

Supplementary Online Material

Linking emotional valence and anxiety in a mouse insula-amygdala circuit. Nicolas C, Ju A, Wu Y, Eldirdiri H, Delcasso S, Jacky D, Supiot L, Fornari C, Vérité A, Masson M, Rodriguez-Rozada S, Wiegert JS, Beyeler A*.

Content: Online methods, Extended data figures and legends, Extended video legend, References.

Online methods

Experimental animals and housing conditions

All the experiments were performed with adult male and female C57Bl6/J mice, aged 9-10 weeks at the beginning of the experiments from Charles River Company. The animals were housed in controlled conditions of temperature and humidity environment, on a reversed 12 h light/dark cycle and with *ad libitum* access to food and water. Animal maintenance, treatments and experimental procedures were conducted according to French governmental regulations, and approved by the ethical committee and the Ministry of Education, Research and Innovation (Saisine #16579) in accordance with the guidelines of the European Communities Council Directives.

Stereotaxic surgeries

Before the surgery, mice were injected with metacam (5mg/kg) to relieve the pain. Mice were anesthetized with isoflurane (5% induction, 1.5%-2.0% maintenance) in a stereotaxic frame (Kopf) throughout the entire surgery. During the whole surgical procedures, the animals' eyes were protected with Ocry-gel (Laboratoire TVM) to maintain lubrication, and the body temperature was kept at physiological levels, by heating pads.

Injections: Intracranial injections were performed using glass pipettes (3-000-203-G/X, Drummond) made by a puller (PC-100, Narishige) to deliver the retrograde tracer or viral vectors at a rate of 5-8 nL/s using a Nanoject III (3-000-207, Drummond). After completion of the injection, the pipette was raised 100 μ m, left for additional 10 min to allow diffusion of the retrograde tracer or the viral vector at the injection site, and then slowly withdrawn. After surgery, the mouse body temperature was maintained using a heat lamp until the animal fully recovered from anesthesia.

Optic fiber implantation: For fiber photometry and optogenetic experiments, the optic fiber was implanted after the viral vector injections. An optic fiber (fiber photometry: 400 μm diameter, >90% efficiency, 0.39 numerical aperture (NA), optogenetics: 300 μm diameter, >80% efficiency, Thorlabs) inserted in a metal ferrule was implanted 50 μm or 200 μm above the viral vector injection site for fiber photometry and optogenetic experiments respectively. Biocompatible cement and resin were used to keep the implanted fiber fixed in the brain.

At the end of the surgery, the incision was sutured, and the mouse body temperature was maintained using a heating lamp until the animals are fully recovered from anesthesia. Mice returned to their home cages for 1 week to allow CTB retrograde transport or 4/6 weeks to allow viral expression. For details about the coordinates, neuronal tracing and viral vectors used for each experiments see Table 1.

Experiments	Coordinates AP/ML/DV from Bregma (mm)	Viral vector	Volume injected	Titer	Provider
Glutamatergic specific fiber photometry	aIC: 1.7/3.1/-3.5 pIC:-0.35/4.0/-4.2	AAV9-CaMKIIa -GCaMP6f-WPRE	300 nL	1.0x10 ¹³ vg/mL	Addgene
Projection specific fiber photometry	aIC: 1.7/3.1/-3.5	AAV9-syn-Flex-GCaMP6m-WPRE	300 nL	6.3x10 ¹³ GC/mL	UPENN
	BLA:-1.6/3.3/-4.9	CAV2-Cre	300 nL	1.1x10 ¹³ pp/mL	IGMM
Glutamatergic specific chemogenetic	aIC: 1.7/3.0/-3.5	AAV9-CaMKIIa -hM4Di-mcherry or AAV9/2-CaMKIIa -mcherry	250 nL	1.0x10 ¹³ GC/mL 6.3x10 ¹² vg/mL	Addgene ETH Zürich
Downstream targets	aIC: 1.7/3.1/-3.5 pIC:-0.35/4.0/-4.2	AAV9-CaMKIIa a-ChR2-eYFP	300 nL	2.2x10 ¹³ GC/mL	Addgene
Ex vivo electro-physiology	IC: 0.0/4.0/-4.0	AAV9/2-CaMKIIa -hChr2(E123A)-eYFP-WPRE (CheTA 2.0 variant for ultrafast optogenetic control)	250 nL	8.8x10 ¹² GC/mL	UPENN
	BLA:-1.6/3.3/-4.9	CTB AF555	200 nL	NA	Fischer Scientific
	CeM:-0.8/2.35/-5.2	CTB AF647	100 nL	NA	Fischer Scientific
somBiPOLES optogenetic	aIC: 1.7/3.1/-3.5	AAV9-hSyn-DIO-somBiPOLES-mCerulean or AAV9-CAG-Flex-mCerulean	300 nL	6.3x10 ¹⁴ GC/mL	donated from Simon Wiegert
	BLA:-1.6/3.3/-4.9	CAV2-Cre or AAVrg-hSyn-Cre	300 nL	1.12x10 ¹³ pp/mL 1.2x10 ¹³ GC/mL	IGMM Addgene
Optogenetic inhibition	aIC: 1.7/3.1/-3.5	AAV1-hSyn1-SIO-stGtACR2-FusionRed or AAV1-hSyn-DIO-mCherry	300 nL	1.9x10 ¹³ GC/mL 1.0x10 ¹³ vg/mL	Addgene
	BLA:-1.6/3.3/-4.9	CAV2-Cre	300 nL	1.1x10 ¹³ pp/mL	IGMM
Optogenetic excitation	aIC: 1.7/3.1/-3.5	AAV5-Ef1a-DIO-hChr2(E123T/T159C)-eYFP or AAV5-EF1a-DIO-eYFP	300 nL	4x10 ¹² GC/mL	UNC GTC Vector core
	BLA:-1.6/3.3/-4.9	CAV2-Cre	300 nL	1.12x10 ¹³ pp/mL	IGMM

Table 1: Details of coordinates, neuronal tracing and viral vectors used for each experiment.

Surgeries for fiber photometry recordings

For region-specific fiber photometry, AAV9-CaMKIIa -GCaMP6f-WPRE was injected into aIC or pIC and an optical fiber (400 μ m core, 0.39 NA, Thorlabs) was then implanted 50 μ m above the injection sites.

For projection-specific fiber photometry, a dual virus strategy was used, an adeno associated virus 9 (AAV9) carrying the GCaMP6m gene, under the control of the human synapsin promotor (hSyn), for exclusive neuronal expression, and in a double-floxed inverted open reading frame (DIO), to be expressed in a cre-dependent manner was used. AAV9-syn-Flex-GCaMP6m-WPRE and CAV2 vector expressing cre recombinase (CAV2-Cre) were injected in aIC and BLA, respectively.

Surgeries for chemogenetic manipulations

For region-specific chemogenetic inhibition, AAV9-CaMKIIa-hM4Di-mCherry or the control virus AAV9/2-CaMKIIa-mCherry was injected into aIC.

