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Optogenetic characterization of CeA CRF pathways in alcohol dependence

Giordano de Guglielmo[1], Marsida Kallupi[1], Matt Pomrenze[2], Elena Crawford[1], Sierra Simpson[1], Paul Schweitzer[1], George F. Koob[3], Robert O. Messing[2], Olivier George[1*]

[1]Department of Neuroscience, The Scripps Research Institute, La Jolla, CA 92037 USA

[3]Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA

[2]National Institutes on Drug Abuse, National Institutes of Health, Baltimore, MD 21224, USA

*Corresponding author:

Dr. Olivier George, Department of Neuroscience, The Scripps Research Institute, 10550 North Torrey Pines Road, SP30-2400, La Jolla, CA 92037, USA. Tel: +1 858 784 7102. Fax: +1 858 784 7405. E-mail: ogeorge@scripps.edu

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Abstract

In alcohol dependent animals, withdrawal from alcohol activates a neuronal ensemble in the central nucleus of amygdala (CeA) that is responsible for escalation of alcohol drinking. However, the neuronal phenotype and neuronal pathways controlled by these neurons is unknown. We investigated the cellular identity of this CeA neuronal ensemble and found that most neurons express corticotropin releasing factor (CRF). Using *Crh*-Cre transgenic rats combined with *in vivo* optogenetics, we tested if inactivation of CeA CRF neurons prevents excessive alcohol self-administration during withdrawal. Rats were injected with AAV-DIO-NpHR-eYFP or AAV-DIO-eYFP (control) and implanted with optical fibers in the CeA. Animals were then exposed to chronic intermittent ethanol (CIE) to induce alcohol dependence. Inactivation of CeA CRF neurons decreased alcohol drinking in dependent rats to levels observed before the animals became dependent, and completely prevented the activation of the entire CeA neuronal ensemble (Fos+ neurons) during withdrawal. No effect was observed in the AAV-DIO-eYFP control group or on water or saccharin self-administration in either group. In a second experiment, rats were injected with AAV-DIO-NpHR-eYFP in the CeA and optical fibers were implanted into downstream projection regions of CeA CRF neurons including the bed nucleus of the stria terminalis (BNST), lateral hypothalamus (LH), parasubthalamic (pSTN), substantia innominata (SI) or parabrachial nuclei (PBN). Optogenetic inactivation of CRF terminals in the BNST recapitulated the effect on alcohol drinking observed with inactivation of CeA CRF cell bodies, whereas inactivation of the LH, SI or PBN had no effect. Taken together, these results demonstrate that activation of CeA CRF neurons during withdrawal is required for the recruitment of the CeA neuronal ensemble that is

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responsible for excessive alcohol drinking in dependent rats, and that the CRF projection to the BNST, but not the LH, pSTN, SI, and PBN is the main downstream pathway responsible for excessive alcohol drinking in dependent rats.

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Introduction

Alcoholism is a chronic relapsing disorder associated with compulsive drinking, loss of control over intake, and emergence of a negative emotional state during abstinence from the drug (Koob *et al*, 2004). Activation of a specific neuronal ensemble in the Central Amygdala (CeA) during alcohol withdrawal is causally related to the excessive alcohol drinking observed in alcohol dependent rats (de Guglielmo *et al*, 2016). However, the cellular phenotypes of these neurons and the brain regions that are controlled by this neuronal ensemble are unknown. Moreover, while it is known that activation of corticotropin releasing factor (CRF) type 1 receptors (CRF_1) in the CeA is required for excessive alcohol drinking in dependent rats, there is to date no direct evidence that activation of CeA CRF neurons is required for alcohol drinking. Indeed, extracellular levels of CRF in the CeA are increased during withdrawal from chronic alcohol exposure (Koob, 2008; Koob *et al*, 2004; Merlo Pich *et al*, 1995; Olive *et al*, 2002), and systemic and intra-CeA administrations of specific CRF1 antagonists reduce both the negative emotional states of alcohol withdrawal and alcohol drinking in dependent rats (Funk *et al*, 2006; Funk *et al*, 2007; Gehlert *et al*, 2007; Koob, 2008). However, no study to date has shown that inactivation of CeA CRF neurons *per se* (not CRF_1 receptors) is responsible for excessive alcohol drinking during acute withdrawal. This is critical because activation of CeA CRF_1 receptors could results from activation of CRF neurons located in other brain regions (BNST, LH, pSTN) that project to the CeA.

We first hypothesized that CRF neurons are critical for recruitment of the CeA neuronal ensemble during alcohol withdrawal. To test this hypothesis, we used *Crh*-Cre transgenic rats combined with *in vivo* optogenetics and immediate early gene brain mapping. We

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then dissected the role of the different downstream CeA-CRF pathways in alcohol dependence using optogenetics inactivation of CRF terminals from CeA neurons projecting to the bed nucleus of the stria terminalis ($CRF^{CeA\text{-}BNST}$), lateral hypothalamus and parasubthalamic nucleus ($CRF^{CeA\text{-}LH/pSTN}$), substantia innominata ($CRF^{CeA\text{-}SIB}$) and parabrachial nucleus ($CRF^{CeA\text{-}PBN}$) (pomrenze 2015).

Materials and Methods

Subjects

Adult male *CRF-cre* rats, weighing 200-225g at the beginning of the experiments, were housed in groups of two per cage (self-administration groups) in a temperature-controlled (22°C) vivarium on a 12 h/12 h light/dark cycle (lights on at 10:00 PM) with *ad libitum* access to food and water. All of the behavioral tests were conducted during the dark phase of the light/dark cycle. All of the procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Operant self-administration

Self-administration sessions were conducted in standard operant conditioning chambers (Med Associates, St. Albans, VT, USA). For the alcohol self-administration studies, the animals were first trained to self-administer 10% (w/v) alcohol and water solutions until a stable response pattern (20 ± 5 rewards) was maintained. The rats were subjected to an overnight session in the operant chambers with access to one lever (right lever) that delivered water (fixed-ratio 1 [FR1]). Food was available *ad libitum* during

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this training. After 1 day off, the rats were subjected to a 2 h session (FR1) for 1 day and a 1 h session (FR1) the next day, with one lever delivering alcohol (right lever). All of the subsequent alcohol self-administration sessions lasted 30 min. The rats were allowed to self-administer a 10% (w/v) alcohol solution (right lever) and water (left lever) on an FR1 schedule of reinforcement (i.e., each operant response was reinforced with 0.1 ml of the solution). For the saccharin self-administration study, the rats underwent daily 30 min FR1 sessions. Responses on the right lever resulted in the delivery of 0.1 ml of saccharin (0.04%, w/v). Lever presses on the left lever delivered 0.1 ml of water. This procedure lasted 13 days until a stable baseline of intake was reached.

Alcohol vapor chambers

The rats were previously trained to self-administer alcohol in the operant chambers. Once a stable baseline of alcohol intake was reached, the rats were made dependent by chronic intermittent exposure to alcohol vapors. They underwent cycles of 14 h on (blood alcohol levels during vapor exposure ranged between 150 and 250 mg%) and 10 h off, during which behavioral testing for acute withdrawal occurred (i.e., 6-8 h after vapor was turned off, when brain and blood alcohol levels are negligible). In this model, rats exhibit somatic and motivational signs of withdrawal (Vendruscolo and Roberts, 2014).

Operant self-administration and withdrawal scores during alcohol vapor exposure

Behavioral testing occurred three times per week. The rats were tested for alcohol or saccharin (and water) self-administration on an FR1 schedule of reinforcement in 30-

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min sessions. Behavioral signs of withdrawal were measured using a rating scale adapted from a previous study (Macey *et al*, 1996) and included ventromedial limb retraction (VLR), irritability to touch (vocalization), tail rigidity, abnormal gait, and body tremors. Each sign was given a score of 0-2, based on the following severity scale: 0 = no sign, 1 = moderate, 2 = severe. The sum of the four observation scores (0-8) was used as an operational measure of withdrawal severity.

Intracranial surgery

For intracranial surgery, the animals were anesthetized with isoflurane. For behavioral experiments, cre-dependent adeno-associated virus carrying inhibitory opsins, AAV-DIO-eNpHR3.0-EYFP or AAV-DIO-EYFP (UNC Vector Core, University of North Carolina, Chapel Hill) were injected bilaterally or unilaterally in the CeA (coordinates from Bregma: - 2.6 mm AP, ±4.2 mm ML, -6.1 mm DV from skull surface). The injection was made through a stainless-steel injector 2 mm longer than the guide cannula (so that its tip protruded into the area) connected to a 10 µl Hamilton syringe, which was controlled by an UltraMicroPump (WPI Inc, Sarasota, FL). Virus was injected (1.0 µl, 100 nl/min) over 10 min followed by additional 10 min to allow diffusion of viral particles. For the rats destined for behavioral experiments, chronic fiber optics were then implanted bilaterally or unilaterally above the CeA (coordinates from Bregma: - 2.6 mm AP, ±4.2 mm ML, -8.1 mm DV from skull surface) and unilaterally above the BNST (coordinates from Bregma: - 0.1 mm AP, ±1.4 mm ML, -6.7 mm DV from skull surface), the LH/pSTN (coordinates from Bregma: - 4.2 mm AP, ±1.2 mm ML, -8.7 mm DV from skull surface), the SI (coordinates from Bregma: - 0.1 mm AP, ±1.8 mm ML, -8.5 mm

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DV from skull surface) and the PBN (coordinates from Bregma: - 9.2 mm AP, ±1.8 mm ML, -6.5 mm DV from skull surface). Following surgery, the rats were allowed to recover for 1 week.

Locomotor activity

Locomotor activity and anxiety-like behavior was evaluated in an opaque open field apparatus (100 × 100 × 40 cm) divided in 20 × 20 cm squares. On the experimental day, the animals were placed in the center of the open field and locomotor activity (number of lines crossed), time in the center total distance traveled, and entries in to the center were scored during 10 minutes. The behavior was videotaped and the Any Maze program was used to analyze the recordings.

