 minutes (Cohort 2) before being removed and returned to their

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283 triads. While only two sessions of the competition were run for our Cohort 1 males, three sessions
284 were run for Cohort 2 in an attempt to obtain clearer dominance hierarchies.

285 Behavioral Scoring

286
287 All behavioral scoring for this experiment was completed using the Behavioral Observation
288 Research Interactive Software (BORIS) [36].

289 Play dominance scoring

290
291 Behavior was scored for the full play session, with both offensive play behaviors (i.e., play
292 initiations or attacks) and defensive play behaviors (i.e., response to play initiations, specifically
293 nape contact) being scored (see [13,35,37]). The following offensive play behaviors were scored:
294 (1) Nape contact, contact of a rat's snout with the nape of another rat and (2) Boxing, which
295 occurred when rats reared and punched at each other with their front legs. The defensive
296 behaviors scored for were: (1) Counter, in which the attacked rat turns to face the attacking animal
297 to launch an attack of their own; (2) Evasion, in which the attacked rat flees from the attacker; (3)
298 Full rotation, in which the target rotates fully into a supine position; (4) Half rotation, in which the
299 targeted animal responds to the attack by shifting their body laterally to break contact without fully
300 losing their feet; (5) No response, in which the target either freezes or carries on at a normal pace
301 in response to attack. The identity of both the initiating rat and their target was noted for every
302 instance of play behavior. Across all sessions, the total nape contacts received for each individual
303 rat was tallied and divided by the total number of nape contact initiated in the cage to determine
304 the percent of contacts each rat had received. If a rat had received a disproportionate amount of
305 contact (>40%) they were deemed the dominant animal.

306 Milk competition scoring

307

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308 Behavior for milk competition began to be scored as soon as all rats were in the bin and
309 the experimenter had exited the footage. Behavior was scored in 1-minute bins for 10-12 minutes.
310 The duration of each subject drinking from or monopolizing the milk jar (i.e., drinking from or
311 having paws/body on the jar and preventing the other rats' access) was scored for each 1-minute
312 interval. To calculate percent monopolization of the resource, the total time all rats spent drinking
313 in each bin was summed and the time spent drinking for individual rats was divided by that value.
314 The amount of time spent drinking was then plotted based on play behavior dominance
315 assignments for all rats.

316 Fear conditioning by-proxy social contact scoring

317
318 Past research from our lab has indicated that there is a strong relationship between the
319 amount of fear displayed by observers at the long-term memory test and the time spent interacting
320 with their Demonstrator during the CS in males [13] and after the CS in females [14,26]. As such,
321 videos of the social acquisition phase of fear-conditioning by proxy were scored for social
322 interaction between the Observer and Demonstrator for each 20 second period during the CS
323 presentation and the 20 second period immediately following each CS presentation to provide a
324 secondary index of fear acquisition. Social contact was scored when Observer and Demonstrator
325 animals made contact other than in passing during the cue period (during CS contact) or in the
326 20 seconds following the CS (post CS contact). The percentage of each score period spent in
327 contact with the Demonstrator was calculated. Data for percent contact during the cue period for
328 males and data for the percent contact immediately following the cue period for females was
329 pulled and combined into a single "relevant contact" measure to be used in all final statistical
330 analyses.

331 Choice test scoring

332

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333 Videos of the choice test to initiate recall of a socially transmitted food preference were
334 scored for the amount of time that a given rat spent interacting with a food cup based on whether
335 it contained the demonstrated/already consumed diet (diet Cin) or the novel diet (diet Co). This
336 was done as a potential secondary measure of food preference, as we anticipated that due to the
337 choice test being abnormally short (10 minutes) by necessity that we might be unable to detect
338 preferences based on amount eaten alone. Interaction with the food cup was scored for whenever
339 a rat was physically in contact with and not actively moving away from the cup (i.e., front paws in
340 contact with the jar, head inside jar, climbing on top of the jar, or actively eating from the jar). For
341 statistical analysis, we calculated the percent of time spent interacting with a cup containing a
342 given diet based on the total amount of time spent interacting with either cup (e.g., for diet Cin,
343 Percent time = $Time_{Diet\ Cin}/(Time_{Diet\ Co} + Time_{Diet\ Cin})$). The full 10-minute choice test session was
344 scored for all rats that underwent the choice test with the exception of one rat whose video was
345 unavailable due to recording equipment failure.

346 Tissue analysis

347
348 To minimize degradation of mRNA by ribonuclease (RNase), all equipment and surfaces used
349 during brain preparation and processing were sanitized regularly with either RNase AWAY™
350 (Thermo Scientific; Waltham, MA, USA) or RNAseZap™ (Ambion; Grand Island, NY, USA).

351 Brain Preparation

352
353 Immediately following euthanasia, subjects were perfused intracardially using a 4%
354 paraformaldehyde (PFA) solution. Brains were then removed and submerged in the 4% PFA
355 solution to allow post-fixation for 24-48 hours. Once post-fixation was complete, brains were
356 transferred to a solution of 30% sucrose in phosphate buffered saline for cryoprotection. Once
357 brains had sunk to the bottom of the vial, indicating sufficient sucrose uptake for cryoprotection,

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358 they were flash frozen in powdered dry ice and moved to a -80ºC freezer for storage until
359 sectioning. Brains were then sectioned coronally on a sliding microtome at 30 µm thickness into
360 six series (so subsequent sections in a single series were 180 µm apart) and immediately
361 mounted and allowed to air dry before being placed in a vacuum chamber with humidity sponges
362 where they were left to dry fully for 24 hours. Only hippocampal sections (approximately -3.2 to -
363 5.2 from bregma) or prefrontal regions (approximately +3.7 to +1.4 from bregma) containing the
364 areas of interest were sectioned and processed. Mounted sections were then placed in a sealed
365 slide box and stored in a -80ºC freezer until processing.

366 ## Tissue processing

367

368 All procedures were modified from the protocols used in Lee et al. [38] and Petrovich et
369 al. [39]. Prior to tissue processing, a cRNA probe for *Arc* mRNA was constructed starting with a
370 plasmid containing a full-length cDNA (~3.0 kbp) of the *Arc* transcript. To create the probe, the
371 DNA was first cut by mixing the plasmid with a 10x digestion buffer (NEBuffer; Biolabs; Ipswich,
372 MA, USA), a 10x EcoRI restriction enzyme (Biolabs), and purified nuclease free water (Ambion)
373 before being incubated at 37ºC for 2 hours. Proper cutting of the DNA was verified using
374 electrophoresis, after which the DNA was purified overnight in ethanol. Following purification, the
375 DNA pellet was spun out in a centrifuge, washed in EtOH, fully dried, and resuspended in a TE
376 buffer. To verify that the DNA was properly linearized, calculate *Arc* concentration, and check that
377 no contaminants were present, a sample of the DNA was tested via spectrophotometry (Nanodrop
378 Lite; Thermo Scientific, Waltham, MA, USA). The Digoxigenin (DIG) labelled probe was
379 transcribed by combining the linearized DNA with RNase free water (Ambion), a 10x transcription
380 buffer (Ambion), RNAse block (Ambion), DIG RNA labelling mix (Roche Applied Science;
381 Indianapolis, IN, USA), and a T7 RNA polymerase (Ambion) before incubating the solution at
382 37ºC for 2 hours. Finally, the probe was diluted in nuclease free water and purified in a mini Quick-
383 Spin column (Roche).

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384 Once the cRNA probe had been constructed, slides containing tissue from the male rats
385 were submerged for 40 minutes in a 4% PFA solution to increase tissue integrity throughout *in*
386 *situ* processing. Tissue from female rats were processed without this PFA wash. Slides were then
387 washed and incubated in a proteinase K (PK) buffer at 37ºC before being treated with a 0.5%
388 acetic anhydride/1.5% triethanolamine solution containing glacial acetic acid for permeabilization.
389 Slides were then washed in a saline-sodium citrate (SSC) buffer before being dehydrated by
390 submersion in ascending concentrations of ethanol and air dried. Finally, each slide was covered
391 in 300 µl of a hybridization buffer containing yeast tRNA (Invitrogen; Carlsbad, CA, USA), salmon
392 sperm DNA (Ambion), dithiothreitol (Sigma; St. Louis, MO, USA), and the cRNA probe. Each slide
393 was cover slipped and temporarily sealed using a DPX mountant (Electron Microscopy Sciences;
394 Hatfield, PA, USA) before being incubated in the hybridization solution for 20 hours at 60ºC.