Surgeries for optogenetic manipulations

For somBiPOLES experiments, an adeno-associated virus 9 (AAV9) carrying the soma-targeted BiPOLES (somBiPOLES) gene under the control of the human synapsin promotor (hSyn) and in a double-floxed inverted open reading frame, (AAV9-hSyn-DIO-somBipoles-mCerulean, donated from Simon Wiegert, University of Hamburg) was injected bilaterally in the aIC. AAV9-CAG-Flex-mCerulean (donated from Simon Wiegert, University of Hamburg) was injected bilaterally in aIC, as control. In the BLA, CAV2-Cre or AAVrg-hSyn-Cre were injected bilaterally and counterbalanced between the mice receiving somBiPOLES or the control virus.

For optogenetic excitation, an adeno-associated virus 5 (AAV5) carrying the Channelrhodopsin 2 (ChR2) gene under the control of elongation factor 1a (EF1a) and in a double-floxed inverted open reading frame, (AAV5-Ef1a-DIO-hChR2(E123T/T159C)-EYFP) was injected in the aIC and CAV2-Cre was injected in the BLA. For control mice, an AAV5 EF1a-driven expressing eYFP in cre-dependent manner was injected bilaterally in aIC and CAV2-Cre in BLA.

For optogenetic inhibition, a synapsin-driven, cre-dependent, adeno-associated virus 1 (AAV1); carrying the soma-targeted Anion conducting Channelrhodopsin 2 (stGtACR2) gene, in a single-inverted floxed open reading frame (SIO) fused to FusionRed (AAV1-hSyn1-SIO-stGtACR2-FusionRed) were injected bilaterally in the aIC and CAV2-Cre was injected bilaterally in the BLA. For control mice, an

AAV1- hSyn-driven expressing mCherry in cre-dependent manner was injected bilaterally in aIC and CAV2-Cre in BLA.

Surgeries for anatomical characterization of IC downstream targets

To label the projections, an anterograde viral vector carrying the gene coding for a green fluorescent protein (AAV9-CaMKIIa-ChR2-eYFP) has been injected in the aIC or in the pIC (Fig. 3a).

Surgeries for ex vivo electrophysiology

To measure the dynamic properties of the IC-BLA or IC-CeM neurons, AAV9/2-CaMKIIa-hChR2(E123A)-eYFP-WPRE was injected in the IC (Fig. 4a). For the reciprocal connection between the IC and BLA, mixture of AAV9/2-CaMKIIa-hChR2(E123A)-eYFP-WPRE and CTB (1:1) was injected in the IC or the BLA, respectively (Fig. 4h,i). hChR2(E123A) is the hChR2 variant CheTA 2.0 for ultrafast optogenetic control^{1,2}. To analyze the intrinsic property of the insular neurons projecting to the BLA and CeM, CTB coupled to AF555 or AF647 (Fischer Scientific) were injected in the BLA or CeM, respectively, to label the insular neurons projecting to the BLA or CeM (Fig. 4j,k,l).

Behavioral assays

One week before the fiber photometry and optogenetic experiments, the animals were handled for 30 minutes a day, at least once a day, and habituated to being connected to the optic fiber. All tests are performed during the dark phase of the reversed light/dark cycle, using red light to observe the behavior (15 ± 3 lux near the behavioral setups). The behavioral mazes were always cleaned with acetic acid 2%, and then distilled water, before each animal session. The animal weight was measured every day, before the test. One camera on top of the experimental maze was used for all the tests, to track the animal's position. Video recordings were synchronized with the photometry signal recordings. An additional camera was put on the side of the apparatus, in the tests that involved drinking or pain response. Temperature and humidity of the experiment room were controlled at 20 to 24 Celsius degrees and 45% to 65%, respectively.

Anxiety assays: We used two classical anxiety tests, the elevated plus maze (EPM) and the open field test (OFT). In these tests, the apparatus is divided into an anxiogenic area and a non-anxiogenic area.

- *Elevated plus maze*: The arena has a plus shape (75 x 75 cm), consisting of two open arms (anxiogenic space) and two closed arms (non-anxiogenic space). Each animal was placed at the center of the maze and left to explore it for 15 minutes.

- *Open field test*: In this test, the arena is squared (60 x 60 cm), and the anxiogenic space is the center, while the non-anxiogenic space are the borders the field. The center is defined as 50% of the total OFT arena. Each animal was put in the open field and left to explore it for 15 or 20 minutes.

Valence assays: For the following tests, food deprivation was done 20 hours before the end of the experiment. For food deprivation, the litter was always changed to remove food scrubs. In those tests, animals' weight was measured after 2 hours of free access to food.

- *Positive valence*: sucrose rewards (15% sucrose in tap water) were available in a 60 x 60 cm squared arena with transparent walls, after 20h of food deprivation. The sucrose consumption was measured using a LED that blinked each time the animal licked from the sucrose port. The test duration was 15 minutes.

- *Negative valence*:

[1] *Quinine (1 mM) consumption*: quinine is a bitter substance very aversive to mice. As mice will taste it only once, we developed a device that will present either sucrose or quinine every 30 seconds, in order to entice the animals to consume quinine multiple times. Mice were food-deprived 20 h before the test, and the test lasted 15 minutes.

[2] *Mild footshock*: in order to assess the neuronal response to another negative valence stimulus, we applied 10 mild footshocks at 300 μ A for 1 second every minute.

[3] *Tail suspension*: the tail suspension test (TST) was used as an aversive experience. The mice were suspended by the hand of the experimenter at approximately 40 cm from the floor for 1 minute, while the calcium signal of aIC-BLA neurons was recorded.

Real-time place preference/aversion (RTPP/A)

This assay was performed over two days for 20 min session each day, in a squared area (60 x 60 cm) divided into two equal chambers. Mice have free access to both chambers through an opening in the central separation. To differentiate the chambers, one was assigned alternating black and white vertical stripes on its walls, while the other with a black circle on the wall. For light optogenetic stimulation protocols see optogenetics section.

Fiber photometry recordings

Signal recordings: To record the fluorescence signals, the fiber photometry system was custom-made and configured as previously described^{3,4}. The optical fiber implanted in the aIC or pIC transmitted the light emitted by the LEDs to excite GCaMP6 chromophore. The 20x objective was connected to a CMOS camera that detected the fluorescence level, which is indicative of the calcium levels and neural activity *in vivo*. The optimal wavelength for GCaMP6 excitation is 470 nm, while the isobestic wavelength is 405 nm (Extended data Fig.1a). This 405 nm channel was used as a negative control, since it is independent of calcium concentration, and allowed us to remove motion-related artifacts and signal unrelated to neuronal activity. Two excitation LEDs (470 nm and 405 nm, Thorlabs) were bandpass filtered and used to excite calcium-dependent and -independent fluorescence from GCaMP6. The filtered excitation lights were reflected by a 495 nm long-pass dichroic mirror and then delivered to the target brain region through a patch cord (0.29 NA, 200 μ m) coupled with the implanted fiber. To minimize the photobleaching effect of the recording, the light intensities in the tip of the patch cord were adjusted to \sim 140 μ W for the 470 nm channel and \sim 58 μ W for the 405 nm channel. The GCaMP6 emission light was bandpass filtered and focused on complementary metal-oxide semiconductor (CMOS) camera sensor for fluorescence detection. A custom Matlab script was used to synchronize fiber photometry and video recordings (revised from: <https://github.com/deisseroth-lab/multifiber>), combined with a programmed Arduino UNO board. The sampling rate was settled at 20 Hz for both photometry and video recording.