Optical inhibition

Optical probes were constructed in which the fiber optic (200 µm core, multimode, 0.37 NA) was inserted and glued into ceramic ferrule. The fiber optic and the ferrule were then glued to a metallic cannula closed with a dust cap. The fiber optic length was then adjusted based on the brain area of interest in order to not leave a gap between the skull and the cannula during surgery. The other end of the fiber optic (FC/PC connection) was attached to a fiber splitter (2x1) that permitted simultaneous, bilateral illumination. The single end of the splitter was attached to a rotating optical commutator (Doric Lenses) to permit free movement of the rat. The commutator connected to a fiber that connected to a laser (DPSS, 200 or 300mW, 532nm, with a multimode fiber coupler for an FC/PC connection, OEM Laser Systems). Prior to the experiments, the light output of the fiber optic was adjusted to approximately 10 mW, as measured by an optical power meter.

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Based on measurements made in mammalian brain (Deisseroth, 2012), assuming a geometric loss of light, light output of 10 mW measured by a standard optical power at the tip of a fiber with an NA of 0.37 and a fiber core radius of 200 μm will produce ~ 1 mW/mm^2 of light up to 1 mm directly away from the fiber tip, which is the minimum amount necessary to produce opsin activation (Gradinaru *et al*, 2009; Tye *et al*, 2011). Based on in vivo measurements of the shape of the light output in mammalian brain tissue, these parameters would be expected to provide sufficient light for opsin activation in at least 0.4 mm^3 of tissue (Yizhar *et al*, 2011). Fiber optics were implanted at the same time of the virus injections and animals were habituated to tethering to the commutator for several self-administration sessions before the beginning of the experiments. In the test days, rats received the optical inhibition paired with the alcohol self-administration session, or the locomotor activity or the withdrawal score.

Recruitment of CeA CRF neurons during alcohol withdrawal

The animals were trained to self-administer alcohol 10% and made dependent on alcohol using the CIE model. After the stabilization of excessive drinking, animals were transcardially perfused and brains harvested, post-fixed and sectioned at 40 μm.

Effect of optogenetic inhibition of CeA CRF neurons on alcohol and saccharine self-administration in alcohol non-dependent and dependent rats.

Animals were injected with AAV-DIO-eNpHR3.0-EYFP or AAV-DIO-EYFP and implanted with bilateral fiber optics in the CeA. One week after the surgery, rats were trained to self-administer 10% alcohol until a stable baseline of intake. At this point the

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experiment started and rats were tested for their alcohol self-administration intake during optical inhibition of the CeA CRF neurons. Sham optical inhibition sessions were performed before and after the test session. The animals were then placed in the alcohol vapor chambers and 3 weeks later underwent the alcohol self-administration escalation phase. When escalation of alcohol intake was achieved, animals were tested for the effects of optical inhibition of CeA CRF neurons as described above. The effect of optical inhibition on somatic withdrawal signs was also assessed.

At the end of this phase the rats were trained to self-administer saccharine for several sessions until a stable baseline of responses, and tested for the effects of optical inhibition of CeA CRF neurons on saccharine self-administration. The effects of optical inhibition were also assessed on the locomotor activity.

Dissection of the role of the different CeA CRF pathways on alcohol and saccharine self-administration in alcohol dependent rats.

CRF-Cre rats were bilaterally infused with an AAV-DIO-NpHR-eYFP in the CeA, implanted unilaterally with fiber optics in the CeA, BNST, PBN, SI and LH and made dependent on alcohol using the CIE model. After escalation of drinking, animals were tested for the effect of optogenetic inhibition of the CeA-CRF projections on alcohol drinking, alcohol withdrawal signs and saccharine self-administration.

Immunohistochemistry

CRF-Cre rats were implanted with unilaterally in the CeA and tested for the effect of inhibition of the CeA for 30 minutes during withdrawal (8h) from alcohol (CIE). 90

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minutes later animals were deeply anesthetized and perfused with 100 ml of phosphate-buffered saline (PBS) followed by 400 ml of 4% paraformaldehyde. The brains were postfixed in paraformaldehyde overnight and transferred to 30% sucrose in PBS/0.1% azide solution at 4°C for 2-3 days. Brains were frozen in powdered dry ice and sectioned on a cryostat. Coronal sections were cut 40 µm thick between bregma +4.2 and -6.48 mm (Paxinos and Watson, 2005) and collected free-floating in PBS/0.1% azide solution. Following three washes in PBS, the sections were incubated in 1% hydrogen peroxide/PBS (to quench endogenous peroxidase activity), rinsed three times in PBS, and blocked for 60 min in PBS that contained 0.3% TritonX-100, 1 mg/ml bovine serum albumin, and 5% normal donkey serum. Sections were incubated for 24 h at 4°C with a rabbit monoclonal anti-Fos antibody (Cell Signaling Technologies #2250) diluted 1:1000 in PBS/0.5% Tween20 and 5% normal donkey serum. The sections were washed again with PBS and incubated for 1 h in undiluted Rabbit ImmPress HRP reagent (Vector Laboratories). After washing in PBS, the sections were developed for 2-6 min in Vector peroxidase DAB substrate (Vector Labs) enhanced with nickel chloride. Following PBS rinses, the sections were mounted onto coated slides (Fisher Super Frost Plus), air dried, dehydrated through a graded series of alcohols, cleared with Citrasolv (Fisher Scientific), and coverslipped with DPX (Sigma).

Quantitative analysis to obtain unbiased estimates of the total number of *Fos+* cell bodies was performed on a Zeiss Axiophot Microscope equipped with MicroBrightField Stereo Investigator software (Colchester, VT, USA), a three-axis Mac 5000 motorized stage (Ludl Electronics Products, Hawthorne, NY, USA), a Q Imaging Retiga 2000R color digital camera, and PCI color frame grabber.

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Immunohistochemistry

Immunohistochemistry was used to characterize the type (CRF) and number (NeuN) of neurons activated (cFOS) during the withdrawal from alcohol. Coronal sections were cut 40 µm thick between Bregma -2.0 and -5.0mm (Paxinos *et al*, 2005). We determined the proportion of all neurons expressing cFos during the alcohol withdrawal by double-labeling for cFos and the neuron-specific protein NeuN, as well as the population activated CRF neurons in the CeA by double labeling for cFOS and CRF.

For cFos:NeuN and cFos:CRF labeling, 40 µm sections were washed three times in phosphate buffered saline (PBS) and permeabilized/blocked for 60 min in PBS with 0.2% Triton X-100, 5% NDS, 3% BSA (Blocking Solution). Sections were incubated in primary antibodies diluted in blocking solution for 24 h on a shaker at RT. Primary antibodies were used at the following concentrations, anti-cFos (Millipore, AB4532 (1:500) and anti-NeuN (Millipore, MAB377, 1:1000). Sections were washed three times 10 minutes in PBS and incubated with fluorescently labeled secondary antibodies diluted in PBS with 2 h on a shaker at RT. Secondary antibodies were Alexa Fluor 488-labeled donkey anti-rabbit (1:500 dilution, A-10042, Invitrogen) and Alexa Fluor 568-labeled donkey anti-mouse (1:500 dilution, S-11249, Invitrogen) and Alexa Fluor 647-labeled donkey anti-goat (1:500 dultion, A-21447) . After labeling, sections were washed three times ten minutes in PBS, mounted onto Fisher Superfrost Plus slides (12-550-15) , and coverslipped with PVA-Dabco (Sigma).

Three sections were bilaterally analyzed for each rat. Cells were identified as neurons based on standard morphology, and only neurons with a focused nucleus within the

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boundary of the CeA were counted. Counts from all images from each rat were averaged so that each rat was an *n* of 1.

Confocal Acquisition and 3D Analysis

Three-dimensional stacks of Images were acquired with a 780 Laser Scanning Confocal microscope (Zeiss,Inc.) using either a 20x (1µm imageslice), 40x(0.6µm imageslice), or 63x (0.2µm imageslice) objective to observe the entirety of the CeA. The system is equipped with a stitching stage and Zen software to reintegrate the tiled image stacks. Stitched z series images of the entire CeA were imported into Imaris software (Bitplane-Andor,Inc.) and FIJI for quantification.

Slice Preparation for whole cell recording:

Cre Crf^+ rats (n= 5) with bilateral infusion of rAAV5/Ef1a-DIO eNpHR into the CeA were deeply anesthetized with isoflurane (3%) 4-5 weeks after virus infusion and then transcardially perfused with ice-cold oxygenated sucrose solution. Rats were then decapitated, and the brains were rapidly removed and placed into oxygenated (95% O_2 - 5% CO_2) ice-cold cutting solution containing (in mM): sucrose 206; KCl 2.5; NaH_2PO_4 1.2; $MgCl_2$ 7; $CaCl_2$ 0.5; $NaHCO_3$ 26; glucose 5; HEPES 5. CeA slices (300 um) thick were cut on a Vibratome (Leica VT1000S, Leica Microsystems, Buffalo Grove, IL) and transferred into oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl, 130; KCl, 2.5; NaH_2PO_4, 1.25; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2$, 2.0; $NaHCO_3$, 24; glucose, 10. Slices were first incubated for 30 min at 37°C, then kept at room temperature for the remainder of the experiment. Individual slices were transferred to a

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recording chamber mounted on the stage of an upright microscope (Olympus BX50WI, Waltham MA, USA). Recordings were performed in continuous oxygenated aCSF perfused at a rate of 2-3 ml/min. Neurons were visualized with a 60x water immersion objective (Olympus), infrared differential interference contrast optics and a CCD camera (EXi Blue from QImaging, Surrey, BC Canada). Whole-cell recordings were performed using current clamp mode with a Multiclamp 700B amplifier, Digidata 1440A and pClamp 10 software (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes (4-7 MΩ) were pulled from borosilicate glass (Warner Instruments, Hamden, CT) and filled with the following internal solution (in mM): KMeSO4 70; KCl 55; NaCl 10; $MgCl_2$ 2; HEPES 10; Mg-ATP 2; Na-GTP 0.2.

