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Targeted overexpression of CRH receptor subtype 1 in central amygdala neurons: Effect on alcohol-seeking behavior

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Abstract

Rationale—The corticotropin-releasing hormone (CRH) system is a key mediator of stress-induced responses in alcohol seeking behavior. Recent research has identified the central nucleus of the amygdala (CeA), a brain region involved in the regulation of fear and stress-induced responses that is especially rich in CRH-positive neurons, as a key player in mediating excessive alcohol seeking. However, detailed characterization of the specific influences that local neuronal populations exert in mediating alcohol responses is hampered by current limitations in pharmacological and immunohistochemical tools for targeting CRH receptor subtype 1 (CRHR1).

Objective—In this study, we investigated the effect of cell- and region-specific overexpression of CRHR1 in the CeA using a novel transgenic tool.

Methods—Co-expression of CRHR1 in calcium-calmodulin-dependent kinase II (αCaMKII)-neurons of the amygdala was demonstrated by double immunohistochemistry using a *Crhr1*-GFP reporter mouse line. A Cre-inducible Crhr1 expressing-adeno-associated virus (AAV) was site-specifically injected into the CeA of αCaMKII-Cre^{ERT2} transgenic rats to analyze the role of CRHR1 in αCaMKII-neurons on alcohol self-administration and reinstatement behavior.

Results—48% of CRHR1-containing cells showed co-expression of αCaMKII in the CeA. AAV mediated gene transfer in αCaMKII-neurons induced a 24-fold increase of *Crhr1* mRNA in the CeA which had no effect on locomotor activity, alcohol self-administration or cue-induced

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reinstatement. However, rats overexpressing *Crhr1* in the CeA increased responding in the stress-induced reinstatement task with yohimbine serving as a pharmacological stressor.

Conclusion—We demonstrate that CRHR1 overexpression in CeA-αCaMKII neurons is sufficient to mediate increased vulnerability to stress-triggered relapse into alcohol seeking.

Keywords

Alcohol; transgenic rats; AAV mediated gene transfer; Corticotropin-releasing hormone receptor subtype 1; calcium-calmodulin-dependent kinase II neurons; amygdala; stress-induced reinstatement

Introduction

One of the key brain regions that mediate stress and stress-induced relapse to alcohol-seeking is the amygdala, particularly the central nucleus (CeA) (Simms et al. 2012). Through projections to other brain regions, e.g. the bed nucleus of the stria terminalis (BNST), the hippocampus, and the hypothalamus (Pitkanen et al. 2000), the CeA relays information concerning stress and anxiety-like behavior. The CeA is also involved in the acquisition and expression of conditioned fear responses, and is a key nucleus of the emotional reward circuitry (Davis and Shi 2000; LeDoux 2000; Pare et al. 2004; Shinnick-Gallagher et al. 2003). Within the amygdala, both glutamatergic (excitatory) and GABAergic (inhibitory) connections mediate signaling and output from this region. The basolateral amygdala (BLA), which receives most of the sensory input to the amygdala, sends glutamatergic projections to the lateral part of the CeA (CeL) (Dong et al. 2001; Ehrlich et al. 2009; McDonald 1982). Within the CeA, the majority of neurons are GABAergic (Cassell et al. 1986; Ehrlich et al. 2009; McDonald 1982; Swanson and Petrovich 1998), as are projections from intercalated cell masses (ITCs), which surround the BLA and act as an interface between the BLA and CeA (Ehrlich et al. 2009; Millhouse 1986).

Alpha calcium/calmodulin-dependent protein kinase II (αCaMKII) plays a crucial role in the modulation of synaptic function via calcium-dependent activation (Yamauchi 2005). It is generally considered as a marker of excitatory neurons (Benson et al. 1992), although recent studies suggest the involvement of αCaMKII in inhibitory transmission within the amygdala (Huang et al. 2014). αCaMKII has been found in GABAergic cells (Beckerman et al. 2013; Huang et al. 2014; Meins et al. 2010) in the amygdala including the CeA (Wang et al. 2013), and a recent proteomics study has shown that moderate alcohol drinking in mice induces adaptation in αCaMKII-AMPA receptor pathway in the amygdala that regulates the positive reinforcing effect of alcohol (Salling et al. 2016).

For the modulation of CeA transmission and functional activity, the corticotropin releasing hormone (CRH) system plays an important role. CRH and CRH receptor subtype 1 (CRHR1) (Bale and Vale 2004; Hauger et al. 2006), are present in glutamatergic and GABAergic neurons in many brain regions (Bonfiglio et al. 2011; Lemos et al. 2012; Refojo et al. 2011). Activation of CRHR1 is central in the initiation of behavioral responses to stress (Coste et al. 2006; Hauger et al. 2006; Janssen and Kozicz 2013). There are several studies suggesting that CRH and CRHR1 play a key role in stress-induced alcohol seeking behavior

(Bjork et al. 2010; Hansson et al. 2006; Le et al. 2002; Marinelli et al. 2007; Meinhardt and Sommer 2015; Noori et al. 2014; Spanagel et al. 2014) and the effects of alcohol on stress-related behavior (Lee et al. 2004; Lee et al. 2001; Ogilvie et al. 1998; Pastor et al. 2008). *Crhr1* mRNA abundance is increased in the amygdala of alcohol dependent rats (Sommer et al. 2008). This translates into increased protein and function, as CRHR1-containing neurons in the CeA show an enhanced activity in response to alcohol (Herman et al. 2013). It has also been suggested in several studies that the CRH system mediates the effects of acute and chronic alcohol exposure via modulation of glutamatergic and GABAergic signaling (Nie et al. 2004; Nie et al. 2009; Roberto et al. 2010; Silberman et al. 2015; Silberman and Winder 2015). Together, these studies demonstrate that amygdala CRHR1 plays a key role in the expression of relapse-like behavior, particularly the stress-induced reinstatement of responding for alcohol. A remaining question is whether increased CRHR1 expression is sufficient to elicit relapse-like behavior.

To address this issue, we assessed the role of CRHR1 in the CeA in cue- and stress-induced reinstatement of alcohol responding. We employed a novel approach for time-, region-, and cell type specific CRHR1 overexpression using alpha calcium/calmodulin-dependent protein kinase II (αCaMKII) Cre ^{ERT2}-transgenic rats (Schonig et al. 2012) and adeno-associated virus (AAV) gene transfer to investigate the role of CRHR1 in CeA neurons during reinstatement behavior.

Material and Methods

Experimental animals.