Data analysis: After the behavioral tests, photometry recordings were analyzed using custom Matlab scripts achieving the following functions:

- [1] Remove the first minute of the recording to avoid LED stabilization and photobleaching artefacts. Each recorded fluorescence image was synchronized with the GCaMP6 light excitation made by the LEDs (470 nm and 405 nm).
- [2] Normalize the 470 nm and 405 nm signals over the test, by subtracting the mean fluorescence from the fluorescence recorded at each time point and dividing this value by the mean fluorescence $((F-F_{mean})/F_{mean}) = \Delta F/F$. The mean fluorescence was calculated over a 60 seconds sliding window, in order to remove photobleaching effects occurring over time.

[3] Subtract the calcium independent signal (405 nm) from the normalized GCaMP6 signal (470 nm), to eliminate unspecific fluorescence (including potential movement artifacts): $\Delta F/F = \Delta F/F_{Ca^{2+}} - \Delta F/F_{isosbestic}$.

The obtained result was the global signal ($\Delta F/F$) that was used as an estimate of tonic activity of the recorded neurons.

[4] Measure calcium transients: the global signal was bandpass filtered (Low threshold 0.2 Hz, and high threshold 6 Hz). Filtered peaks were detected as high-amplitude events (defined as events with amplitudes two median absolute deviation (MAD) above the median of the sliding window), were then filtered out from the signal, and the median of the trace re-calculated excluding those peaks. Peaks with a local maxima greater than two MADs of the resultant trace, are identified as transients, which is used as an estimate of phasic activity of the recorded neurons. Average peak amplitude and peak frequency were compared across groups (adapted from Muir et al.,)⁴.

[5] Synchronization of the fluorescence signal with the location of the animal in the maze (EPM or OFT), recorded by the behavior camera and detected using the Bonsai software.

[6] Map the global calcium signal depending on the location of the animal in the entire mazes (EPM or OFT, Fig. 1 and 5).

[7] Average the global calcium signal depending on the location of the animal in the maze: open arm, closed arms and center of the EPM, or center and borders of the OFT.

In the end, we obtain three variables as results: the global signal, the transients' frequency, and the transients' amplitude. For event-based tests (sucrose and quinine consumption, footshock and tail suspension tests) we performed an event-based analysis (per-stimulus average), where the signal was averaged and compared between defined periods: before and after event onset. All results were statistically compared using student paired t-tests.

Chemogenetics

Behavior: A week before the EPM and OFT tests mice received one intraperitoneal (ip) injection of saline for two days for habituation. On test days, mice received an ip injection of clozapine-N-oxide (CNO, 3 mg/kg) 30 min before the test. On test day 1, mice were placed in the EPM for 15 minutes. On test day 2, mice were place in the OFT for 15 minutes.

Data analysis: The software Bonsai was used to track the mouse location and the data were analyzed with custom Python script to extract the time spent in the open and closed arms of the EPM and in the center and the boarders of the OFT. The distance travelled and the time speed were also measured for both tests. Two mice were excluded from the EPM analysis because they spent less than 3% of the total time in the open arms.

Optogenetics

somBiPOLES experiments

Six weeks after surgery, mice were habituated to be handled and to be connected to the optic fiber. Videos were recorded using a webcam (Logitech). Bilateral stimulation of aIC neurons was achieved by connecting the fiber implant to a 1x2 Step-index multimode fiber optic coupler (200 μ M diameter, 0.39 NA, Thorlabs, Germany) in turn connected to a 1x2 Fiber-optic Rotary Joints - Intensity Division (RJ-ID, 400-700nm, Doric Lenses, Canada 200 μ M diameter). The rotary joint splitter was fixed on top of the arena and connected on the other side through a patch cord to a mini-cube wavelength divider (DMC_1x2w_FC, [470nm:590nm,470], Doric Lenses, Canada) which functions as a laser combiner system housing patch cords from a 473 nm laser source [MBL-III-473-100mW, Opto-Engine, USA] and a 593 nm diode laser [MGL-F-593.5 – 50mW, Opto-Engine, USA] for activation of the *GtACR2* and Chrimson components of somBiPOLES, respectively. Coupling to the implant was done with zirconia mating sleeves (1.25, Doric lenses, Canada). Stimuli were generated according to regions of interest and time epochs defined in custom-written code in bonsai software and laser was triggered using Arduino-Uno board.

For activation of Chrimson, pulse trains (593 nm, ~10 mW at each fiber end, 20 ms pulse duration, 20 Hz repetition rate) of 3 mins epoch were presented, while *GtACR2* was activated by pulse trains (473 nm, ~20 mW at each fiber end, 5 ms pulse duration, 20 Hz repetition rate) of 3 mins epoch. Light emitted was tested to ensure, an inter-train interval of 2 s between each epoch and to not overlap with laser lights, through a custom-written Arduino code.

For EPM, the experiment was with 3 mins epochs, in which 1 epoch of orange light stimulation was followed by an epoch of blue light stimulation and an epoch of no stimulation. This was repeated once, making a

total duration of 18 minutes. For OFT, the same epochs design was used, but for 4 mins each, a total duration of 24 minutes.

ChR2 and GtACR real-time place preference/aversion (RTPP/A)

On the first day, mice were allowed to freely explore the two chambers with no light stimulation. The natural preference of the animal for one side is characterized by the side where the mice spend more time and is defined as the preferred chamber.

On the second day, according to the preference exhibited, half of the mice were stimulated in their preferred side, and the other half in the non-preferred side. At the start of the 20 min session, individual mice were placed in the unstimulated chamber. For the optogenetic inhibition (GtACR), each time the mouse entered the stimulated side of the arena, light stimulation (5 ms pulses, 20 Hz, 20 mW, 473 nm laser) was delivered until the mouse crossed back into the non-stimulated chamber. For optogenetic activation (ChR2) each time the mouse entered the stimulated chamber, light stimulation (20 ms pulses, 20 Hz, 10 mW, 593nm laser) was delivered until the mouse crossed back into the non-stimulated chamber.