To study neuronal inhibition elicited by optogenetics, a green laser (532 nm) was switched on for 6.5 seconds, by a Master-8 stimulator (AMPI, Jerusalem, Israel) to generate trains at a frequency of 15 Hz.

Neurons expressing eNpHR within the CeA were visualized with differential interference contrast and widefield fluorescence imaging using an Olympus 60X immersion objective. To identify eNpHR-expressing neurons, a Lambda DG-4 light source was used with an in-line EX540/EM630 filter set. A Mosaic 3 pattern illuminator (Andor Instruments, Belfast, UK) coupled to a 532nm light emitting diode (CoolLED Limited, Andover, UK) was attached to the microscope and used for light delivery (10 mW/mm^2, to approximate output in the behavioral experiments) through the objective within the slice preparation.

Histology

Following the completion of behavioral experiments, rats were deeply anaesthetized and transcardially perfused. Brains were removed and 40 μm coronal slices containing the

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CeA, the BNST and the PBN were cut on a cryostat. Collected brain slices were mounted on microscope slides and expression of viral vectors and/or optical fiber placements was examined for all rats under fluorescent microscopy. Rats showing no YFP expression in the CeA in result of faulty microinjections, or showing fiber placements failing to target the regions of interest were excluded from behavioral analysis.

Statistical analysis

Data are presented as means ± SEM. For comparisons with only two groups, *p* values were calculated using paired or unpaired t-tests as described in the figure legends. Comparisons across more than two groups were made using a one-way ANOVA, and a two-way ANOVA was used when there was more than one independent variable. A Newman Keuls post hoc test was used following significance with an ANOVA. Withdrawal signs were analyzed by the nonparametric Mann-Whitney *U* statistic, followed by Dunn's multiple-comparison test. The standard error of the mean is indicated by error bars for each group of data. Differences were considered significant at *p* values below 0.05. All data were analyzed with STATISTICA 7 software.

Results

CeA CRF neurons are recruited during alcohol withdrawal

At the end of the escalation phase and exposure to chronic intermittent ethanol (CIE) vapor, rats averaged 45.1±7.2 responses for alcohol compared with 17.5±3.4 responses before exposure to CIE (p<0.01, Fig 1.A). After stabilization of excessive drinking, animals were transcardially perfused and brains harvested, post-fixed and sectioned at

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40 µm. Withdrawal from alcohol vapor produced a significant recruitment of Fos^+ neurons in the CeA [(df=8); t=6.16; p<0.001] (Fig. 1B). This recruitment was limited to a small subpopulation of neurons in the CeA as double labeling with Fos/Neun revealed that only 7-8% of all CeA neurons ($Neun^+$) were also Fos^+ [(df=8); t=22.4; p<0.01] (Supplementary Fig. 1). Double Fos-CRF IHC showed that while the total number of CRF neurons did not change between withdrawal rats and naïve [(df=8); t=0.58; p=NS] (Fig. 1C), the number of Fos^+/CRF^+ neurons in the CeA dramatically increased during withdrawal [(df=8); t=6.24; p<0.001] (Fig. 1D) and represented the majority of the FOS^+ neurons (80%).

Whole cell patch recording

To confirm the functionality of NpHR in the local circuitry, electrophysiological recordings of neuronal activity from single neurons were performed in acute CeA slices from *crf-cre* rats. CeA neurons were depolarized by current injection to evoke sustained firing of action potentials, and CRF^+ neurons were identified by visualization of YFP fluorescence (Zhao *et al*, 2011). In six neurons showing fluorescence that were held at -52.9 +/- 0.4 mV, exposure of the slice to a green laser (532 nm) using a train (6.5 sec) at 15 Hz (Haubensak *et al*, 2010) elicited a hyperpolarization of 6.5 +/- 1.0 mV, concomitantly suppressing the firing of action potentials (Fig. 2C). In another six neurons that did not show fluorescence, a similar exposure to the laser (6.5 sec at 15 Hz) did not elicit any significant effect on resting potential (held -51.8 +/- 0.5 mV, hyperpolarization 0.5 +/- 0.3 mV; Fig.2 D).

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Optogenetic inhibition of CeA CRF neurons reduces alcohol self-administration selectively in alcohol dependent rats.

In the non-dependent animals the inhibition of the CeA CRF neurons induced by the activation of the green laser didn't show any effect in controls and NpHR injected rats.

The ANOVA didn't show any effect in term of groups [$F_{(1,14)}$=1.30; *p*=NS], treatment [$F_{(1,28)}$=0.03; *p*=NS] or the groups x treatment interaction [$F_{(1,28)}$=2.10; *p*=NS]. Water self-administration was not affected by the green laser in both groups both in terms of groups [$F_{(1,14)}$=0.17; *p*=NS], treatment [$F_{(1,28)}$=0.82; *p*=NS] and groups x treatment [$F_{(1,28)}$=1.07; *p*=NS](Fig 3A) interactions.

After 6 weeks of vapor exposure (CIE), animals escalated their alcohol consumption as shown by the one-way ANOVA [$F_{(1,28)}$=0.82; $p<0.001$]. Newman Keuls *post hoc* tests demonstrated a significant increase in alcohol self-administration starting from session 8 to session 12, compared with the first day vapor exposure ($p<0.05$, Fig. 3B). The figure 3C shows the effects of the optogenetic inhibition of the CeA CRF neurons on alcohol (panel a) and water (panel b) self-administration in alcohol dependent rats. The mixed factorial ANOVA demonstrated that both Controls and NpHR rats escalated their alcohol intake after x weeks of vapor exposure [$F_{(1,14)}$=14.9; $p<0.01$] (p<0.05 vs baseline pre-vapor using Newman Keuls *post hoc* test). Activation of the green laser selectively reduced alcohol self-administration in NpHR injected rats. The ANOVA showed a significant effect of treatment: [$F_{(2,28)}$=12.7; $p<0.001$] and a groups x treatment interaction [$F_{(2,28)}$=8.38; $p<0.01$]. No significant main effect of groups was detected: [$F_{(1,14)}$=1.74; *p*=NS]. The Newman Keuls *post hoc* test demonstrated a selective reduction of alcohol intake in the NpHR inhibited rats. No effect of inhibition of CeA CRF neurons

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was detected on water self-administration: groups [$F_{(1,14)}$=0.7; *p*=NS], treatment: [$F_{(2,28)}$=0.22; *p*=NS], groups x treatment interaction [$F_{(2,28)}$=2.44; *p*=NS].

To ensure that the effect observed was not due to non-specific effect caused by prolonged (30 min) illumination of the CeA with the green laser, we measured alcohol drinking after only 5 min of illuminations. Results showed that there was no difference in the magnitude of the effects between the first 5 minutes and the entire 30 minutes session (see supplementary figure 2A).

To further test the behavioral specificity of this effect, animals were trained to self-administer saccharine and tested for the effects of inhibition of CeA CRF neurons on saccharine intake. The mixed factorial ANOVA didn't show any effect of the activation of the green laser in controls and NpHR rats neither in terms of saccharine intake (groups: [$F_{(1,14)}$=0.27; *p*=NS], treatment: [$F_{(2,28)}$=0.48; *p*=NS], groups x treatment interaction [$F_{(2,28)}$=0.06; *p*=NS]) nor in terms of water intake (groups: [$F_{(1,14)}$=0.57; *p*=NS], treatment: [$F_{(2,28)}$=1.13; *p*=NS], groups x treatment interaction [$F_{(2,28)}$=0.27; *p*=NS]).

The animals were also tested in an open field and the t-test didn't show any significant effect of exposure to the green laser between controls and NpHR injected rats when measuring the total distance traveled [(df=8) t=0.36; *p*=NS] and the time spent in the center [(df=8) t=0.36; *p*=NS].

Effect of unilateral optogenetic inhibition of CeA CRF neurons on alcohol self-administration and CeA Fos^{+} neurons.

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A group of *crf-cre* rats was bilaterally infused with AAV-DIO-NpHR-eYFP and implanted with unilateral fiber optics in the CeA (the right or left CeA was randomly used for each rats and evenly distributed between groups). The effect of optogenetic inhibition of the CeA CRF neurons on alcohol drinking, somatic signs and saccharine self-administration were measured using the same CIE protocol than in the first experiments. *Alcohol drinking*. The Two-way ANOVA repeated measures with time and laser illumination as within factors showed a significant effect of the time [$F(2,22)=16.36$]; $p<0.001$], laser [$F(1,11)=6.62$]; $p<0.05$] and laser x time [$F(2,22)=13.15$]; $p<0.001$] interaction. Newman Keuls *post hoc* test showed that there was a significant reduction of ethanol self-administration when the green laser was turned on ($p<0.01$ vs laser OFF, fig. 4A).

A separate one-way ANOVA demonstrated that animals showed escalation of alcohol intake (compared to baseline pre-vapor) [$F(6,66)=9.23$; $p<0.001$]. The escalation of alcohol drinking was observed only in days when the laser was off ($p<0.001$) and was completely blocked by the optogenetic inhibition of CeA-CRF neurons, as demonstrated by the Newman Keuls *post hoc* analysis. *Withdrawal signs*. Immediately before the last 2 alcohol self-administration sessions, the rats were observed for somatic signs. As shown in Fig. 4B, the Laser ON group exhibited significant decreases in ventromedial limb retraction (Mann-Whitney $U = 9.00$, $p < 0.05$) and abnormal gait ($U = 3.000$, $p < 0.01$). The sum of the five rating scores revealed a significant decrease in overall withdrawal severity ($U = 4.5$, $p < 0.05$; Fig. 4B, inset) after inhibition of CeA-CRF neurons.

Saccharine self-administration. The t-test didn't show any effect of inhibition of the CeA CRF neurons on saccharine self-administration [(df=11); t=0.321; p=NS] (Fig 4C).

