

1 **Title: An insula-central amygdala circuit for behavioral inhibition**

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3 **Abbreviated title: A circuit for behavioral inhibition**

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41

42 **Abstract**

43 Predicting which substances are suitable for consumption during foraging is critical for
44 animals to survive. While food-seeking behavior is extensively studied, the neural circuit
45 mechanisms underlying avoidance of potentially poisonous substances remain poorly
46 understood. Here we examined the role of the insular cortex (IC) to central amygdala
47 (CeA) circuit in the establishment of such avoidance behavior. Using anatomic tracing
48 approaches combined with optogenetics-assisted circuit mapping, we found that the
49 gustatory region of the IC sends direct excitatory projections to the lateral division of the
50 CeA (CeL), making monosynaptic excitatory connections with distinct populations of
51 CeL neurons. Specific inhibition of neurotransmitter release from the CeL-projecting IC
52 neurons prevented mice from acquiring the “no-go” response, while leaving the “go”
53 response largely unaffected in a tastant (sucrose/quinine)-reinforced “go/no-go” task.
54 Furthermore, selective activation of the IC-CeL pathway with optogenetics drove
55 unconditioned lick suppression in thirsty animals, induced aversive responses, and was
56 sufficient to instruct conditioned action suppression in response to a cue predicting the
57 optogenetic activation. These results indicate that activity in the IC-CeL circuit is
58 necessary for establishing anticipatory avoidance responses to an aversive tastant, and is
59 also sufficient to drive learning of such anticipatory avoidance. This function of the IC-
60 CeL circuit is likely important for guiding avoidance of substances with unpleasant tastes
61 during foraging in order to minimize the chance of being poisoned.

62

63 **Significance Statement**

64 The ability to predict which substances are suitable for consumption is critical for
65 survival. Here we found that activity in the insular cortex (IC) to central amygdala (CeA)
66 circuit is necessary for establishing avoidance responses to an unpleasant tastant, and is
67 also sufficient to drive learning of such avoidance responses. These results suggest that
68 the IC-CeA circuit is critical for behavioral inhibition in anticipation of potentially
69 poisonous substances during foraging.

70

71 **Introduction**

72 The ability to suppress actions that can lead to harmful consequences is critical for
73 survival. For example, animals, including humans, stop consummatory behavior when
74 encountering food or liquid with an unpleasant taste, which indicates the existence of a
75 potentially poisonous substance. Animals are also capable of learning to use
76 environmental cues (such as an odor, color, location or context) to predict unpleasant
77 properties (such as a bitter taste, toxicity) of a substance, and subsequently using these
78 predictive cues to guide avoidance of the substance during foraging. While food
79 approaching and reward seeking behaviors are extensively studied (Balleine, 2005, 2011;
80 Kelley, 2004), the neural circuit mechanisms that underlie innate and learned suppression
81 of actions that may lead to food poisoning, or aversive consequences in general, are
82 poorly understood.

83

84 The insular cortex (IC), including the gustatory cortex (GC), plays an important role in
85 processing taste and visceral information (Accolla and Carleton, 2008; Katz et al., 2001;
86 Samuelsen and Fontanini, 2017; Yamamoto et al., 1985). The IC is also engaged in

87 various learning tasks, especially when learning to associate a neutral tastant (also known
88 as a conditioned stimulus, CS) with an intrinsically aversive stimulus (also known as an
89 unconditioned stimulus, US), or to associate a non-gustatory CS with an appetitive or
90 aversive tastant US (Bermudez-Rattoni, 2004; Kusumoto-Yoshida et al., 2015; Vincis
91 and Fontanini, 2016; Yasoshima and Yamamoto, 1998). In one such task, a “go/no-go”
92 task in which auditory “go” and “no-go” CSs predict the delivery of sucrose and quinine,
93 respectively, specific CS-evoked responses develop in the IC as rats learn the predictive
94 value of each CS (Gardner and Fontanini, 2014). IC neurons also show CS-evoked
95 responses in a conditioned food-approaching task (Kusumoto-Yoshida et al., 2015).
96 These findings indicate that associative learning driven by gustatory reinforcement
97 induces plastic changes in the IC, and suggest that the IC may participate in guiding
98 approach or avoidance responses during feeding or foraging behaviors. Consistently,
99 pharmacological and optogenetic inhibition of the IC impairs conditioned food
100 approaching behavior (Kusumoto-Yoshida et al., 2015). Nevertheless, whether the IC is
101 also essential for avoidance of aversive tastes remains unknown.

102

103 The central nucleus of the amygdala (CeA), including its lateral division (CeL), is a
104 prominent downstream structure of the IC. As it is also a direct recipient of taste
105 information from the brainstem (Carter et al., 2013), the CeA is anatomically positioned
106 to process convergent taste information. Indeed, it has been shown that the CeA encodes
107 taste identification and palatability, responses that follow those in the IC (Sadacca et al.,
108 2012). These findings suggest that the CeA may process and use the information
109 originating from the IC to influence taste-motivated behaviors.

110

111 The CeL plays an important role in the learning and expression of defensive behaviors
112 (Ciocchi et al., 2010; Goosens and Maren, 2003; Li et al., 2013; Wilensky et al., 2006), as
113 well as in reward-related behaviors and feeding (Gallagher et al., 1990; Kentridge et al.,
114 1991; Kim et al., 2017; Robinson et al., 2014; Seo et al., 2016). For instance, previous
115 studies showed that the somatostatin-expressing (SOM⁺) subpopulation of neurons in the
116 CeL is essential for the acquisition and expression of conditioned freezing and action
117 suppression (Fadok et al., 2017; Li et al., 2013; Yu et al., 2016). The protein kinase C- δ -
118 expressing (PKC- δ ⁺) CeL neurons, on the other hand, participate in conveying aversive
119 US information, drive avoidance response, and are sufficient to instruct aversive learning
120 (Yu et al., 2017). Furthermore, it has been shown that CeA neurons, including PKC- δ ⁺
121 CeL neurons, are critical for suppression of feeding behavior (Cai et al., 2014; Petrovich
122 et al., 2009). As the CeL is a major direct downstream target of the IC, these findings
123 point to the possibility that the IC controls learning or expression of taste-motivated
124 behavioral inhibition through the IC-CeL circuit.

125

126 To test this hypothesis, we used anatomical, electrophysiological, and circuit based
127 manipulation approaches. We found that an excitatory monosynaptic connection exists
128 between the posterior division of the IC and the CeL in mice, and that activation of the
129 IC-CeL pathway excites specific subtypes of neurons within the CeL. Notably, selective
130 inhibition of CeL-projecting IC neurons specifically impairs the conditioned inhibitory
131 response to a cue predicting an aversive tastant. Furthermore, activation of the IC-CeL
132 circuit with optogenetics produces a powerful suppression of ongoing licking behavior in

133 thirsty mice, induces avoidance behavior, and is sufficient to instruct conditioned lick
134 suppression. These results reveal an important role of the IC-CeL circuit in the
135 establishment of anticipatory behavioral inhibition, in particular the inhibition of
136 consummatory behavior in response to cues predicting an unpleasant taste.

137

138 **Materials and Methods**

139 **Animals**

140 Before surgery, mice were group-housed under a 12-h light-dark cycle (7 a.m. to 7 p.m.
141 light) with food and water freely available. The *Som-cre* (Taniguchi et al., 2011), *Ail4*
142 (Madisen et al., 2010), *Prkcd-cre* (Haubensak et al., 2010), and *Rosa26-stop^{fllox}-tTA* (Li et
143 al., 2010) mice were described previously and were purchased from the Jackson
144 Laboratory. All mice were bred onto C57BL/6J genetic background. The *Som-cre;Ail4*
145 mice, which were heterozygous for both the Cre allele and the Lox-Stop-Lox-tdTomato
146 allele, were bred by crossing homozygous *Som-cre* mice with homozygous *Ail4* reporter
147 mice. Male mice of 40–80 d of age were used for behavioral and anatomical experiments.
148 Male and female mice of 35-45 d of age were used for *in vitro* slice physiology
149 experiments. Behavioral experiments were performed during the light cycle. All
150 procedures involving animals were approved by the Institute Animal Care and Use
151 Committees of Cold Spring Harbor Laboratory and carried out in accordance with US
152 National Institutes of Health standards.