The Place preference index was calculated as below: (T=time)

$$\text{Place preference index (PPI) \%} = \frac{T_{\text{stim chamber}} - T_{\text{non-stim chamber}}}{T_{\text{stim chamber}} + T_{\text{non-stim chamber}}} \times 100$$

Brain extraction, histology and imaging

After fiber photometry recordings, chemogenetic, ChR2 or GtACR optogenetics experiments, the animals were euthanized with pentobarbital (300 mg/kg) and perfused with ringer's solution followed by 4% PFA (antigenfix, F/P0014, MM France) at 4°C. The brains were extracted and left in 4% PFA overnight at 4°C, for post fixation. Then, they were transferred to a 30% sucrose solution, in PBS 1X at 4°C, for cryoprotection. After sinking in the sucrose solution, the brains were embedded in optimum cutting temperature compound (OCT) and sliced into 100 µm coronal slices, in a freezing sliding microtome (12062999, Thermo Scientific). Sections were incubated with a DNA-specific fluorescent probe Hoechst33342 (1:5000; Fischer scientific) for 20 mins, washed with PBS 1X followed by mounting on microscope slides with Polyvinyl alcohol PVA-DABCO mounting medium (Sigma Aldrich, France). Images were acquired at the fluorescence microscope (THUNDER Imager 3D Tissue, Leica), using the de-convolution system.

cFos immunohistochemistry

For somBiPOLES optogenetic experiments, cFos stimulation was performed by connected the mice to a patch cord (200 μ M diameter, 0.39 NA, Thorlabs, Germany), and orange light (593 nm, ~10 mW at each fiber end, 20 ms pulse duration, 20 Hz repetition rate) was delivered for 5 min, while the mice were in their homecage. After an hour and a half, animals were anesthetized with pentobarbital (300 mg/kg) and transcardially perfused. After cryoprotection in PBS 1X-buffered sucrose (30%) solution, brains were sliced to 50 μ m coronal brain sections using a frozen sliding microtome (12062999, Thermo-Scientific). Brain sections were first washed in PBS 1X (3x 5min) at room temperature (RT) and then were blocked in 3% normal goat serum (NGS) in PBS 1X (0.3% Triton-X-100) for 2 hours at RT. Slices were incubated overnight at 4°C in rabbit polyclonal anti-cFos (1:500, Synaptic System, #226-003). Then slices were washed with PBS 1X (3 x 10 min), followed by incubation with fluorescent secondary antibodies Alexa Fluor 555 Goat anti-rabbit IgG (1:1000, Fischer scientific, #1890860) at RT for 2 hours. After washing with PBS 1X (3 x 10 min), the slices were incubated with Hoechst33342 (1:5000) for 10 mins before a final washing step. Finally, brain slices were mounted, and cover slipped using PVA-DABCO (Sigma Aldrich). Immediate-early gene cFos were used to quantify optogenetic activation in somBiPOLES experiment, as a marker for neuronal activation in aIC-BLA neurons. Images of fiber implantation sites, viral expression, and injection sites were taken using a fluorescence microscope (Leica Microscopy) and were overlaid on the coronal atlas of Paxinos [4th edition]⁵. For confocal microscopy, images were captured through a 10x dry objective (NA 0.70) and a 40x oil-immersed objective (NA 1.30) from a Leica SP5 confocal microscope (Leica Microscopy). For cell counting of cFos and insula projections, z-stacks of ROIs (z-step: ~2-4 μ m, between stacks, 1024 x 1024 pixels) were scanned using the 40x objective and cell counting was only made on the maximum projection of merged pictures. Leica software and Image-J were used to process images and count cells.

Inclusion criteria

Mice were excluded from the analysis when optic fiber placement or viral expression patterns were out of brain targets. For EPM, mice were excluded from the analysis when they fell off the EPM and/or when they spent less than 3% of the total time in the open arms. Mice that exhibited freezing behavior in anxiety and

valence tests were excluded, as they were considered biasedly stressed. Mice in fiber-photometry experiments were excluded when the signal-to-noise ratio was below control mice, or with no signal.

Anatomical characterization of IC downstream targets and aIC-BLA axonal collaterals

Images were acquired with a Leica SP8 confocal microscope (Leica Microscopy). First, all processed brain slices, as well as all slices containing an injection site were imaged using a 10X objective. Z-stacks of the 12 downstream regions have been captured in a picture format of 1024x1024 pixels (Number of z- stacks: 30, z-steps: $\sim 1 \pm 0.10 \mu\text{m}$, 2-3 frame average). All images have been processed using the open source Fiji software (ImageJ, NIH).

Fluorescence was quantified using custom Python script. Fluorescence values from digitized tiff images in 8 bits encoded as a number between 0 and 255 were divided by 255, to bring the fluorescence intensity range between 0 and 1.

For quantification of eYFP-expressing axonal projection from glutamatergic neurons of aIC and pIC in Fig. 3, the images were then z-projected across 30 slices using a maximum intensity projection. A thresholding procedure was used to separate axon fluorescence from background. A threshold of 0.5 was set across all images in the green (eYFP) channel. These thresholds were chosen by an experimenter (blind to the imaged area and animal) observing the results of thresholding with different threshold values across multiple images (~5 images). eYFP fluorescence was quantified as the fraction of pixels in the image above this threshold.

Ex vivo electrophysiological recordings

Preparation of acute brain slice: One week after retrograde tracer injection or 4-5 weeks after viral vector injection, mice were anesthetized with pentobarbital (300 mg/kg) and perfused transcardially, with 20 ml of modified artificial cerebrospinal fluid (ACSF, at $\sim 4^\circ\text{C}$) containing (in mM): 75 sucrose, 87 NaCl, 2.5 KCl, 1.3 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, 25 NaHCO₃ and 5 ascorbic acid. The brain was then extracted and 300 μm thick coronal brain slices containing the insula, BLA or CeM were collected by a semi-automatic vibrating blade microtome (VT1200; Leica) inside ice-cold modified ACSF. Acute coronal sections were incubated in oxygenated ACSF containing (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 1.0 MgCl₂, 2.4 CaCl₂, 26

NaHCO₃, 10 glucose (pH 7.25-7.4; 300±2 mOsm). Recordings were started one hour after slicing and the temperature was maintained between 31–33°C both in the holding chamber and during the recordings. All injection sites were checked and imaged with the microscope (BX51, Olympus).

Whole-cell patch-clamp recording: Recordings were made from CTB-labelled neurons in the insula for the intrinsic property of insular-amygdala projection neurons and from neurons in BLA or CeM or CTB-labelled neurons for channelrhodopsin-assisted circuit mapping (CRACM) and their synaptic properties. Borosilicate glass capillaries were pulled by P-1000 puller (Sutter Instrument) and the recording electrodes (4-6 MΩ) were filled with K-gluconate-based internal solution containing (in mM): 125 potassium gluconate, 20 HEPES, 10 NaCl, 3 MgATP, 8 biocytin and 2 Alexa Fluor 350 (pH 7.25-7.4; 280-290 mOsm). Voltage- and current-clamp recordings were conducted using a Multiclamp 700B amplifier (Molecular Devices). Analog signals were low-pass filtered at 1 kHz and digitized at 10 kHz using a Digidata 1440 and pClamp9 software (Molecular Devices). ACSF and drugs were applied to the slice via a peristaltic pump (Minipuls3, Gilson) at 2 mL/min.