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A separate group of rats was tested for the effect of inhibition of the CeA for 30 minutes during withdrawal (8h) from alcohol (CIE). The ANOVA showed a significant effect of the optogenetic inhibition of CeA CRF neurons [$F_{(3,2)}$=13.61; p<0.01]. Newman Keuls *post hoc* tests showed that after the inhibition (Laser ON), the increase in Fos+ neurons normally observed during withdrawal (Laser OFF, p<0.01 OFF vs Naive) was completely prevented (p<0.01 ON vs OFF). Unilateral inhibition also partially affected the contralateral CeA, as a significant decrease in Fos+ neurons was also observed on the contralateral side (p<0.01 ON/Controlateral vs OFF Fig. 4D).

Dissection of the role of the different CeA CRF pathways on alcohol drinking in dependent rats

BNST

Alcohol drinking. Two-way ANOVA with repeated measures with the time and the laser as within factors showed a significant effect of the time [F(2,22)=9.18]; p<0.01], laser [F(1,11)=29.72]; p<0.001] and laser x time [F(2,22)=5.31]; p<0.05] interaction. Further analysis of the Newman Keuls *post hoc* test showed that there was a significant reduction of ethanol self-administration when the green laser was turned on (p<0.01 vs laser off, fig. 5A.a). A separate one-way ANOVA demonstrated that animals showed escalation of alcohol intake (compared to baseline pre-vapor) [F(6,66)=11.43; p<0.0001]. The Newman Keuls *post hoc* analysis that rats showed escalation of alcohol drinking only in the OFF days (p<0.001), while animals showed a level of responding similar to the baseline pre-vapor (BSL) after optogenetic inhibition of the $CRF^{CeA\text{-}BNST}$ terminals (Fig.

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5A.a). _Withdrawal signs_. Immediately before the last 2 alcohol self-administration sessions, the rats were observed for withdrawal signs 8h into withdrawal. As shown in Fig. 5A.b, the laser ON group exhibited significant decreases in abnormal gait (Mann-Whitney $U = 6.00$, $p < 0.05$), and body tremors ($U = 10.50$, $p < 0.05$). The sum of the five rating scores revealed a significant decrease in overall withdrawal severity ($U = 1.5$, $p < 0.01$; Fig. 5A.b, inset) after the inhibition of $CRF^{CeA\text{-}BNST}$ terminals.

Saccharine self-administration. The t-test didn't show any effect of inhibition of the $CRF^{CeA\text{-}BNST}$ terminals on saccharine self-administration [(df=11); t=0.12; p=NS] (Fig 5A.c).

SIB

Alcohol drinking. The Two-way ANOVA repeated measures with the time and the laser as within factors showed a significant effect of the time [$F(2,10)=15.10$]; $p<0.001$], but no effect of the laser [$F(1,5)=4.39$]; p=NS] or of the interaction laser x time [$F(2,10)=3.66$]; p=NS]. A separate one-way ANOVA demonstrated that animals showed escalation of alcohol intake (compared to baseline pre-vapor) [$F(6,30)=4.28$; $p<0.01$]. Escalation of alcohol intake was not affected by the optogenetic inhibition of the $CRF^{CeA\text{-}SI}$ terminals, as shown by the Newman Keuls post hoc tests. In fact the operant responding for alcohol remained high in both ON and OFF days ($p<0.05$ or $p<0.01$ vs BSL,Fig. 5B.a). Optogenetic inhibition of the CeA-SIB-CRF terminals didn't show any effect on alcohol drinking [(df=11); t=0.49; p=NS]. _Withdrawal signs_. Immediately before the last 2 alcohol self-administration sessions, the rats were observed for

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withdrawal signs. As shown in Fig. 5B.b, the inhibition of $CRF^{CeA\text{-}SI}$ terminals didn't affect the overall withdrawal severity (U = 15.50, p =NS; Fig. 5B.b, inset).

Saccharine self-administration. The t test didn't show any effect of inhibition of the $CRF^{CeA\text{-}SI}$ terminals on saccharine self-administration [(df=5); t=0.22; p=NS], (Fig. 5B.c).

LH/pSTN

Alcohol drinking. The Two-way ANOVA repeated measures with the time and the laser as within factors showed a significant effect of the time [F(2,10)=8.87]; p<0.01], but no effect of the laser [F(1,5)=0.78]; p=NS] or of the interaction laser x time [F(2,10)=0.16]; p=NS] (Fig. 5C.a). A separate one-way ANOVA demonstrated that animals showed escalation of alcohol intake (compared to baseline pre-vapor) [F(6,30)=4.28; p<0.01]. Escalation of alcohol intake was not affected by the optogenetic inhibition of the $CRF^{CeA\text{-}LH/pSTN}$ terminals, as shown by the Newman Keuls post hoc tests. In fact, the operant responding for alcohol remained high in both ON and OFF days (p<0.05 vs BSL,Fig. 5C.a). *Withdrawal signs*. Immediately before the last 2 alcohol self-administration sessions, the rats were observed for withdrawal signs. As shown in Fig. 5C.b, the inhibition of $CRF^{CeA\text{-}LH/pSTN}$ terminals neurons didn't affect the overall withdrawal severity (U = 14.00, p =NS; Fig. 5C.b, inset). *Saccharine self-administration*. The t test didn't show any effect of inhibition of the $CRF^{CeA\text{-}LH/pSTN}$ terminals on saccharine self-administration [(df=5); t=1.024; p=NS] (Fig. 5C.c).

PBN

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Alcohol drinking. The Two-way ANOVA repeated measures with the time and the laser as within factors showed a significant effect of the time [F(2,22)=7.14]; p<0.01], but no effect of the laser [F(1,11)=0.73]; p=NS] or of the interaction laser x time [F(2,22)=1.99]; p=NS]. A separate one-way ANOVA demonstrated that animals showed escalation of alcohol intake (compared to baseline pre-vapor) [F(6,66)=4.75; p<0.001]. Escalation of alcohol intake was not affected by the optogenetic inhibition of the $CRF^{CeA\text{-}PBN}$ terminals, as shown by the Newman Keuls post hoc tests (Fig. 5D.a). *Withdrawal signs*. Immediately before the last 2 alcohol self-administration sessions, the rats were observed for withdrawal signs. As shown in Fig. 5D.b, the inhibition of $CRF^{CeA\text{-}PBN}$ terminals didn't affect the overall withdrawal severity (U = 18.50, p =NS; Fig. 5D.b, inset).

Saccharine self-administration. The t test didn't show any effect of inhibition of the $CRF^{CeA\text{-}PBN}$ terminals on saccharine self-administration [(df=11); t=0.77; p=NS] (Fig. 5D.c).

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Discussion

This report demonstrate that CRF neurons represent the majority of the neurons composing the CeA neuronal ensemble that is recruited during withdrawal in alcohol dependent rats. We further demonstrate that activation of CRF neurons in the CeL is required for the recruitment of a CeA neuronal ensemble located in the CeL, CeM and CeC. Optogenetic inhibition of CeL CRF neurons fully reversed the escalation of alcohol drinking and partly alleviated the somatic signs of withdrawal in dependent rats, without affecting water or saccharine self-administration. Finally, selective optogenetic inhibition of the $CRF^{CeA\text{-}BNST}$ pathways, but not $CRF^{CeA\text{-}SI}$, $CRF^{CeA\text{-}LH/pSTN}$ or $CRF^{CeA\text{-}PBN}$, recapitulated the behavioral effects of inactivation of CeA CRF neurons.

Numerous studies have demonstrated that the chronic intermittent exposure (CIE) to alcohol vapor model has robust predictive validity for alcoholism and construct validity for the neurobiological mechanisms of alcohol dependence (Heilig and Koob, 2007; Koob, 2009). In fact, rats made dependent with chronic intermittent exposure to alcohol vapor (14 h on/10 h off) increase their alcohol drinking if tested during early and protracted withdrawal with clinically relevant BALs (150-250 mg% range) and compulsive alcohol intake despite adverse consequences (Vendruscolo *et al*, 2012). The compulsive drug seeking associated with alcoholism can be derived from multiple neuroadaptations, but a key component involves the construct of the negative reinforcement, defined as alcohol drinking for alleviating a negative emotional state (Koob, 2014). The recruitment of brain stress systems, such as corticotropin-releasing factor (CRF), in the extended amygdala has been hypothesized having a central role on generating the negative emotional state that drives such negative reinforcement.

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We recently demonstrated that withdrawal from alcohol produces a significant recruitment of Fos^+ neurons in the CeA (de Guglielmo *et al*, 2016). Here, we show that the recruitment of the CeA neuronal ensemble is limited to a small subpopulation of neurons in the CeA, as double labeling with Fos and NeuN revealed that only 7-8% of neurons (NeuN+) were Fos+ (Suppl 1). No difference was observed between the different subregions of the CeA, although the lateral CeA (which contains the majority of CRF neurons (Pomrenze *et al*, 2015)) exhibited the largest increase in Fos^+ neurons (Suppl 1). Moreover, double Fos-CRF IHC showed that while the total number of CRF neurons did not change between groups (Fig.1), the number of Fos^+/CRF^+ neurons in the CeA dramatically increased during withdrawal from alcohol. Finally, neuronal phenotyping showed that the CeA withdrawal neuronal ensemble was mostly composed of both CRF+ and CRF- neurons with CRF+ representing the majority (~80%) of the total number of Fos+ neurons.