153

154 **Viral vectors**

155 Most of the adeno-associated viruses (AAV) were produced by the University of North
156 Carolina vector core facility or the University of Pennsylvania vector core and have
157 previously been described (Ahrens et al., 2015; Penzo et al., 2015; Stephenson-Jones et
158 al., 2016; Yu et al., 2016): AAV9-Ef1a-DIO-eYFP, AAV9-Ef1a-DIO-hChR2(H134R)-
159 eYFP, AAV9-CAG-ChR2-GFP, AAV9.CAG.Flex.TeLC-eGFP.WPRE.bGH, and AAV-
160 TRE-hGFP-TVA-G. The AAV8.2-hEF1 α -DIO-synaptophysin-mCherry was produced by
161 the MIT Viral Gene Transfer Core. The EnvA-pseudotyped, protein-G-deleted rabies-
162 EnvA-SAD- Δ G-mCherry virus was produced by the Viral Vector Core Facility at Salk
163 Institute (Penzo et al., 2015). CAV2-Cre was purchased from Montpellier vector platform
164 (Plateforme de Vectorologie de Montpellier (PVM), Biocampus Montpellier,
165 Montpellier, France) (Penzo et al., 2015; Stephenson-Jones et al., 2016). All viral vectors
166 were stored in aliquots at -80°C until use.

167

168 **Histology**

169 Animals were deeply anesthetized and transcardially perfused with PBS, followed by
170 perfusion with 4% paraformaldehyde (PFA) in PBS. Brains were dissected out and
171 postfixed in 4% PFA at 4°C for three hours followed by cryoprotection in a PBS-buffered
172 sucrose (30%) solution until brains were saturated (~ 36 h). $50\ \mu\text{m}$ coronal brain sections
173 were cut on a freezing microtome (SM 2010R, Leica). Brain sections were first washed in
174 PBS (3 x 5 min) at room temperature (RT) and then were blocked in 3% normal goat
175 serum (NGS) in PBST (0.3% Triton X-100) for 30 min at RT, followed by incubation
176 with primary antibodies overnight at 4°C . Sections were then washed with PBS (4 x 15
177 min) and incubated with fluorescent secondary antibodies at RT for 2 hours. After

178 washing with PBS (4 x 15 min), sections were mounted onto glass slides with
179 Fluoromount-G (Beckman Coulter). Images were taken using a LSM 780 laser-scanning
180 confocal microscope (Carl Zeiss).

181

182 **Electrophysiology**

183 For electrophysiological experiments, mice were anesthetized with isoflurane,
184 decapitated and their brains quickly removed and chilled in ice-cold dissection buffer
185 (110.0 mM choline chloride, 25.0 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 0.5
186 mM CaCl₂, 7.0 mM MgCl₂, 25.0 mM glucose, 11.6 mM ascorbic acid and 3.1mM
187 pyruvic acid, gassed with 95% O₂ and 5% CO₂). Coronal slices (300 μm) containing the
188 amygdala complex were cut in dissection buffer using a HM650 Vibrating-blade
189 Microtome (Thermo Fisher Scientific). Slices were immediately transferred to a storage
190 chamber containing artificial cerebrospinal fluid (ACSF) (118 mM NaCl, 2.5 mM KCl,
191 26.2 mM NaHCO₃, 1 mM NaH₂PO₄, 20 mM glucose, 2 mM MgCl₂ and 2 mM CaCl₂, at
192 34 °C, pH 7.4, gassed with 95% O₂ and 5% CO₂). After 40 min recovery time, slices
193 were transferred to RT (20–24°C) and perfused with ACSF constantly.

194

195 Simultaneous whole-cell patch-clamp recordings from pairs of SOM⁺ and SOM⁻ CeL
196 neurons were obtained with Multiclamp 700B amplifiers (Molecular Devices).
197 Recordings were under visual guidance using an Olympus BX51 microscope equipped
198 with both transmitted light illumination and epifluorescence illumination, and SOM⁺ cells
199 were identified based on their fluorescence (tdTomato). To evoke IC-driven synaptic
200 transmission onto CeL neurons, the AAV-ChR2-YFP was injected into the IC of *Som-*

201 *Cre;Ail4* mice and allowed to express for 3 weeks. Acute brain slices were prepared, and
202 a blue light was used to stimulate ChR2-expressing axons in the CeL. The light source
203 was a single-wavelength LED system ($\lambda = 470$ nm; CoolLED.com) connected to the
204 epifluorescence port of the Olympus BX51 microscope. 1 ms light pulses were triggered
205 by a TTL signal from the Clampex software to drive synaptic responses. Light pulses
206 were delivered every 10 seconds and synaptic responses were low-pass filtered at 1 KHz
207 and recorded at holding potentials of -70 mV (for AMPA-receptor-mediated responses)
208 and $+40$ mV (for NMDA-receptor-mediated responses). NMDA-receptor-mediated
209 responses were quantified as the mean current amplitude from 50-60 ms after stimulation.
210 Evoked EPSCs were recorded in ACSF with 100 μ M picrotoxin to block inhibitory
211 synaptic transmission. The internal solution for voltage-clamp experiments contained 115
212 mM cesium methanesulphonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl₂, 4 mM
213 Na₂-ATP, 0.4 mM Na₃GTP, 10 mM Na-phosphocreatine and 0.6 mM EGTA (pH 7.2).
214 To assess presynaptic function, a paired-pulse stimulation protocol (50 ms inter-stimulus
215 interval) was used to evoke double-EPSCs, and paired-pulse ratio (PPR) was quantified
216 as the ratio of the peak amplitude of the second EPSC to that of the first EPSC.

217

218 **Monosynaptic tracing with pseudotyped rabies virus**

219 Retrograde tracing of monosynaptic inputs onto genetically-defined cell populations of
220 CeL was performed and described in our previous study (Penzo et al., 2015), and the data
221 presented here were generated from the same study but were not published previously.
222 Briefly, the *Som-cre;Rosa26-stop^{fllox}-tTA* mice and the *Prkcd-cre;Rosa26-stop^{fllox}-tTA*
223 mice, which express tTA in SOM⁺ cells and PKC- δ ⁺ cells, respectively, were injected

224 into the CeL with the AAV-TRE-hGFP-TVA-G (0.2–0.3 μ l) that expresses the following
225 components in a tTA-dependent manner: a fluorescent reporter histone GFP (hGFP);
226 TVA (which is a receptor for the avian virus envelope protein EnvA); and the rabies
227 envelope glycoprotein (G). Two weeks later the mice were injected in the same location
228 with the rabies-EnvA-SAD- Δ G-mCherry (1.2 μ l), a rabies virus that is pseudotyped with
229 EnvA, lacks the envelope glycoprotein, and expresses mCherry. This method ensures that
230 the rabies virus exclusively infects cells expressing TVA. Furthermore, complementation
231 of the modified rabies virus with envelope glycoprotein in the TVA-expressing cells
232 allows the generation of infectious particles, which then can trans-synaptically infect
233 presynaptic neurons.

234

235 **Stereotaxic surgery**

236 Standard surgical procedures were followed for stereotaxic injection (Li et al., 2013;
237 Penzo et al., 2015). Briefly, mice were anesthetized with ketamine (100 mg per kg of
238 body weight) supplemented with dexmedetomidine hydrochloride (0.4 mg per kg) and
239 positioned in a stereotaxic injection frame (myNeuroLab.com). A digital mouse brain
240 atlas was linked to the injection frame to guide the identification and targeting (Angle
241 Two Stereotaxic System, myNeuroLab.com).

242

243 Viruses (\sim 0.4 μ l) were delivered with a glass micropipette (tip diameter, \sim 5 μ m) through
244 a skull window (1–2 mm²) by pressure applications (5–20 psi, 5–20 ms at 0.5 Hz)
245 controlled by a Picospritzer III (General Valve) and a pulse generator (Agilent). The
246 injection was performed at the following stereotaxic coordinates for CeL: 1.18 mm

247 posterior, 2.9 mm lateral, and 4.6 mm ventral from bregma; and for IC: 0.10 mm
248 posterior, 3.90 mm lateral, and 4.20 mm ventral from bregma. For optogenetic
249 experiments, immediately after viral injection, an optical fiber (core diameter, 105 μm ;
250 Thorlabs, Catalog number FG105UCA) was implanted 300 μm above the center of viral
251 injection. The optical fiber together with the ferrule (Thorlabs) was secured to the skull
252 with C&B-Metabond Quick adhesive luting cement (Parkell Prod), followed by dental
253 cement (Lang Dental Manufacturing).

254

255 Following the above procedures, a small piece of metal bar was mounted on the skull,
256 which was used to hold the mouse in the head fixation frame during behavior
257 experiments.

258

259 **Behavioral tasks**

260 *Licking Behavior*

261 Water deprivation started 23 hours before training. Mice were trained in the head fixation
262 frame for 10 minutes daily. A metal spout was placed in front of the animal's mouth for
263 water delivery. The spout also served as part of a custom "lickometer" circuit, which
264 registered a lick event each time a mouse completed the circuit by licking the spout while
265 standing on a metal floor. The lick events were recorded by a computer through custom
266 software written in LabView (National Instruments). Each lick triggered a single opening
267 of a water valve calibrated to deliver 0.3 μl water.

