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Anterior cingulate inputs to nucleus accumbens control the social transfer of pain and analgesia

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Materials and Methods

Animals

All procedures were approved by the Stanford University Administrative Panel on Laboratory Animal Care in accordance with American Veterinary Medical Association Guidelines and the International Association for the Study of Pain. Unless otherwise specified, adult C57Bl6/J mice (strain 000664, Jackson Laboratory; aged 7 weeks at the start of experiments) were used. For targeted recombination in active populations (TRAP) experiments, we used male and female second generation of *fos*-TRAP mice ($Fos^{2A\text{-}iCreER}$; TRAP2, which were generously donated by the Luo lab at Stanford University), aged 7-16 weeks for all experiments. These mice were on a B6.129 background and, when necessary, were crossed with Ai14-TdTomato Cre-reporter mice (strain 007914, Jackson Laboratory) to visualize active neurons.

Housing

Mice were housed 2-4 per cage on a 12 h light/dark cycle with food and water ad libitum. Cagemates were used in all experiments. At the start of each experiment, mice were housed 4 per cage. Following the first day of experimental manipulation mice were housed 2 per cage with experimentally matched cagemates (fig. S1).

Drugs

4-hydroxytamoxifen (Sigma, #H6278) was prepared in a solution of castor/sunflower oil and administered i.p. (50 mg/kg). Morphine was prepared in saline and administered s.c. (10 mg/kg, Sigma #M8777). Complete Freund's Adjuvant (10 µL, Sigma #F5881) or capsaicin (10 µg, Sigma, M2808) was injected into the left hindpaw.

Viral Reagents

Unless otherwise noted, all viral reagents were purchased from the Stanford Neuroscience Gene Vector and Virus Core. AAVDJ-*CaMKIIα*-NpHR3.0-eYFP, AAVDJ-*CaMKIIα*-eYFP, or AAVDJ-*CaMKIIα*-ChR2-eYFP were used for all optogenetic experiments except those involving $FosCreER^{T2}$ in which AAVDJ-*CaMKIIα*-DIO-NpHR3.0-eYFP or AAVDJ-*CaMKIIα*-DIO-eYFP were used. For retrograde tracing, a 1:1 volume mixture of AAV-CAG-$FLEx^{loxP}$-TVA-mCherry and AAV-CAG-$FLEx^{loxP}$-RG was injected followed by EnvA-pseudotyped

RVΔG-GFP+EnvA (custom prep, L. Luo lab, 1.3×10^9 colony forming units/ml) into the same location 3 weeks later.

Stereotactic Surgeries

All surgeries were conducted under aseptic conditions using a small animal digital stereotaxic instrument (David Kopf Instruments). Mice were anesthetized with isoflurane (5% induction, 1% maintenance), a small incision was made in the scalp and burr holes were drilled in the brain surface at the appropriate stereotaxic coordinates (AP and ML relative to bregma; DV relative to brain surface at target coordinate): +0.98 AP, 0.278 ML, -0.78 DV for ACC; +0.98 AP, 1.12 ML, -4.15 DV for NAc. Viruses (0.3 µL) were infused at a rate of 0.1 µL/min using a glass micropipette connected to a Hamilton syringe via tubing back-filled with mineral oil. The injector tip was lowered an additional 0.1 mm below the planned injection site and then raised to the final coordinate prior to infusion to facilitate virus diffusion at the site of injection, instead of along the needle track. Following infusion, pipettes were raised 100 µm for 5 min to allow for diffusion and then were removed slowly.

Optogenetic fibers (ferrules) were implanted ~10-50 µm above the sites of interest, (0.98 AP, 0.278 ML, -0.3 DV unilaterally for ACC; 0.98 AP, 1.12 ML, -3.75 DV bilaterally for NAc core; -1.75 AP, 3.0 ML, -3.55 DV bilaterally for BLA). Ferrules were made in-house using 1.25 mm diameter multimode ceramic ferrules (Thorlabs), 200 µm fibre optic cable with numerical aperture (NA) 0.39 (Thorlabs) and blue dye epoxy (Fibre Instrument Sales). Ferrules were secured to the skull using miniature screws (thread size 00–90 × 1/16, Antrin Miniature Specialties) and light-cured dental adhesive cement (Geristore A&B paste, DenMat). Mice recovered from anesthesia individually on a heating pad before being placed into group housing.

Monosynaptic tracing

Cell specific monosynaptic tracing studies were carried out as previously described (*46*) with minor modifications. A 1:1 volume mixture of AAV-CAG-FLEx^{loxP}-TVA-mCherry and AAV-CAG-FLEx^{loxP}-RG (200 nL) was injected into the NAc core of TRAP2 mice. One week later, mice were habituated in a behavior room and injected with saline intraperitoneally for 2 consecutive days, and then injected with 4-hydroxytamoxifen (4-OHT) prior to the social interaction for 4 h. Mice were then housed with treatment-matched cagemates for 3 weeks at

which time 200 nL of RVΔG-GFP+EnvA was injected into the same location. Mice were subsequently housed for 1 week prior to sacrifice and tissue processing.

Optogenetic manipulations

For optogenetic photostimulation of ChR2, ferrules were connected to a 473 nm laser diode (OEM Laser Systems) through a FC/PC adaptor and a fiber optic rotary joint (Doric Lenses). Laser output was controlled using a Master-8 pulse stimulator (A.M.P.I.), which delivered 5 ms light pulses at 20 Hz. Light output through the optical fibers was adjusted to ~5 mW (somatic) or ~15 mW (terminals) using a digital power meter console (Thorlabs). For activation of NpHR3.0, the optical fiber was connected to a 532 nm laser diode (Shanghai Dream Lasers Technology Co, Ltd) via a FC/PC adaptor and a fiber optic rotary joint (Doric Lenses). Laser output was again controlled using a Master-8 pulse stimulator (A.M.P.I.) and adjusted to ~5 mW (somatic) or ~10-15 mW (terminals). Mice received cycles of 8 s light on and 2 s light off. Mice were acclimated to optogenetic tethers during the acclimation/habituation periods prior to the test day.