For behavioral experiments αCaMKII-Cre^{ERT2} rats were used. In our hands rats are a better model system to study behaviors related to alcohol addiction (Vengeliene et al. 2014). Due to the lack of a specific antibody directed to CRHR1, the Crhr1-GFP transgenic reporter mouse (Bernardi et al. 2017; Justice et al. 2008) was used for immunohistochemical assays. Male *Crhr1*-GFP mice with a mixed BALB/cJ x C57BL/6J genetic background (Justice et al. 2008) were kept single housed with water and food available *ad libitum*, under a 12 h light/dark cycle (light off at 6:00 pm). αCaMKII-Cre^{ERT2} rats (Sprague-Dawley genetic background, (Schonig et al. 2012)) weighing 190 – 290 g at the beginning of the experiment were bred at the Central Institute of Mental Health (Mannheim, Germany). They were housed in standard cages of four rats per cage with *ad libitum* access to food and water, under a 12 h light/dark cycle (lights off at 5:00 am). All behavioral testing was performed during the dark phase. Experiments were carried out in accordance with the ethical guidelines for the care and use of laboratory animals, and were approved by the local animal care committee (Regierungspräsidium Karlsruhe, Germany, AZ 35-9185.81/G-183/09, AZ 35-9185.81/G-221/15).

Double Immunohistochemistry.

Crhr1-GFP mice were deeply anesthetized and perfused with 0,9% NaCl solution with 10000 IE/1 heparin followed by fixation with phosphate solution containing 4% paraformaldehyde and 14% saturated picric acid. Brains were isolated, post-fixed for 1-2h, dehydrated in 1xPBS solution with 10% sucrose for 3 d and quickly frozen at −80°C. For

fluorescent double-labeling 16 μm coronal sections (Bregma −0.7 mm to −1.7 mm, (Paxinos 2004)) were cut with a cryostat (Leica, Germany), mounted on gelatin-coated slides and stored at −20°C. Sections were rehydrated, dipped in 0.01 M PBS buffer, and incubated with a mixture of anti-GFP (rabbit, 1:500 dilution, ThermoFisher Scientific, USA) and anti-αCaMKII (mouse, 1:500 dilution, Pierce Biotechnology, USA) primary antibodies at 4°C overnight (Pfarr et al. 2015). Brain sections were rinsed in 0.01 M PBS/0.3%Triton solution containing the secondary antibody Alexa Fluor 488-labeled donkey anti-rabbit (1:1000 dilution, ThermoFisher Scientific, USA) and Alexa Fluor 594-labeled donkey anti-mouse (1:1000 dilution, ThermoFisher Scientific, USA) and incubated for 1–2 h at room temperature. Sections were rinsed briefly, mounted and coverslipped. Slides were analyzed using a Leica TCS SP2 imaging system mounted on a DM IRE2 microscope (Leica Microsystems, Germany) using x20 and x63 oil planchromat lenses, and recorded with argon ion laser (458-514 nm) and green neon laser (543 nm). Images were acquired with a Z-stack of 0.49 μm from 3 Crhr1-GFP mouse brains. For quantification, the number of cells at 2 different levels of the CeA (between AP: −1.22 and −1.46 mm according to (Paxinos and Watson 1998)) was counted in each hemisphere. Both GFP-stained cells and GFP-αCaMKII co-localized cells were quantified using the cell counter analysis macro of Image J. The percentage of co-localization was calculated for each recording and the mean ± SEM was calculated.

DNA constructs and AAV vector generation.

A rat cDNA encoding Crhr1 was cloned using BamHI/EcoRI from pCRII into an AAV plasmid containing the cytomegalovirus immediate early enhancer/chicken β-actin (CAG) promoter, the woodchuck hepatitis virus posttranscriptional regulatory element and the bovine growth hormone polyadenylation sequence flanked by AAV2 inverted terminal repeats. A transcriptional terminator element, flanked by loxP sites preceding the cDNA (pAAV-Stop-*Crhr1*). was employed to enable cell type-selective transgene expression following Cre recombinase-mediated recombination (Guggenhuber et al. 2010). The same expression cassette, carrying the humanized renilla green fluorescent protein (hrGFP) reporter (Guggenhuber et al. 2010), was used as a control. In addition a plasmid encoding Cre recombinase fused to a nuclear localization signal (pAAV-Cre) was used for in vitro validation of pAAV-Stop-*Crhr1*.

Transfection.

Co-transfection of pAAV-Stop-*Crhr1* and pAAV-*Cre* in human embryonic kidney cells (HEK) gown on coverslips was performed by standard calcium phosphate precipitation. Forty-eight hours later, cells were fixed and subjected to immunocytochemistry by permeabilization in 0.1 %Triton-X100 in 1% normal goat serum followed by incubation in primary anti-CRHR1 antibody (1:500; Santa Cruz Biotechnology, USA, sc-1757) overnight. Cells were washed and then incubated for 1h with the appropriate Alexa488-conjugated goat IgG (1:1000; Invitrogen, USA). Before the third wash in PBS, cells were counterstained with the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. Coverslips were then mounted and fluorescence was visualized using a Zeiss Z1 AxioExaminer NLO710 confocal microscope (Carl Zeiss Microimaging, Germany). Production of pseudotyped rAAV1/2

mosaic vectors and determination of genomic titers was performed as described (Klugmann et al. 2005).

Intra-amygdala virus administration.

Rats were injected with either the Crhr1 overexpression virus or the control virus. They were anaesthetized with isoflurane and immobilized in a Kopf stereotaxic frame. A WPI microinjection pump with a 33 gauge blunt needle was used to deliver 500 nl of the virus (5 x 10^8 vector genomes) bilaterally into the central amygdala (CeA; AP:−2.3 mm, ML: ± 4.2 mm, DV: −7.8 mm according to (Paxinos and Watson 1998)) at a flow rate of 100 nl/min. The needle was left in place for 5 min after the end of the injection to avoid backflow.

Induction of Crhr1 overexpression by tamoxifen injection.

5 d after virus injection, tamoxifen was injected ip for 5 consecutive days, with one injection on day 1, 3 and 5 and two injections on day 2 and 4. After the injections were completed, the rats were allowed to recover for 6 weeks, during which the overexpression of CRHR1 take effect.

Open field locomotor test.

Locomotor activity was measured 6 weeks after virus injection in an open field arena (51 cm x 51 cm x 50 cm) made of dark PVC, at 10.5 lux for 15 min. A center zone (24.9 cm χ 22.2 cm) was defined in the middle of each box. For the test, the animal was placed in the center of the box and the experimenter then left the room while a camera above recorded the animal's movements. The observation program Viewer^2 (Biobserve GmbH, Bonn, Germany) was used for tracking and analysis of locomotor activity (Distance traveled [cm], center time [s] and velocity [cm/s]).