268

269 It took mice 4–7 days to achieve stable licking, the criterion for which was 10-minute
270 continuous licking with no interval between licking longer than 10 seconds. We used a
271 lick suppression index to quantify animals' degree of photostimulation-evoked
272 suppression of licking behavior: Lick suppression index = $(L_{\text{PRE}} - L_{\text{CS}}) / (L_{\text{PRE}} + L_{\text{CS}})$,
273 where L_{PRE} is the number of licks in the 5 s period before CS onset, and L_{CS} is the
274 number of licks in the 5 s CS period (Yu et al., 2016).

275

276 ***Go/no-go task***

277 Water deprivation started 23 hours before training, and mice were habituated to the head
278 fixation frame for 20 minutes on the first day of training with access to water through the
279 metal spout. On following days, animals underwent 2 training sessions each day, one in
280 the morning and the other in the afternoon. The 2 sessions were at least 4 hours apart,
281 with each consisting of 100 trials. For the subsequent 3-6 sessions, mice were exposed
282 only to the “go” cue (a 1-s, 5-kHz pure tone) followed by the delivery of 4.5 μl of water.
283 After mice successfully retrieved water on at least 80% of the trials, they moved to the
284 next training phase, in which they were required to lick the spout at least 1 time during
285 the go cue in order for the water to be released. This phase took an additional 3-6 sessions
286 until the animals reached the criteria of 80% correct responses. Following this phase,
287 animals received 1 training session consisting of the go cue paired with the delivery of
288 sucrose solution (100 mM) instead of water.

289

290 The next phase consisted of 10 sessions of go/no-go training. During this phase, 50
291 presentations of the go cue were delivered randomly intermixed with 50 presentations of

292 the “no-go” cue (a 1-s white noise), with the constraint that either cue could not appear
293 more than 5 times in a row, and that the first trial was always a go cue. Licking the spout
294 during the no-go cue resulted in the delivery of quinine solution (4.5 μ l, 5 mM). The mice
295 were required to lick at least once the spout during the 1 s window of cue presentation in
296 order to receive the US. During all phases of the experiment, brief suction (500 ms in
297 duration) near the spout was applied 3.5 s after tone onset to remove any residual solution
298 from the previous trial.

299

300 For analysis, trials were sorted into go trials and no-go trials. A correct response during a
301 go trial (“hit”) occurred when the mouse successfully licked the spout during the go cue
302 and subsequently received sucrose. A correct response during a no-go trial (“correct
303 reject”) occurred when the mouse successfully omitted the lick response during the no-go
304 cue and thus avoided quinine. The overall performance over the entire session was
305 calculated as the total correct responses divided by the total trials: overall performance =
306 (hits + correct rejects) / (total trials).

307

308 For the optogenetics experiments we used a modified version of this go/no-go task, in
309 which licking during the go cue led to water delivery (4.5 μ l), whereas licking during the
310 no-go cue resulted in water delivery (4.5 μ l) accompanied by laser stimulation. The laser
311 was delivered coincidentally with water delivery (50 ms after CS offset) at 20- or 30-Hz
312 for 2.5 seconds (the duration that the water would be available if the animal licked during
313 the no-go cue presentation). Suction was applied to remove any unconsumed water.
314 Animals received 8 training sessions in the final phase of this task.

315

316 ***Real time place aversion (RTPA)***

317 As previously described (Stephenson-Jones et al., 2016), one side of a custom chamber
318 (23 × 33 × 25 cm; made from plexiglass) was assigned as the stimulation zone,
319 counterbalanced among mice. Mice were placed individually in the middle of the
320 chamber at the onset of the experiment, the duration of which was 30 min. Laser
321 stimulation (5-ms pulses delivered at 20 or 30 Hz) was triggered when mice entered the
322 stimulation zone, and lasted until mice exited the stimulation zone. Mice were videotaped
323 with a CCD camera interfaced with the Ethovision software (Noldus Information
324 Technologies), which was used to control the laser stimulation and extract the behavioral
325 parameters (position, time, distance, and velocity).

326

327 ***In vivo optogenetics***

328 For bilateral optogenetic stimulation in the CeL, a branched patch-cord (Doric Lenses,
329 Catalog number BFP(2)_105/125/900-0.22_1m_FC-2xZF1.25) for light delivery was
330 connected at one end to a laser source ($\lambda = 473$ nm, OEM Laser Systems) and at the other
331 end, which was composed of two terminals, to two CeL-implanted optical fibers through
332 sleeves (Thorlabs). For photostimulation-induced lick suppression, the stimuli were 5-ms
333 30-Hz light pulses (or across a range of frequencies) delivered for 5 s. For
334 photostimulation during RTPA, 5-ms 20- or 30-Hz light pulses were delivered. Laser
335 intensity was 10 mW measured at the end of optical fiber.

336

337 **Statistics and data presentation**

338 All data are presented as mean \pm s.e.m. All statistics are indicated where used. Data were
339 analyzed with GraphPad Prism. Behavioral tests were performed by an investigator with
340 knowledge of the identity of the experimental groups. All behavior experiments were
341 controlled by computer systems, and data were collected and analyzed in an automated
342 and unbiased way. Virus-injected animals in which the injection or optical fiber
343 implantation was misplaced were excluded.

344

345 **Results**

346 To investigate the function of the IC-CeL circuit, we began by characterizing how IC
347 neurons innervate the major CeL cell types. We first used a modified rabies virus system
348 to trace the monosynaptic inputs onto CeL neurons (Callaway and Luo, 2015; Penzo et
349 al., 2015). This approach revealed a monosynaptic projection from the IC to the CeL
350 (Fig. 1A, B). In particular, IC neurons innervate both of the two major populations of the
351 CeL, the SOM⁺ neurons and PKC- δ ⁺ neurons (Fig. 1A, B). Notably, the IC was the only
352 cortical region identified by this approach to send monosynaptic projections to the CeL.
353 The CeL-projecting IC neurons were preferentially localized in the posterior part of the
354 IC, which overlaps at least partially with the GC (Fig. 1B).

355

356 As the IC also sends projections to the BLA (Allen et al., 1991), we next determined
357 whether the same IC neurons project to both the CeL and the BLA. To this end we
358 injected the CeL and BLA with the retrograde tracer AlexaFluor-488- or 555-conjugated
359 cholera toxin (CTB-488 or CTB-555), respectively (Fig. 1C). We found that CTB

360 reliably labeled CeL-projecting IC neurons (Fig. 1D), with a distribution pattern similar
361 to that of CeL-projecting IC neurons labeled by the rabies virus (Fig. 1B). CTB also
362 labeled a prominent population of BLA-projecting IC neurons, the vast majority of which
363 has a distribution pattern distinct from that of CeL-projecting IC neurons (Fig. 1D). This
364 result demonstrates that CeL-projecting neurons and BLA-projecting neurons in the IC
365 are largely non-overlapping populations.

366

367 To selectively target the CeL-projecting IC neurons and visualize their axonal
368 projections, we injected the CeL with a retrograde canine adenovirus expressing Cre
369 recombinase (CAV2-Cre) (Bru et al., 2010), followed by injecting the IC with an adeno-
370 associated virus harboring a double-floxed inverted open reading frame (AAV-DIO) that
371 expresses, in a Cre-dependent manner, a presynaptic protein synaptophysin tagged with a
372 fluorescent protein mCherry (AAV-DIO-synaptophysin-mCherry) (Fig. 1E). This
373 strategy led to the labeling of IC neurons that sent dense axonal fibers to the CeL (Fig.
374 1F). Sparse labeling of axon fibers in the BLA can also be detected (Fig. 1F), which
375 likely was caused by spillover of the CAV2-Cre to the BLA and thus the labeling of
376 BLA-projecting IC neurons. Though the ipsilateral CeL had the densest projections, we
377 also observed mCherry⁺ axon terminals in the contralateral CeL, consistent with results
378 from retrograde tracing with CTB (data not shown). Altogether, the anatomical tracing
379 results demonstrate that the IC sends robust projections to the CeL, and that the IC-CeL
380 and IC-BLA are distinct circuits.