The intrinsic properties (passive and active) of neuronal membrane were measured from all patched neurons. The passive properties of neuronal membranes were analyzed by seal test (5 mV, 1 sec) and voltage-step mode (-110-+30 mV for 1 sec, 10 mV step). In the current clamp recording, the current was injected in ramp mode (0-300 pA for 1 sec) for measuring the firing threshold and rheobase and step mode (0-300 pA for 2 sec, 20 pA step) for acquiring injected current-action potential frequency relationship.

For CRACM of insular inputs onto BLA or CeM neurons or reciprocal connection between the insula and BLA, ChR2 expressed in the axonal fibers was activated in the downstream regions using a LED light source (470 nm, CoolLED p4000) and the average power to trigger a light response in the patched neurons was 0.17±0.06 mW/mm². To test if the response of ChR2 terminal activation was monosynaptic, optogenetically-evoked excitatory/inhibitory postsynaptic current (oEPSC/oIPSC) was measured with holding potential clamped at -70mV during the bath application of the sodium channel blocker, tetrodotoxin (TTX, 1 μM) and the potassium channel blocker, 4-aminopyridine (4AP, 100 μM)^{6,7}. NMDA-R (glutamate receptor selectively activated by N-methyl-D-aspartate) antagonist, D-(-)-2amino-5-phosphonopentanoate (AP5), and AMPA-R (glutamate receptor selectively activated by 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoate) antagonist, 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide

(NBQX), were applied to confirm whether the monosynaptic contacts were abolished by blocking all the glutamatergic receptors. At a holding potential of -70 mV for EPSCs and of 0mV for IPSCs, paired and ten trains of light stimulations (2 ms, 50 ms interval) were given to test short-term synaptic plasticity.

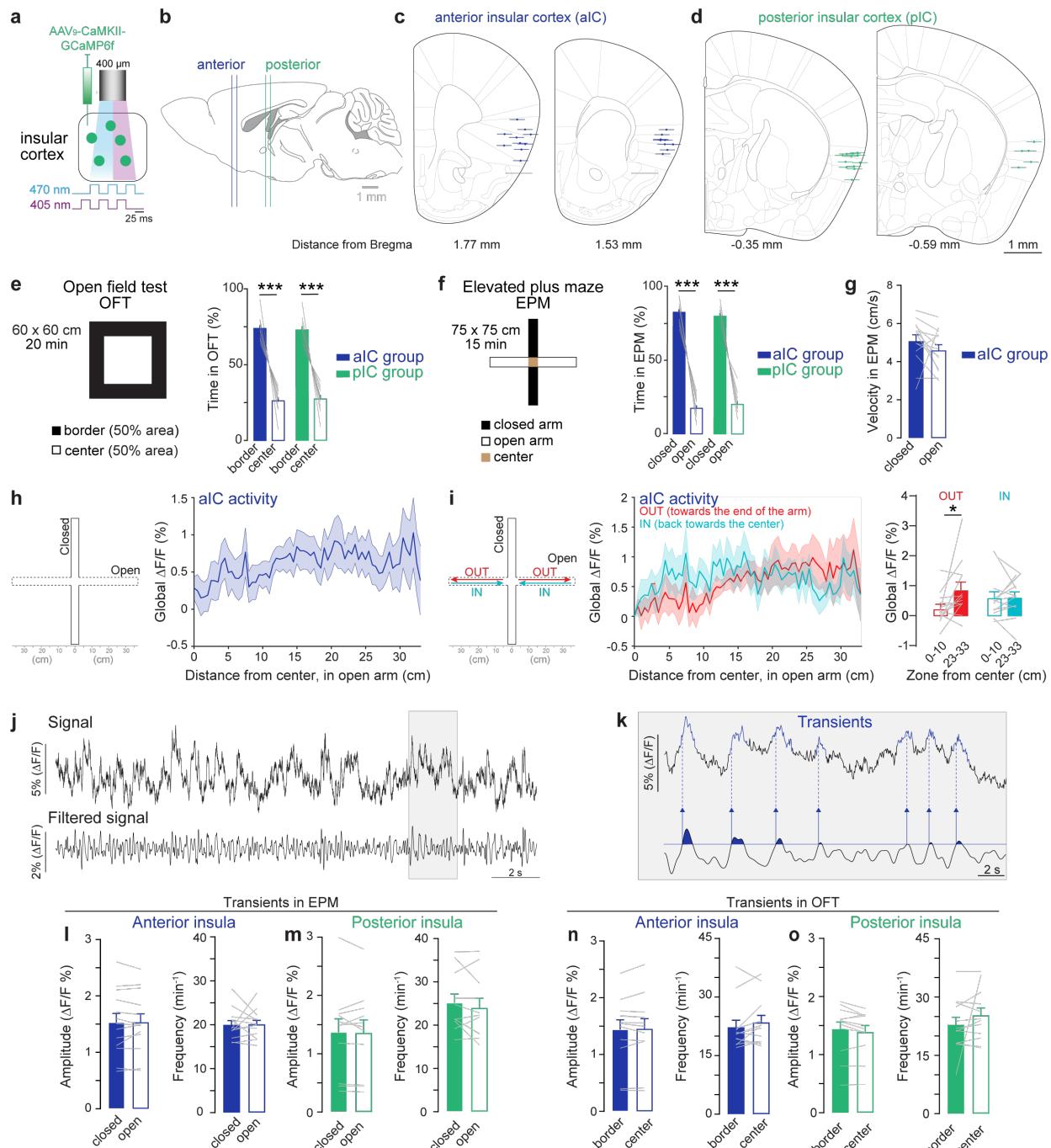
The location of all recorded neurons was checked after the recording (Extended data Fig. 2e) and only the cells located in the aIC, pIC, BLA or CeM were kept for further analysis.

Data analysis: All the electrophysiological data were analyzed by Clampfit software (Molecular Devices) and custom-made python script.