Social transfer of pain

Mice were acclimated to the testing room for 40 min for two days prior to beginning of experiments (see timeline in Fig. 1A). On the test day, mice were lightly restrained (Control, Bystander) or lightly restrained and injected with 10 μL of an inflammatory medium, Complete Freund's Adjuvant (CFA), into the intraplantar surface of the left hind paw. CFA is known to rapidly and reliably produce long lasting localized inflammation (*24, 25*). Immediately following this quick handling, paired mice were placed into a clean housing cage (without food or water) for a 1 h social interaction, at which time they were subjected to mechanical threshold testing and then the other requisite behavioral testing (Fig. 1A). For time course experiments, following the initial behavioral assays, mice were housed as pairs with treatment-matched cagemates (and intermittently subjected to mechanical threshold assays). On the second test session/second week, mice were lightly restrained (Control, Bystander) or lightly restrained and pricked with a 26 g needle on the surface of the left hind paw (CFA) and subjected to behavioral testing. For experiments with optogenetic manipulations, mice were tethered to optogenetic cables during acclimation.

Mechanical sensitivity

All assays were performed without knowledge of the experimental manipulation or viral injection performed on each individual subject in a given group. However, control mice were run in separate cohorts to limit exposure to mice that had been given noxious stimuli, as even brief exposure to CFA and BY mice caused significant variance in mechanical thresholds (e.g. fig. S2A, B). Furthermore, in CFA mice, significant swelling in the CFA-injected hindpaw occurred several hours after injection, and often made blinding to this group impossible at later timepoints. Responses (withdrawal, shaking, or licking the paw) to (1-2 s) mechanical stimulation of the plantar surface of the left hindpaw were determined with von Frey hairs (0.01-2 g plastic fibers, Touch Test) using the up-down technique (*26*). This method uses stimulus oscillation around the response threshold to determine the median 50% threshold of response. Mice were allowed to acclimate to homemade plexiglass enclosures on top of a homemade wire testing rack for 20 min on 2 days before the start of the experiment, and for 10-30 min before each test session. The testing rack was located within each testing room and illuminated with a dim lamp. Unless otherwise noted, mechanical sensitivity was assessed before treatment exposure (baseline, represented by a dotted line "---" on mechanical sensitivity graphs), and then testing occurred 24 h later. For timecourse experiments and certain optogenetic experiments, testing occurred at 0, 4, 24, 48, 72 h and 1 week after social interaction. For optogenetic experiments, mechanical thresholds were taken with the light off and the light on in the following pattern: OFF, ON, OFF, ON. The light was turned on or off for the duration of each threshold test, which took less than 1 minute per mouse. Data are displayed as the first light OFF session (Fig. 3B, D, F; Fig. 4G, J and Fig. 5F, H) followed by the average of the light ON and light OFF sessions (Fig. 3C, E, G).

Thermal place test (TPT)

The thermal place test occurred on a dual hot/cold plate (Bioseb, BIO-T2CT) with opaque plexiglass walls surrounding the 2 compartments (13 in long; 3 in wide; 9.5 in high). One floor/side of the chamber was set to room temperature (30℃), and the other side/floor was set to (40℃). Mice were allowed to freely explore the chamber for 10 minutes, data are displayed as an average time spent during minutes 6-10, as mice were acclimating to the novel environment during minutes 1-5. Pilot behavioral experiments determined that mice do not show thermal place aversion to the warm area (40 ℃) versus the reference area (30℃) 1 h after CFA

injection, but do display an avoidance of the warm floor 1 week after CFA injection (Fig. 5C). Additionally, pilot tests determined that mice could not be placed on the hot plate repeatedly, as they quickly learned to avoid the warm floor almost entirely.

Tail immersion

Mice were tested for thermal nociceptive sensitivity using the heat-evoked tail withdrawal reflex. Two days before the first test session, mice were habituated to handling (light restraint in a soft cloth) and the tip of their tail (5 cm from the end) was immersed into room temperature water. On the test days, mice were lightly restrained, and the tail was submerged into 46° C water to measure the latency of the response (flicking the tail out of water) using a stopwatch. Two tail withdrawal measurements were taken 10 min apart and averaged for a single data point for each animal.

Emotional discrimination

Briefly [described in detail in (*27*)], mice were habituated to the enclosures for 10 min, 3 days prior to the test day. Test observer mice were allowed to explore the entire chamber during habituation (free of other mice), while CFA, Bystander and Neutral "demonstrator" mice were habituated for 10 min under the wire cups. The enclosure included walls (35.5 x 23.5 x 19 cm), and a separator (11 x 14cm) made with black plexiglass and two black cylindrical wire pencil cups (10.5 cm in height, bottom diameter 10.2 cm, bars spaced 1 cm apart). A plastic cup was placed on the top of the wire cups to prevent the observer mice from climbing. On the test day, mice were subjected to a 1 h social interaction to create CFA, Bystander and Control pairs (interaction was the same as that used in social transfer paradigms). Immediately following the social interaction, pairs of mice were placed under the cups: either a Bystander mouse paired with a Control mouse, a CFA mouse paired with a Control mouse or two Control mice. An age and sex-matched stranger test mouse was allowed to explore the entire chamber for 6 minutes. The sessions were video recorded and manually score with a stopwatch after the fact by an experimenter blind to the treatment conditions.