381

382 To assess the synaptic connectivity between the IC and CeL, we used the *Som-cre;Ai14*
383 mice, in which SOM⁺ cells can be identified by their red fluorescence, and injected the IC
384 of these mice with an AAV expressing the light-gated cation channel channelrodhopsin-2
385 (AAV-ChR2-YFP) that allows photostimulation of axonal projections (Zhang et al.,
386 2006) (Fig. 2A). Approximately 3 weeks after the AAV injection, we prepared from these
387 mice acute brain slices containing the CeL, in which we recorded synaptic transmission
388 onto simultaneously patched pairs of adjacent SOM⁺ (red-fluorescent) and SOM⁻ (non-
389 fluorescent) CeL neurons in response to optogenetic stimulation of the IC inputs. Brief
390 light pulses evoked fast excitatory synaptic transmission in nearly all the recorded CeL
391 neurons (Fig. 2B). Notably, the AMPA receptor-mediated component of synaptic
392 transmission onto SOM⁺ neurons was significantly greater than that onto SOM⁻ neurons
393 (Fig. 2B-C). The NMDA receptor-mediated synaptic transmission onto these two
394 neuronal populations was not different. In a subset of these pairs, we also examined
395 paired-pulse ratio (PPR; see Methods) and found no significant difference between the
396 two cell types (Fig. 2C). These results indicate that IC inputs can activate both SOM⁺
397 neurons and SOM⁻ neurons in the CeL, with the latter being mainly PKC- δ^+ neurons (Li
398 et al., 2013).

399

400 We reasoned that the IC-CeL circuit could have a role in behavioral inhibition, because
401 SOM⁺ CeL neurons are essential for the generation of passive defensive responses,
402 including freezing behavior and action suppression (Fadok et al., 2017; Li et al., 2013;
403 Penzo et al., 2015; Yu et al., 2016), while PKC- δ^+ CeL neurons convey aversive US
404 information and are sufficient to instruct aversive learning (Yu et al., 2017). To test this

405 hypothesis, we set out to inhibit the CeL-projecting IC neurons in mice and subsequently
406 trained these mice in a go/no-go task that engages the IC. We first injected the CeL with
407 the CAV2-Cre (Fig. 3A), and then injected the IC in the same mice with an AAV
408 expressing the tetanus toxin light chain (TeLC), which blocks neurotransmitter release
409 (Murray et al., 2011), or GFP (as a control) in a Cre-dependent manner (AAV-DIO-
410 TeLC-GFP or AAV-DIO-GFP, respectively) (Fig. 3A). As described above (Fig. 1E, F),
411 this strategy led to selective targeting of the IC-CeL circuit (Fig. 3B, C).

412

413 Four to five weeks following viral injections, we began training these mice in the go/no-
414 go task (see Methods), in which an auditory stimulus (go cue) predicts the delivery of a
415 palatable liquid (sucrose), while a different auditory stimulus (no-go cue) predicts the
416 delivery of an unpleasant liquid (quinine) (Fig. 3D). Mice need to learn to produce an
417 instrumental response (lick) during the go cue to receive sucrose, and inhibit that
418 response during the no-go cue to avoid quinine (Fig. 4A). Learning in this task has
419 previously been shown to be paralleled by the development of cue-specific responses in
420 IC neurons (Gardner and Fontanini, 2014).

421

422 We found that bilateral inhibition of synaptic transmission from CeL-projecting IC
423 neurons with TeLC markedly affected animals' behavior in the no-go trials, but left that
424 in the go trials of this task largely unaffected (Fig. 4A, B). Specifically, in the go trials,
425 mice in both the GFP group and the TeLC group showed stimulus-evoked licking (Fig.
426 4A), leading to similar performance (Fig. 4B); although closer inspection of these
427 animals' behavioral patterns revealed that the TeLC mice did not allocate their licking to

428 the CS and US period as much as the GFP control mice, especially on the final training
429 session (Fig. 4A,B). By contrast, in the no-go trials, while the GFP mice gradually
430 learned to withhold licking in response to the no-go cue, and thus successfully avoid
431 quinine in most of the trials towards the end of the training sessions (Fig. 4A, B), the
432 TeLC mice showed no sign of learning and thus were markedly impaired in performance
433 even at the end of the training sessions (Fig. 4A, B). The overall performance of the GFP
434 mice also showed a learning effect, evidenced by a gradual increase in performance over
435 the first 8 training sessions followed by asymptotic performance (Fig. 4B). On the other
436 hand, the animals expressing TeLC showed no such improvement with continued training
437 (Fig. 4B).

438

439 The impairment in behavioral inhibition observed during the no-go trials (or decrease in
440 the “correct reject”) in the TeLC mice could be caused by a general increase in
441 responding. If so, then the performance of these mice in the go trials (measured as the
442 percentage of trials in which the mice made a response; or the “hit” rate) would also
443 increase. However we observed no such increase; in fact, the TeLC group showed a mild
444 decrease in hit rate, in particular in late sessions (Fig. 4B). The TeLC mice also showed a
445 reduced lick rate in response to the go cue, and had lick rate similar to that of the GFP
446 mice following the delivery of sucrose (Fig. 4A, C, D), further arguing against a general
447 increase in responding in these mice. In contrast, these mice showed an increased lick
448 rate specifically to the no-go cue and during the inter-trial interval (ITI) (Fig. 4A, C, E),
449 consistent with the notion that they were impaired in action suppression. We noticed that
450 the TeLC mice had a tendency to show increased lick rate following quinine delivery

451 compared with the GFP mice (Fig. 4D), suggesting that inhibiting the IC-CeL pathway
452 may partially impair the processing of aversive taste information during this task.
453 Alternatively, or additionally, the observations that the TeLC mice showed impaired
454 performance (although mild) and reduced lick rate during the go cue (Fig. 4B,C), as well
455 as impaired performance and increased lick rate during the no-go cue and quinine
456 delivery (Fig. 4B-D) could be explained by an impairment in these mice in anticipation of
457 salient outcomes, a function that has been attributed to the CeA (Balleine and Killcross,
458 2006; Haney et al., 2010; Roesch et al., 2012).

459

460 We also tested a subset of these animals on their sensitivity to increasing concentrations
461 of quinine during a free-licking session (10 minute). Similar to the GFP control group,
462 the TeLC group showed decreased average licking rate during this period (reflecting a
463 reduction in the total volume consumed) with increasing concentrations of quinine (Fig.
464 4F). This result is consistent with previous findings that lesions of the GC in rats do not
465 affect the amount of either quinine or sucrose solutions consumed at varying
466 concentrations (Hashimoto and Spector, 2014), and indicates that inhibition of CeL-
467 projecting IC neurons does not abolish animals' basic ability to process quinine's sensory
468 and aversive properties, at least when there is little cognitive demand. Together, these
469 results indicate that the IC-CeL pathway is required for establishing the learned,
470 anticipatory behavioral inhibition to avoid an aversive tastant.

471

472 Our results suggest that the CeL-projecting IC neurons preferentially regulate the no-go
473 response, consistent with the critical role of the CeL in processing aversive information

474 and in the learning and expression of avoidance behaviors. We therefore tested whether
475 activation of the IC-CeL pathway is sufficient to drive aversive responses as well as
476 instruct learning of an avoidance behavior. For this purpose we delivered the ChR2 or
477 GFP (as a control) specifically into CeL-projecting IC neurons bilaterally, using the
478 retrograde and intersectional strategy based on the CAV2-Cre as described above (Fig.
479 5A), and subsequently bilaterally implanted optical fibers over the CeL (Fig. 5B, C).

480

481 Four to five weeks following surgery, these mice were water deprived and trained to
482 achieve stable licking to a water spout, during which we delivered pulses of blue light
483 into the CeL. Photostimulation in the CeL in which the axon terminals originating from
484 the IC expressed ChR2 elicited robust suppression of licking, an effect that was
485 dependent on the frequency of stimulation (Fig. 5D, E). Furthermore, such optogenetic
486 activation of the IC-CeL pathway induced place aversion in a real time place aversion
487 (RTPA) task (Fig. 5F). By contrast, photostimulation in the CeL in which IC axons
488 expressed GFP induced neither lick suppression nor place aversion (Fig. 5D-F). These
489 results suggest that activation of the IC-CeL circuit is aversive, and is sufficient to induce
490 action suppression and avoidance responses.

491

492 To test whether activation of the IC-CeL circuit can be substituted for an aversive tastant
493 to instruct avoidance learning, similar to learning of the no-go response in the go/no-go
494 task, we trained the same mice as those used in Fig. 5 in a modified go/no-go task, in
495 which licking during one cue led to water delivery alone (the “laser-off” trials), whereas
496 licking during another cue resulted in water delivery coinciding with bilateral

497 photostimulation in the CeL (the “laser-on” trials) (Fig. 6A; also see Methods). We found
498 that the mice in which the IC-CeL pathway expressed Chr2 – and thus could be activated
499 by the photostimulation – showed reduced responding to the cue in the laser-on trials
500 compared with in the laser-off trials (Fig. 6B, C). By contrast, the mice in which the IC-
501 CeL pathway expressed GFP showed similar cue-evoked responding in the laser-on or
502 laser-off trials (Fig. 6B, C). We also verified that the photostimulation did not cause
503 obvious motor effects in either the Chr2 mice or the GFP mice in an open field setting
504 (Fig. 6D).