Statistical analysis

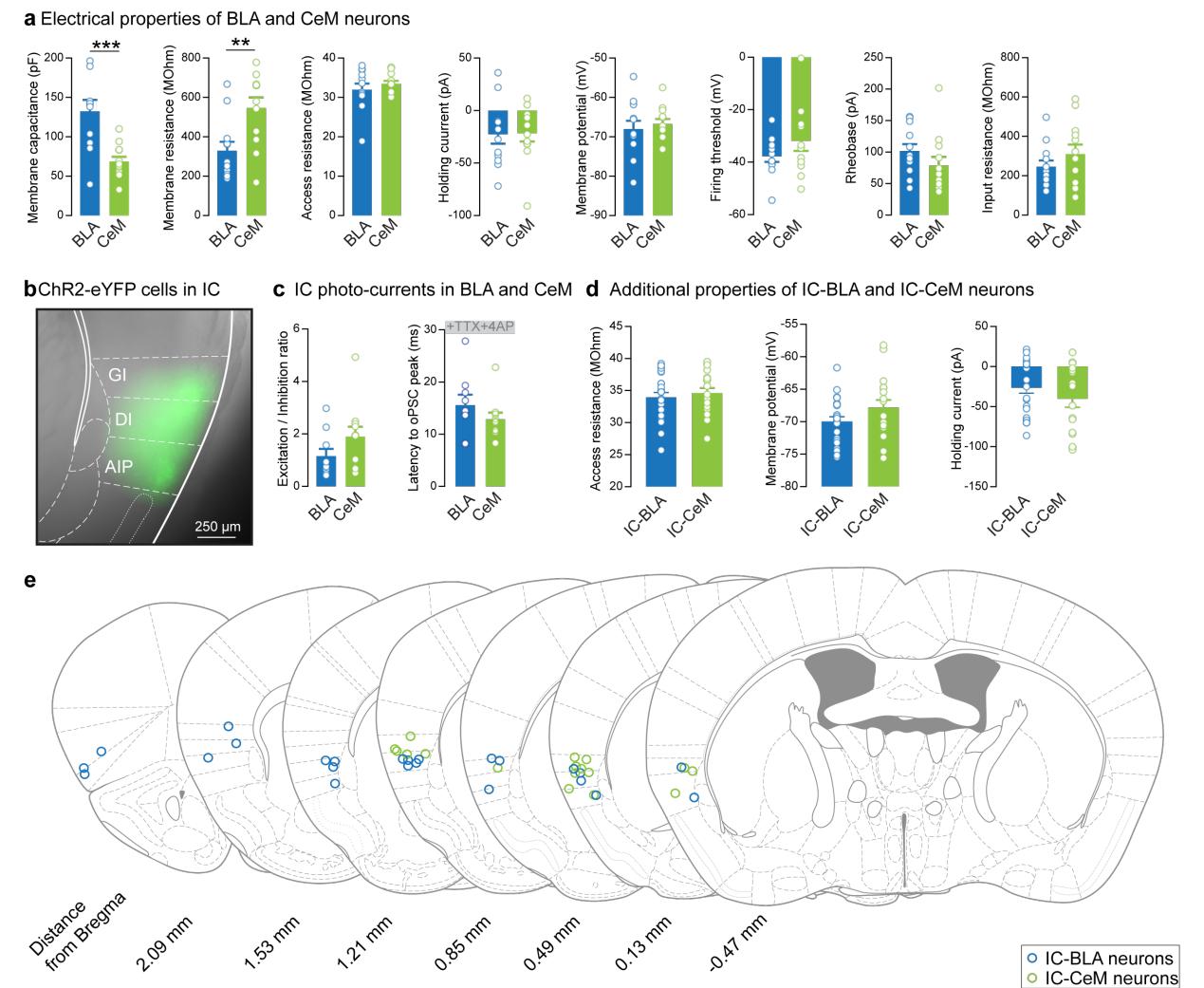
We used factorial ANOVA and t-tests using Matlab or GraphPad Prism 9. When we obtained significant interaction effects ($p < 0.05$, two-tailed), we followed them with post hoc tests (Fisher LSD). Because our multifactorial ANOVA yielded multiple main and interaction effects, we only report significant effects that are critical for data interpretation. For correlation analyses the Pearson's correlation coefficient was calculated (r^2).