We previously demonstrated that inactivation of the withdrawal neuronal ensemble in the CeA reverses the escalation of alcohol drinking in dependent rats and alleviates the somatic signs of withdrawal (de Guglielmo *et al*, 2016). However, it was unknown if the activation of the CeA CRF neurons represent a consequence of the recruitment of the CeA withdrawal neuronal ensemble or a cause of its recruitment. The results show that inactivation of CeA CRF neurons completely prevented the recruitment of the CeA neuronal ensemble that includes both CRF+ and CRF- neurons. These results are line with our previous work demonstrating the chemogenetic stimulation of CeA CRF neurons recruits a population of CRF+ and CRF- neurons in the CeL and CeM that can be prevented (for the CRF- neurons) by a CRF1 receptor antagonist (Pomrenze et al,

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2015). While CeA CRF neurons are GABAergic, it has been shown that activation of CRF1 receptor in the CeL increases glutamate release locally (Silberman and Winder, 2013) suggesting that the recruitment of CRF- neurons in the neuronal ensemble may be mediated by activation of CR1 receptor on glutamatergic terminals. It also appears that the unilateral inhibition might also affect the contralateral CeA, suggesting a partial synchronization of bilateral CeAs. We next evaluated the effect of optogenetic inhibition of CeA CRF neurons on animals trained to self-administer alcohol and made dependent by chronic intermittent exposure to alcohol vapor. *crf-cre* rats were infused with an AAV-DIO-NpHR-eYFP (eNpHR) or AAV-DIO-eYFP (eYFP), implanted bilaterally with fiber optics in the CeA. Inactivation of CeA CRF neurons decreased alcohol drinking in dependent rats (**Fig. 3**). This effect was reversible and the inactivation reversed the level of drinking to pre-escalation levels before induction of dependence. To our knowledge, this is the first direct demonstration that CeA CRF neurons are responsible for activation of the CeA neuronal ensemble and that CeA CRF neurons are actually driving excessive alcohol drinking in dependent rats. The inactivation of CeA CRF neurons had no effect on saccharine self-administration or locomotor activity, and no effect of the laser was observed in the control group (AAV-DIO-eYFP, fig 3). These results suggest that the decreased drinking observed with NpHR is specific of alcohol drinking in dependent rats and not due to non-specific alterations of other behaviors. While we have not observed any non-specific effects of 30 min of inactivation using 532nm/NpHR on behavioral measures, one may be concerned about potential neuroadaptations at the cellular levels. To test this hypothesis, we tested the effect of only 5 min of green laser in NpHR rats since it has been shown that inhibition for 5 min using

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532nm/NpHR do not produce aberrant or non-specific neuronal response (Mahn *et al*, 2016). We did not observe any qualitative or quantitative differences between 5 and 30 min exposures. Indeed, the same effect-size between NpHR and YFP rats was observed after 5 min compared to 30 min.

Next, we wanted to test if the inactivation of the population of CeA CRF neurons during withdrawal would modulate the recruitment of Fos+ neurons in the CeA. In this experiment, we implanted *crf-cre* rats with unilateral fiber optics in the CeA and tested the effect of unilateral inhibition of the CeA for 30 minutes during withdrawal (8h) from alcohol (CIE) as in the previous experiment. Unilateral inhibition of the CeA CRF neurons during withdrawal led to the same reduction of alcohol intake observed with the bilateral inhibition. We also found that the somatic withdrawal signs were significantly reduced. We recently identified that CeA CRF neurons send dense projections to the BNST, SIB, pLH/pSTN and PBN, however, the role of each CRF projection on alcohol drinking was unknown.

Inactivation of the $CRF^{CeA\text{-}BNST}$ fully recapitulated the effects observed after inactivation of CeA CRF neurons, demonstrating that activation of the $CRF^{CeA\text{-}BNST}$ projection is critical for excessive alcohol drinking in dependent rats (**Fig. 5**). The effect was specific for alcohol drinking and withdrawal, while saccharine self-administration was not modified.

The BNST is considered to be a connective center between stress regions including BLA, CeA and PVN and brain reward centers such as VTA and NAc (Brog *et al*, 1993; Georges and Aston-Jones, 2001, 2002; Silverman *et al*, 1981). Importantly, the BNST is a critical modulator of addiction-like behavior (10421107). Several neuropeptides

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including CRF innervate and modulate activity within the BNST (Kash *et al*, 2015). A substantial body of evidence supports the role of CRF signaling in the BNST during anxiety induced by withdrawal from drug of abuse (Huang *et al*, 2010; Overstreet *et al*, 2003). These effects on withdrawal are considered to drive the effects of CRF in the BNST on stress-induced reinstatement of drug seeking behavior (Erb *et al*, 2001; Erb and Stewart, 1999). The CeA sends dense CRF projections to the ventral and dorsal BNST (Pomrenze *et al*, 2015; Sakanaka *et al*, 1986). While a clear role of BNST CRF signaling in stress-induced reinstatement has been described, it is less clear what role CRF signaling in the BNST plays in alcohol addiction. For instance, although intra-CeA injections of CRF antagonists post CIE can block CIE-induced increases in EtOH self-administration (Funk *et al*, 2006; Varodayan *et al*, 2017), post-CIE intra-BNST injections of the same antagonist have been shown not effective (Funk *et al*, 2006). In this report we clearly show the pivotal role of $CRF^{CeA\text{-}BNST}$ projections for the excessive alcohol drinking in dependent rats. In particular we think that the effects on the reduction of alcohol intake observed here are mediated by the ventral BNST projections, since they were the target of our fiber optics. In fact, we have previously shown the lack of CRF cells from the ventral BNST in our *crf-cre* rats (Pomrenze et al.).

No effect was observed in the $CRF^{CeA\text{-}SI}$, $CRF^{CeA\text{-}LH/pSTN}$ or $CRF^{CeA\text{-}PBN}$ We identified a small portion of fibers projecting from the CeA to the SIB. Previous findings have shown that acute alcohol doesn't affect Fos^{+} immunoreactivity in the SIB (Herring *et al*, 2004), although chronic ethanol may lead to significant cellular loss in the SIB of mice (Beracochea *et al*, 1987). Also, the SIB is a major attentional modulator of prefrontal cortex (PFC) functions (Zaborszky *et al*, 1999). The CeA-SIB-PFC circuit has not been

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studied in the context of drug addiction, but there is converging evidence that it is involved in attentional processes, salience and learning. We hypothesized that the CeA-SI-PFC pathway could promote alcohol drinking through increased salience of the negative emotional state and facilitation of learning to obtain relief from such emotional states through alcohol drinking. However, this hypothesis was not supported by the results as alcohol drinking and withdrawal signs were not altered by the inhibition of $CRF^{CeA\text{-}SI\ terminals}$. A second hypothesis, that needs further study, might be that the $CRF^{CeA\text{-}SI\ terminals}$ might be responsible instead for the cognitive impairment induced by excessive alcohol drinking, given that PFC neurons recruitment during alcohol withdrawal predicts cognitive impairment and that we observed a functional disconnection between the CeA and the infralimbic/prelimbic cortex during abstomemce from alcohol binge-like drinking (George *et al*, 2012).

The LH/pSTN area is a major input of the Medial Habenula (MH)-Interpeduncular nucleus (IPN) circuit, which has been recently demonstrated to contribute to withdrawal-induced nicotine intake (Grieder *et al*, 2014; Zhao-Shea *et al*, 2015). Considering the critical role of the MH-IPN pathway for compulsive nicotine intake as well as behavioral signs of withdrawal, we expected that optogenetic inhibition of $CRF^{CeA\text{-}LH/pSTN}$ terminals would prevent excessive alcohol drinking and somatic signs of withdrawal, but we didn't find any effect. Such results demonstrate that activation of this pathway during withdrawal is not causal to the excessive drinking and expression of the somatic signs of withdrawal. An alternative hypothesis is that the $CRF^{CeA\text{-}LH/pSTN}$ pathway is recruited during withdrawal, but is specifically involved in reinstatement of alcohol seeking in absence of drinking. Indeed, the LH has been shown to be critical for context-induced

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reinstatement of alcohol seeking (Moorman *et al*, 2016). Future experiments in context-induced reinstatement of alcohol seeking after extinction of alcohol drinking will test this hypothesis.

The largest and densest projection from the $CRF^{CeA\text{-}PBN}$ neurons was identified in the lateral PBN. The CeA receives nociception-related information via a direct monosynaptic pathway from the external part of the lateral parabrachial nucleus (Sarhan *et al*, 2005), which is the predominant target of the ascending nociceptive spino-parabrachio-amygdaloid pathway (Jasmin *et al*, 1997; Todd, 2010). Previous studies have demonstrated that nociceptive stimuli increase neuronal activity (Hermanson and Blomqvist, 1996) and Fos immunoreactivity (Bester *et al*, 1997) in the PBN. Since inhibition of the $CRF^{CeA\text{-}PBN}$ terminals didn't affect alcohol drinking or the somatic signs of withdrawal, one possibility is that the $CRF^{CeA\text{-}PBN}$ projection mediates hyperalgesia during withdrawal from alcohol (de Guglielmo *et al*, 2017; Edwards *et al*, 2012). Further studies will be required to test this hypothesis.

A large body of evidence shows that CRF and GABA systems in the CeA play an important role in alcohol dependence (Koob, 2008; Menzaghi *et al*, 1994; Merlo Pich *et al*, 1995; Valdez and Koob, 2004). At a cellular level, acute ethanol enhances evoked $GABA_A$ receptor-mediated inhibitory postsynaptic currents ($IPSC_S$) by increasing GABA release in rats (Roberto *et al*, 2003; Roberto *et al*, 2004) and mice (Nie *et al*, 2004). These effects are mediated by CRF and can be blocked by a CRF_1 antagonist (Roberto *et al*, 2010). The effects on alcohol intake observed here are specific of CRF neurons but we cannot rule out the hypothesis they might be mediated by the inactivation of other neurotransmitters such as CRF, GABA, dynorphin or somatostatin considering the high

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degree of colocalization and overlap between these populations of neurons (Gilpin and Roberto, 2012; Kang-Park *et al*, 2015; Marchant *et al*, 2007; Pomrenze *et al*, 2015).

In summary, this report identifies that activation of CeA CRF neurons is required for the recruitment of the CeA neuronal ensemble responsible for excessive alcohol drinking in dependent rats. Moreover, we demonstrated that inactivation of the $CRF^{CeA\text{-}BNST}$ pathway completely reverses excessive alcohol drinking during withdrawal and partially prevent the somatic signs of withdrawal while inactivation the $CRF^{CeA\text{-}SI}$, $CRF^{CeA\text{-}LH/pSTN}$ and $CRF^{CeA\text{-}PBN}$ does not appear to be involved in alcohol drinking or the somatic signs of withdrawal. Future studies will be important to identify if the $CRF^{CeA\text{-}SI}$, $CRF^{CeA\text{-}LH/pSTN}$ and $CRF^{CeA\text{-}PBN}$ are instead involved in mediating other aspects of alcohol dependence including cognitive impairments, alcohol seeking and hyperalgesia. These results suggest the role of the CeA CRF pathways in addiction can be dissociated and that targeting the the CRF CeA neuronal ensemble or the $CRF^{CeA\text{-}BNST}$ may facilitate the development of novel therapeutic approaches for the treatment of alcohol-use disorders.