395 Once hybridization was complete, cover slips were carefully removed, and slides were
396 incubated in a 4xSSC buffer mixed with sodium thiosulfate (ST) at 60ºC for an hour before being
397 treated with an ethylenediaminetetraacetic acid-based solution to inhibit RNAse activity at 37ºC.
398 Following this, slides were washed in descending concentration of SSC solution mixed with ST
399 again at 60ºC. Tissue was then washed in a detergent solution (Tween20; Sigma) before being
400 stained with the PerkinElmer TSA Fluorescein system (NEL701001KT; PerkinElmer, Waltham,
401 MA, USA). Slides were placed in a humid chamber and treated with blocking buffer followed by
402 an anti-DIG-HRP conjugate for 2 hours. Slides were then briefly washed in the detergent solution
403 before being returned to a dark humid chamber and coated with a solution containing fluroscein
404 tyramide reagent (FITC) and allowed to sit for 30 minutes. Finally, slides were washed, allowed
405 to air dry, and cover slipped with a mountant containing the nuclear stain 4',6-diamidino-2-
406 phenylinodole (DAPI) (Vectashield; Vector Lab, Burlingame, CA, USA). Slides were stored in the
407 dark at -20ºC until imaging.

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408 **Imaging**

409

410 All imaging was completed using an Axio Scope A1 microscope (Zeiss; Thornwood, NY,
411 USA). Regions of interest were identified via DAPI staining using a 10x objective with the
412 assistance of the Paxinos and Watson brain atlas [40] and then imaged under a 40x objective
413 (actual magnification ~900 X). Images were taken for both DAPI and FITC stains and later
414 colorized and merged automatically using a custom macro in the ImageJ software with FIJI (NIH,
415 Bethesda, MD). Due to tissue damage occurring over the course of *in situ* not all sections or areas
416 of potential interest were viable. As such, images were not able to be z-stacked reliably and,
417 instead, were taken on a single plane. The following regions were imaged and counted: the
418 prelimbic cortex (+3.72 to +2.52 from bregma), the infralimbic cortex (+3.52 to +2.2 from bregma),
419 the lateral (+3.72 to +3.2 from bregma) and ventral (+3.72 to +3.0 from bregma) orbitofrontal
420 cortex, the CG1 region of the anterior cingulate cortex (+3.72 to +2.52 from bregma), and the CA3
421 region of the ventral hippocampus (-4.3 to -4.8 from bregma) (See Fig 3). Though the amygdalar
422 nuclei were also of particular interest for their well-established role in fear learning, the
423 aforementioned tissue damage tended to be particularly severe in this area. As such, we were
424 not able to obtain a sample size large enough to include that region (a minimum of 6 viable
425 images/region was required for a rat to be included in the statistical analysis of a given area).

426 Counts were completed region by region and all image files were assigned a random
427 numerical code to blind the experimenter completing the counts from any details concerning the
428 image at the time of counting. All cell counts were taken in ImageJ with the FIJI package and
429 were made using the cell counting tool. Cells were counted for nuclear and cytoplasmic *Arc* mRNA
430 expression separately and cells showing overlapping expression were counted as dual
431 expressing. The final counts for nuclear *Arc* expressing and cytoplasmic *Arc* expressing cells
432 included only those cells expressing in only that region (i.e., did not include dual expressing cells).
433 Full counts for DAPI stained cells were taken and the percent of cells showing expression in each

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434

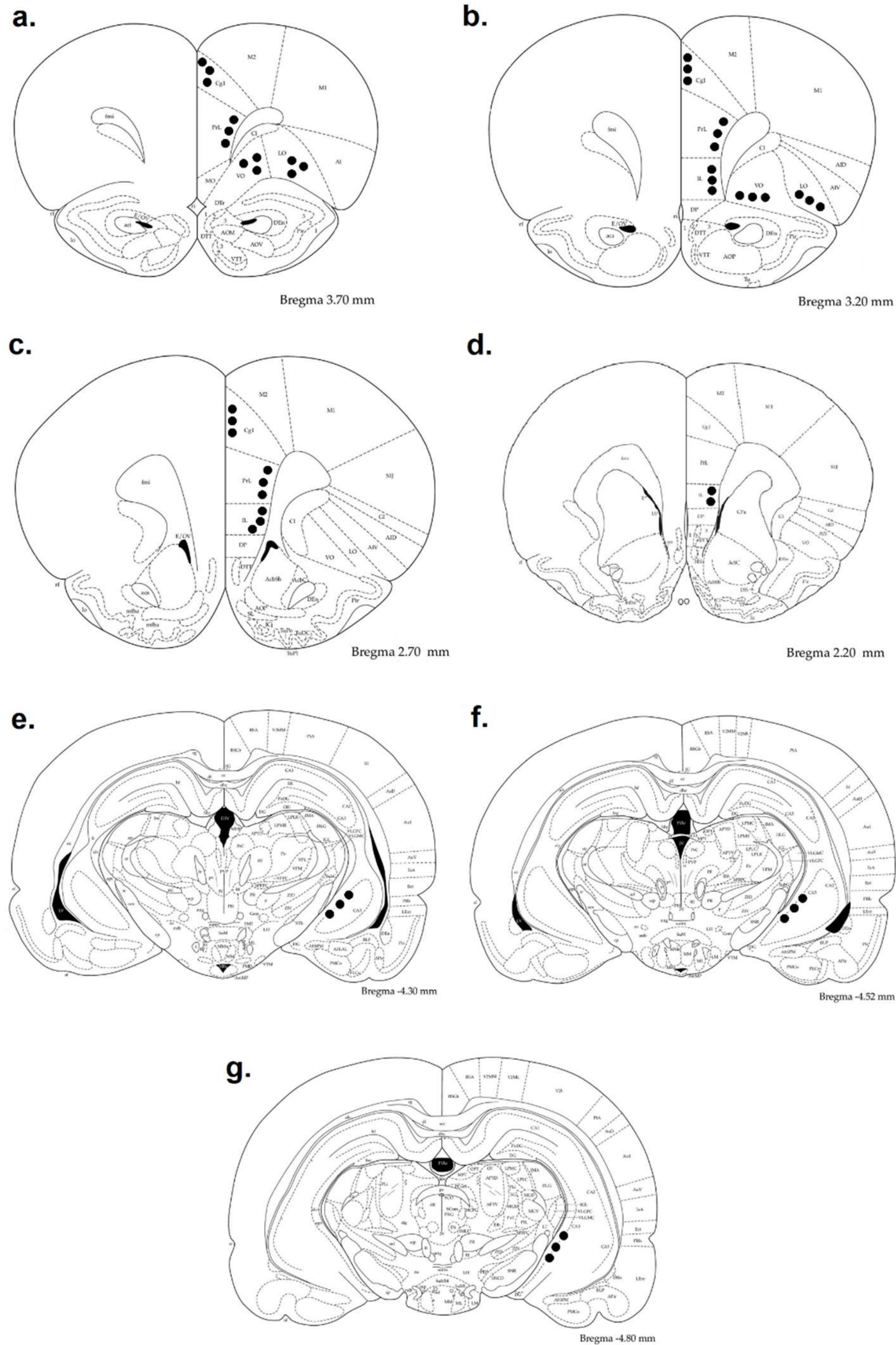

Fig 3. Representation of sampled areas. Images of coronal rat brain sections adapted from Paxinos and Watson (2006). The blacked-out circles indicate the approximate areas sampled from each plane for (a-d) the prelimbic, infralimbic, CG1 region of the anterior cingulate cortex, and the ventral and lateral orbitofrontal cortices, and (e-g) the CA3 region of the ventral

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435 given area was calculated followed by the average percent of cells showing each type of activation
436 in individual rats. To prevent the scores of rats with larger numbers of images from having a
437 disproportionate effect on our statistics and to prevent an inflation of sample size only these
438 averages were used in our final analysis.

439 Results

440
441 All statistical analyses were completed using the R coding software. The full code is freely
442 available to view at our data repository at
443 https://dataverse.tdl.org/dataverse/MonfilsFearMemoryLab. Unless otherwise stated, the cutoff
444 for a test to be considered statistically significant was set to $p < 0.05$.