505

506 Of note, although optogenetic activation of the IC-CeL pathway was less effective than
507 quinine reinforcement, we observed a pattern of responding in these mice qualitatively
508 similar to that of the control animals trained on the standard sucrose/quinine go/no-go
509 task (Fig. 4B), in which the mice showed low levels of responding to the no-go cue and
510 high levels of responding to the go cue. Together, these results indicate that activation of
511 the IC-CeL pathway is sufficient to instruct the learning of anticipatory action
512 suppression. This mechanism is likely engaged when learning to avoid an aversive
513 outcome, such as an unpleasant tastant.

514

515 **Discussion**

516 In this study we examined the role of the IC-CeL circuit in the establishment of
517 behavioral inhibition for avoiding an unpleasant tastant. Using retrograde anatomic
518 tracing approaches, including the modified rabies virus-assisted tracing, together with
519 Chr2-based circuit mapping, we found that the GC region of the IC sends direct

520 excitatory projections to the CeL, which make monosynaptic connections with both the
521 SOM^+ and SOM^- CeL neurons, with the latter being mainly $PKC-\delta^+$ neurons (Li et al.,
522 2013; Penzo et al., 2014). Specific inhibition of the CeL-projecting IC neurons with
523 TeLC prevented mice from acquiring the no-go response, and only mildly affected the go
524 response in a tastant (sucrose/quinine)-reinforced go/no-go task. Furthermore, selective
525 activation of the IC-CeL pathway with optogenetics drove unconditioned lick suppression
526 in thirsty animals, induced avoidance behavior, and was sufficient to instruct conditioned
527 action suppression in response to the cue that predicts the optogenetic activation. These
528 results demonstrate that activity in the IC-CeL circuit is necessary for establishing
529 anticipatory avoidance responses to an unpleasant tastant, and is also sufficient to drive
530 learning of such anticipatory avoidance responses.

531

532 The IC has been shown to contain regions with selective responses to distinct tastants
533 (Chen et al., 2011). As measured in anesthetized animals, these “hotspots” occupy non-
534 overlapping regions within the GC. The region with preferential responses to bitter tastes
535 resides in the posterior part of the GC, which corresponds approximately to the area of IC
536 we identified that sends the strongest projections to the CeL (also see (Allen et al.,
537 1991)). These findings suggest that the IC may preferentially convey information about
538 bitter/aversive tastants to the CeL, which in turn drive behavioral inhibition. Interestingly,
539 although the IC has been shown to be required for identifying and discriminating between
540 tastants (Katz et al., 2001; Peng et al., 2015; Sammons et al., 2016), we did not observe
541 an effect on quinine sensitivity when we selectively silenced the IC-CeL pathway with
542 TeLC (Fig. 4F), suggesting that the ability for bitter tastant identification and that for

543 processing the basic reinforcing properties of bitter tastants are preserved in these mice,
544 at least when cognitive demand is low.

545

546 The CeA, including the CeL, receives aversive information of different modalities
547 directly from the brainstem parabrachial nucleus (PBN) (Carter et al., 2013; Han et al.,
548 2015; Norgren et al., 1989; Sato et al., 2015). Previous studies have shown that the CeA
549 responds to noxious stimuli, such as footshocks that induce somatic pain (Han et al.,
550 2015; Radulovic et al., 1998), colorectal distension that induces visceral pain (Myers and
551 Greenwood-Van Meerveld, 2012), and lithium chloride, which induces malaise and is the
552 most commonly used US for inducing conditioned taste aversion (Lamprecht and Dudai,
553 1995). The CeA has also been implicated in taste processing (Sadacca et al., 2012) and
554 feeding behaviors (Cai et al., 2014). Thus, the CeA is anatomically poised to process
555 convergent somatosensory, visceral, gustatory and aversive information and may be
556 recruited by multiple neural circuits for action suppression in a variety of tasks, in which
557 the goal is to avoid unwanted consequences.

558

559 More recent studies, including those of our own, indicate that the CeA contains
560 functionally heterogeneous neuronal populations. SOM^+ CeL neurons control passive
561 defensive responses, such as freezing and action suppression (Fadok et al., 2017; Li et al.,
562 2013; Penzo et al., 2014; Penzo et al., 2015; Yu et al., 2016), whereas $PKC-\delta^+$ CeL
563 neurons are involved in conveying aversive US information and instructing aversive
564 learning (Yu et al., 2017). The $PKC-\delta^+$ CeL neurons have also been linked to suppression
565 of feeding (Cai et al., 2014). As both of these CeL populations receive direct excitatory

566 inputs from the IC (Fig. 1), they may contribute to distinct aspects of the IC-CeL circuit
567 function described in the current study. An intriguing possibility is that the SOM⁺
568 population induces action suppression when excited by IC inputs, while the PKC- δ ⁺
569 population regulates learning and potentially aversion when activated by the same inputs.
570 Some of the functions mediated by CeL neurons, in particular those by PKC- δ ⁺ neurons,
571 are consistent with the findings that the CeA plays a role in alerting or attentional
572 processes, and can explain our observations suggesting that the IC-CeL circuit may have
573 a more general function in behavior, i.e., it influences performance and actions during not
574 only the no-go trials, but also the go trials of the go/no-go task, although the impact on
575 the no-go trials is much stronger.

576

577 **Figures**

578 **Figure 1. The IC sends monosynaptic projections to the amygdala.** (A) A schematic
579 of the experimental approach (see Methods). (B) Representative images of the tracing
580 result for SOM⁺ (left) and PKC- δ ⁺ (right) CeL neurons. Top, retrogradely labeled neurons
581 in the IC. Bottom, starter neurons in the CeL are identified by their co-expression of
582 mCherry and histone GFP (cells in yellow). Data in A & B were replicated in 3 mice for
583 each group, and were from the same injections reported in (Penzo et al., 2015). (C) Top:
584 a schematic of the experimental approach. Bottom: a representative image of the injection
585 sites. (D) The distribution of the BLA-projecting (red) and the CeL-projecting (green)
586 neurons in the IC. AIC, agranular insular cortex; GIC, granular insular cortex; DIC,
587 disgranular insular cortex. Data in C & D were replicated in two mice. (E) A schematic
588 of the experimental approach. (F) Representative images showing the mCherry⁺ axons

589 (top), which originated from the CeL-projecting IC neurons (bottom). In the insets are
590 enlarged images of the boxed areas in the right, which are located in the amygdala (top)
591 and the IC (bottom).

592

593 **Figure 2: Functional connectivity between IC neurons and CeL neurons.**

594 (A) A schematic of the experimental design. AAV-ChR2-YFP was injected into the IC of
595 a *SOM;Ai14* mouse (left), and patch clamp recording was performed in acute slices
596 containing the amygdala (right). (B) Sample traces of EPSCs recorded from a SOM^+ and
597 a SOM^- CeL neuron, which were recorded simultaneously. The EPSCs were evoked by
598 optogenetic stimulation of the IC axons terminating in the CeL, with the AMPA receptor-
599 mediated EPSCs being stimulated with two pulses (50 ms inter-pulse interval) protocol.

600 (C) Quantification of the EPSC amplitude evoked by the first pulse mediated by AMPA
601 receptors is shown in the left panel, NMDA receptors is shown in the middle, and the
602 paired-pulse ratio (PPR) of AMPA receptor-mediated EPSCs is shown on the right. T-
603 tests revealed that the AMPA receptor-mediated EPSC was larger in SOM^+ than SOM^-
604 CeL neurons (AMPA, $t(18) = 3$, $*P = 0.0085$, NMDA, $t(18) = 0.7$, $P = 0.47$, $n = 10$ pairs,
605 t test; PPR, $t(12) = 1.8$, $P = 0.1$, $n = 7$ pairs, t test). Data in C are presented as mean \pm
606 s.e.m.

607

608 **Figure 3. Experimental design to test the role of the IC-CeL circuit in a go/no-go**

609 **task.** (A) A schematic of the experimental design to selectively inhibit the IC-CeL
610 circuit. (B) Representative images showing the cells in the IC infected with GFP (left
611 panels) and TeLC-GFP (right panels) virus. In the lower panel are enlarged images of the

612 boxed areas in the images in the upper panel. (C) Axon terminals expressing GFP (left)
613 or TeLC-GFP (right), which originated from CeL-projecting IC neurons. (D) A schematic
614 of the go/no-go task.