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Figure Legends

Figure1. Alcohol withdrawal recruits CRF neurons in the CEA. **A)** Escalation of alcohol drinking after CIE exposure. ** p<0.01 vs baseline. **B)** Number of Fos+ nuclei per mm^2 in the CeA. **C)** Double Fos-CRF IHC showed that while the total number of CRF neurons did not change between withdrawal rats and naïve **D)** the number of Fos+/CRF+ neurons in the CeA dramatically increased during withdrawal. **E)** Representative pictures of CeA double Fos-CRF IHC in Naïve and **F)** alcohol withdrawal rats. Data are expressed as Mean ± SEM. ** p<0.01 and ***p<0.001 vs naïve.

Figure 2. Validation of the CRF Cre rat. **A)** Cre dependent eYFP expression in the CeA. **B)** Cre-dependent eYFP colocalizes with CRF-immunoreactivity in the CeA. **C)** Infrared (left) and YFP fluorescence (middle) images of a CeA neuron. *Right:* current clamp recording of the same neuron (held at -50 mV) depicting the hyperpolarization obtained in response to delivery of 6.5 sec train (asterisks) of green light flashes. **D)** In this CeA neuron (held at -52 mV) that did not present fluorescence, similar light stimulation did not elicit a response.

Figure 3. A) Effect of optogenetic inhibition of CeA CRF neurons on alcohol (panel a) and water (panel b) self-administration in non-dependent rats. **B)** Escalation of alcohol self-administration during alcohol vapor exposure. * p<0.05 vs baseline **C)** Effect of optogenetic inhibition of CeA CRF neurons on alcohol (panel a) and water (panel b) self-administration in alcohol dependent rats. # p<0.05 vs BSL, ** p<0.01 vs Escalation. **D)** Effect of optogenetic inhibition of CeA CRF neurons on saccharine (panel a) and water (panel b) self-administration. **E)** Effect of optogenetic inhibition of CeA CRF neurons on total distance traveled and **(F)** time spent in the center after the open field test. Data are expressed as Mean ± SEM.

Figure 4. A) Representative images of NpHR injection in the CeA. **B)** Time-course of the effect of unilateral optogenetic inactivation of CeA CRF neurons. # p<0.05 vs BSL,

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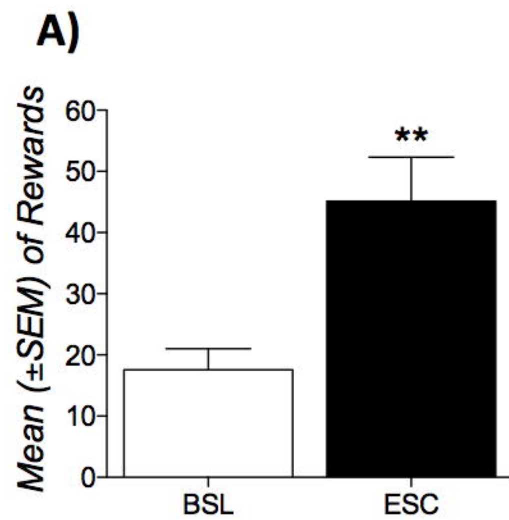
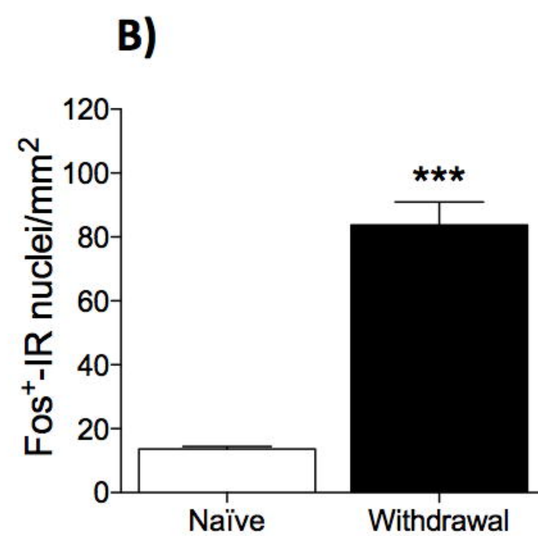
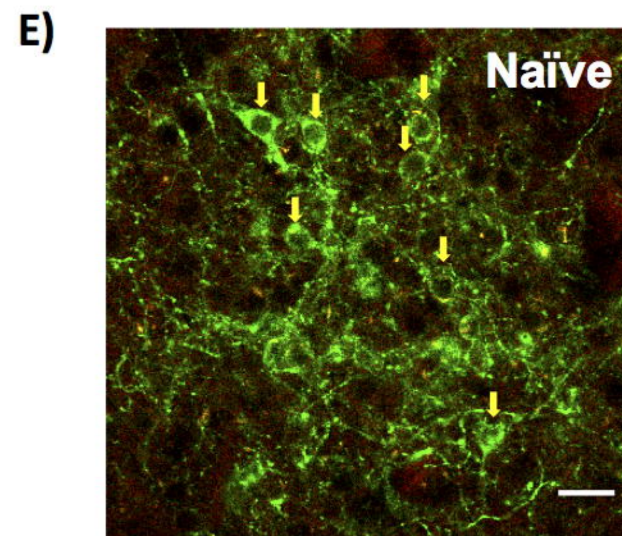
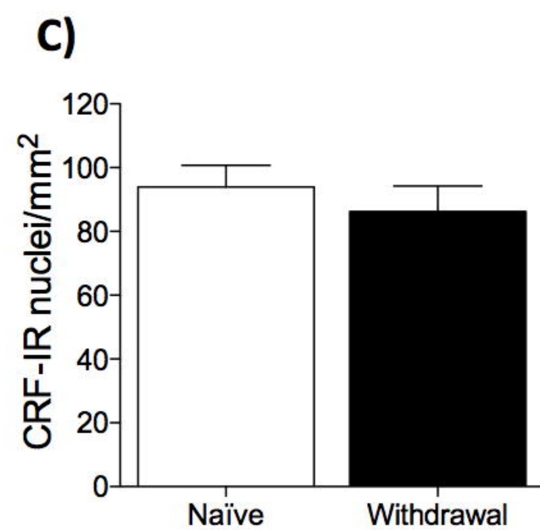
** p<0.01 vs Laser OFF **C)** Effect of CeA CRF neurons inhibition on somatic withdrawal signs. * p<0.05 vs Laser OFF. **D)** Effect of unilateral optogenetic inhibition of the CeA-CRF neurons on saccharine self-administration. E) effect of CeA CRF neurons inhibition for 30 minutes during withdrawal (8h) from alcohol (CIE) on CeA Fos immunoreactivity. ## p<0.01 vs naïve; * p<0.05 and ** p<0.01 vs Laser OFF; + p<0.05 vs controlateral. **F), G), H), I)**: representative pictures of the different experimental conditions.

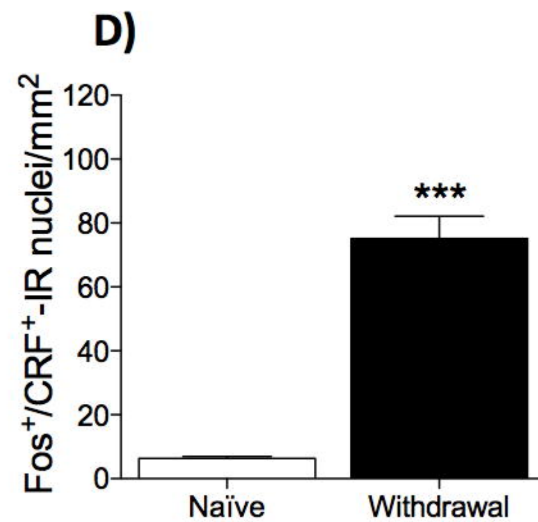
Figure 5. Shema: Representative images of the area of injection (CeA) and the area of optogenetic inhibition (BNST, SIB, LH and PBN) **A.a)** Time-course of the effect of unilateral optogenetic inactivation of CeA CRF BNST terminals. # p<0.05 vs BSL, ** p<0.01 vs Laser OFF **A.b)** Effect of CeA CRF BNST terminals inhibition on somatic withdrawal signs. * p<0.05 vs Laser OFF. **A.c)** Effect of unilateral optogenetic inhibition of the CeA-CRF BNST terminals on saccharine self-administration. **B.a)** Time-course of the effect of unilateral optogenetic inactivation of CeA CRF SIB terminals. # p<0.05 vs BSL **B.b)** Effect of CeA CRF SIB terminals inhibition on somatic withdrawal signs. **B.c)** Effect of unilateral optogenetic inhibition of the CeA-CRF SIB terminals on saccharine self-administration. **C.a)** Time-course of the effect of unilateral optogenetic inactivation of CeA CRF LH terminals. **C.b)** Effect of CeA CRF LH terminals inhibition on somatic withdrawal signs. **C.c)** Effect of unilateral optogenetic inhibition of the CeA-CRF LH terminals on saccharine self-administration. **D.a)** Time-course of the effect of unilateral optogenetic inactivation of CeA CRF PBN terminals. # p<0.05 vs BSL, ** p<0.01 vs Laser OFF **D.b)** Effect of CeA CRF PBN terminals inhibition on somatic withdrawal signs. * p<0.05 vs Laser OFF. **D.c)** Effect of unilateral optogenetic inhibition of the CeA-CRF PBN terminals on saccharine self-administration.