445 Behavioral Results

446 Dominance tests results

447
448 As only Cohort 2 rats were assigned conditions based on dominance rank, Cohort 1 males
449 were not included in these analyses, resulting in data from nine triads (n = 27 rats) being included.
450 To verify our dominance assignments, we ran a two-way ANOVA (Type 2) with the percent of
451 total nape contacts in the cage received as the dependent variable and engaging rat rank and
452 responding rat rank as independent variables. The interaction had to be tested separated using
453 a one-way ANOVA. We found an overall effect of both engaging ($F_{(2,49)} = 16.409$, $p < 0.0001$) and
454 responding ($F_{(2,49)} = 19.490$, $p < 0.0001$) rank and an interaction between the two ($F_{(5,48)} = 9.84$, p
455 < 0.0001). A post-hoc Tukey HSD found that, as expected, dominant ($p = 0.0082$) and S1 ($p =$
456 0.043) were significantly more likely to engage than S2 rats, and dominants were more likely to
457 be the responder when compared to both S1 ($p = 0.0031$) and S2 ($p = 0.002$) rats. S1 rats were
458 also significantly more likely to contact the dominant rat than the S2 rat ($p = 0.00031$). We also

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459 examined the percent of times a rat responded to a nape contact with a counter, a behavior that
460 has previously been found to be more likely in dominant rats [37]. Differences in likelihood of
461 counter response was tested using a series of Kruskal-Wallace tests due to violations of ANOVA
462 assumptions. We found that while there was no detected effect of engaging rank ($H_2 = 3.23$, $p = 0.199$)
463 there was a significant effect of responding rank ($H_2 = 7.92$, $p = 0.0191$) and a post-hoc
464 Dunns test with Holm's p-adjustment found that dominant assigned rats did counter significantly
465 more than rats assigned to the S1 condition ($p = 0.0162$) but not rats assigned to the S2 condition
466 ($p = 0.156$). A mixed-effects ANOVA run to examine performance during the milk dominance
467 assessment with percent of time monopolizing the milk cup as the dependent variable, assigned
468 rank as the between-subjects variable, and minute of scoring as the within-subjects variable. We
469 found a significant overall effect of rank ($F_{(2,24)} = 4.83$, $p = 0.0172$) but no effect of minute ($F_{(9,216)}$
470 $= 0$, $p > 0.99$) and no interaction between the two ($F_{(18,216)} = 1.189$, $p = 0.272$). Post-hoc pairwise
471 comparisons across the various ranks averaged across minute found that S1 ranks rats spent
472 significantly more time monopolizing the milk cup than S2 rats ($p = 0.0165$) and dominant rats
473 also trended in that direction ($p = 0.09$), but there was no significant difference between dominant

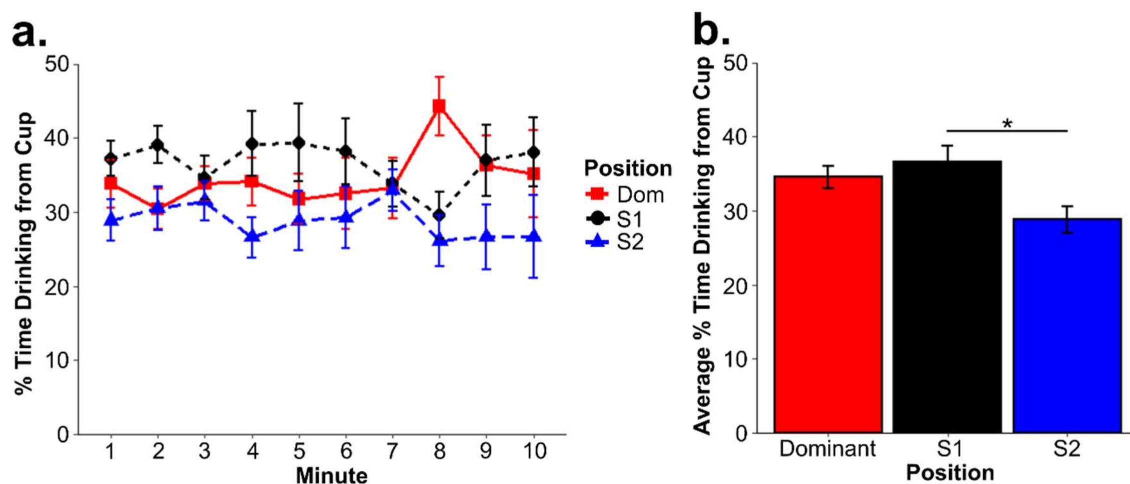

Fig 4. Milk dominance test results. The above figures show the average percent of total time that rats assigned a given rank spent monopolizing the milk cup (a) across the first ten minutes of the dominance test and (b) averaged across each minute by rank. $*p < 0.05$

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474 rats and S1 rats ($p = 0.713$) (see Fig 4). Notably, these results are counter to earlier findings from
475 our lab [13], which might be attributable to differences in the container used to hold the milk during
476 testing as the lid of our container was slightly wider (4.45 cm Diameter vs. 3.75 cm diameter)
477 making the milk more easily accessible.

478 ## Fear conditioning and fear conditioning by-proxy

479
480 To ensure that Demonstrators had sufficiently acquired the CS-US association, their
481 freezing on day 2 (during the FCbP observation period) was run through a one-way within-
482 subjects ANOVA with timepoint (pre-CS, CS1, CS2, and CS3) as the within-subjects factor. We
483 found a significant effect of cue ($F_{(3,87)} = 77.96$, $p < 0.0001$) and a post-hoc pairwise testing using
484 a Bonferroni adjustment for multiple comparisons confirmed that freezing during the CS was
485 significantly higher than at baseline (all $p < 0.001$) (see Fig 5a). A set of Kruskal-Wallace analyses
486 were run on freezing on to the CS presentation on the terminal day (day 4) of the experiment as
487 a nonparametric alternative to an ANOVA due to violations of ANOVA assumptions by the
488 untransformed dependent variable. Kruskal-Wallace analyses were run on sex, experimental
489 condition, and a factor containing all combinations of the two (to detect potential interactions) as
490 independent variables. It found that while there was no overall effect of sex ($H_1 = 1.55$, $p = 0.2132$)
491 on its own, there was a significant effect of experimental condition ($H_2 = 35.1$, $p < 0.0001$) and a
492 significant effect of the combined factors ($H_5 = 37.38$, $p < 0.0001$). Post-hoc Dunn's tests using
493 the Holm adjustment for multiple comparisons found that rats in the Demonstrator condition froze
494 significantly more to the CS than both Observers ($p < 0.0001$) and Controls ($p < 0.0001$), but,
495 surprisingly, Observers and Controls did not significantly differ in their freezing from each other
496 ($p = 0.814$). Dunn's testing on the combined sex and condition variable found that the overall
497 effect detected via Kruskal-Wallace was driven entirely by the Demonstrator condition, i.e., no
498 interaction effects were detected (see Fig 5b). Notably, our Demonstrators also displayed an
499 unusually low percentage of freezing to this final CS (mean = 25.2) that we were unable to

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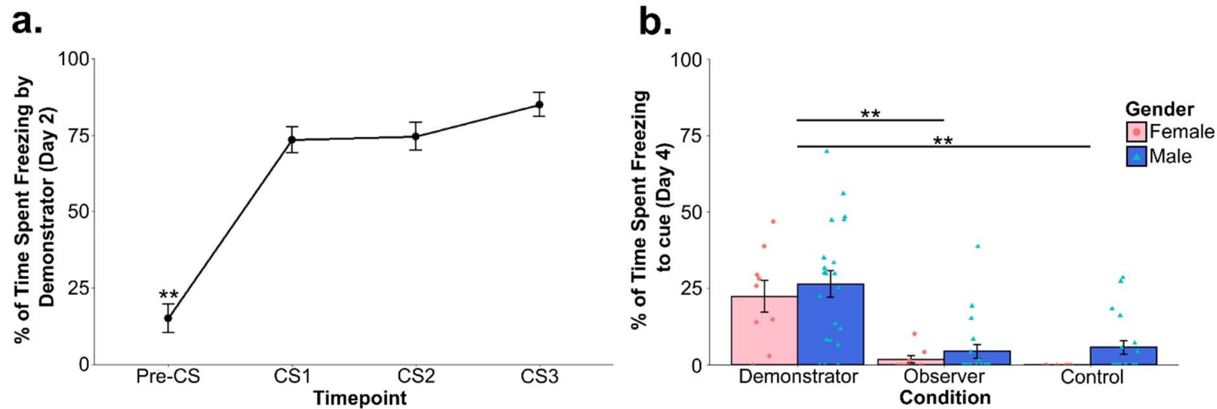

Fig 5. Fear conditioning and fear conditioning by-proxy behavioral results. The above figures show the average percent of total time that rats froze during or prior (Pre-CS) to the CS presentation for (a) Demonstrators on day 2, during FCbP interactions and (b) all rats to the single CS presentation on the terminal day of the experiment. *$**p < 0.005$*