Social transfer of analgesia

On the test day, mice were lightly restrained and injected with 10 μL of CFA into the intraplantar surface of the left hind paw, immediately followed by a subcutaneous (s.c.) injection of either 10 mg/kg morphine (CFA-MOR) or saline (CFA-Control or CFA-Analgesia-Bystander). Pairs of mice were then placed into a clean housing cage for 1 h, immediately followed by mechanical testing (Fig 5A). Following mechanical testing, mice were separated and housed for 1 week with treatment-matched cagemates, before being subjected to a second set of assays. On this second test day, mice were lightly restrained and pricked with a 26 gauge needle on the surface of the left hind paw, followed by s.c. injection of either 10 mg/kg morphine (CFA-Mor) or saline (CFA-Control or CFA-Analgesia-Bystander). Mice were then placed back in their housing cage with their previous partner for 1 h, followed by mechanical testing and the thermal place test. Following the social transfer and behavioral test, all mcie were separated and housed with treatment matched cagemates (fig. S1). For optogenetic experiments, laser stimulation was given during the entire social interaction as described above and during every other minute during the thermal place test.

Observational fear response

Observational fear response assays were performed as described previously (*19*) with slight modifications. Two fear-conditioning shock chambers, made by different manufacturers, in two entirely separate rooms were used as the two separate contexts (Context A: Ugo Bassile passive avoidance chamber, and Context B: Med Associate self-administration chambers with shock floor additions) . To enhance the magnitude of the social transfer of fear (*36*, *44*), 24 h prior to the social transfer procedure (Day 1; Fig. 4A), BY mice were administered a shock experience by being placed in one of the fear-conditioning chambers (context A, Fig 4A) for 5 minutes at which time they received 2 unpredicted, un-cued, footshocks (0.4 mA, for 2 sec, 1 min interval) and then transferred back to their home cage with treatment matched cagemates. The following day (Day 2), a naive mouse was placed in the other fear-conditioning chamber (Context B) while a BY mouse was placed in an adjacent homemade plexiglass observation chamber, which allowed for the communication of visual, auditory and olfactory cues through a transparent, perforated plexiglass divider. After a 5 min habituation period, the naive mouse received 24 unpredicted, un-cued, footshocks (0.7 mA, for 2 sec, 10 sec intervals over 4 min). The entire

session was recorded with a high definition camera (Logitech) and the time spent freezing was scored manually using a stop watch. Mice were then housed in pairs for 24 h with treatment-matched cagemates. The following day (Day 3), contextual observational fear memory was assessed by placing the BY mice back into the observation chamber in the same context (Context B) for 10 min.

For optogenetic manipulations, fiber optic patch-cords were connected to the BY mice before transfer to the observation chamber on Day 2 and Day 3. On Day 2, the laser was turned on for the entire 4 min shock period. On Day 3, the laser was turned off and on every other minute. One week after completion of the social transfer of fear experiments, shocked and BY mice were subjected to the social transfer of pain test (Fig. 4D). There was no difference in baseline mechanical thresholds between shocked and BY mice, which were counterbalanced into CFA and BY groups.

Real time place preference test

A 35.5 x 23.5 x 19 cm chamber was divided into 2 compartments by a partial barrier. The left and right compartments had different visual cues and the side initially paired with stimulation was randomly assigned each day. Optical stimulation was controlled by a computer running Biobserve software, which tracked animal position and triggered light delivery via video tracking location. Initially, the mouse was placed in the non-stimulated compartment with the rest of the arena closed off. The mouse was free to explore the entire arena for the remainder of the test. Every time the mouse crossed to the stimulation-paired side of the chamber, pulsed light was delivered (473 nm laser, 5 ms light pulses at 20 Hz, ~10-15 mW output) until the mouse crossed back into the other side. Immediately following the initial test, a reversal test was conducted and the side paired with stimulation was switched; there was no interruption between the initial and the reversal phases of the experiment. The average time of the initial and reversal sessions were used for analysis.

General histological procedures, cell counting, and imaging

Mice were deeply anesthetized with sodium pentobarbital, transcardially flushed with cold PBS and perfused with 4% paraformaldehyde/PBS. Brains were removed, post-fixed for 24 h and then cryoprotected in 20% sucrose/PBS for 24 h; followed by 30% sucrose for 24 h, at which

point they were either transferred to PBS for storage or immediately sectioned on a cryostat (40 µm slices). TRAP2;Ai14 brain slices did not require immunohistochemistry to visualize TdTomato positive neurons, which were manually counted by a researcher blind to experimental condition. Sections containing 12 brain regions of interest were selected for analysis. Brain regions were defined using the Mouse Brain Atlas (*47*) parameters. Photos from each slice were overlayed onto corresponding brain atlas slices, and all TdTomato+ cells within the designated brain region were manually counted by a researcher blind to experimental condition. Averages for each region were determined from across 3 to 8 slices from 6-9 mice in each group. Both males and female TRAP2 mice were used, as no sex dependent differences were detected ($P>0.05$).

For localization of viral injections, sections were rinsed between steps with PBS, and blocked in 5% normal donkey serum/PBS (The Jackson Laboratory) for 45 min. The tissue was then incubated overnight with 1:1000 rabbit anti-GFP antibody (Aves, GFP-1020). This was followed by 1 h incubations with Alexa Fluor 488-labeled secondary antibodies (raised in donkey, Invitrogen catalog number A-11055). Finally, slices were washed with PBS, mounted on charged slides and coverslipped with Prolong Gold (Invitrogen). Viral infusions were considered accurate when neuronal expression of the virus was limited to the boundaries of the chosen brain region [as defined by (*47*)]. When visualization of the virus was not possible or spread of the virus was beyond the designated target, data were not included. This occurred in less than 5% of the injected mice.

For analysis of monosynaptic rabies tracing, sections were processed via immunohistochemistry for GFP (described above), injection location was verified in the NAc core via presence of GFP and mCherry fluorescence and cells were manually counted from 3 slices throughout the ACC from 3 mice in each group (CFA, BY).