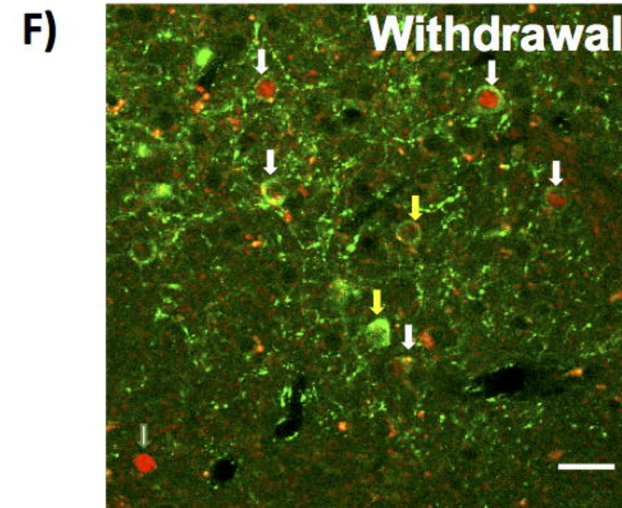

A)
Mean (±SEM) of Rewards
0
10
20
30
40
50
60
**
BSL
ESC

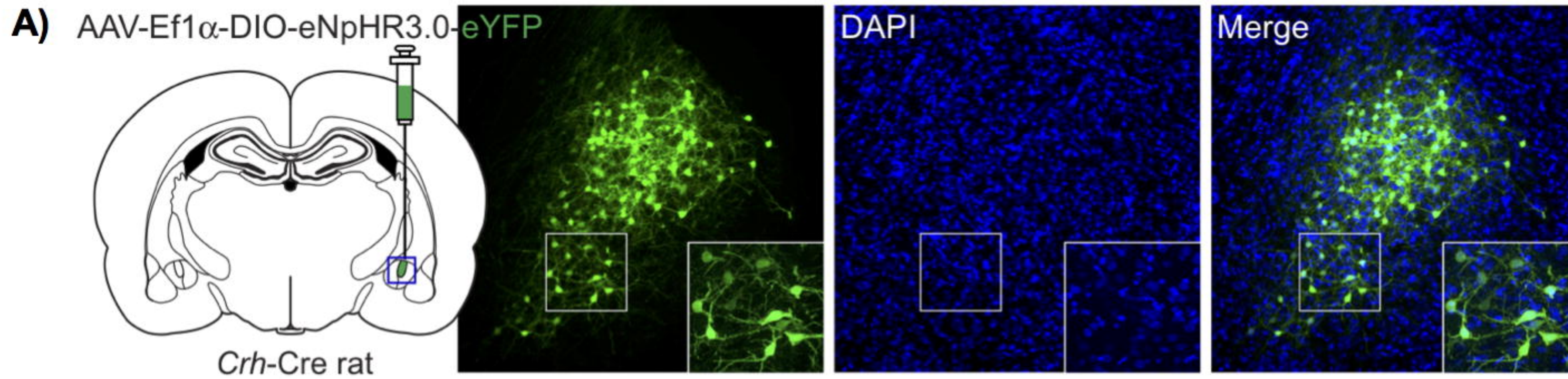

B)
Fos+-IR nuclei/mm2
0
20
40
60
80
100
120
***
Naïve
Withdrawal

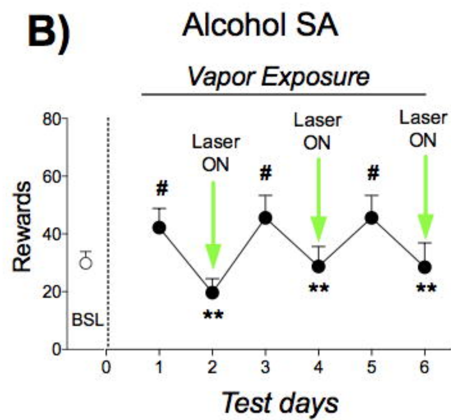

E)
Naïve

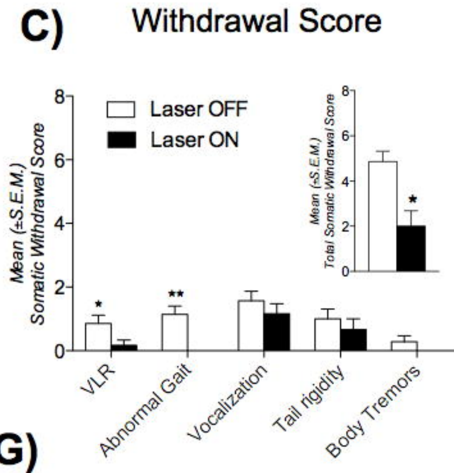

C)
CRF-IR nuclei/mm2
0
20
40
60
80
100
120
Naïve
Withdrawal

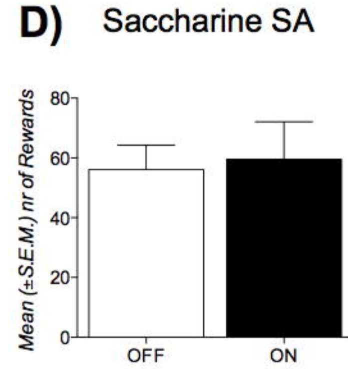

D)
Fos+/CRF+-IR nuclei/mm2
0
20
40
60
80
100
120
***
Naïve
Withdrawal

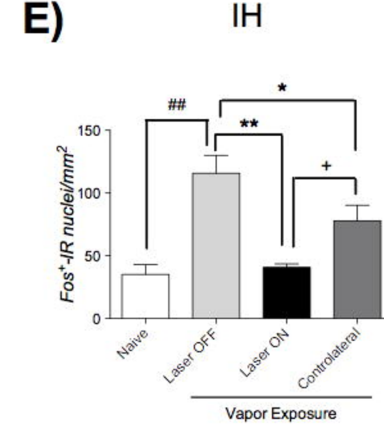

F)
Withdrawal

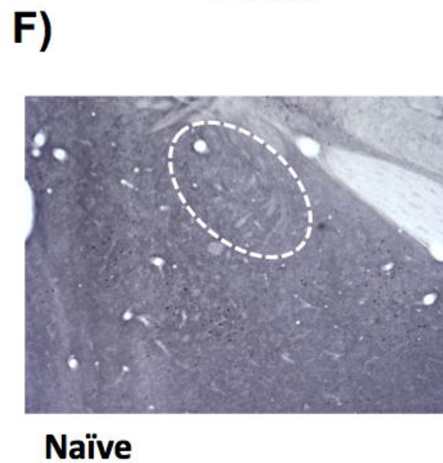

A)
AAV-Ef1α-DIO-eNpHR3.0-eYFP
DAPI
Merge
Crh-Cre rat

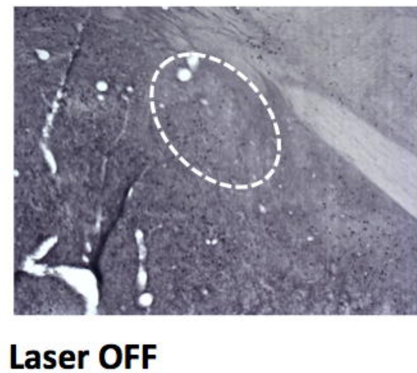

B)
Alcohol SA
Vapor Exposure
Rewards
Laser ON
BSL
Test days

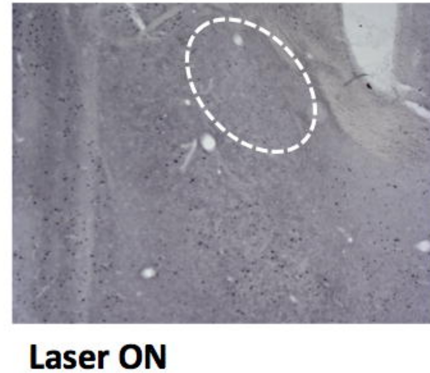

C)
Withdrawal Score
Laser OFF
Laser ON
Mean (±S.E.M.) Somatic Withdrawal Score
Mean (±S.E.M.) Total Somatic Withdrawal Score
VLR
Abnormal Gait
Vocalization
Tail rigidity
Body Tremors

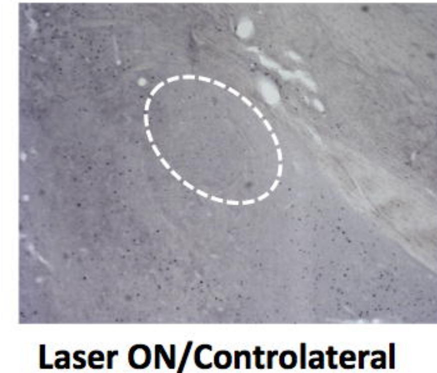

D)
Saccharine SA
Mean (±S.E.M.) nr of Rewards
OFF
ON


E)
IH
Fos+-IR nuclei/mm2
Naive
Laser OFF
Laser ON
Controlateral
Vapor Exposure

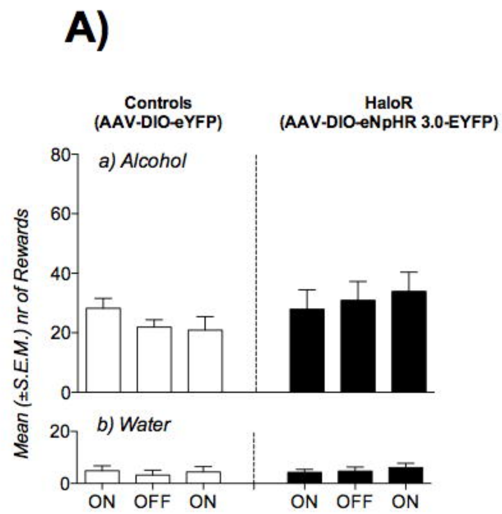
F)

Naïve

G)