Drugs.

Alcohol (10% v/v) was prepared using 96-97% ethyl alcohol (Sigma-Aldrich, Germany) and tap water. Tamoxifen (Sigma-Aldrich, Germany) was dissolved in medium-chain triglycerides (Stadtklinik Frankenthal, Frankenthal, Germany) for intraperitoneal (ip) injection at a concentration of 20 mg/ml and administered at a dose of 40 mg/kg. Yohimbine hydrochloride (Sigma-Aldrich, Germany) was dissolved in distilled water for ip injection and administered at a dose of 1.25 mg/kg and a volume of 1 ml/kg.

Cue- and stress-induced reinstatement of alcohol-seeking behavior.

The cue- and stress-induced reinstatement procedure was performed as recently described by (Hansson et al. 2006; Hansson et al. 2017; Hirth et al. 2016; Meinhardt et al. 2013; Pfarr et al. 2015; Pfarr et al. 2018; Uhrig et al. 2017).

Operant self-administration.

The alcohol-seeking experiment was performed during the dark phase in operant conditioning chambers (MED Associates) enclosed in ventilated sound-attenuating cubicles. Each chamber was equipped with a retractable response lever on each side panel of the chamber. Pressing the left lever activated a syringe pump, which delivered 30 μl of fluid into

a liquid receptacle next to the lever. A light stimulus (house light) was mounted above the response lever. The light was only illuminated in response to left lever presses. An IBM-compatible computer controlled the delivery of fluids, presentation of stimuli, and data recording.

Saccharin-fading procedure and alcohol self-administration training.

Rats were trained to differentiate between active and inactive levers using a saccharin-fading procedure (Suppl. Figure 1), as Sprague-Dawley rats do not learn this behavior well if trained only with alcohol reward. A saccharin-fading procedure was employed according to Tolliver et al. (1988). During the first six training sessions, left lever presses were rewarded with the delivery of 30 μl of 0.2% saccharin on a fixed-ratio 1 (FR1) schedule, while right lever presses were recorded, but did not have any programmed consequences. The rats were water-deprived 16 hours before each of the first three training sessions, after which they had access to water *ad libitum*. Each of six 0.2% saccharin sessions were followed by 1 d of 0.2% saccharin + 5% (v/v) alcohol, 1 d of 5% alcohol, 1 d of 0.2% saccharin + 8% alcohol, 1 d of 8% alcohol, 1 d of 0.2% saccharin + 10% alcohol. In the final two sessions only 10% alcohol was delivered. Once rats were trained to press the active lever to receive a 10 % alcohol fluid reward, responses to the left lever also activated the house light (1 light flash per second for 3 s), which also indicated a 3 s “time-out” period, during which additional lever presses of the active lever were recorded but did not lead to further delivery of 10% alcohol. In addition to the conditioned light stimulus (discrete cue), orange odor was presented during the entire session as a contextual cue, by adding 6 drops of orange extract to the bedding of the operant chambers. The bedding was changed and the trays cleaned at the end of each training session (Ciccocioppo et al. 2003; Ciccocioppo et al. 2002). After 10 self-administration sessions, a stable baseline was reached. A criterion for a stable baseline is met when the mean number of the reward lever presses in each of the last three sessions not varies by more than 20%.

Extinction of alcohol self-administration behavior.

During extinction training, responses on the left lever did not result in delivery of alcohol or activation of the light flashing, and the environmental stimulus was omitted. The extinction criterion was set to <10 % of baseline active lever presses, calculated from the final 3 retraining sessions after virus injection (Fig. 2A).

Cue-induced Reinstatement.

The conditions during cue-induced reinstatement were identical to training sessions, except that the pressing of the left lever did not result in alcohol delivery. The conditioned discrete and contextual cues were presented.

Yohimbine-induced Reinstatement

Yohimbine was injected ip 30 min before the start of the test session to serve as a stressor. The conditions in the operant conditioning chamber were identical to the extinction phase.

Cue- and Yohimbine-induced Reinstatement

Rats were injected with yohimbine (ip) 30 min before the start of the test sessions. During cue-induced reinstatement trials, the conditioned discrete and contextual cues were presented, but active lever pressing did not result in the delivery of alcohol (Le et al. 2005).

In situ hybridization.

At the end of behavioral testing, rats were decapitated, brains quickly removed then snap-frozen in isopentane at −40°C. Brains were stored at −80°C before the preparation of serial 12 μm coronal brain slices at Bregma −1.8 mm to – 2.8 mm according to The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 1998). Sections were mounted on SuperFrost glass slides and stored at −80°C until further use. Antisense and sense RNA probe generation (Table 1) and *in situ* hybridization was performed as previously described (Bernardi et al. 2014; Hansson et al. 2006; Sommer et al. 2008; Uhrig et al. 2017). *In situ* hybridization of viral *WPRE, αCaMKII* and *Crhr1* were used to verify the injection sites and the successful *Crhr1* mRNA overexpression. Animals in which the injection did not lead to *Crhr1* mRNA overexpression in the CeA were excluded from the analysis.

Statistical analysis.

Data are expressed as mean ± SEM. For statistical evaluation, the Statistica software (StatSoft, Germany) was used. *In situ* hybridization and open field data were analyzed by one-way ANOVA (control vs. overexpression virus). A repeated measures ANOVA (with the factor treatment, i.e. control vs. overexpression virus, and with session, i.e. self-administration sessions or extinction and reinstatement, as repeated measures, and considering active and inactive levers) was used. In cases where the ANOVA revealed significant differences, Newman-Keuls post-hoc test was used for self-administration sessions and reinstatement tests.

Results

CRHR1 and αCaMKII are co-localized in the CeA.

Because it has been difficult to obtain specific CRHR1 immunostaining, we used BAC transgenic mice expressing GFP under the control of the *Crhr1* genomic locus (Justice et al. 2008). Using these reporter mice, we were able to determine if and to what extent CRHR1 is expressed in αCaMKII-expressing CeA cells. Double fluorescence immunostainings were performed on coronal amygdala brain sections according to Pfarr et al. (2015). αCaMKII-ir neurons were distributed throughout the amygdala with the highest expression levels in the BLA and the CeA. CRHR1-GFP positive neurons were found mainly along the ITC clusters and in the CeL (Figures 1A–B). Quantification revealed that 48% of αCaMKII-ir- positive cells within the CeA co-express CRHR1-GFP (Figure 1C).