500 replicate using near identical behavioral procedures (see Supporting Information methods). We
501 did, however, confirm that the Demonstrators' freezing during the CS period was not just context
502 based by using a Wilcoxon signs-rank test (due to violation of the assumption of normality
503 because of a floor effect for pre-CS freezing) to compare freezing during the CS to their freezing
504 prior to CS presentation ($Z = 49$, $p = 0.0013$). That Observer rats did not show higher freezing
505 than Control rats during the final CS presentation, while somewhat concerning, is likely the result
506 of our using only a single CS presentation. While past research in our lab has found that FCbP
507 observer rats will freeze over controls on the first CS presentation of a long-term memory test
508 [12], there were some methodological changes (pre-exposure of controls to the CS and rats being
509 run during their dark cycle) that resulted in slight changes in behavior. This was confirmed in a
510 follow-up experiment run under similar conditions where we ran a full three CS recall test (see
511 Supporting Information text).
512

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513 **Choice test/food tasks**

514

515 Choice test performance using either percent of time spent interacting the food cup
516 containing diet Cin or percent of all eaten that was diet Cin was compared between Demonstrators
517 and Observers using a two-sample t-test. We found no significant difference between the two
518 groups on time spent at the diet Cin food cup ($t_{31} = 0.97$, $p = 0.3404$) or on the percent of total
519 eaten that was diet Cin ($t_{31} = 0.74$, $p = 0.4636$). To determine whether this lack of an effect was
520 due to both groups showing a preference for diet Cin, we ran a set of one-sample t-tests
521 comparing the percent of total eaten that was diet Cin against the case in which rats showed no
522 preference for either diet ($\mu = 50$). We found that while both Demonstrator ($t_{16} = 2.204$, $p =$
523 0.04265) and Observer ($t_{16} = 3.105$, $p = 0.0068$) rats showed a significant preference for the diet
524 Cin based on the percent eaten, neither Demonstrators ($t_{16} = 0.476$, $p = 0.641$) nor Observers (t_{15}
525 $= 1.885$, $p = 0.079$) spent significantly more time interacting with the diet Cin food cup (see Fig
526 6a,b). The lack of difference between Observers and their Demonstrators can likely be explained
527 by: (1) a slight innate preference for diet Cin over diet Co, as past research in our lab has found
528 in Sprague-Dawleys [14], and (2) our decision to only use diet Cin as the demonstrated flavor in
529 an attempt to decrease variance in the behavioral experience of our observers and (3) the brevity
530 of the choice test compared to our standard design (10 minutes vs 1 hour). It is also worth noting
531 that the Cohen's d effect size for the Observer's preference towards cinnamon ($d = 0.75$) is larger
532 than the effect size calculated for Demonstrators ($d = 0.53$), though both fall into the category of
533 medium effect sizes. Finally, to determine whether experimental condition influenced the total
534 amount of food eaten, we ran a two-way ANOVA with total grams of food eaten during the choice
535 as the dependent variable and experimental condition and sex as the independent variables. We
536 found that while, as expected, there was a significant effect of sex ($F_{(1,82)} = 35.66$, $p < 0.0001$)
537 (see Fig 6c) with females eating less than males, there was no significant effect of experimental

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538 condition ($F_{(2,81)} = 0.334$, $p = 0.717$) (see Fig 6d) and no interaction between the two ($F_{(2,81)} = 1.02$,
539 $p = 0.365$).

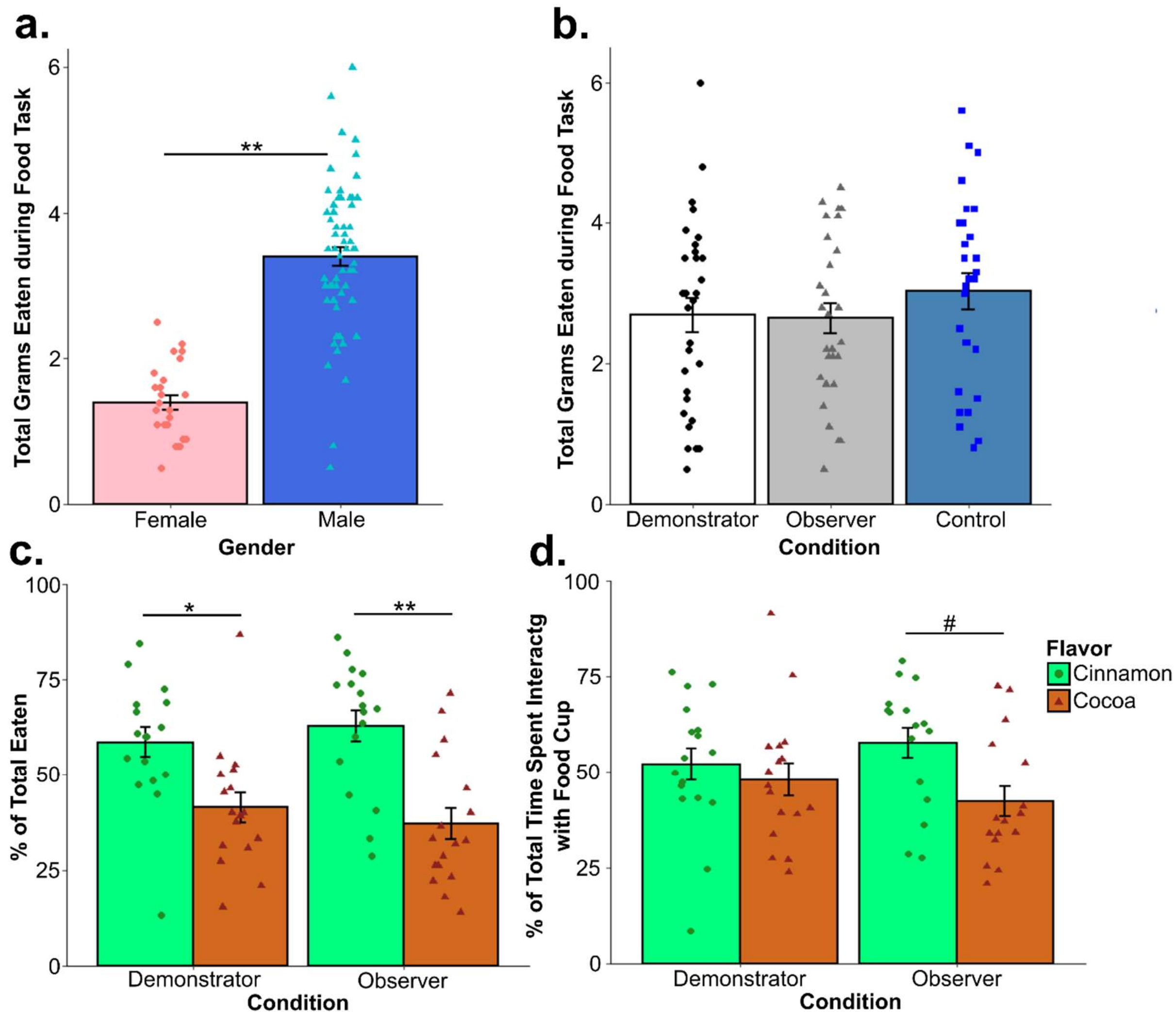

Fig 6. Day 4 food task behavioral results. For rats that went through the choice test on the final day of experimentation, we found that (a) while Observers and Demonstrators did not differ significantly from each other in the percent of diet Cin (the demonstrated flavor) eaten, they did both show a significant preference for the diet. However, (b) neither group spent significantly more time interacting with the food cup containing diet Cin. Examining the total amount eaten during the final food task for all rats we predictably found that (c) females overall ate significantly less than males but (d) experimental condition has no overall effect on the total amount eaten. *#p< 0.1*, **p < 0.05, **p < 0.01*

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540 # Arc Results

541 ## Arc Statistical analysis overview

542
543 All of our *Arc* results, unless otherwise mentioned, were tested for significance using a
544 series of two-way ANOVAs (type 2) containing sex and condition as between subject variables
545 (Sex and Condition) with an individual ANOVAs run for each area of expression (nucleus,
546 cytoplasm, and dual). Similarly, a series of one-way ANOVAs were run with a combined variable
547 containing the food task (diet Cin only or Choice test for Demonstrators and Observers; plain
548 chow only for all Controls) for each area of expression. Sex was not included as a secondary
549 variable as the relatively low number of female rats made sample sizes too small for certain
550 condition/food task combinations. When ANOVA assumptions were violated, data were
551 transformed using either a log(y+1) function or by taking the inverse square root. As these
552 transforms did not always succeed in bringing ANOVAs in line with assumptions, Kruskal-Wallace
553 tests were performed on datasets where transforms were not effective. Pairwise t-tests were
554 performed for post-hoc analyses against a Bonferroni-corrected alpha value when ANOVAs
555 indicated a significant effect of condition ($\alpha = 0.017$) or a significant sex and condition interaction
556 ($\alpha = 0.008$; conditions tested against each other within each sex only) with between-group effect
557 sizes calculated using Cohen's d. To provide a better gauge of variability for our smaller group
558 sizes, the MS_{Error} obtained from our ANOVA was used in the denominator of post-hoc t-tests.
559 Effect sizes for ANOVAs were calculated using the standard partial η^2 formula and for Kruskal-
560 Wallace tests using the formula $\eta^2_H = (H - k + 1)/(n – k)$. For simplicity of data presentation, unless
561 the addition of the food task grouping variable resulted in a significant effect or unless a significant
562 contribution of sex was detected all data were presented graphically split up by area of expression
563 and overall experimental condition only. Any rats that had fewer than 6 viable images counted in
564 a given brain region were excluded from the analysis for that area.