Laser OFF

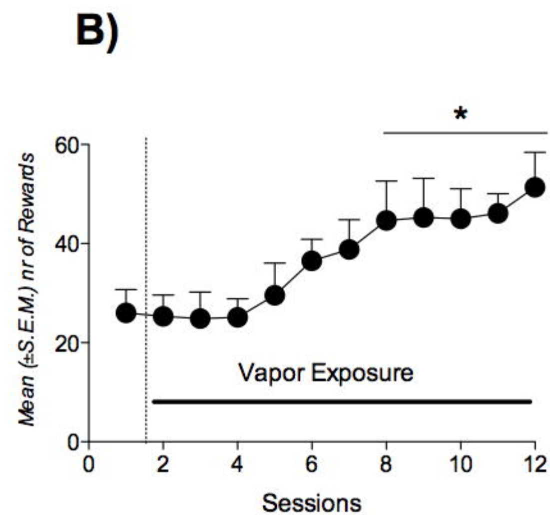
H)

Laser ON

I)

Laser ON/Controlateral

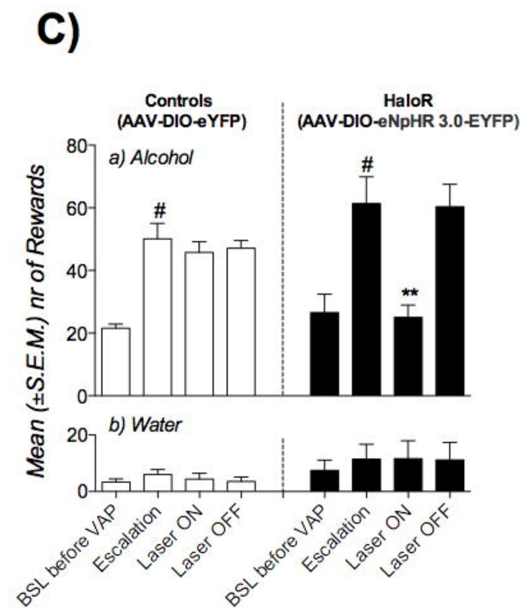
A)



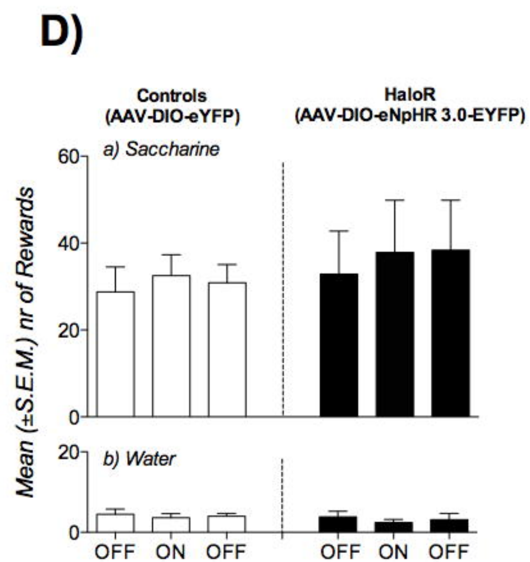
B)



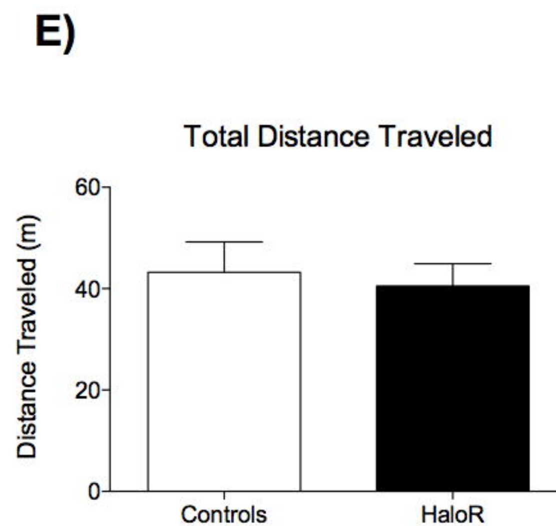
C)



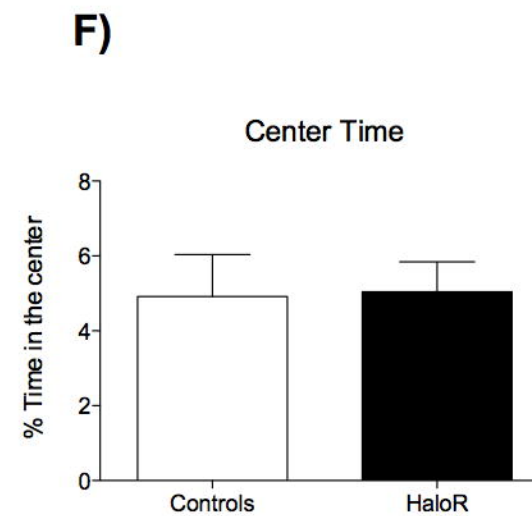
D)



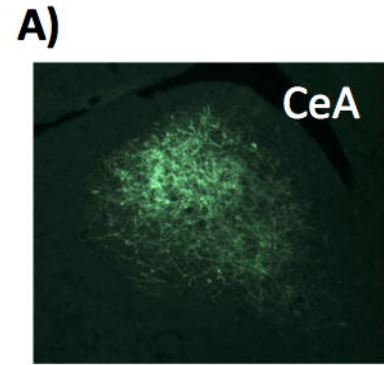
E)



F)



A)



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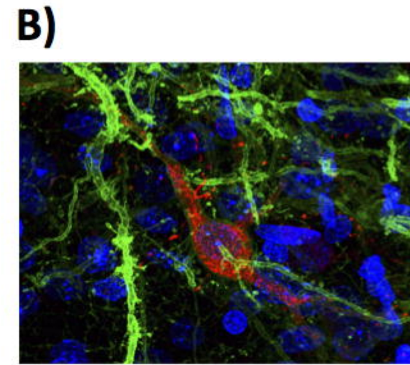
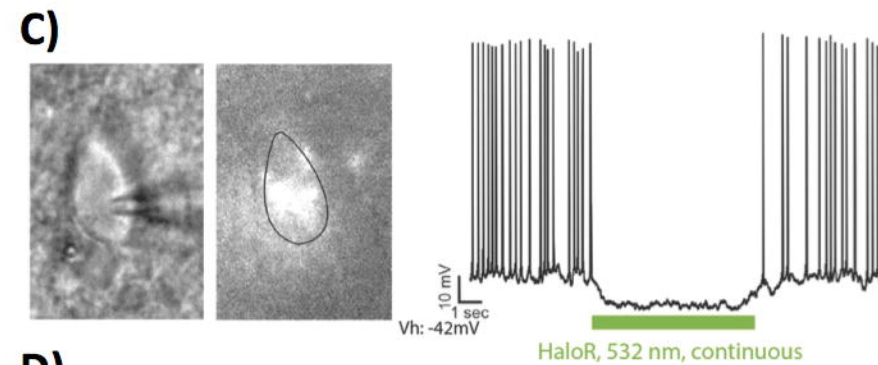


C)



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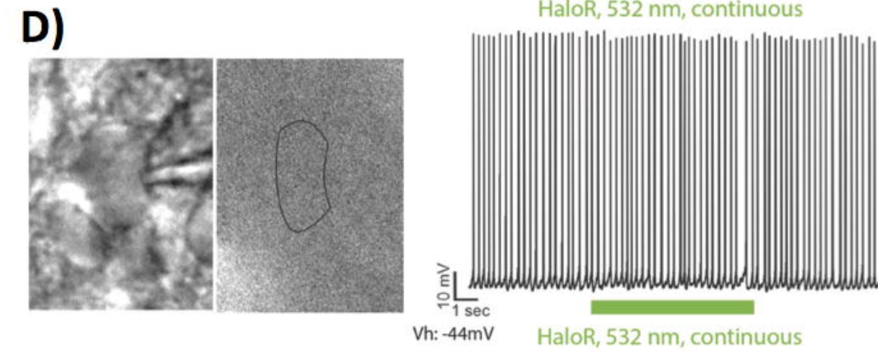

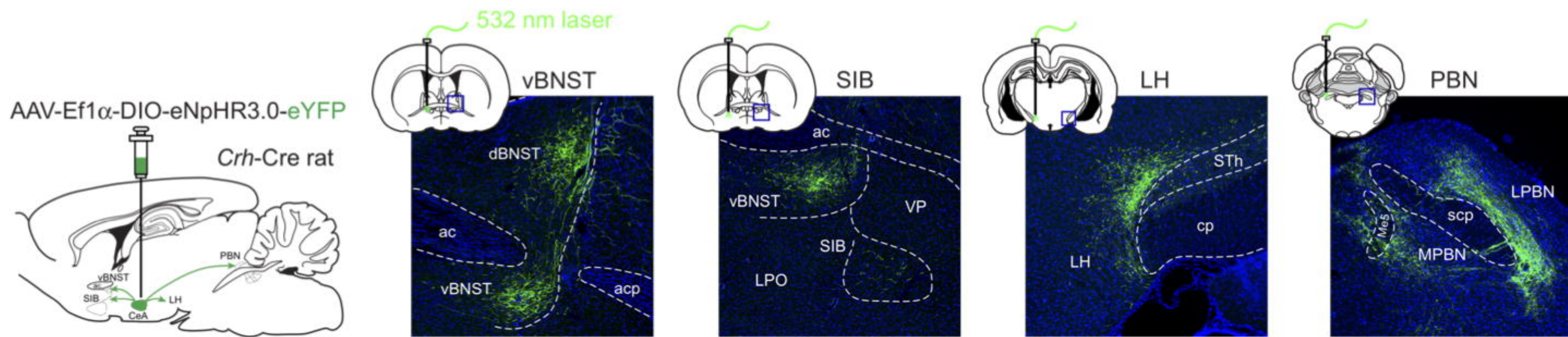

Alcohol SA

Somatic Signs

Saccharine SA

BNST

A.a Vapor Exposure

Laser ON
Rewards
BSL
Test days

A.b
Laser OFF
Laser ON
Mean (±S.E.M.) Somatic Withdrawal Score
VLR
Abnormal Gait
Vocalization
Tail rigidity
Body Tremors

A.c
Mean (±S.E.M.) nr of Rewards
OFF
ON

SIB

B.a Vapor Exposure

B.b

B.c

LH

C.a Vapor Exposure

C.b

C.c

PBN

D.a Vapor Exposure

D.b

D.c