Behavioral effects of *Crhr1* overexpression in the CeA

Crhr1 overexpression in the CeA does not alter alcohol self-administration.— The timeline of the behavioral experiments is shown in Figure 2A. Self-administration training, extinction and reinstatement were analyzed using repeated measures ANOVA

followed by Newman-Keuls post-hoc analysis using virus treatment as a between group factor and time points (baseline before virus injection, baseline after injection, extinction, reinstatement) as well as active vs. inactive lever presses as within group factors. αCaMKII-Cre^{ERT2} rats were trained to self-administer 10% alcohol and assigned to prospective treatment groups (baseline: 84.1 ± 10.1 vs. 77.0 ± 11.7 active lever presses, designated *Crhr1* overexpression and control group, respectively). No differences in active or inactive level presses were found between the assigned groups. After virus injection and tamoxifen treatment, rats had a six-week recovery period after which they were tested in the open field to check for effects of the vector on basal locomotor behavior. No differences between *Crhr1* overexpression and control vector injected rats were found in the open field test (velocity: controls: 4.6±0.5 cm/s, *Crhr1* overexpression: 5.2±0.5 cm/s; distance traveled: controls: 4143.7±405.3 cm, *Crhr1* overexpression: 4717.9±460.8 cm; activity: controls: 21.2±1.8 %, *Crhr1* overexpression: 24.2±2.2 %; center time: controls: 47.6±6.9 s, *Crhr1* overexpression: 44.9±8.3 s). Next, rats underwent 10 self-administration sessions. The baseline of both groups increased significantly compared to lever presses before surgery (controls: 200.0±16.3 active lever presses, $F[1,22]=37.78$, $p=0.000003$; *Crhr1* overexpression: 218.9±18.7 active lever presses, $F[1,24]=40.45$, $p=0.000001$), but there was no difference in ethanol self-administration between active and control virus injected rats (Figures 2B–D).

Yohimbine- but not cue-induced reinstatement is increased by intra-amygdala *Crhr1* overexpression.—After extinction, rats were tested successively for cue-induced, yohimbine-induced, and combined cue- and stress-induced reinstatement of alcohol seeking. Both groups showed a significant reinstatement in all tests (repeated measures analysis with extinction and reinstatement as within group effect: Cue-induced reinstatement: $F[1,23]=200.1$, $p<0.001$; yohimbine-induced reinstatement: $F[1,23]=39.2$, $p<0.001$; Cue- and yohimbine-induced reinstatement: $F[1,23]=287.3$, $p<0.001$). Furthermore, overall effect on lever presses showed that animals clearly distinguished between the active and inactive levers (repeated measures analysis lever effect: Cue-induced reinstatement: $F[1,23]=264.9$, $p<0.001$; yohimbine-induced reinstatement: $F[1,23]=84.1$, $p<0.001$; Cue- and yohimbine-induced reinstatement: $F[1,23]=292.3$, $p<0.001$). *Crhr1* overexpression in the CeA had no effect on cue-induced reinstatement (Figure 2E). However, post-hoc analysis revealed that animals with *Crhr1* overexpression showed increased responding at the active lever during yohimbine-induced reinstatement compared to control rats ($p=0.025$), as well as in the combined cue- and yohimbine-induced reinstatement ($p=0.011$, Figures 2F–G).

Confirmation of AAV mediated Cre-dependent *Crhr1* overexpression In vitro validation of Cre-dependent Crhr1 overexpression.—To demonstrate the functionality of the Cre-dependent *Crhr1* expression construct, human embryonic kidney (HEK293) cells were transfected with the Cre-inducible *Crhr1* AAV-vector plasmid. As expected, immunostaining for the receptor could only be detected after co-transfection of the *Crhr1* construct with a *Cre*-expression plasmid (Figure 3A).

In vivo validation of Cre-dependent Crhr1 overexpression.—After behavioral experiments, *Crhr1* mRNA expression levels were determined in the CeA of active and control AAV injected αCaMKII-Cre^{ERT2} rats (Figure 3B). *In situ* hybridization for *Crhr1*

mRNA in the amygdala (Figure 3C) showed a strong, 24-fold increase in the overexpression vector injected rats compared to control vector injected rats ($F[1,23]=31.6$; $p<.001$, Figure 3D). Placement of intra-amygdala injections were estimated by localization of the viral WPRE-sequence via *in situ* hybridization (Figure 3C), and a map of the injection site was generated for each rat (Figure 3E). Animals in which the injection did not accurately target the CeA were excluded from the entire study. Thus, 13 overexpression and 12 control AAV rats were used for the analysis.

Discussion

Stress is an important precipitating factor in the reinstatement of alcohol-seeking after prolonged abstinence. As the CRH system is one of the major stress response systems in the brain, many studies have investigated the effects of alcohol on CRH and its receptors, as well as the role of the CRH system in alcohol consumption and dependence. In the present study we show for the first time that overexpression of CRHR1 in a distinct population of αCaMKII- positive CeA cells is sufficient to drive increased yohimbine-induced reinstatement. This was accomplished using a transgenic αCaMKII-Cre^{ERT2} rat driver line and AAV-mediated gene transfer. We demonstrated strong co-expression of CRHR1 in αCaMKII neurons of the CeA, and validated the *Crhr1* overexpression induced by our conditional gene transfer approach both *in vitro* and *in vivo*. When analyzing reinstatement behavior, we found that *Crhr1* overexpression in the CeA increased yohimbine-induced reinstatement, but not alcohol self-administration or cue-induced reinstatement of alcohol-seeking.

Defining the distinct role of the CRH system in alcohol dependence is challenging. Although there are many reports dedicated to this topic, study designs vary widely, as well as the interpretation of results (Phillips et al. 2015). *Crhr1* knockout mice have been reported to be more sensitive to alcohol concentration (Pastor et al. 2011), and voluntary alcohol consumption is not altered in *Crhr1* knockout mice in a long-term continuous two-bottle, free-choice access paradigm (Molander et al. 2012; Sillaber et al. 2002). However, alcohol consumption was reduced in female *Crhr1* knockout mice when assayed using a short-term, single-bottle drinking-in-the-dark paradigm (Kaur et al. 2012). Moreover, stress-induced alcohol intake was increased in global *Crhr1* knockout mice (Sillaber et al. 2002), but was decreased in conditional brain-specific knockouts (Molander et al. 2012). These latter experiments suggest opposite effects of peripheral and central CRHR1 on stress responses. In alcohol dependent animals, CRH and CRHR1 expression is chronically increased in the amygdala, and dependence-induced behavioral sensitivity to stress was can be blocked by CRHR1 antagonism (Sommer et al. 2008). More importantly, administration of the CRHR1/2 antagonist D-Phe-CRH into the CeA significantly decreased alcohol self-administration in dependent but not in control rats (Funk et al. 2006), pointing to the CeA as a major site of signaling adaptation to alcohol.