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565 Bivariate correlations were calculated for Observer and Demonstrator animals to assess
566 potential relationships between behavioral measures and *Arc* cell counts for expression occurring
567 at appropriate timepoints (e.g., cytoplasmic *Arc* counts for percent of cinnamon eaten). Pearson's
568 correlation coefficients were used in the event that no outliers in either dataset were detected with
569 a Grubbs test; if outliers were detected, Spearman's correlation coefficient was used instead. To
570 gauge whether a relationship between social learning and *Arc* in dual expressing cells in
571 Observers, an overall metric of social learning – referred to from here on out as the social learning
572 metric (SLM) - was calculated take the mean of the z-score standardized scores for the
573 percentage of total eaten that was the demonstrated food and the percentage of time spent in
574 contact with the Demonstrator during the FCbP social learning phase during the CS presentation
575 (males) or after the CS presentation (females). Notably, percent freezing to the cue on the final
576 day was not used for Observer rats because our results and the results of our follow up experiment
577 (see Supporting Information data) indicated that the conditions of our behavioral testing procedure
578 resulted in some freezing behavior even in Control rats – at least in males - and, as such, might
579 not be the best gauge of the strength of the socially acquired fear response. As such, given our
580 past findings that interactions with the Demonstrator during or after the CS (depending on sex)
581 highly predicted later freezing to the cue [12–14], interaction with the Demonstrator at the sex
582 appropriate timepoint was tested for correlations against nuclear *Arc* activity rather than freezing
583 to the cue on the final day for Observer rats. For Demonstrators, a similar metric was calculated
584 based on standardized freezing to the cue on the final day and the percent of total eaten that was
585 the familiar diet (Diet Cin) and checked against dual *Arc* activity. To correct for the multiple tests
586 run on each behavioral dataset (6, for each brain region), the critical p-value for correlations was
587 Bonferroni adjusted to 0.0083.

588 Arc Results

589 (An overview of statistical results for each area can be found in S1-S4 Tables)

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590

591 No significant effect of condition and no interaction between sex and condition was
592 detected in the vCA3, infralimbic cortex, anterior cingulate cortex, or the lateral and ventral
593 orbitofrontal cortex (all $p > 0.05$) (see Fig 7). Additionally, none of the one-way ANOVAs found a
594 significant effect of condition when rats were further separated based on the food task they were
595 assigned in any of these areas or in the prelimbic cortex (all $p > 0.1$). An overall effect of sex was
596 found in a number of regions including: nuclear *Arc* expression in the ventral orbitofrontal cortex
597 ($F_{(1,64)} = 4.851$, $p = 0.031$; $\eta^2_{partial} = 0.07$); dual expressing cells in the lateral orbitofrontal cortex
598 ($F_{(1,57)} = 6.18$, $p = 0.016$, $\eta^2_{partial} = 0.094$); nuclear ($F_{(1,69)} = 35.470$, $p < 0.001$, $\eta^2_{partial} = 0.325$),
599 cytoplasmic ($F_{(1,69)} = 60.715$, $p < 0.0001$, $\eta^2_{partial} = 0.463$), and dual expressing ($F_{(1,69)} = 9.84$, $p =$
600 0.003, $\eta^2_{partial} = 0.124$) cells in the vCA3 of the hippocampus; dual expressing cells in the CG1
601 region of the anterior cingulate cortex ($F_{(1,66)} = 15.930$, $p < 0.001$, $\eta^2_{partial} = 0.194$); in nuclear
602 expressing cells ($F_{(1,73)} = 18.05$, $p < 0.001$, $\eta^2_{partial} = 0.196$) and dual expressing cells ($F_{(1,73)} =$
603 13.666, $p < 0.001$, $\eta^2_{partial} = 0.15$) in the infralimbic cortex (see Fig 8); and in cytoplasmic
604 expressing cells ($H_1 = 4.3$, $p = 0.038$, $\eta^2_H = 0.045$) and dual expressing cells ($F_{(1,70)} = 18.11$, $p <$
605 0.001, $\eta^2_{partial} = 0.203$) in the prelimbic cortex (see Fig 9b,c). Female rats displayed higher *Arc*
606 counts than males in areas other than the anterior cingulate, infralimbic, and prelimbic cortices,
607 in which male counts were higher across all conditions. Notably, post-hoc analyses found no
608 overall significant effect of condition within the *Arc* counts for across any of the tested regions or
609 areas of cell expression (all $p > 0.1$). The two-way ANOVA examining nuclear expression in the
610 prelimbic cortex found a significant interaction effect between sex and experimental condition
611 ($F_{(2,70)} = 3.96$, $p = 0.023$, $\eta^2_{partial} = 0.102$). Post-hoc testing found a significant difference between
612 nuclear *Arc* expression in female Demonstrators and female Controls only ($t_{9.7} = 3.9$, $p = 0.0032$,
613 $d = 1.22$) (see Fig 9a). Correlational analyses found a significant negative relationship between
614 the SLM score of Observer rats and the percent of cells showing dual *Arc* expression in the ventral
615 orbitofrontal cortex ($t_{10} = -3.41$, $p = 0.0066$, $r = -0.73$) (see Fig 10a). Follow up analyses confirmed

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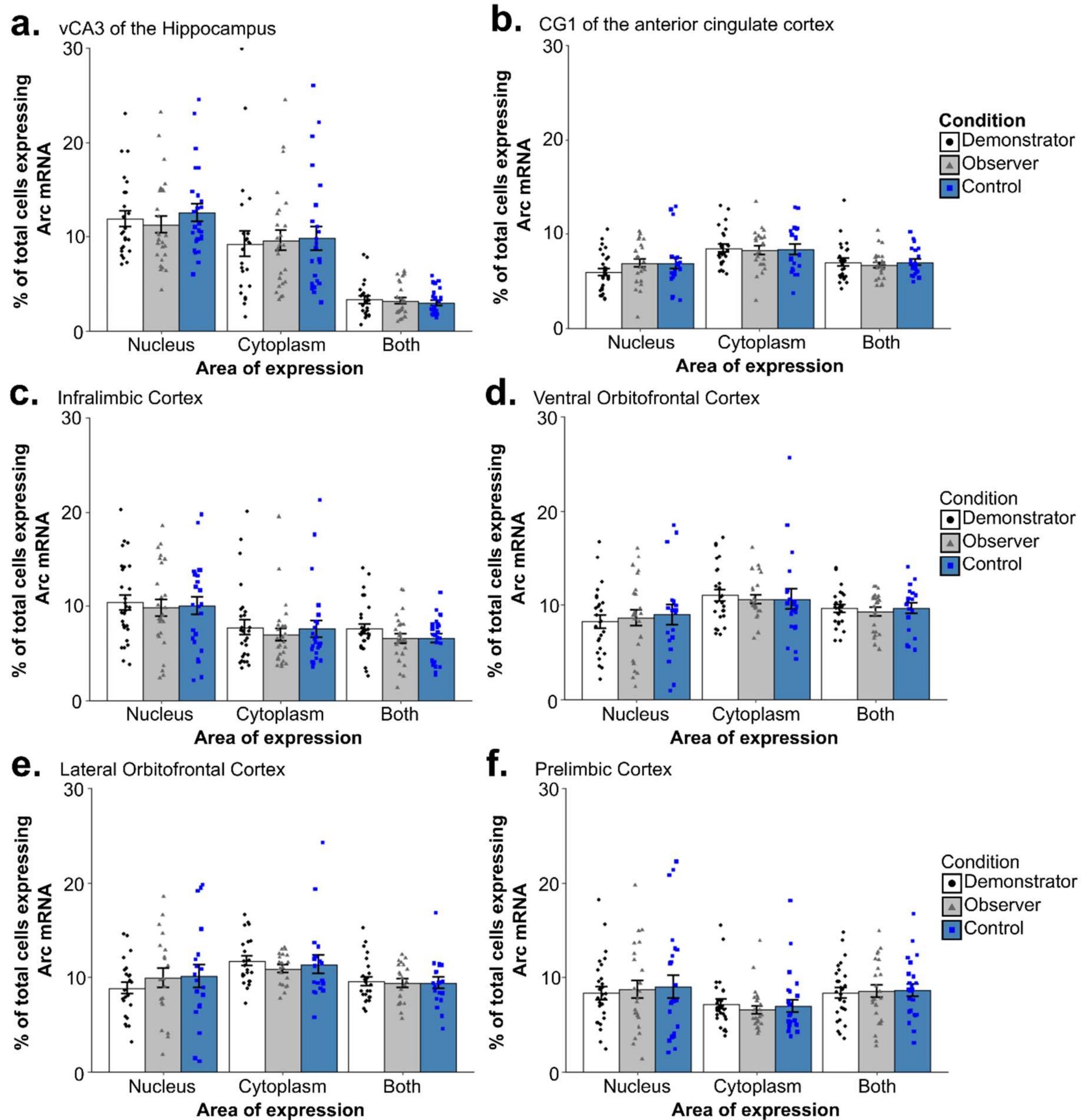