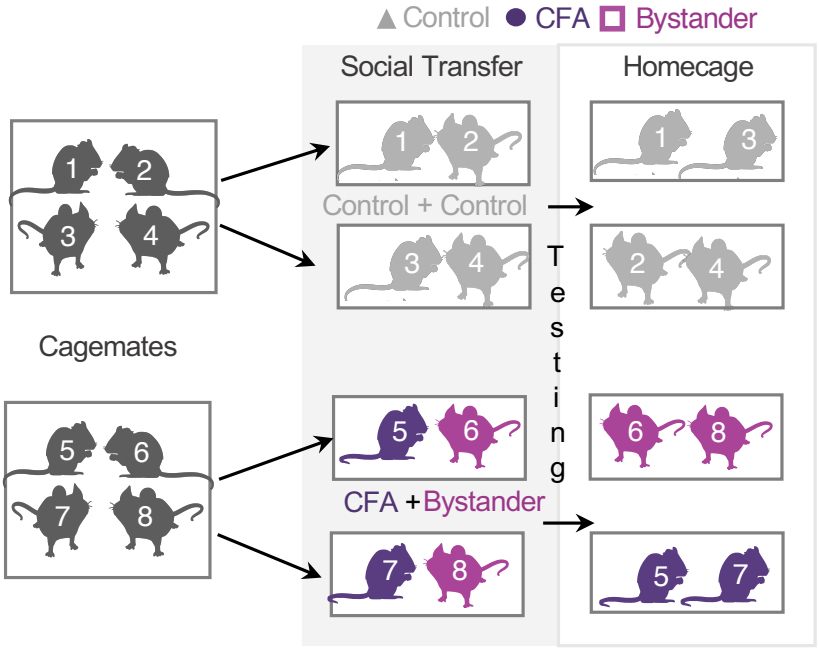

Fig. S1.

Diagram of housing arrangements. Diagram demonstrating splitting of mice into new cages following treatment, social transfer and testing. Immediately following social transfer, mice were split into treatment matched pairs during recovery and for the remainder of the experiment.

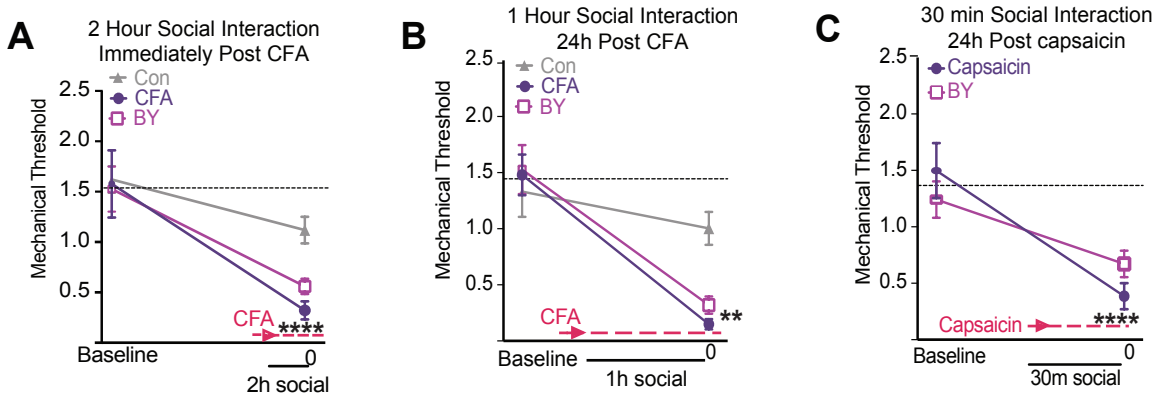

Fig. S2.

Further analysis of social transfer of pain timing. (**A**) Mechanical sensitivity immediately after a CFA injection with a 2 h social interaction in Con/Con and CFA/BY pairs (Main effect of time $F_{1,20} = 29.64$, $P<0.0001$, n=8). Control mice were tested in the same sessions as CFA and BY mice, leading to a decrease in mechanical thresholds. (**B**) Mechanical sensitivity immediately after a 1 h social interaction 24 h after CFA injection in Con/Con and CFA/BY pairs (Time x Treatment interaction $F_{2,27} = 5.728$, $P=0.0084$, n=8), Control mice were tested in the same sessions as CFA and BY mice, leading to a decrease in mechanical thresholds. (**C**) Mechanical sensitivity immediately after a capsaicin injection with a 30 min social interaction in Capsaicin/BY pairs (Main effect of time $F_{1,18} = 36.07$, $P<0.0001$, n=7-14). Data are mean ± s.e.m. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $***P < 0.0001$. Two-way repeated measures ANOVA with Holm-Sidak post hoc tests.

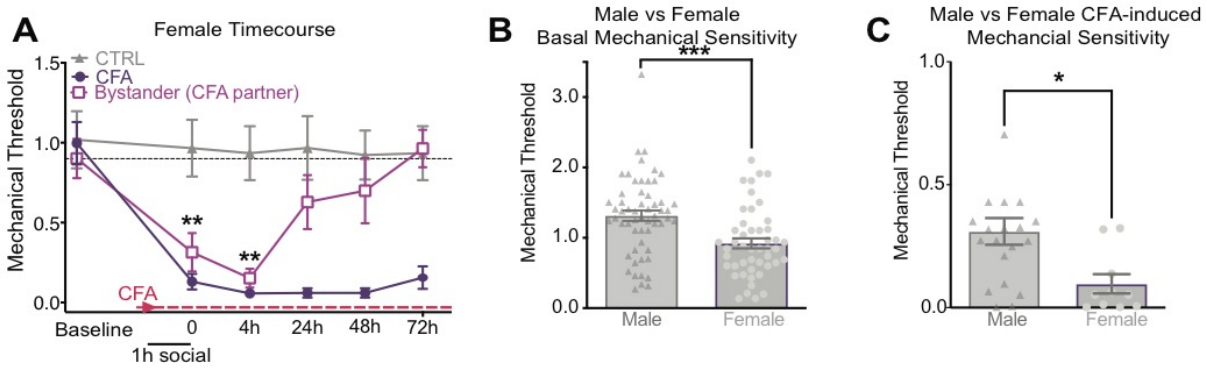

Fig. S3.