An increase in *Crhr1* expression has also been found in a rat line genetically selected for high alcohol consumption, i.e. the alcohol preferring Marchigian-Sardinian Preferring (msP) rats (Ciccocioppo et al. 2006). These animals show an increase of CRHR1 in the CeA that likely triggers their excessive drinking and high stress vulnerability (Hansson et al. 2006;

Kirson et al. 2018; Natividad et al. 2017; Stopponi et al. 2017). An intriguing observation is that in msP rats given *ad lib* access to alcohol, many differentially-expressed genes (e.g. CRHR1) are regulated similarly to control levels, suggesting high alcohol consumption resolves aberrant expression, perhaps serving as a self-medication strategy for alcohol-preferring animals (Hansson et al. 2007). CeA injection of the CRHR1 antagonist antalarmin has been shown to attenuate binge-like alcohol intake in mice (Lowery-Gionta et al. 2012). CRHR1 antagonism also diminishes stress-escalated alcohol consumption in mice with a history of chronic alcohol intake (Albrechet-Souza et al. 2017), and in mice subjected to social defeat induced elevated alcohol drinking (Norman et al. 2015) (Hwa et al. 2016). These experimental findings suggest the importance of CRHR1 in alcohol consumption behavior. However, here we found no changes on baseline alcohol self-administration after intra-CeA *Crhr1* mRNA overexpression in αCaMKII neurons. One possible explanation for this negative result is that CRHR1 signaling in non-αCaMKII-containing CeA CRHR1 neurons is crucial for alcohol drinking escalation.

We found a significant increase in baseline self-administration after surgery during the recovery period. It is currently unclear whether this effect is due to surgery, anesthesia, or some interaction between these or other perioperative variables. Studies in rodents suggest that increased operant performance may be related directly to anesthesia-induced neural activity, which may be selective to tasks that primarily measure spatial learning and memory processes (Walters et al. 2016).

For reinstatement of alcohol-seeking behavior, many studies found decreased active lever pressing after administration of CRHR antagonists, although these results depend highly on the particular animal strain and paradigm used. It has been shown that the CRHR1/2 antagonist D-Phe-CRH and the CRHR1 antagonist CP-154,526 attenuate footshock-induced reinstatement in Wistar rats (Le et al. 2000). In alcohol dependent rats, D-Phe-CRH was effective in reducing active lever pressing in footshock-induced reinstatement and a combination of footshock- and cue-induced reinstatement, while having no effect on cue-induced reinstatement alone (Liu and Weiss 2002). Another study found that the CRHR1 antagonist antalarmin blocked footshock-induced reinstatement of alcohol-seeking in msP rats, with no effect in Wistar rats (Hansson et al. 2006). Likewise, the CRHR1 antagonist MTIP reduced footshock-induced reinstatement in msP rats and alcohol dependent rats, while not affecting Wistar control rats (Gehlert et al. 2007). Antalarmin (ip) and D-Phe-CRH (intramedian raphe nucleus) were also found to reduce yohimbine-induced reinstatement of alcohol drinking in Wistar rats (Le et al. 2013; Marinelli et al. 2007), which has been proven to be a stable stressor that reliably induces reinstatement of drug-seeking (Le et al. 2005; Shepard et al. 2004). Overall, our findings are consistent with these studies. As CRHR1 antagonism decreases stress-induced reinstatement, we show that overexpression of *Crhr1* mRNA in the CeA increases stress- but not cue-induced reinstatement.

In contrast to most previous pharmacological studies that applied CRHR1 antagonists either systemically or by i.c.v. injection, we used local overexpression of CRHR1 in the CeA, a region critical for stress and anxiety-like behavior (reviewed in (Ehrlich et al. 2009)), alcohol intake in dependent rats (Funk et al. 2006) and stress-induced alcohol seeking (Simms et al. 2012), and assayed reinstatement of alcohol self-administration. Two

subregions of the CeA can be distinguished, the CeL and the medial (CeM) division of the CeA (McDonald 1982), which both use GABA as a neurotransmitter, but play different functional roles in determining amygdalar output (Duvarci and Pare 2014; Lopez de Armentia and Sah 2004). The CeL acts as the major input region of the CeA, receiving projections from the lateral amygdala and from other regions, such as sensory and higher-order cortical areas (Ehrlich et al. 2009; McDonald 1998; Savander et al. 1995). In addition to sending GABAergic projections to the BNST, the lateral hypothalamus, and the parabrachial nucleus (Veinante and Freund-Mercier 1998), the CeL is thought to inhibit the CeM, the major output region of the CeA, through GABAergic projections (LeDoux et al. 1988; Petrovich and Swanson 1997; Pitkanen et al. 2000; Sun et al. 1994; Veening et al. 1984). An important difference between these two subdivisions of the CeA is the high density of CRH peptide in the CeL, while significantly less CRH peptide is present in the CeM (Cassell et al. 1999; Cassell et al. 1986; Veening et al. 1984). In addition, the CeL is enriched for expression of the dopamine receptor D2, another system that contributes to the generation of anxiety-like behavior (Perez de la Mora et al. 2012) via action in the CeA. In line with these findings, we report immunoreactivity for the CRHR1 in the CeL. Strong CRHR1-ir was also present in the ITCs. Although, not part of the CeA, the ITCs project to the CeL and CeM, as well as other nuclei that mediate anxiety- and stress-related behavior (Amir et al. 2011; Koob and Le Moal 2008). Our targeting approach did not distinguish between these different CRHR1 expressing amygdala subregions. Thus, an alternative explanation of our results is that overexpression occurs in cells that do not normally express the receptor, perhaps accounting for the behavioral effects observed here, which potentially represents a pathologic mechanism in stress related disorders including alcohol addiction.