Fig 7. Arc counts across primary experimental condition. The above graphs show the percent of total DAPI stained cells that displayed *Arc* expression in the nucleus, cytoplasm, or in both area (dual) across the primary experimental conditions in (a) the vCA3 of the hippocampus, (b) the CG1 region of the anterior cingulate cortex, (c) the infralimbic cortex, (d) the ventral orbitofrontal cortex, (e) the lateral orbitofrontal cortex, and (f) the prelimbic cortex. Across all regions and areas of cell expression examined, no group differences were found between any of the conditions (all $p > 0.1$).

616

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617

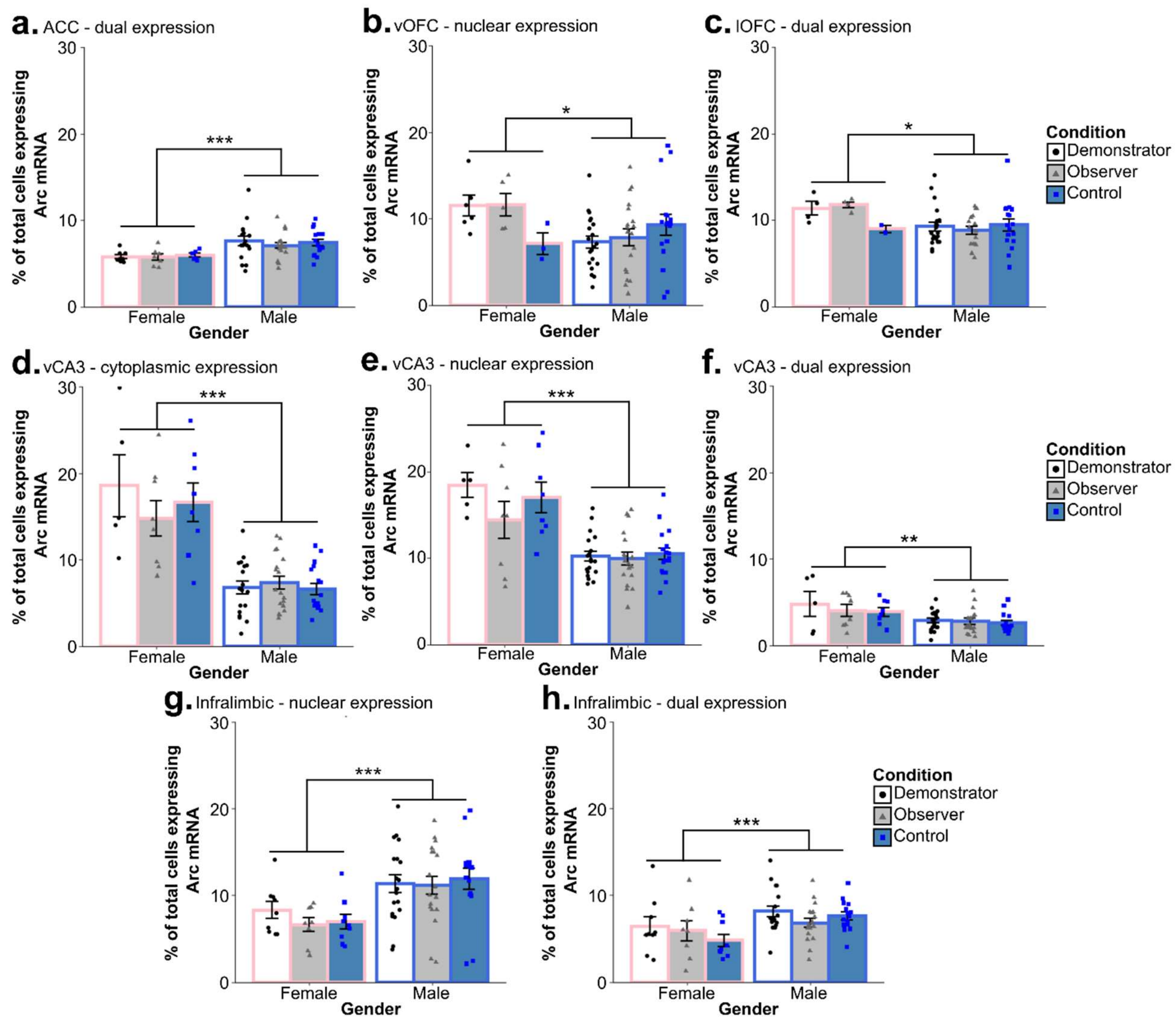

Fig 8. Differences in *Arc* expression between male and female rats. Significant differences in *Arc* expression were between male and female subjects when comparing (a) dual *Arc* expression in the CG1 region of the anterior cingulate cortex, (b) nuclear *Arc* expression in the ventral orbitofrontal cortex, (c) dual expression in the lateral orbitofrontal cortex, (d) cytoplasmic, (e) nuclear, and (f) dual *Arc* expression in the vCA3 of the hippocampus, and (g) nuclear and (h) dual *Arc* expression in the infralimbic cortex.

*+p < 0.1, *p < 0.05, **p < 0.01, ***p < 0.001*

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618

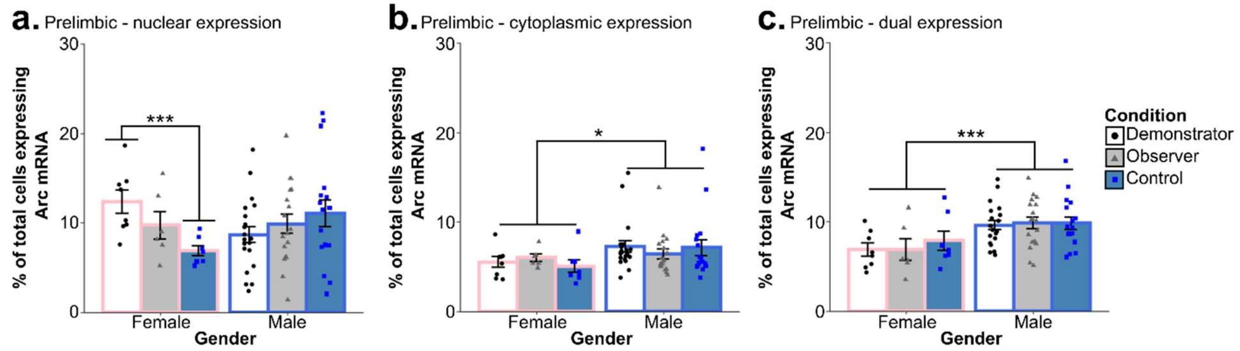

Fig 9. Differences in *Arc* expression between male and female rats across the prelimbic cortex. While initial ANOVA analysis found a significant sex and condition interaction in (a) the nuclear prelimbic counts, with female Demonstrators showing significantly more *Arc* expression than female Controls. Females did show lower overall (b) cytoplasmic and (c) dual *Arc* expression as compared to males in the prelimbic cortex, however. **p < 0.05, ***p < 0.001*

619

620

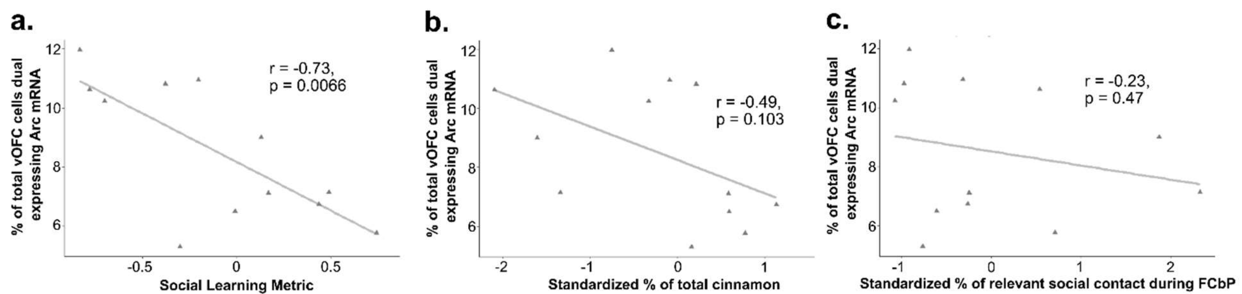

Fig 10. Relationship between social learning measures dual *Arc* expression in the vOFC. (a) A significant negative relationship was found between a social learning metric calculated by summing standardized measures of social acquisition of the STFP and socially acquired fear association in Observers and the percent of *Arc* dual-expressing cells in the ventral orbitofrontal cortex. This relationship was not significant when looking at either (b) the standardized measure of STFP or (c) the standardized measure sex relevant social contact during FCbP – used as a proxy for social fear learning - alone. Notably, both male and female animals were included in this dataset.