615

616 **Figure 4. The IC-CeL circuit is required for suppression of behavioral responding.**

617 (A) Population histogram of licking behavior during the first and final sessions of go/no-
618 go training. Licking behavior is represented separately according to go (top panels) or no-
619 go (bottom panels) trials for both GFP (blue; $n = 9$) and TeLC (red; $n = 7$) mice. Dashed
620 line denotes the time window of CS delivery. US was delivered 50 ms after CS offset.
621 Shaded regions represent s.e.m. (B) Quantification of performance (the percentage of
622 correct responding trials) on the go/no-go task. For the go trials, the TeLC animals
623 showed a mild reduction in performance compared to GFP animals (two-way repeated
624 measures (RM) ANOVA revealed a significant interaction, but no main effects: main
625 effect of session, $F(9,126) = 1.4$; $P = 0.2$; main effect of virus treatment, $F(1,14) = 2.4$; P
626 $= 0.1$; interaction, $F(9,126) = 2.8$; $P < 0.01$; $*P < 0.01$, Post hoc Sidak's multiple
627 comparisons test). For the no-go trials, the TeLC animals showed a marked reduction in
628 performance compared to GFP animals (two-way RM ANOVA revealed a main effect of
629 session ($F(9,126) = 2.84$; $P = 0.005$), a main effect of virus treatment ($F(1,14) = 17.56$; P
630 < 0.001), and a significant interaction ($F(9,126) = 4.8$; $P < 0.001$); $*P < 0.05$, Post hoc
631 Sidak's multiple comparisons test). For the overall performance, the TeLC animals
632 showed a reduction in performance compared to GFP animals (two-way RM ANOVA
633 revealed a main effect of session ($F(9,126) = 2.41$; $P = 0.01$), a main effect of virus
634 treatment ($F(1,14) = 24.28$; $P < 0.01$), and a significant interaction ($F(9,126) = 9.8$; $P <$

635 0.01). * $P < 0.01$, Post hoc Sidak's multiple comparisons test). (C) TeLC animals showed
636 a mild reduction in licking in response to the go cue (two-way RM ANOVA revealed a
637 significant interaction, but no main effects (main effect of session, $F(9,126) = 1.9$; $P =$
638 0.06 ; main effect of virus treatment, $F(1,14) = 4.1$; $P = 0.06$; interaction, $F(9,126) = 3.5$;
639 $P < 0.001$; * $P < 0.05$, Post hoc Sidak's multiple comparisons test). TeLC animals showed
640 an increase in licking in response to the no-go cue (two-way RM ANOVA revealed a
641 main effect of session ($F(9,126) = 3.76$; $P < 0.001$), a main effect of virus treatment
642 ($F(1,14) = 15.8$; $P = 0.001$), and a significant interaction ($F(9,126) = 4.45$; $P < 0.001$; * P
643 < 0.05 , Post hoc Sidak's multiple comparisons test). (D) TeLC inhibition of the IC-CeL
644 circuit did not affect US-evoked licking rate ($P > 0.05$, two-way RM ANOVA). (E)
645 TeLC inhibition of the IC-CeL circuit increased the inter-trial licking rate (two-way RM
646 ANOVA revealed a main effect of session ($F(9,126) = 4.13$; $P < 0.001$), a main effect of
647 virus treatment ($F(1,14) = 15.73$; $P = 0.001$), and a significant interaction ($F(9,126) =$
648 2.08 ; $P = 0.037$); * $P < 0.05$, Post hoc Sidak's multiple comparisons test). (F) Quinine
649 sensitivity test showing lick rate during a 10-minute free-licking session for various
650 concentrations of quinine followed by a final session of water. TeLC inhibition of the IC-
651 CeL circuit did not affect quinine sensitivity (two-way RM ANOVA main effect of
652 quinine concentration, $F(7,84) = 16.3$, $P < 0.0001$; main effect of virus, $F(1,12) = 0.78$, P
653 $= 0.4$; interaction, $F(7,84) = 0.24$, $P = 0.97$). All data are presented as mean \pm s.e.m.

654

655 **Figure 5. Optogenetic activation of the IC-CeL circuit is sufficient to induce action**
656 **suppression and avoidance behavior. (A & B)** Schematics of the experimental design.
657 (C) Representative images showing the locations of optical fiber implantation and green

658 axon fibers in the CeL, which originated from CeL-projecting IC neurons expressing
659 ChR2-YFP (left) or GFP (right). **(D)** Raster plots showing licking behavior for a GFP
660 (left) and a ChR2 (right) animal. Blue bars and shaded areas indicate the time window of
661 laser stimulation. **(E)** Quantification of the effect of the photostimulation on licking
662 behavior (suppression index) at different stimulation frequencies ($F(4,14) = 4.375$, $P =$
663 0.017 ; $*P = 0.024$, $**P = 0.007$; one-way ANOVA followed by Sidak's multiple
664 comparisons test; 5-20Hz, $n = 3$ mice; 30Hz, $n = 6$ mice). **(F)** In a real-time place
665 avoidance behavioral paradigm, the ChR2 animals avoided the stimulation side (two-way
666 RM ANOVA main effect of stimulation side, $F(2,16) = 4.27$; $P = 0.03$; main effect of
667 virus treatment, $F(1,8) = 1.53$; $P = 0.25$); interaction, $F(2,16) = 6.94$; $P = 0.007$; $**P =$
668 0.005 , Post hoc Sidak's multiple comparisons test; GFP, $n = 4$ mice, ChR2, $n = 6$ mice).

669

670 **Figure 6. Optogenetic activation of the IC-CeL circuit is sufficient to instruct**
671 **avoidance learning.** **(A)** A schematic of the experimental design. **(B)** Animal behavior is
672 quantified as the percentage of trials that animals chose to lick during CS presentation
673 (chosen trials) (GFP animals: two-way RM ANOVA showed a main effect of session
674 ($F(5,30) = 5.59$; $P < 0.001$, no main effect of trial type ($F(1,6) = 2.07$; $P = 0.2$, and a
675 significant interaction ($F(5,30) = 3.05$; $P = 0.024$; Post hoc Sidak's multiple comparisons
676 tests revealed a significant difference in session 1 ($P = 0.004$, $n = 4$ mice). ChR2 mice:
677 two-way RM ANOVA showed a main effect of trial type ($F(1,8) = 5.7$; $P = 0.04$), no
678 main effect of session ($F(7,56) = 1.35$; $P = 0.25$) or interaction ($F(7,56) = 0.71$; $P = 0.66$;
679 $n = 5$ mice). **(C)** Quantification of the chosen trials in the final training session (two-way
680 repeated measures ANOVA revealed a main effect of group (GFP vs. ChR2) ($F(1,7) =$

681 8.19; $P = 0.024$), a main effect of trial type (laser-off vs. laser-on) ($F(1,7) = 6.59$; $P =$
682 0.037), and a significant interaction ($F(1,7) = 5.778$; $P = 0.047$); $*P = 0.015$, Post hoc
683 Sidak's multiple comparisons test). **(D)** In an open field, laser stimulation did not affect
684 movement of mice (GFP or ChR2 mice), measured as average movement velocity over
685 10 trials ($P > 0.05$, two-way RM ANOVA). All data are presented as mean \pm s.e.m.