We used a Crhr1 reporter mouse line to identify CRHR1 expressing cells in the CeA (Justice et al. 2008). We found that 48% of CRHR1-GFP positive cells co-express αCaMKII in the CeA. The available data on *Crhr1* mRNA and CRHR1 protein distribution in the CeA does not suggest substantial differences between mouse and rat (Treweek et al. 2009; Van Pett et al. 2000). We therefore used Crhr1-GFP reporter mice to guide our behavioral experiments in rats using the αCaMKII-Cre^{ERT2} driver line (Schonig et al. 2012) to conditionally overexpression *Crhr1*. αCaMKII is considered a marker of glutamatergic neurons within the hippocampus and cortical regions (Benson et al. 1991). This kinase is expressed throughout the forebrain and plays a crucial role in the modulation of synaptic function due to its calcium-dependent activation (Benson et al. 1992; Benson et al. 1991; Jones et al. 1994; Liu and Murray 2012). Based on *in situ* hybridization studies, αCaMKII is also expressed in GABAergic neurons of the thalamic reticular nucleus, the globus pallidus, cerebellar Purkinje cells, and the commissural nucleus of the stria terminalis (Benson et al. 1992). Furthermore, recent studies suggest αCaMKII is expressed in GABAergic cells of the amygdala, in particular in the ITCs (Huang et al. 2014; Meins et al. 2010) and in a subset of CeA neurons (Beckerman et al. 2013). Thus, αCaMKII is expressed both in excitatory and inhibitory neurons. The high degree of overlap in CRHR1 and αCaMKII expression found in CRHR1-GFP mice suggests that the αCaMKII rat line is a useful tool non-ectopic, cell-specific overexpression of *Crhr1*. Injecting Cre-inducible-*Crhr1* encoding AAV into this region increased expression of *Crhr1* mRNA approximately 24-fold, without causing nonspecific behavioral effects as tested in the open field paradigm. Because the CRH system

is considered an 'alarm'-system and is thought to be silent under non-stimulated conditions (reviewed in (Heilig and Koob 2007)), we expected and confirmed that *Crhr1* overexpression had no effect on non-stimulated, i.e. non-stressed, behaviors such as locomotion and alcohol self-administration. Several brain regions, including the BNST, the CeA, and the nucleus accumbens shell, are key regions involved in stress-induced reinstatement (reviewed in (Shaham et al. 2003). Here, we manipulated CRHR1 expression in only one of these regions (CeA), perhaps explaining the relatively low effect size observed.

A key consideration is that CRHR1 colocalization with αCaMKII was assessed in healthy animals in a basal state. Under condition of long-term activation (e.g. in alcohol addiction), there is a long-term increase of *Crhr1* expression in the amygdala, and this likely includes an increased expression in a subset of αCaMKII neurons. In addition, recent studies identified distinct sets of neurons, so called neuronal ensembles within the amygdala region (de Guglielmo et al. 2016) and the prefrontal cortex area (George and Hope 2017; Pfarr et al. 2015; Pfarr et al. 2018) that are functionally involved in the reinstatement to alcohol-seeking behavior. Thus during the transition into dependence, we hypothesize that neuroadaptations occur in these ensembles similar to those experimentally-induced by overexpression of CRHR1. Future studies are needed to more thoroughly characterize the role of CRHR1 in specific neuronal ensembles involved in dependence-induced drinking and reinstatement behavior.

A potential limitation of the study is that repeated reinstatement sessions were performed without randomizing the sequence of session (i.e. cue-induced, yohimbine-induced, cue +yohimbine-induced). Although we have used a similar experimental design in the past without noticeable effects on behavioral performance (Pfarr et al. 2015; Pfarr et al. 2018) we cannot exclude potentially carry-over effects between trials. However, the goal here was not to compare different aspects of reinstatement behaviors, but to demonstrate the utility of a novel technical tool that allows to interfere with CRHR1 expression in a time, region and cellular manner.

Overall, our study further supports the importance of CRHR1 in stress-induced relapse to alcohol seeking. Using a new technical approach to drive CRHR1 overexpression in specific neuronal populations of the amygdala, we demonstrated that increased expression of this receptor is sufficient to cause exaggerated stress dependent alcohol consumption, which serves as a potential mechanism that determines higher vulnerability to excessive alcohol consumption in humans. This virus-based conditional expression approach employing available transgenic Cre-driver rat lines will be key to assessing the function of different populations of amygdalar CRHR1 neurons in alcohol-seeking and relapse-like behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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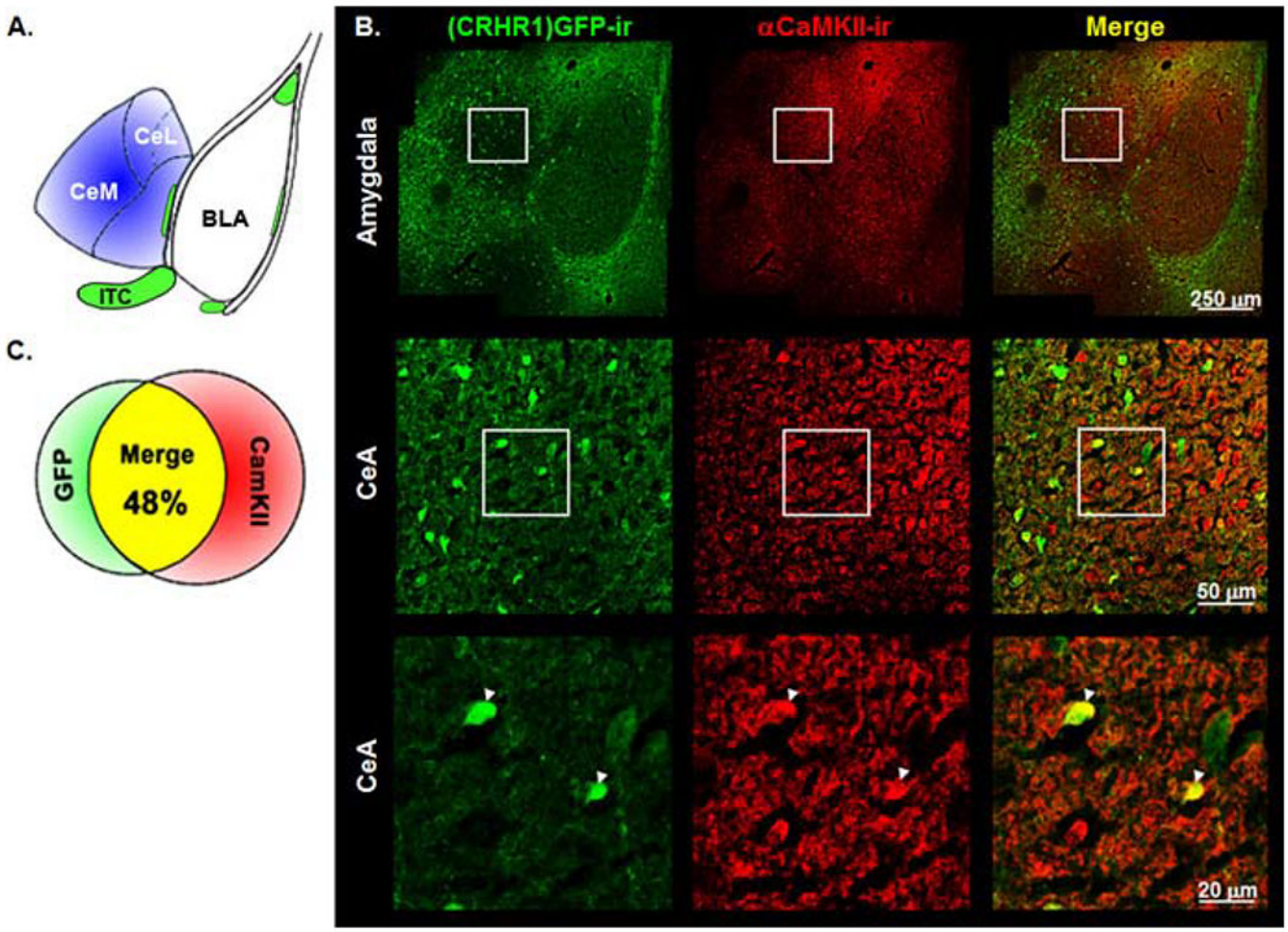