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621 that this relationship was not significant when looking at either the standardized measure of
622 percent cinnamon eaten (t_{10} = -1.795, p = 0.103, r = -0.49) or the standardized measure of sex
623 relevant contact during FCbP (t_{10} = -0.75, p = 0.47, r = -0.23) alone (see Fig 10b,c). All other
624 correlational analyses were not significant beyond our Bonferroni corrected alpha value (all $p > 0.01$).
625

626 Discussion

627
628 Contrary to our expectations, our results did not show any differences in *Arc* expression
629 following long term memory recall based on whether the subject had acquired reward- and fear-
630 based information by means of direct learning or social learning. Even more puzzlingly, Control
631 rats that were put through analogous behavioral procedures prior to euthanasia but that had not
632 been through any explicit fear- or reward-based training did not differ in *Arc* expression across
633 the CG1 region of the ACC, the infralimbic cortex (IL), the vCA3 of the hippocampus, or the ventral
634 or dorsal orbitofrontal cortex (OFC) when compared Demonstrators or Observers. Overall, the
635 only differences in *Arc* expression that were detected were driven by subjects' sex and showed
636 no interaction with experimental condition. Though it is true that recall processes may not
637 necessarily induce as many of the long-term changes in neural activity and connectivity that *Arc*
638 is thought to be involved in [41] as learning procedures do, past research has found certain recall
639 procedures to be sufficient to induce increased *Arc* activity [42,43]. As such, the lack of an effect
640 across conditions that we see cannot be attributed only to our choice to examine learning at the
641 recall timepoint. In the following sections, we will first examine our overall findings in the context
642 of past research into the brain mechanisms underlying recall processes in the STFP paradigm,
643 fear-conditioning and observational fear-conditioning procedures, and our findings in the ventral
644 orbitofrontal cortex in the context of past research. We will then cover our findings – and,

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645 importantly, the limitations around our ability to interpret these findings – on sex effects on *Arc*
646 expression.

647 *Arc* in the recall of a socially transmitted food preference

648
649 Past research examining expression of the IEG c-Fos has found that a number of the
650 areas we examined, specifically the orbitofrontal cortex, vCA3, infralimbic cortex, and the
651 prelimbic cortex [22,23] show activation at the 48 hour recall timepoint for a socially transmitted
652 food preference. It is also notable that these results from Smith et al. [23] were obtained using the
653 same STFP control paradigm as was used in this study, indicating that though STFP recall
654 induced activity in these regions may have been detectable with c-Fos, this may not be the case
655 at this timepoint when examining *Arc*. This interpretation is backed up by the findings of Pilarzyk
656 et al. [43], who examined *Arc* mRNA activity following STFP recall in Pde11a knockout mice,
657 which displayed impaired recent STFP and enhanced remote STFP compared to Pde11a wild-
658 type controls. They found that both animals showed increases in *Arc* expression over home-cage
659 controls at this timepoint in the ventral and dorsal CA1, the ventral and dorsal subiculum, and in
660 the CG1 and CG2 of the ACC. Moreover, while Pde11a knockout mice showed decreased *Arc*
661 expression following a recall procedure for a recently acquired (24 hours post) STFP memory
662 when compared to Pde11a wild-types in the vCA1, no difference between the two genetic lines
663 was evident in any of the other regions examined. At a more remote recall timepoint (7 days post),
664 knockout animals showed higher *Arc* activity post-recall in the CG1 and CG2 of the ACC but not
665 in the vCA1 as compared to the wildtype controls, with home cage animals showing no baseline
666 difference regardless of genetic line. Given that these differences in ACC *Arc* activity were not
667 observed during early recall and the differences in vCA1 *Arc* activity was not seen during remote
668 recall, it is reasonable to assume that this *Arc* activity was specific to both the experience of STFP
669 recall and the recall timepoint. These findings are particularly interesting in light of prior research

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670 examining c-Fos activity in the vCA1 and the ACC at the respective timepoints at which enhanced
671 *Arc* activity was seen in these animals, as past studies have found no differences in c-Fos activity
672 in these areas when recall was induced on the exact same timeframe [22,23]. With this in mind,
673 it is perhaps unsurprising that we also observed no recall induced changes in *Arc* expression in
674 the various regions we examined despite their consistently being shown to be active using c-Fos
675 as an activity marker. Exactly what the implications of this are - outside of the obvious conclusion
676 that not all IEGs are equal – is hard to say when working with mostly null findings. That said, the
677 high sensitivity of cellular compartment analysis of temporal activity by fluorescence *in situ*
678 hybridization (catFISH) and our large group sizes for the primary behavioral conditions
679 (Demonstrator, Observer, and Control) does lend validity to the non-significance of our findings.
680 One caveat to our design that future experimenters might want to consider is the possibility that
681 Demonstrators may also have acquired a STFP simply through exposure to the scent of the
682 consumed food on their own breath and carbon disulfide from the nasal cavity of the Observer
683 with whom they were interacting.

684 ***Arc* in the recall of direct and socially acquired fear**
685 **associations**

686
687 Our ability to interpret our findings regarding our rats undergoing recall of fear acquired
688 via direct learning is significantly aided by how well-characterized the system underlying fear
689 learning and recall is. A number of the areas we examined are well established as being involved
690 in fear or extinction learning (the latter of which we would assume to be initiated in Demonstrators,
691 as they had undergone non-reinforced CS presentation during FCbP) specifically the ACC, the
692 prelimbic cortex, and the infralimbic cortex [30–32,44]. Though a much smaller pool of research
693 is available regarding the neural mechanisms of social fear, the proposed models of social fear
694 learning posit a system similar to that underlying recall of directly acquired fear associations also

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695 underlies the fear learning and recall processes for social fear learning [28]. Our findings indicate
696 no overall role of the ACC, prelimbic cortex (PL), or IL in either recall of a socially acquired fear
697 association or a directly acquired fear association (though see also discussion of sex differences
698 in PL activity below). However, as covered in the previous section, this likely just indicates that
699 *Arc* does not serve as a reliable indicator of activity in this case. Examination of these areas post-
700 fear acquisition would likely tell a different story. Though explicit research in *Arc* activity following
701 fear recall is limited, there is some past research to draw from. Chia & Otto [42] found that when
702 Arc protein expression was examined following the presentation of a CS that a rat had acquired
703 a fear association for via trace fear conditioning (i.e., fear conditioning with a delay between CS
704 termination and shock delivery) rats were found to have significantly higher Arc expression in both
705 the dorsal and ventral hippocampus when compared to unconditioned controls that were exposed
706 to the chamber but not the CS. Notably, Arc was quantified by Western Blot analysis of the
707 homogenized ventral and dorsal HPC in this experiment, so precise localization of HPC activity
708 was not available. These findings likely indicate that, like in STFP, *Arc* transcription might be
709 induced in certain areas of the hippocampus at the 48-hour recall timepoint for a cued fear
710 memory.