Extended data figure legends

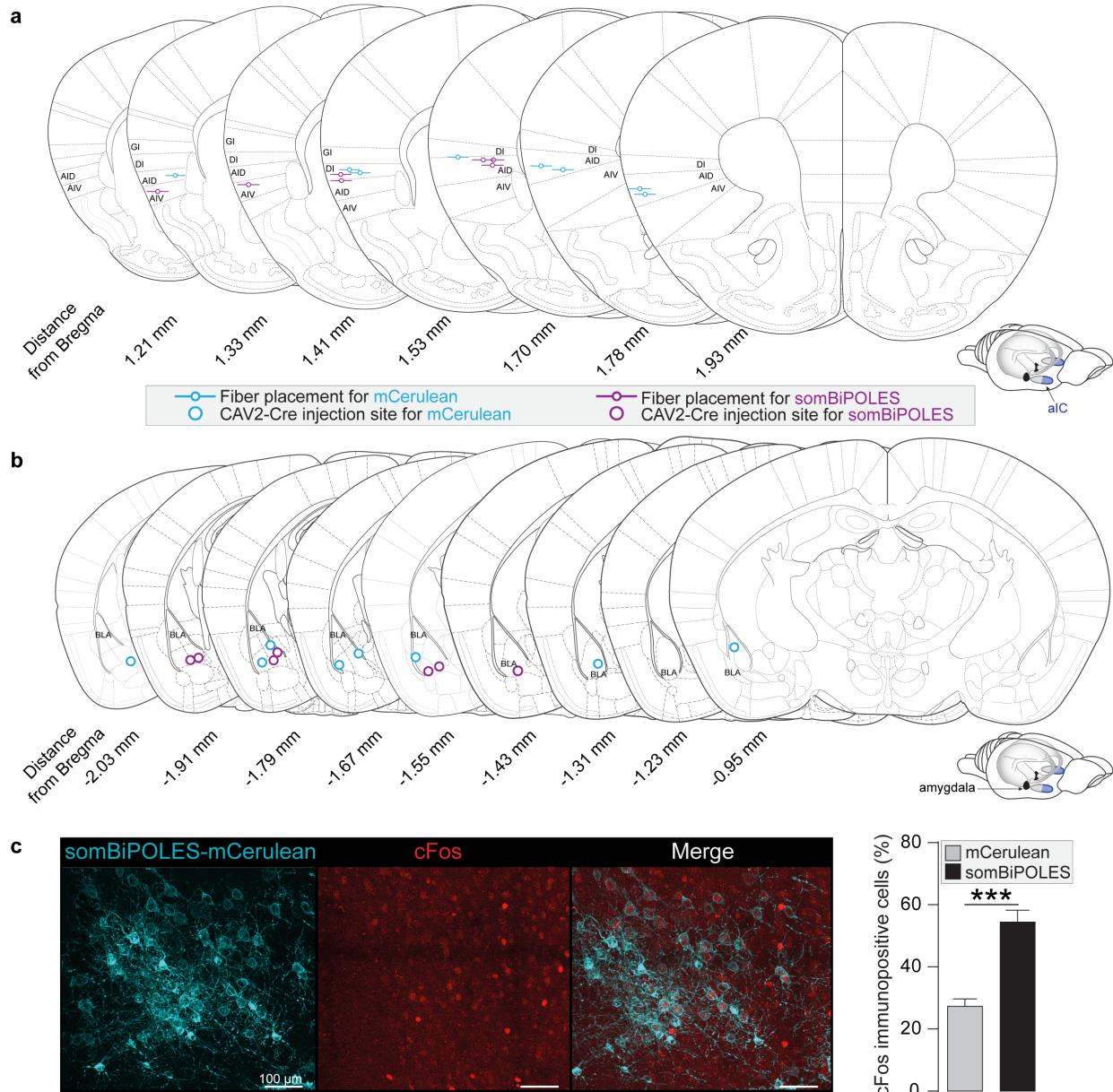


Extended data Figure 1: Histological verification, and complementary anxiety responses of the aIC and pIC. **a.** Schematic of calcium imaging. **b.** Histology verification of fiber implantation sites in aIC (**c**) and pIC (**d**). **e.** Percentage of time spent in borders and center of the OFT (Two-tailed paired t-test, ***p<0.0001, aIC n=13, pIC n=11). **f.** Percentage of time spent in closed and open arms of EPM (Two-tailed paired t-test, ***p<0.0001, aIC n=13, pIC n=11). **g.** Locomotion velocities in closed vs open arms (n=13). **h.** Left. Schematic of EPM. Right. Mean calcium signal along the distance from the center to the end of EPM open arms. **i.** Left. Schematic of EPM open arms exploration (OUT) and retreat (IN) analysis. Middle. Calcium signal along the distance from the center to the end of the open arms while the animal is navigating OUT to the end of the arms or back IN. Right. Average of IN and OUT calcium signal for the space bin of 0-10 cm.

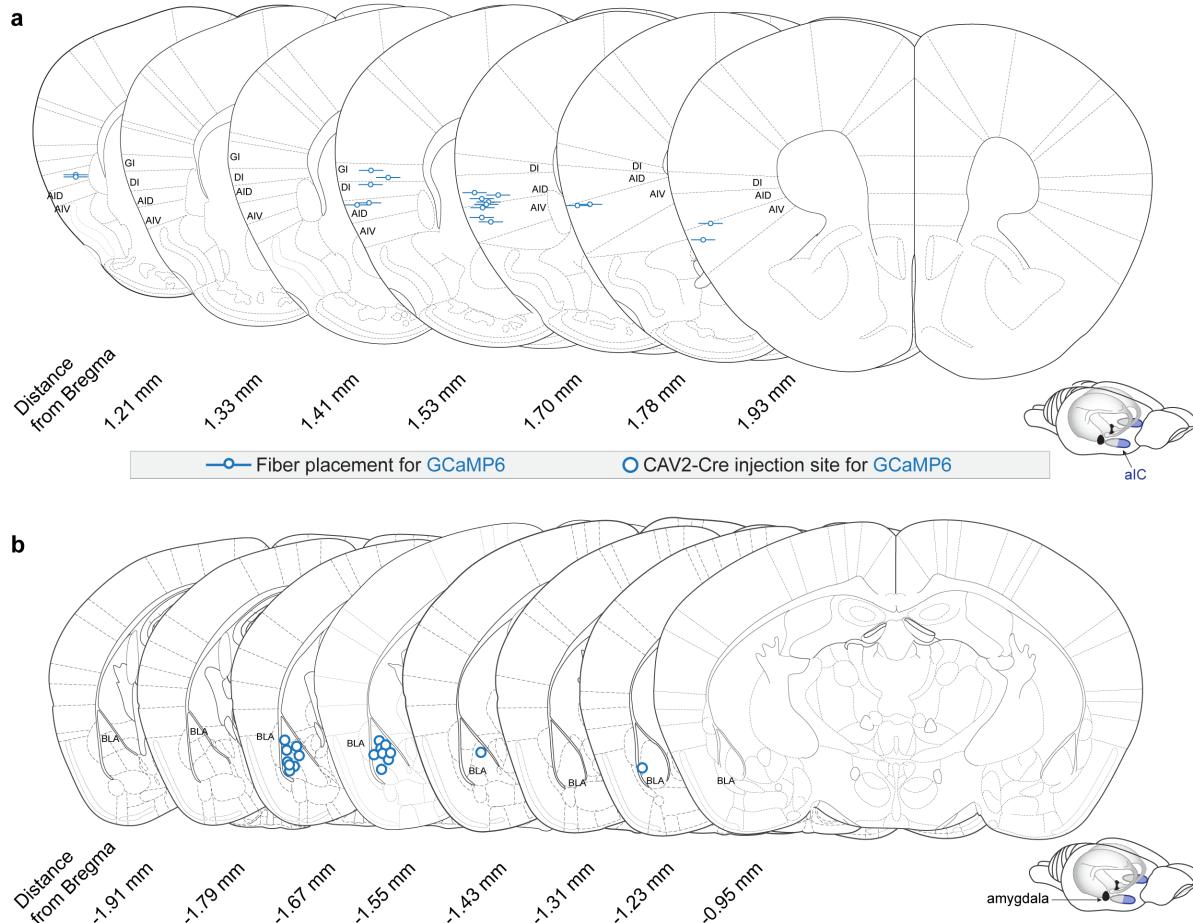
cm and 23-33 cm of the open arms. Global calcium signal during open arms OUT is significantly increased at the extremity of the open arms (23-33 cm) compared to the beginning of the arms (0-10 cm) (Two-tailed paired t-test, $*p=0.034$, $n=13$). **j.** Fiber photometry signal recorded from aIC neurons, (Top) Bulk GCaMP6f signal, and (Bottom) filtered GCaMP6f signal for calcium transients' detection. $\Delta F/F$ represents the fluorescent changes from the mean level of the entire recording time series. **k.** Representation of automated transients' detection. Filtered GCaMP6f peaks exceeding the threshold (horizontal line in the lower trace) were identified as the transients. **l.** (aIC) Left: Mean calcium transients amplitude between open and closed arms ($n=13$). Right: Mean calcium transients frequency in the open compared to closed arms ($n=13$). **m.** (pIC) Mean calcium transients amplitude (left, $n=12$) and frequency (right, $n=12$) in open and closed arms. **n.** (aIC) Left: Mean calcium transients amplitude in the center and the borders of the OFT ($n=13$); Right: Mean calcium transients frequency in the center and the borders of the OFT ($n=13$). **o.** (pIC) Mean calcium transients amplitude (left, $n=12$) and frequency (right, $n=12$) in the center and the borders of the OFT. All the results are represented as mean \pm SEM.



