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The central amygdala corticotropin-releasing hormone (CRH) neurons modulation of anxiety-like behavior and hippocampus-dependent memory in mice

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Abstract

The encoding, consolidation and retrieval of memories is a multifaceted process that depends strongly on the optimal level of arousal but high levels of arousal may trigger anxiety, which negatively impacts the memory processing by the brain. We investigated the role of CRH neurons in the central amygdala (CeA) for their capacity to modulate both, the anxiety-like behavior and hippocampus-dependent memory. First, we activated the CRH neurons in CeA using cre-dependent AAV-DREADD in CRH-cre mice. The activation of CeA CRH neurons increased the anxiety-like behavior in Elevated-O maze (O-maze) and Light-Dark box (LDB). The activation of the CeA CRH also decreased Y-maze memory performance and the discrimination index in novel object recognition test (NOR). The inhibition of CeA CRH neurons with AAVDREADD had the opposite effects on the anxiety-like behavior and the memory tests. Next, we used a combination of retrograde cre virus injected into locus ceruleus (LC) and cre-dependent AAV-DREADD injected into the CeA. While the excitation of the CeA neurons that project to LC increased the anxiety-like behavior, it also led to a better performance on the memory tests. The behavioral and memory effects were accompanied by increased c-Fos expression in the LC region. Pretreatment with CRH1receptor antagonist antalarmin hydrochloride blocked the effects that were observed after the activation of the CeA projections to LC. Our findings highlight the role of CeA CRH neuronal population not only as a generator of anxiety but also demonstrate their role in the control of hippocampus-dependent memory.

Introduction

The lifetime prevalence of anxiety disorders is 28.8%, which is the highest among the mood disorders in the United States (Kessler, Berglund et al. 2005). Anxiety disorders often present with cognitive deficits such as changes in attention, impairment of cognitive abilities

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Conflicts of interest

No potential conflicts of interest exist.

and memory retrieval (Coles and Heimberg 2002). Recent research has identified several neuroanatomical pathways in the limbic system that are responsible for anxiogenic or anxiolytic behavior, alluding to a pathway specific control of behavior (Tye, Prakash et al. 2011, Felix-Ortiz, Beyeler et al. 2013, Jennings, Sparta et al. 2013, Kheirbek, Drew et al. 2013, Kim, Adhikari et al. 2013). However, the effects of various neuropeptides, which serve as neurotransmitters in these pathways for control of animal behavior, are not well studied (Raskind, Peterson et al. 2013). Additionally, very few studies have considered the cognitive effects of the activation of these pathways during stress. A better understanding of the circuit-based pathology for anxiety disorders and comorbid cognitive deficits is essential for our understanding of the nature of anxiety and for the development of novel therapeutics for anxiety related disorders.

CRH is a ubiquitous CNS neuropeptide with dense clusters of CRH expressing cells in the CeA, bed nucleus of the stria terminalis (BNST) and paraventricular nucleus of the hypothalamus (PVN) (Bloom, Battenberg et al. 1982). The main CRH function is the control of the hormonal, autonomic and behavioral response to stressors (Koob 1999). The physiological and behavioral effects of CRH neuronal activation follow the activation of hypothalamic-pituitary-adrenal axis (HPA) by hypothalamic CRH populations and extrahypophyseal regions such as the CeA and BNST, which project heavily to brain stem centers of the autonomic nervous system (Charlton, Ferrier et al. 1987, Gray 1993, Pomrenze, Millan et al. 2015). The CRH actions are mediated by CRH1 (Chen, Brunson et al. 2000) and CRH2 receptors (Lovenberg, Liaw et al. 1995). Behavior studies in CRH1 deficient mice indicate that the CRH1 receptor is predominantly involved in the control of anxiety-like behavior (Smith, Aubry et al. 1998, Timpl, Spanagel et al. 1998). According to the literature, CRH administered intracerebroventricularly or at specific brain sites within the limbic regions including the CeA produces a variety of behavioral effects, such as an increased arousal, decreased exploration of the open field, decreased time in the open arms of the elevated plus maze, increased respiratory and cardiovascular effects, all indicative of an anxiogenic effect (Berridge and Dunn 1986, Berridge and Dunn 1987, Dunn and Berridge 1987, Liang and Lee 1988, Butler, Oliver et al. 2016). The modulation of animal behavior by the activation of CRH neurons is channeled through the activation of the dopaminergic and catecholaminergic system in several brain regions (Dunn and Berridge 1987, Valentino and Wehby 1988).

The CeA is a brain region that is essential for the initiation of the stress response (Adhikari 2014). The dominant GABAergic neuronal population of the CeA also expresses a great variety of neuropeptides including CRH. The CRH neurons are the predominant peptidergic group in the medial subdivision of the CeA, which also functions as the main output of the nucleus (Cassell, Freedman et al. 1999). The CRH projections from the CeA to LC, and more specifically to the rostromedial dendritic zone, which receives a great majority of afferent projections, have been shown to modulate the LC neuronal activity (Van Bockstaele, Chan et al. 1996, Van Bockstaele, Peoples et al. 1999). The LC is a small tightly packed pontine structure that is involved in a broad number of physiological functions including arousal, memory, cognition, pain processing, behavioral flexibility, and stress reactivity (Berridge and Waterhouse 2003, Snyder, Wang et al. 2012, Hickey, Li et al. 2014). Noradrenergic neurons within the LC exhibit distinct activation profiles. A pattern of low to