Figure 1. Co-located CRHR1 and αCaMKII in the CeA.

(A.) Schematic illustration of the amygdala brain region of the mouse (Paxinos 2004). **(B.)** Immunofluorescence staining in the amygdala for (CRHR1)GFP-ir (green), αCaMKII-ir (red) and with merged channels (yellow) in *Crhr1*-GFP reporter mouse line. White arrow heads indicate (CRHR1)GFP-ir and αCaMKII-ir co-localization. **(C.)** Quantification of co-located (CRHR1)GFP-ir- and αCaMKII-ir -positive cells in the CeA. BLA, basolateral amygdala; CeA, central amygdala; CeM, medial division of the CeA; CeL, lateral division of the CeA; ITC, intercalated cell cluster. For more details, see Material and Methods.

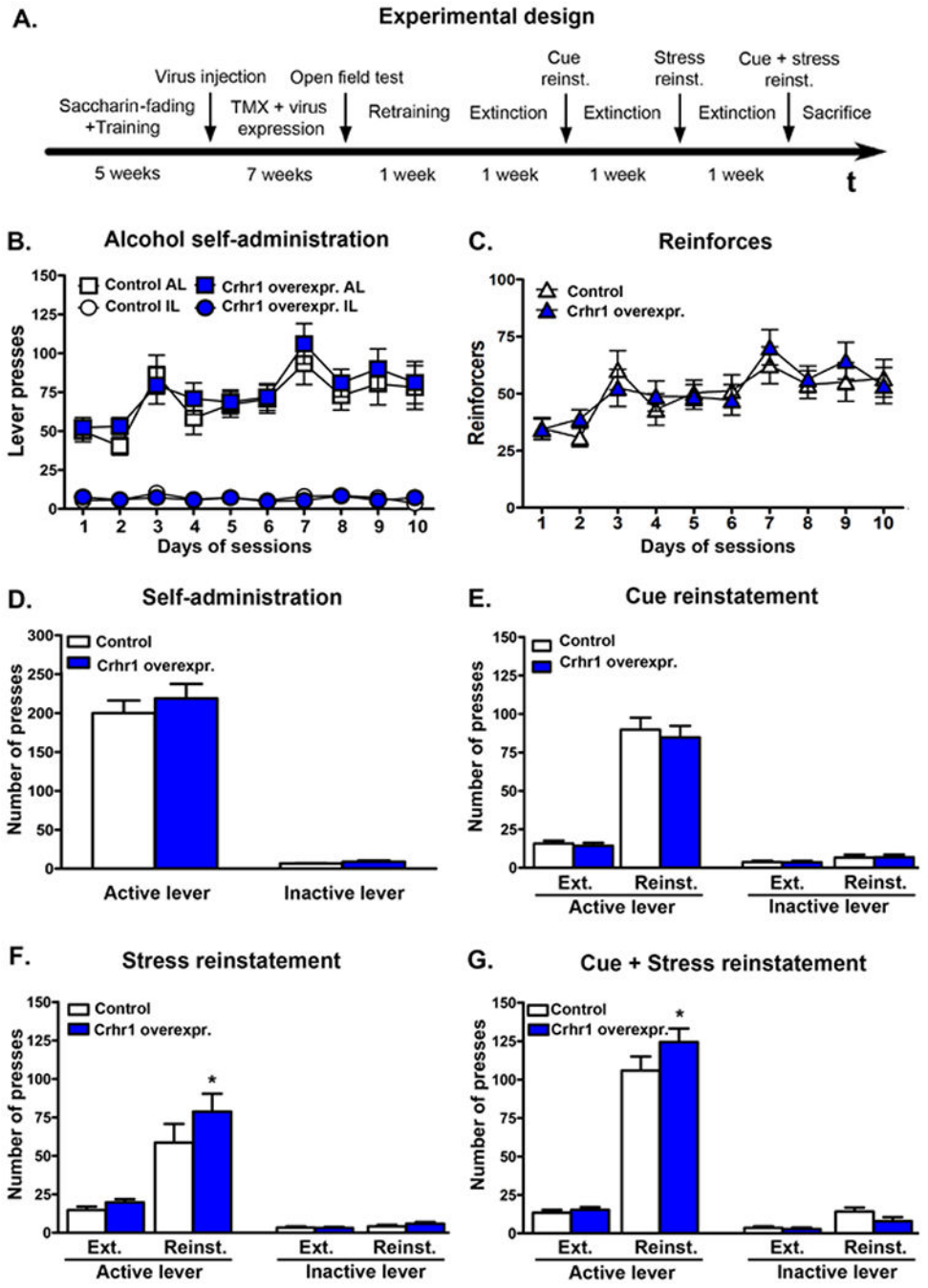

Figure 2. Stress-induced alcohol seeking behavior in αCaMKII^{CreERT2} rat injected with Crhr1-overexpression virus.

(A.) Experimental design and time line of the operant behavior. **(B.)** Active and inactive lever presses and **(C.)** number of reinforcers during training sessions with conditioned and environmental cue within the prospective treatment groups. **(D.)** Baselines of the last three training sessions after intra-amygdala injection of *Crhr1* overexpression or control virus. **(E.)** Cue-induced, **(F.)** yohimbine-induced and **(G.)** combined cue- and yohimbine-induced reinstatement of alcohol-seeking behavior. Bar graphs illustrate number of lever presses of

control AAV rats (white bars) and rats with *Crhr1* overexpression in the CeA (dark-blue bars). Stress was induced by yohimbine (1.25 mg/kg, ip). Statistical analysis was performed by repeated measures ANOVA, followed by Newman-Keuls post-hoc test if appropriate; n=12-13; p values: *p<0.05 *Crhr1* overexpression vs control AAV rats. TMX, tamoxifen; AL, active lever; IL, inactive lever; Ext, extinction; Reinst., reinstatement; Yoh., yohimbine; ind, induced.