686

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688

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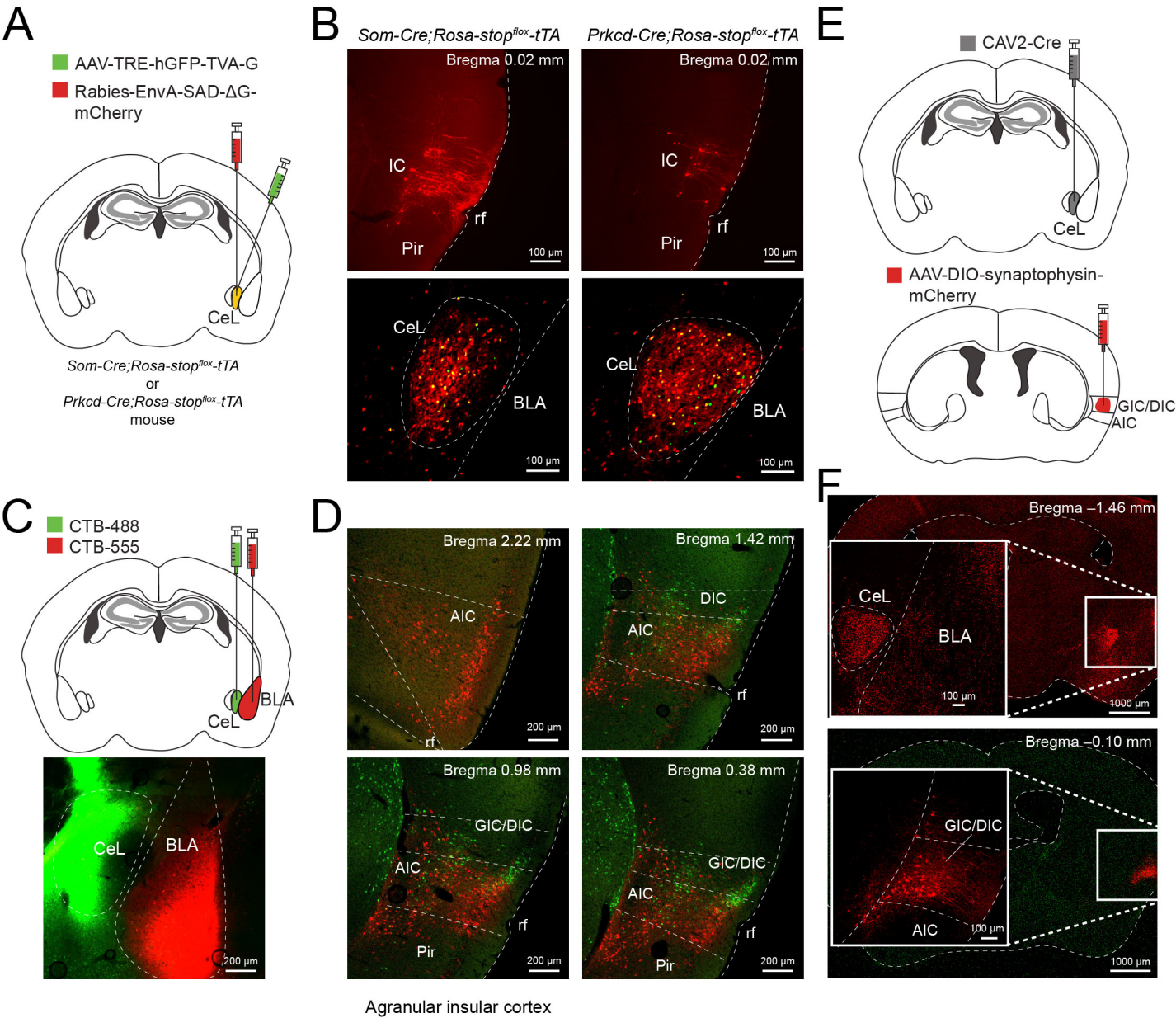
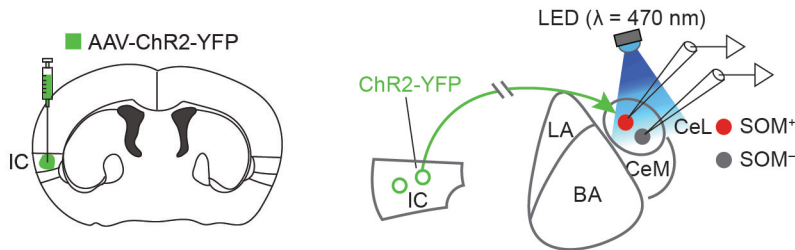
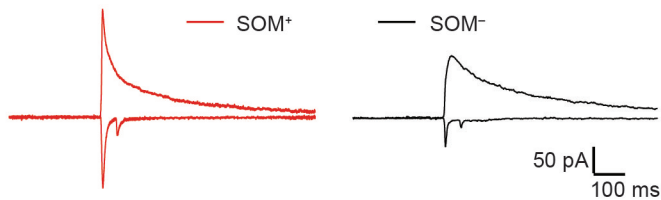
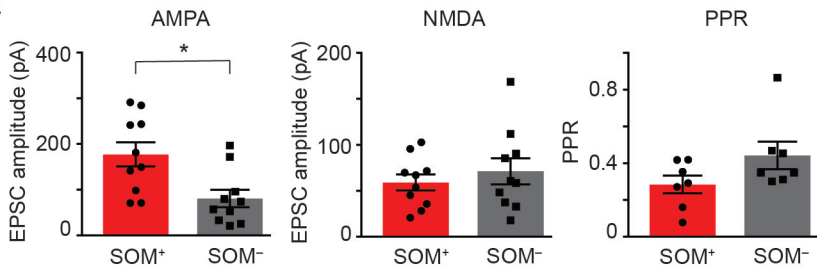