Social transfer of pain in females. (**A**) Time course of mechanical von Frey (VF) sensitivity at 0, 4, 24, 48, and 72 h post 1 h social interaction in Con/Con and CFA/BY pairs of female mice (Time x Treatment interaction $F_{6,137} = 3.014$, $P=0.0085$, n=8). (**B**) Comparison of basal mechanical sensitivity in males and females ($t_{102}=3.76$, $P=0.003$). (**C**) Comparison of CFA-induced mechanical sensitivity 1 h post CFA injection ($t_{28}=2.602$, $P=0.0147$). Data are means ± s.e.m. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ comparing Con and BY. Two-way repeated measures ANOVA (A) with Holm-Sidak post hoc tests, or unpaired t-test (B, C).

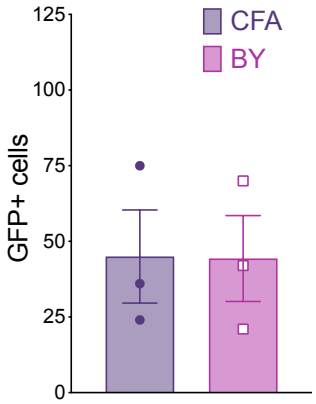

Fig. S4. Monosynaptic rabies quantification in TRAP mice. ACC neurons that were monosynaptically connected to NAc neurons that were "TRAP'd" during social transfer were labelled via monosynaptic rabies virus tracing in TRAP2-BY and TRAP2-CFA mice. GFP positive (+) cells (± SEM) in the ACC were manually counted from 3 slices taken through the ACC to produce an average for each animal (n=3/group).

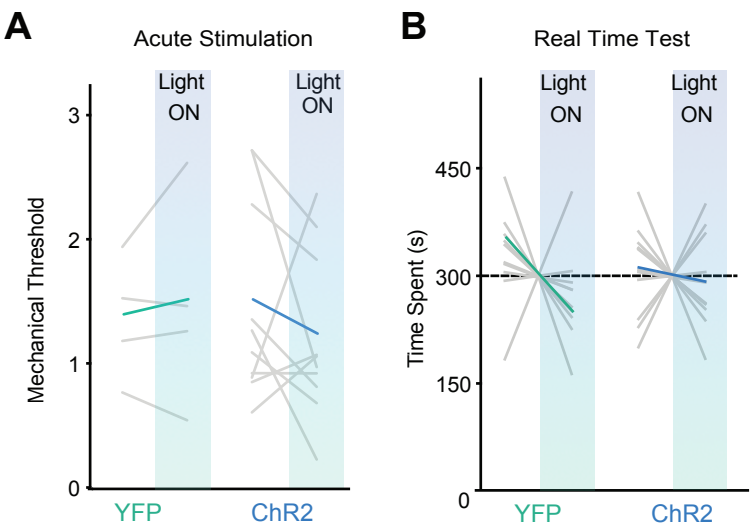

Fig. S5.

Effects of activating ACC→NAc projections. (**A**) Acute stimulation of ACC→NAc projections in YFP and ChR2 mice during mechanical testing, prior to any other treatment. (**B**) Real time place preference test in YFP and ChR2 mice prior to social transfer of pain testing.

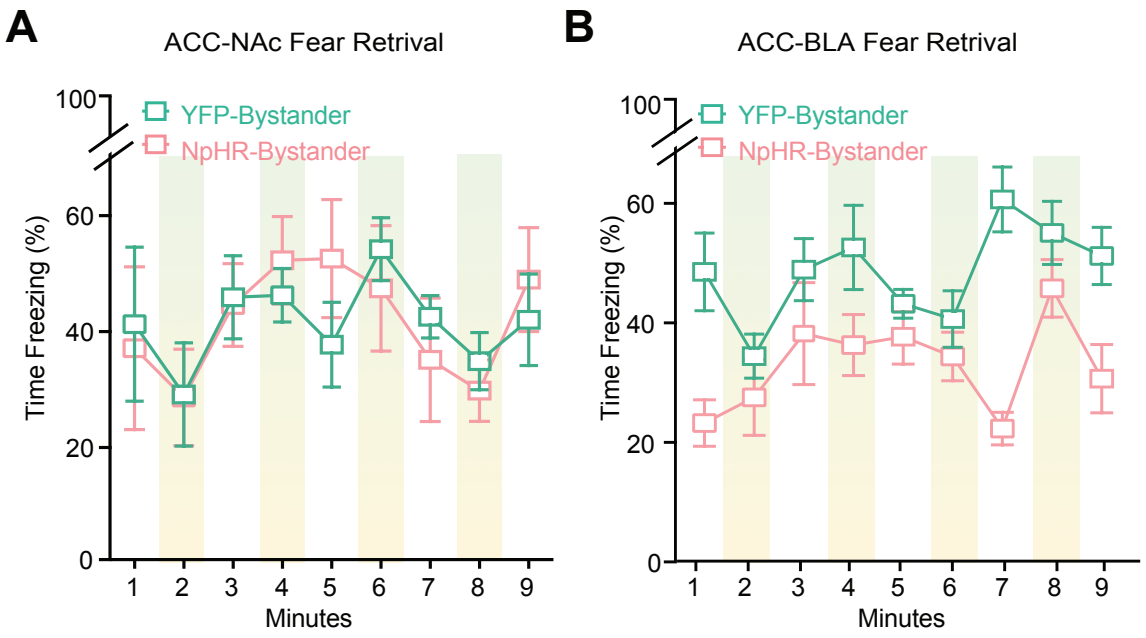

Fig. S6.