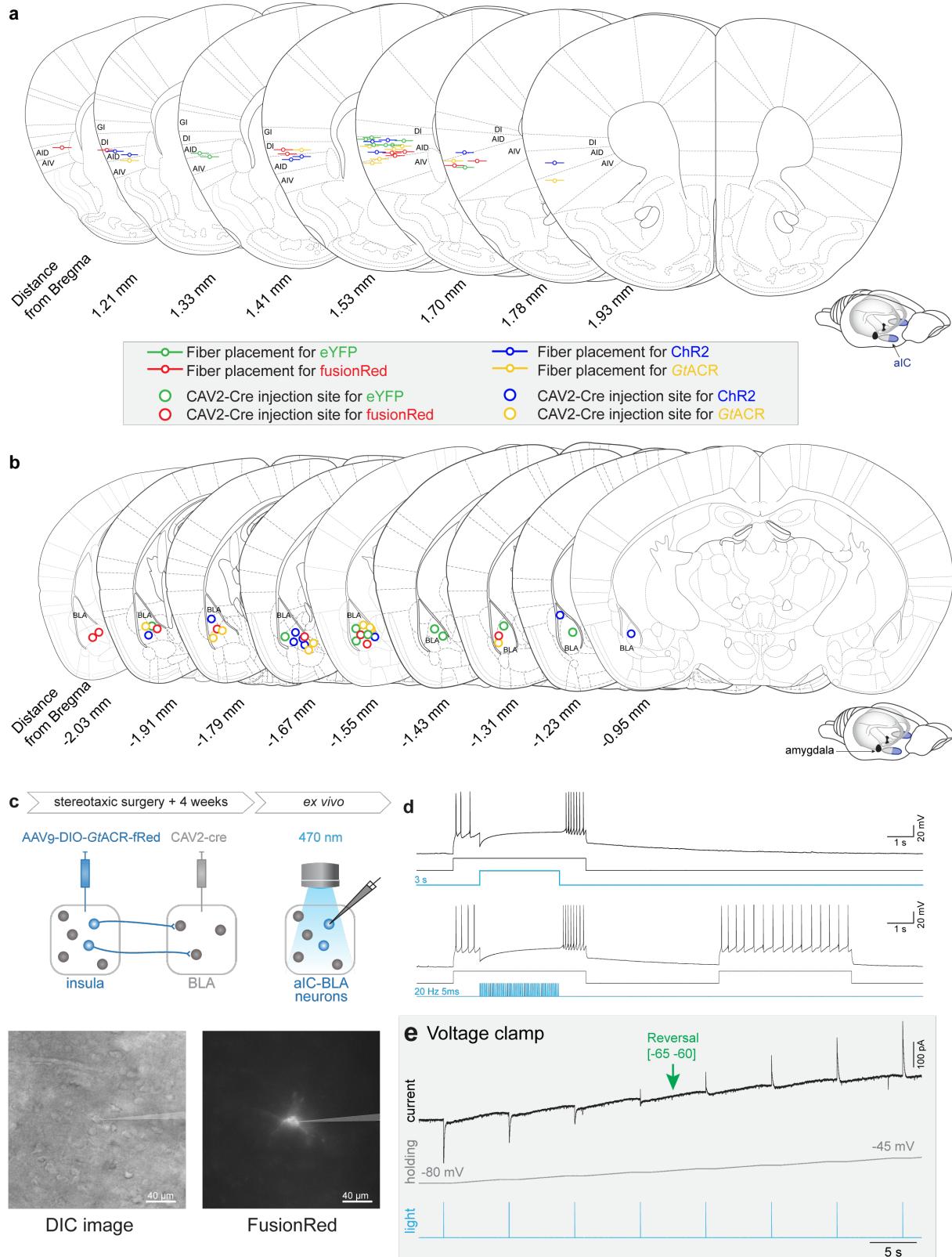
Extended data Figure 2: Additional electrophysiological properties. **a.** Intrinsic properties of neurons in the BLA and CeM recorded for synaptic properties upon the optogenetic activation of the insular terminals in Fig. 3a-g. **b.** Expression of ChR2-eYFP under the promoter of CaMKIIα by AAV strategy in the IC. **c.** Excitation/inhibition ratio and the latency to oEPSC peak by TTX and 4AP application from IC photo-current in the BLA and CeM. **d-e.** Location of patched cells (**d**) and additional electrophysiological properties (**e**) from IC-BLA and IC-CeM neurons for Fig. 3 j-p. All the results are represented as mean \pm SEM.



Extended data Figure 3: Histological verification of viral vector injections and optic fiber placement for somBiPOLES optogenetic experiments. **a.** Bars represent the optic fiber tip in the aIC for mice who received injection of the virus carrying the gene coding for somBiPOLES or the virus control mCerulean. **b.** Circles represent CAV2-Cre injection site in the BLA. Antero-posterior levels (distance from Bregma, in millimeters) are indicated at the bottom of each slice. **c.** Fluorescent images of neurons expressing somBiPOLES-mCerulean, cFos and the merge of the two images. The percentage of cFos-immunoreactive cells is significantly higher in the aIC neurons projecting to BLA expressing somBiPOLES compared to the control mCerulean (Two-tailed unpaired t-test, ***p<0.001, mCerulean n=8, somBiPOLES n=7). AID: agranular insula, AIV: agranular insula, DI: dysgranular insula, GI: granular insula, BLA: basolateral amygdala. Coronal sections from Franklin and Paxinos⁵. All the results are represented as mean \pm SEM.



Extended data Figure 4: Histological verification of viral vector injections and optic fiber placement for fiber photometry experiments. **a.** Bars represent the optic fiber tip in the aIC for mice who received injection of the virus carrying the gene coding for GCaMP6. **b.** Circles represent CAV2-Cre injection site in the BLA. Antero-posterior levels (distance from Bregma, in millimeters) are indicated at the bottom of each slice. AID: agranular insula, AIV: agranular insula, DI: dysgranular insula, GI: granular insula, BLA: basolateral amygdala. Coronal sections from Franklin and Paxinos⁵.



Extended data Figure 5: Histological verification of viral vector injections and optic fiber placement for optogenetic experiments. a. Bars represent the optic fiber tip in the aIC for mice who received injection of the virus carrying the gene coding for ChR2 and GtACR and their respective control eYFP and fusionRed.

b. Circles represent CAV2-Cre injection site in the BLA. Antero-posterior levels (distance from Bregma, in millimeters) are indicated at the bottom of each slice. **c.** Injection of a viral vector carrying *GtACR* gene in the aIC. After 6 weeks, the brain was sectioned into acute slices for whole-cell patch-clamp recordings. **d.** Representative whole-cell patch-clamp recordings in current-clamp mode of a *GtACR2*-expressing cell silenced by light application. Illuminating with 473 nm light delivered for 3 sec or in 5 ms pulses at 20 Hz inhibit action potential generation induced by a current injection. The cell firing returned to normal current-induced firing pattern after light stimulation. **e.** Representative whole-cell patch-clamp recordings in voltage-clamp mode. AID: agranular insula, AIV: agranular insula, DI: dysgranular insula, GI: granular insula, BLA: basolateral amygdala. Coronal sections from Franklin and Paxinos⁵. All the results are represented as mean \pm SEM.

Extended Video 1: aIC-BLA neuronal activity in the elevated plus maze test (EPM). Real-time recording of calcium signals in aIC-BLA neurons of a representative mouse injected with AAV9-syn-Flex-GCaMP6m-WPRE in the aIC and CAV2-Cre in the BLA, during exploration of the EPM.

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