Figure 1

A**B****C****Figure 2**

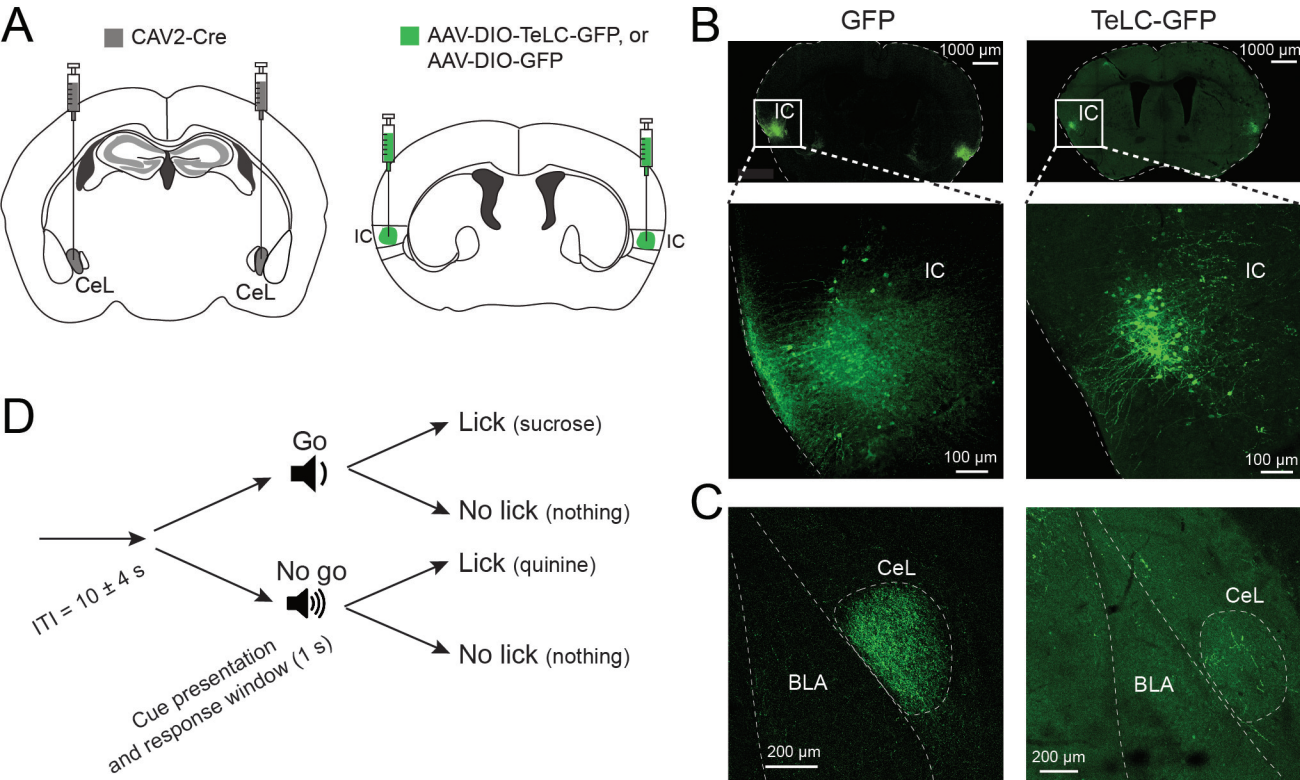


Figure 3

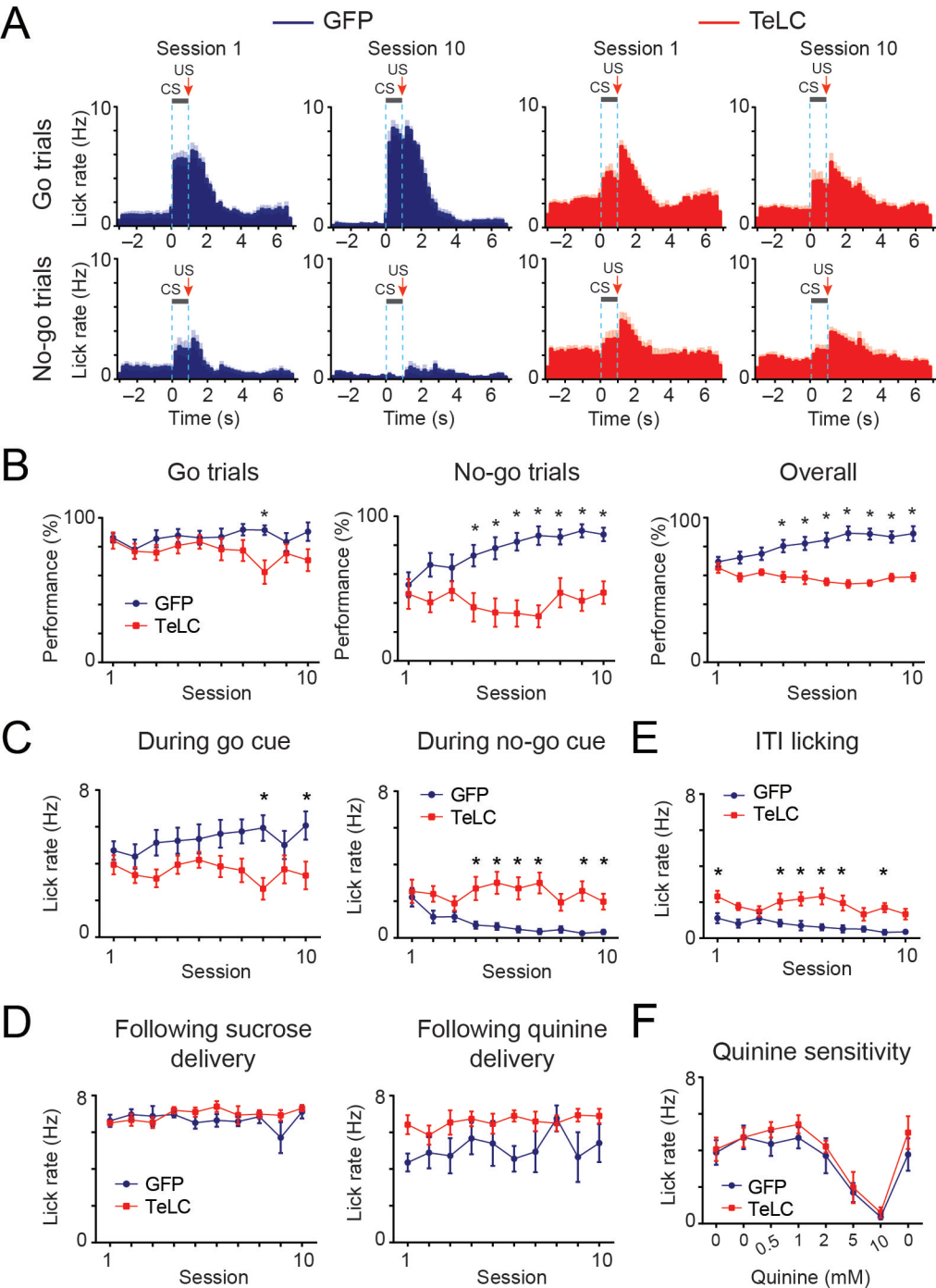


Figure 4

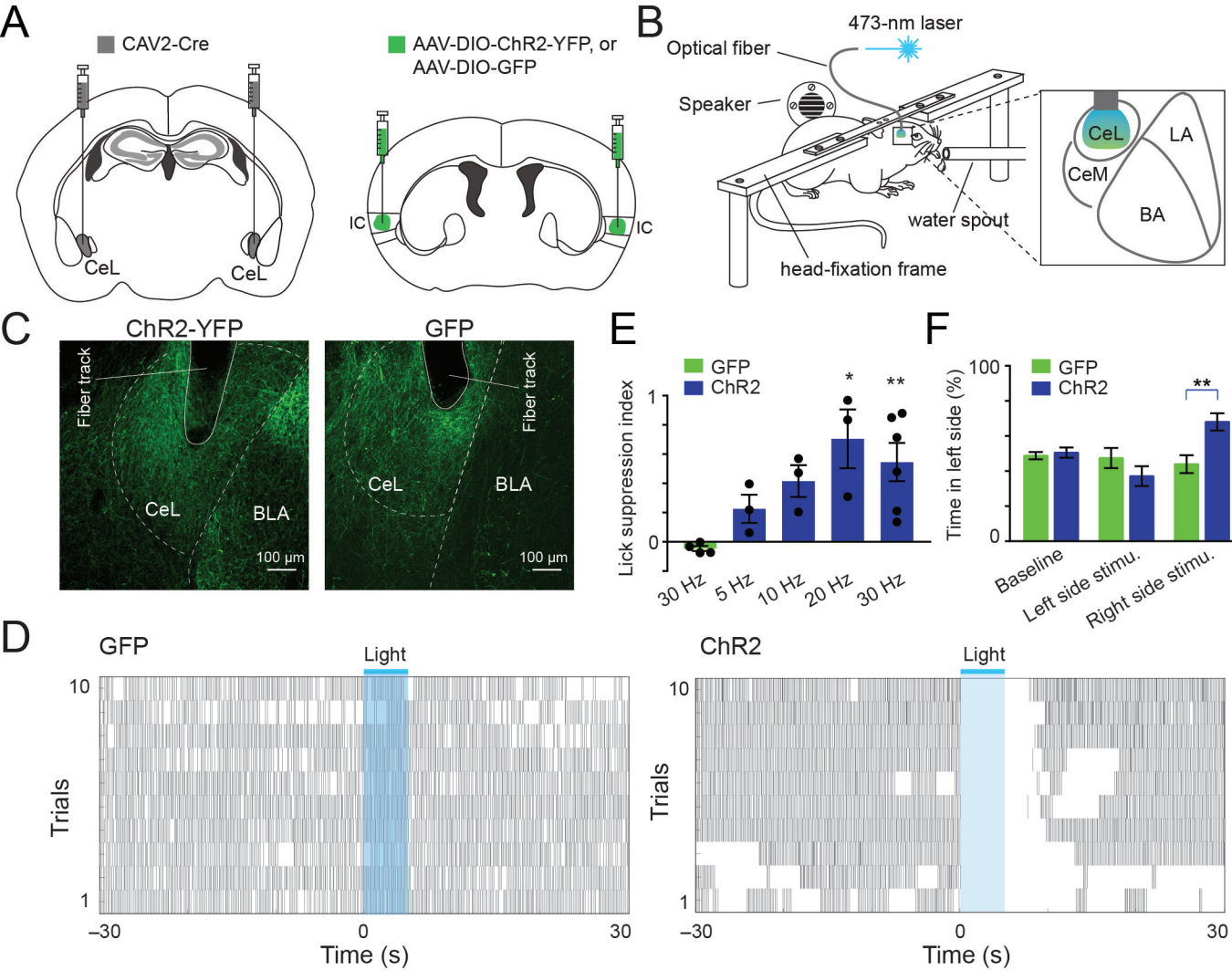


Figure 5

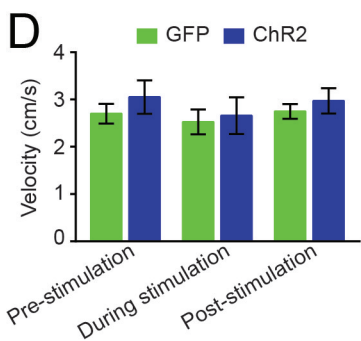
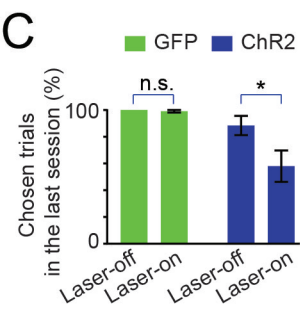
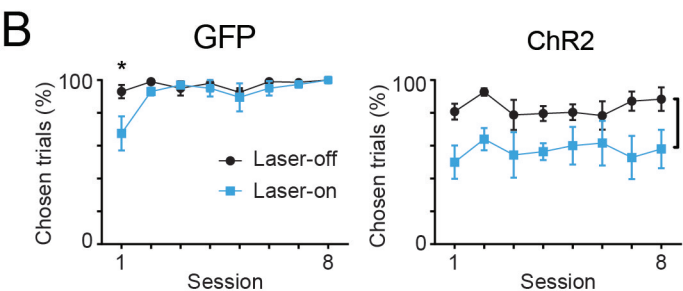
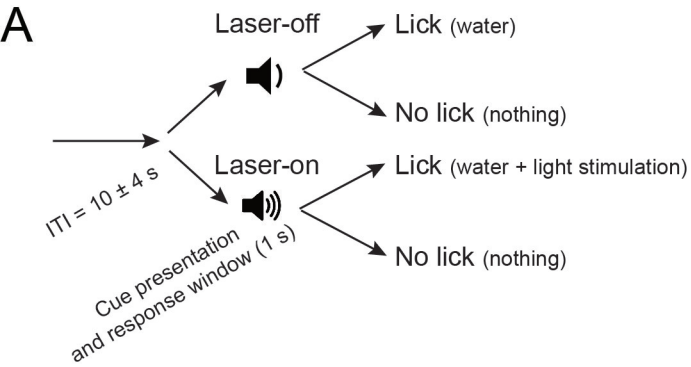


Figure 6