Effects of inhibiting ACC→NAc or ACC→BLA projections on freezing behavior. (**A**) Freezing behavior during light on and light off sessions during fear memory retrieval in YFP- and NpHR-Bystander mice with implants above the NAc. (**B**) Freezing behavior during light on and light off sessions during fear memory retrieval in YFP- and NpHR-Bystander mice with implants above the BLA. Data are means ± s.e.m.

A	Figure	Data/Comparison	N, n/group	Primary statistic	Post-hoc test	Comparison	p value	Notation	F statistic
	1B	Timecourse of mean mechanical thresholds. Treatment group x time.	N=57 mice, 17-20/group	Two-way Mixed Effects Model		Group x time interaction	.0012	**	F(8,178) = 3.277
					Holm-Sidak	Con vs. CFA at time 0	.0004	***	
					Holm-Sidak	Con vs. BY at time 0	.0044	**	
					Holm-Sidak	Con vs. CFA at 4h	.0013	**	
					Holm-Sidak	Con vs. BY at 4h	.0077	**	
					Holm-Sidak	Con vs. CFA at 24h	.0001	***	
					Holm-Sidak	Con vs. BY at 24h	.065	NS	
					Holm-Sidak	Con vs. CFA at 48h	.0022	**	
	1C	Mean mechanical thresholds immediately after social transfer (time 0). Treatment group.	N=57 mice, 17-20/group	One-way ANOVA		Main effect of group	.0001	***	F(2, 54) = 14.26
					Holm-Sidak	Con vs. CFA	<.0001	****	
					Holm-Sidak	Con vs. BY	.0003	****	
	1D	Mean mechanical thresholds of ipsilateral and contralateral paws. Paw x group.	N=48 paws, 24 mice, 12/group	Two-way ANOVA		Main effect of paw	<.0001	****	F(3,33) = 12.30
					Holm-Sidak	CFA ipsi vs. contra	<.0001	****	
	1E	Mean tail withdrawal latency. Treatment group.	N=22 mice, 6-8/group	One-way ANOVA		Main effect of group	.0007	***	F(2,19) = 10.88
					Holm-Sidak	Con vs. CFA	.0004	***	
					Holm-Sidak	Con. vs. BY	.0037	**	
	1F	Time spent on warm floor. Treatment group.	N=26, 8-9/group	One-way ANOVA		Main effect of group	.0024	**	F(2,23) = 7.933
					Holm-Sidak	Con vs. CFA	.0032	**	
					Holm-Sidak	Con vs. BY	.0032	**	
	1G	Time spent sniffing each group. Treatment group.	N=18, 6/group	One-way ANOVA		Main effect of group	.0402	*	F(2,15) = 4.010
					Holm-Sidak	Con vs. CFA	.0208	*	
					Holm-Sidak	Con vs. BY	.0364	*	

B

Figure	Data/Analysis	n	Primary statistic	Post-hoc test	Comparison	p value	Notation	F statistic
2C	TRAP cell count. Experimental group vs. brain region	N=24 mice, 6-9/group, 3-8 slices/region	One-way ANOVA		ACC Main effect of group	.0003	***	F(2, 25) = 11.47
				Holm-Sidak	Con vs. CFA	.0936	NS	
				Holm-Sidak	Con vs. BY	.0001	***	
					INS Main effect of group	.0001	***	F(2, 16) = 16.15
				Holm-Sidak	Con vs. CFA	.1338	NS	
				Holm-Sidak	Con vs. BY	<.0001	****	
					NAc Main effect of group	.0003	***	F(2, 24) = 8.832
				Holm-Sidak	Con vs. CFA	.2415	NS	
				Holm-Sidak	Con vs. BY	.0011	**	
					MD Thal Main effect of group	.0018	**	F(2, 15) = 9.95
				Holm-Sidak	Con vs. CFA	.0042	**	
				Holm-Sidak	Con vs. BY	.0015	**	
					IL Thal Main effect of group	.0105	*	F(2, 15) = 6.263
				Holm-Sidak	Con vs. CFA	.0987	NS	
				Holm-Sidak	Con vs. BY	.0059	**	
					VB Thal Main effect of group	.0043	**	F(2, 15) = 8.001
				Holm-Sidak	Con vs. CFA	.0242	*	
				Holm-Sidak	Con vs. BY	.0025	**	
					L Hb Main effect of group	.4318	NS	F(2, 25) = .8886
					CeA Main effect of group	.0005	***	F(2, 16) = 12.46
				Holm-Sidak	Con vs. CFA	.0657	NS	
				Holm-Sidak	Con vs. BY	.0003	***	
					BLA Main effect of group	.0028	**	F (2, 16) = 8.693
				Holm-Sidak	Con vs. CFA	0.053	NS	
				Holm-Sidak	Con vs. BY	0.001	**	
					BNST Main effect of group	.00692	**	F (2, 9) = 3.646
				Holm-Sidak	Con vs. CFA	0.056	NS	
				Holm-Sidak	Con vs. BY	.0562	NS	
					ZI Main effect of group	.1549	NS	F (2, 8) = 2.376
					PAG Main effect of group	<0.0001	****	F (2, 15) = 21.99
				Holm-Sidak	Con vs. CFA	0.022	*	
				Holm-Sidak	Con vs. BY	<0.0001	****	