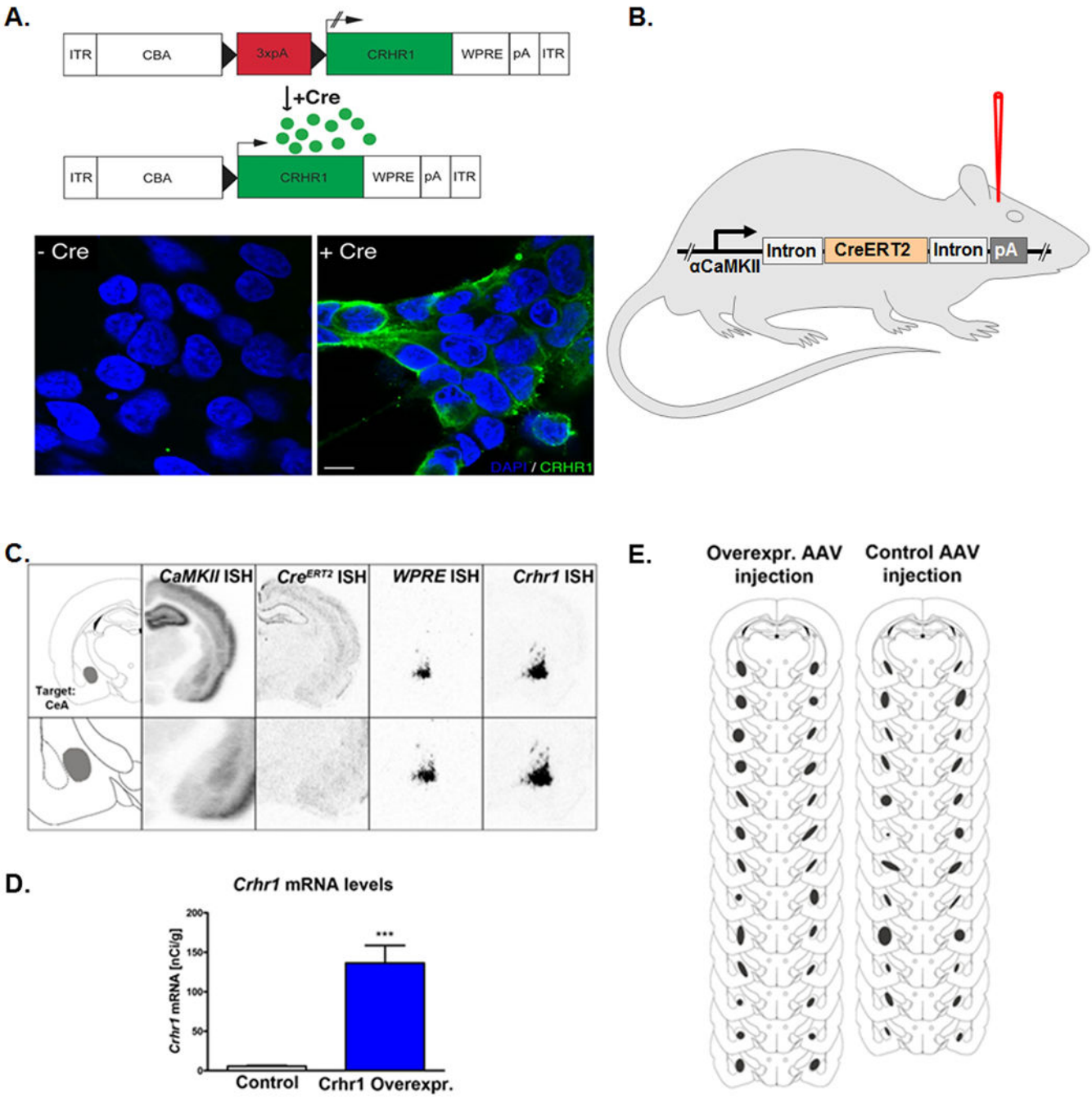

Figure 3. *In vitro* and *in vivo* validation of AAV-Crhr1 overexpression.

(A.) Cre-recombinase-activated overexpression of CRHR1 *in vitro*. Co-transfection of HEK cells with pAAV-Stop-*Crhr1*/control and pAAV-*Cre* resulted in strong CRHR1 at the cell surface, while no transgene expression could be detected in the absence of Cre expression. Blue color; cell nucleus staining with DAPI; green color: CRHR1-ir; scale bar 10 μm. **(B.)** *Crhr1* overexpression virus or control virus were bilaterally injected into the CeA of αCaMKII-Cre^{ERT2} transgenic rats. **(C.)** Schematic outline of the target region (left, Bregma −2.8 mm according to (Paxinos and Watson 1998), and representative images of *in situ*

hybridization for *αCaMKII*, *Cre*^{ERT2}, *WPRE* and *Crhr1*mRNA. **(D.)** AAV overexpression induced a 24-fold increase of *Crhr1*mRNA as compared to control AAV injected rats. **(E.)** Schematic illustration showing quality of intra-amygdala injected AAV. Virus injection sites were confirmed by *WPRE in situ* hybridization of each injected rat. For more details, see Material and Methods.

Table 1.

Primers used for RNA probe generation

mRNA	DNA	FW-Primer	RV-Primer
Cre^{ERT2}	Plasmid (Feil et al. 1997)	5′-GGGCTGCCA CGACCAAG-3′	5′-GCTACACCAG AGACGGAAATC-3′
αCaMKII	NM_009792.3	5′-AGGAAGTCTCTC GCTGGTTG-3′	5′-AACTGAACGC TGGAACTGGAC-3
WPRE	Plasmid #021_pAM/CBA-CRHR1-WPRE-bGH	5′-TGGTTGCTGTCTCTT TATGAGGAGTTGTGGC CCGTTGTCAGGCAACG TGGCGTGGTGTGCACT GTGTTTGCTGACGCAA CCCCCACTGGTTGG-3′	5′-GGCATTGCCACC TGTCAGCTCCTTTCCGG GACTTTCGCTTTCCCCC TCCCTATTGCCACGGC GGAACTCATCG-3′
Crhr1	NM_030999	5′-CGCTGTGAGA ACCTGTCCCTG-3′	5′-TAGGATGAA AGCCGAGATG-3′