711 Potential Role of the Ventral Orbitofrontal Cortex in Recall of 712 Socially Acquired Information

713
714 In a landmark study, Lesburguères et al. [24] were able to demonstrate that while dorsal
715 hippocampal (dHPC) activity was necessary for acquisition and short-term recall of an acquired
716 STFP, the STFP memory was eventually offloaded to the OFC for long-term storage. Additionally,
717 Lesburguères et al. were able to demonstrate that that tagging of neurons in the orbitofrontal
718 cortex during STFP acquisition is necessary for long-term storage of socially transmitted food
719 preferences and that interference with the OFC activity following acquisition impairs remote

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720 memory recall (30 days post-acquisition) (though see also [45]). These findings would suggest
721 ongoing communication between the dHPC and the OFC in the first days or weeks post-STFP
722 acquisition and, furthermore, would suggest ongoing reorganization of the OFC at this timepoint
723 to accommodate the long-term storage of the STFP memory. While the lack of overall differences
724 in ventral or lateral OFC *Arc* expression between Demonstrators, Controls, and Observers in this
725 study would challenge that interpretation somewhat, we did detect a significant negative
726 correlation between our combined measure of overall social learning performance and dual-*Arc*
727 expressing cells in the vOFC. Furthermore, this correlation was not observed between a similar
728 metric formed for Demonstrators based on their choice test performance and their freezing to the
729 cue. As reliance on socially acquired information can be thought of as making the choice between
730 potentially unreliable social information and the potential dangers of learning through direct
731 experience, it is possible that this apparent inhibitory role of the vOFC on expression of socially
732 acquired information might be connected to the OFC's broader role in value-based decision
733 making [46–49].

734 ## Sex Differences in *Arc* Transcription

735
736 Prior to this discussion, it should be stated that our ability to interpret our sex-related
737 results is hindered for a number of statistical and methodological reasons. First, our occasionally
738 low sample size for females, with group size for sex/condition combinations ranging from n = 2 to
739 n = 9 following removal of rats without enough viable sections (though notably an n < 5 was only
740 present for female Controls in the vOFC and lOFC and female Demonstrators and Observers in
741 the lOFC). Additionally, our lack of entirely undisturbed controls means that we have no way to
742 determine whether these sex differences are the result of baseline or task-specific differences in
743 *Arc* mRNA production. Finally, because the pre-*in situ* PFA wash was not introduced until all
744 female sections had been processed, it is possible that this difference in tissues processing might

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745 have affected the overall stain. That said, if this were the case, we might expect to see a broader
746 and more consistent effect of sex across regions and types of *Arc* expression (nuclear,
747 cytoplasmic, and dual). As it is, 18 regions/cellular areas of *Arc* expression combinations are
748 examined and only 10 display a significant overall effect of sex. Furthermore, this effect is not
749 uniform in its direction, with males displaying greater overall *Arc* expression in 5 cases and
750 females displaying greater expression in the other 5. Regardless, we feel that our findings here
751 should serve only to inform possible future research into sex differences in *Arc* expression. As it
752 is, the limitation of the current study would make drawing definitive conclusions regarding sex
753 effects on *Arc* expression inappropriate. This should be kept in mind in reading the following
754 discussion.

755 Although there has been little investigation into sex differences in *Arc* expression, there
756 are some findings indicating that female rats may show higher levels of *Arc* expression in certain
757 regions of the dorsal hippocampus following repeated exposure to a relatively enriched
758 environment [50], though a trend in the opposite direction has also been observed in animals
759 tested without prior behavioral intervention [50]. Our findings may indicate that sex differences in
760 *Arc* transcription may be present following certain general behavioral tasks or experiences. In the
761 CG1 region of the ACC we found that males, overall, had more cells active at both timepoints,
762 possibly due to higher baseline *Arc* transcription in the ACC of males or increased transcription
763 following context changes/re-exposure (home cage → STFP testing room → conditioning
764 chamber) as there is some evidence – though limited – for a role of the ACC in long-term recall
765 of contextual memories [51]. Male rats also displayed higher nuclear and dual *Arc* counts in the
766 infralimbic (IL) cortex. It is possible that the higher IL *Arc* counts in males might be explained by
767 the role of the infralimbic cortex in extinction and fear inhibition [31,52,53] and the well
768 documented impairments in the inhibition and extinction of learned fear in females [54–56]. If this
769 is the case, however, it does raise the question of why no overall differences were observed

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770 between our Control, Observer, and Demonstrator animals if *Arc* expression was being triggered
771 by CS-elicited infralimbic activity.

772 Females showed higher levels of *Arc* expression for all counts in the vCA3. The difference
773 in nuclear counts could potentially have been the result of greater activation following exposure
774 to the CS or re-exposure to the conditioning chamber in females, while the higher levels of
775 cytoplasmic *Arc* expression in the vCA3 following the food task may indicate a sex differences in
776 the role of *Arc* in the vCA3 either the recognition of "familiar" food (even for Observers the scent
777 would be familiar due to their prior interaction with the Demonstrator) or reward/general
778 consummatory processes. That females also showed significantly higher dual labelling in the
779 vCA3 – though this effect was small – might also indicate generalized increases in vCA3 *Arc*
780 transcription in females. Female rats also displayed higher nuclear *Arc* transcription in the ventral
781 OFC and higher dual levels of *Arc* mRNA in the lateral OFC, though these results are more difficult
782 to interpret due to the low number of female Control rats whose brain tissue was intact enough to
783 take OFC counts (n = 2 and 3 for the lateral and ventral OFC, respectively). Data from the Control
784 rats we do have indicate a possible sex mediated increase in OFC *Arc* mRNA production, but it
785 is just as possible that this effect would not persist with a higher n. It is notable that some past
786 research has indicated structural differences in the OFC and functional differences in OFC-
787 mediated behaviors between female and male rodents [57–59].

788 Possibly our most interesting sex differences in *Arc* mRNA were detected in the prelimbic
789 cortex. In the prelimbic cortex (PL), males showed overall higher numbers of cells expressing *Arc*
790 in the cytoplasm and in both the cytoplasm and nucleus (dual expressing) than females. While no
791 within-sex differences across condition assignments were detected for these counts, we did find
792 a significant sex/condition interaction in our nuclear prelimbic counts. Specifically, it appears that
793 while male Demonstrators and Observers did not show increases in *Arc* transcription over
794 Controls at the fear-recall timepoint, female Demonstrators showed significantly higher nuclear
795 *Arc* transcriptions than Controls while female Observers fell in the middle between the two. This

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796 sex-effect may be driven by the aforementioned deficits observed in learned fear inhibition and
797 extinction that are observed in females [54–56], as past research has suggested that the PL is
798 critically involved in stimulating fear behavior [52,60,61], essentially serving an opposing role to
799 the IL. Furthermore, a number of studies have implicated differences in PL signaling and structure
800 as potential driving factors for these sex-specific impairments in fear-inhibition and extinction [62–
801 65]. While we found no significant difference in female and male freezing behavior to the cue, the
802 upregulation of *Arc* mRNA in response to a non-reinforced fear associated CS in specifically
803 female Demonstrators may be indicative of differential neural restructuring in the PL that could
804 ultimately lead to sex differences in fear expression.

805
Conclusions

806
807 While the findings of this study did not broaden our understanding of the brain
808 mechanisms involved in the retrieval of socially acquired memories as much as we had hoped,
809 our results do provide some potential insights on sex differences in *Arc* expression as well as the
810 role (or lack thereof) of *Arc* in long-term memory recall. Our findings suggest that - at least in the
811 prefrontal cortex and vCA3 – the induction of brain activity through recall of socially acquired
812 information does not appear to be sufficient to cause increases in *Arc* expression over those
813 caused by the testing procedure alone. However, the validity of this takeaway is certainly brought
814 into question by the inconclusive results of our behavioral tests, which might suggest that poor
815 retainment of the socially acquired information was at fault for this lack of effect. We theorize that
816 this may be because minimal neural restructuring is triggered when recall occurs prior to systems
817 consolidation. Further research into the role of the Arc protein in social learning recall processes
818 is still warranted given that our behavioral results do not demonstrate social learning in Observer
819 rats as definitively as we would have hoped. Future research examining overlap in the neural
820 mechanisms governing different forms of social learning might also benefit from the inclusion of

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821 animals undergoing acquisition procedures and animals undergoing remote recall procedures, as
822 these timepoints may be more likely to induce plasticity changes and thus changes in *Arc*
823 expression. Though the short timeframe of *Arc* expression in and around the cell body may make
824 this methodologically difficult to achieve, rapid acquisition of a STFP might be achieved by using
825 multiple demonstrators at once.
826
827 **Declarations**
828 **Author's Contributions:** LAA designed the experiment, gathered and analyzed data, processed
829 and imaged tissue samples, and drafted the manuscript. ENH and JD assisted with tissue sample
830 processing. VN assisted with the experiments documented in Supporting Information. MHM and
831 HJL designed the experiment, gathered data, provided guidance and training for tissue
832 processing and imaging, and approved the final version of the manuscript.
833 **Availability of data and material:** Raw data files are available in The Monfils Lab repository,
834 housed in the Texas Data Repository in Dataverse
835 (https://dataverse.tdl.org/dataverse/MonfilsFearMemoryLab). All other materials are available by
836 request to the authors.
837 **Code availability:** Code is available alongside data and materials in the Monfils Lab repository
838 (see above).

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