C	Figure	Data/Comparison	N, n/group	Primary statistic	Post-hoc test	Comparison	p value	Notation	F statistic
	3B	Mean mechanical thresholds immediately after social transfer (time 0). Treatment group.	N=31 mice, 5-11/group	One-way ANOVA		Main effect of group	<.0001	****	F(3,27) = 11.09
					Holm-Sidak	BY-YFP vs. BY-NpHR	.0025	**	
	3C	Mean mechanical thresholds during light off and light on sessions. Light status x treatment group.	N=31 mice, 5-11/group	Two-way Mixed Effects Model		Main effect of group	<.0001	****	F(3,26) = 13.90
					Holm-Sidak	NS effect of light	>.05	NS	
	3D	Mean mechanical thresholds immediately after social transfer (time 0). Treatment group.	N=21 mice, 4-7/group	One-way ANOVA		Main effect of group	.0001	***	F(3,17) = 14.22
					Holm-Sidak	BY-YFP vs. BY-NpHR	.0004	***	
	3E	Mean mechanical thresholds during light off and light on sessions. Light status x treatment group.	N=21 mice, 4-7/group	Two-way Mixed Effects Model		Main effect of group	.0019	**	F(3,16) = 7.907
					Holm-Sidak	NS effect of light	>.05	NS	
	3F	Mean mechanical thresholds immediately after social transfer (time 0). Treatment group.	N=23, 5-7/group	One-way ANOVA		Main effect of group	.0003	***	F(3,19) = 10.36
					Holm-Sidak	BY-YFP vs. BY-NpHR	.0043	**	
	3G	Mean mechanical thresholds during light off and light on sessions. Light status x treatment group.	N=23, 5-7/group	Two-way Mixed Effects Model		Main effect of group	.0007	***	F(3, 19) = 8.793
					Holm-Sidak	NS effect of light	>.05	NS	
	3H	Timecourse of mean mechanical thresholds. Treatment/virus group baseline x time.	N=50, 8-17/group	Two-way Mixed Effects Model		Group x time interaction	<.0001	****	F(18,197) = 3.780
					Holm-Sidak	YFP-CFA Baseline vs. time 0	.0005	***	
					Holm-Sidak	YFP-BY Baseline vs. time 0	.0005	***	
					Holm-Sidak	ChR2-CFA Baseline vs. time 0	<.0001	****	
					Holm-Sidak	ChR2-BY Baseline vs. time 0	<.0001	****	
					Holm-Sidak	YFP-CFA Baseline vs. 4h	.0048	**	
					Holm-Sidak	YFP-BY Baseline vs. 4h	.0471	*	
					Holm-Sidak	ChR2-CFA Baseline vs. 4h	<.0001	****	
					Holm-Sidak	ChR2-BY Baseline vs. 4h	.0002	***	
					Holm-Sidak	YFP-CFA Baseline vs. 24h	.0056	**	
					Holm-Sidak	YFP-BY Baseline vs. 24h	.8237	NS	
					Holm-Sidak	ChR2-CFA Baseline vs. 24h	<.0001	****	
					Holm-Sidak	ChR2-BY Baseline vs. 24h	.0013	**	
					Holm-Sidak	YFP-CFA Baseline vs. 48h	.0048	**	
					Holm-Sidak	YFP-BY Baseline vs. 48h	.9375	NS	
					Holm-Sidak	ChR2-CFA Baseline vs. 48h	.0002	***	
					Holm-Sidak	ChR2-BY Baseline vs. 48h	.0158	*	
					Holm-Sidak	YFP-CFA Baseline vs. 72h	.0048	**	
					Holm-Sidak	YFP-BY Baseline vs. 72h	.9902	NS	
					Holm-Sidak	ChR2-CFA Baseline vs. 72h	.0027	**	
					Holm-Sidak	ChR2-BY Baseline vs. 72h	.01	*	
					Holm-Sidak	YFP-CFA Baseline vs. 1 week	.0695	NS	
					Holm-Sidak	YFP-BY Baseline vs. 1 week	.9970	NS	
					Holm-Sidak	ChR2-BY Baseline vs. 1 week	.9918	NS	
					Holm-Sidak	ChR2-CFA Baseline vs. 1 week	.0107	*	

D

Figure	Data/Comparison	N, n/group	Primary statistic	Post-hoc test	Comparison	p value	Notation	F/t statistic
4B	Mean freezing over time. Treatment group x time	N=14 mice, 6-8/group	Two-way ANOVA		Group x time interaction	<.0001	****	F(8,96) = 4.787
				Holm-Sidak	Shock min 1 vs. min 6	.0011	**	
				Holm-Sidak	BY min 1 vs. min 6	<.0001	****	
				Holm-Sidak	Shock min 1 vs. min 7	.0002	***	
				Holm-Sidak	BY min 1 vs. min 7	<.0001	****	
				Holm-Sidak	Shock min 1 vs. min 8	.0002	***	
				Holm-Sidak	BY min 1 vs. min 8	.0002	***	
				Holm-Sidak	Shock min 1 vs. min 9	.0009	***	
				Holm-Sidak	BY min 1 vs. min 9	.0010	**	
4C	Mean time spent freezing. Within group: conditioning vs. retrieval.	N=10 mice	Paired t-test		Conditioning vs. Retrieval	<.0001	****	t(9) = 6.904
4E	Mean freezing over time. Treatment group x time.	N=10 mice, 5/group	Two-way ANOVA		Main effect of time	<.0001	****	F(3.99, 31.92) = 10.08
				Holm-Sidak	YFP min 1 vs. min 6	.0038	**	
				Holm-Sidak	NpHR min 1 vs. min 6	.0081	**	
				Holm-Sidak	YFP min 1 vs. min 7	.0001	***	
				Holm-Sidak	NpHR min 1 vs. min 7	.0771	NS	
				Holm-Sidak	YFP min 1 vs. min 8	.0002	***	
				Holm-Sidak	NpHR min 1 vs. min 8	.0046	**	
				Holm-Sidak	YFP min 1 vs. min 9	.0384	*	
				Holm-Sidak	NpHR min 1 vs. min 9	.0294	*	
4F	Mean time spent freezing. Between groups.	N=10 mice, 5/group	Unpaired t-test		YFP vs. NpHR	.9724	NS	t(8) = .03571
4G	Mean mechanical thresholds immediately after social transfer (time 0). Treatment group.	N=20 mice, 5/group.	One-way ANOVA		Main effect of group	.0001	***	F(3,16) = 13.25
					BY-YFP vs. BY-NpHR	.0004	***	
4H	Mean freezing over time. Treatment group x time.	N=18 mice, 9/group	Two-way ANOVA		Main effect of time	<.0001	****	F(8,128) = 23.88
				Holm-Sidak	YFP min 1 vs. min 6	<.0001	****	
				Holm-Sidak	NpHR min 1 vs. min 6	<.0001	****	
				Holm-Sidak	YFP min 1 vs. min 7	<.0001	****	
				Holm-Sidak	NpHR min 1 vs. min 7	<.0001	****	
				Holm-Sidak	YFP min 1 vs. min 8	<.0001	****	
				Holm-Sidak	NpHR min 1 vs. min 8	.0019	**	
				Holm-Sidak	YFP min 1 vs. min 9	<.0001	****	
				Holm-Sidak	NpHR min 1 vs. min 9	<.0001	****	
4I	Mean time spent freezing. Between groups.	N=20 mice, 10/group.	Unpaired t-test		NS YFP vs. NpHR	.0005	***	t(18) = 3.908
4J	Mean mechanical thresholds immediately after social transfer (time 0). Treatment group.	N=36 mice, 9/group.	One-way ANOVA		NS Main effect of group	.8626	NS	F(3,32) = .2474

E	Figure	Data/Comparison	N, n/group	Primary statistic	Post-hoc test	Comparison	p value	Notation	F/t statistic
	5B	Timecourse of mean mechanical thresholds. Treatment group x time.	N=29 mice, 14-15/group	Two-way Mixed Effects Model		Group x time interaction	<.0001	****	F(7,125) = 5.634
					Holm-Sidak	CFA-Con vs. CFA-Analg-BY at time 0	<.0001	****	
					Holm-Sidak	CFA-Con vs. CFA-Analg-BY at 4h	.0003	***	
	5C	Mean time spent on warm floor 1hr or 1 week post CFA. Treatment.	N=28 mice, 8-12/group	One-way ANOVA		Main effect of treatment	.001	**	F(2,25) = 9.142
					Holm-Sidak	No injection vs. 1 week post CFA	.0126	*	
	5D	Mean time spent on warm floor. Treatment.	N=42 mice, 12-15/group	One-way ANOVA		Main effect of group	<.0001	****	F(2,39) = 25.80
					Holm-Sidak	CFA-Con vs. CFA-Mor	<.0001	****	
					Holm-Sidak	CFA-Con vs. CFA-Analg-BY	<.0001	****	
	5F	Mean mechanical thresholds immediately after 1 week social transfer (time 0). Treatment group.	N= 31 mice, 7-8/group	One-way ANOVA		Main effect of treatment	<.0001	****	F(3,27) = 15.04
					Holm-Sidak	YFP-CFA-Con vs. YFP-Analg-BY	<.0001	****	
	5G	Mean time spent on warm floor immediately post 1 week social transfer. Treatment group.	N=45 mice, 7-8/group	One-way ANOVA		Main effect of treatment	<.0001	*****	F(5,39) = 21
						YFP-Analg-BY vs. YFP-CFA-Con	.0064	**	
						YFP-Analg-BY vs. YFP-CFA-Mor	.0092	**	
						YFP-Analg-BY vs. NpHR-CFA-Con	.0064	**	
						YFP-Analg-BY vs. NpHR-CFA-Mor	.0008	***	
						YFP-Analg-BY vs. NpHR-Analg-BY	.0092	**	
	5H	Mean mechanical thresholds immediately after 1 week social transfer (time 0). Treatment group.	N= 32 mice, 8/group	One-way ANOVA		Main effect of treatment	<.0001	****	F(3,27) = 15.04
					Holm-Sidak	YFP-CFA-Con vs. YFP-Mor-BY	<.0001	****	
	5I	Mean time spent on warm floor immediately post 1 week social transfer. Treatment group.	N=48 mice, 8/group	One-way ANOVA		Main effect of treatment	<.0001	****	F(5,42) = 26.93
						YFP-Mor-BY vs. YFP-CFA-Con	.0045	**	
						YFP-Mor-BY vs. YFP-CFA-Mor	.0002	***	
						YFP-Mor-BY vs. NpHR-CFA-Con	.0045	**	
						YFP-Mor-BY vs. NpHR-CFA-Mor	.002	**	
						YFP-Analg-BY vs. NpHR-Analg-BY	.0045	**	

Table S1.

Detailed statistical tables for figures 1-5. (**A**) Statistical tests to compare behavioral results between control (Con), CFA pain (CFA) and bystander (BY) mice in Fig. 1B-G (**B**) Statistics tests used to compare TRAP/TdTomato+ cell counts in slices from Con, BY and CFA mice in Fig 2C. (**C**) Statistical tests comparing Con, CFA and BY mice injected with either AAV-YFP or AAV-NpHR (Fig. 3B-G) or AAV-YFP or AAV-ChR2 (Fig. 3H). (**D**) Statistical tests comparing demonstrator mice given shock (Shock) to observer/bystander (BY) mice in Fig. 4B,C,and Shock and BY mice injected with AAV-YFP or AAV-NpHR, Fig. 4E-J. (**E**) Statistical tests comparing CFA-Con, CFA-Morphine (CFA-Mor) and CFA-Analgeisa-BY (CFA-Analges-BY) in Fig 5B-D, injected with either AAV-YFP or AAV-NpHR, Fig. 5F-I. From left to right: Figure #,

Data/comparison, N, Statistical test, Post hoc analysis, Comparison and effect, p values, notation (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; NS, not significant), and F/t statistics.