moderate spontaneous discharge (1–2 Hz) (tonic mode), strong biphasic activation by sensory stimuli (phasic mode) and synchronous firing is associated with focused attention, optimal arousal and memory performance. Exposure to stress or the stress related neuropeptide CRH is thought to drive high tonic activity (3–8 Hz) and simultaneously decrease the phasic responses (Redmond and Huang 1979). Blocking CRH1 receptors in LC is sufficient to prevent stress-induced anxiety (McCall, Al-Hasani et al. 2015). Thus, exposure to CRH biases LC activity towards high tonic activity resulting in anxiety-like behavior and inability to concentrate and maintain performance on tasks that require focused attention. This suggests that the behavioral component of emotional arousal through the CeA engages the attention and memory circuitry of LC. In addition, the activation of LC with subsequent release of norepinephrine in the hippocampus results in input specific synaptic plasticity (Haring and Davis 1985, Mello-Carpes, da Silva de Vargas et al. 2016). The arousal and focused attention that are required for memory processing and learning also depend on the LC activity (Manahan-Vaughan and Kulla 2003, Sara and Bouret 2012).

In the present study, we altered the activity of CRH neurons in CeA using chemogenetic technique and conducted a series of behavior tests for anxiety-like behavior and hippocampus-dependent memory. Because the LC is a part of CRH modulatory circuitry, we also examined how the chemogenetic activation of CeA projections to LC will affect the mice behavior and hippocampus-dependent memory.

Experimental procedures

Animals

Thirty transgenic B6(Cg)-Crh^{tm1(cre)}Zjh/J (CRH-cre) and twenty wild type C57BL/6J male mice (30–35g), age 9 to 10 weeks were purchased from Jackson Laboratories, Bar Harbor, ME. The mice were group-housed in five animals per cage and given food and water ad libitum. All experiments including stereotaxic surgery and behavioral assays were conducted in accordance with the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996) and the guidelines of the Animal Care and Use Committee at Rosalind Franklin University of Medicine and Science. All behavior experiments were conducted between 9:00 am and 1:00 pm after at least 1 hour of acclimatization to the testing room. Multiple days of resting time between behavior tests assured that there are no carryover effects from prior tests. The order of tests in which mice were tested was the same for each group and each mouse was tested only once. During the test trials, the experimenter was separated from the testing area by a curtain and the animal behavior was video recorded for subsequent analysis.

Stereotaxic surgery

The stereotaxic surgeries were performed under aseptic techniques and 2% isoflurane anesthesia with flow rate of 400 ml/min. A longitudinal skin incision was done to expose the bregma and lambda sutures of the skull. Small holes (2 mm diameter) were drilled into the skull and the viral solution was injected using a 32-gauge needle connected to an infusion pump (Micro syringe pump, World Precision Instruments, Sarasota, FL) in volume of 0.2 µl over 5 minutes. Cre-activated adeno-associated virus with excitatory Gs receptor pAAV-

hSyn-DIO-rM3D(Gs)-mCherry or inhibitory Gi receptor (pAAV-hSyn-hM4D(Gi)-mCherry) in titer of 5.4×10^{12} particles/ μ l were purchased from UNC Vector core, Chapel Hill, NC. The viral solution was injected bilaterally into the CeA with coordinates: anteroposterior – 1.3 mm, lateral $\pm$ 2.5 mm and dorsoventral – 4.5 mm relative to bregma. The CeA projections to LC were labeled by bilateral injection of rAAVCAG-eGFP-F2A-Cre (NINDS Viral Production Core Facility, Bethesda, MD) into LC in volume of 100 nL, titer 1.06×10^9 particles/ μ l with coordinates: anteroposterior – 5.4 mm, lateral $\pm$ 0.8 mm and dorsoventral – 4.0 mm. The successful transfection with rAAV-CAG-eGFP-F2A-Cre assured Cre-recombinase activity in retrogradely labeled cell bodies in the CeA. The LC injections were followed by injections of the excitatory pAAV-hSyn-DIO-rM3D(Gs)-mCherry virus into the CeA in the same manner described above. The pAAV-hSyn-DIO-rM3D(Gs)-mCherry contains the floxed inverted sequence of rM3D(Gs)-mCherry, which is reoriented in the presence of Cre, prompting the expression of rM3D(Gs)-mCherry. This ensures that rM3D(Gs)-mCherry is expressed exclusively in CeA neurons that project to the LC. Following each surgery, the skin was closed with wound clips and analgesia (ketoprofen 2 mg/kg, i.p.) and fluids were provided for 3 days after the surgery.

Drugs

Clozapine N-oxide [CNO] (Cat. No: 4936, Tocris, Ellisville, MO) was first dissolved into DMSO and then brought up to volume with distilled water. The working solution was aliquoted and stored at -200C until further use. The mice were injected intraperitoneally with 0.2 ml of CNO (1 mg/kg dose) or vehicle 30 to 40 minutes before the behavior tests. Previous behavioral studies have used CNO in doses that range between 0.2 mg/kg to 10 mg/kg (Ferguson, Eskenazi et al. 2011, Qiu, Chen et al. 2016, Sengupta, Winters et al. 2016, Wicker and Forcelli 2016). The choice of 1 mg/kg dose was based on our preliminary behavior experiments and c-Fos expression studies in mice without DREADDs as well as studies by Jann et al., showing that there is no retro conversion of CNO to clozapine at this dose (Jann, Lam et al. 1994) and studies by Manvich et al., showing that there is no clozapine-like behavior responses to this dose (Manvich, Webster et al. 2018).

Antalarmin hydrochloride was dissolved in DMSO and then diluted to dose of 30 mg/kg in 0.2 ml volume with distilled water. Antalarmin hydrochloride is a non-peptide corticotropin - releasing hormone receptor 1 (CRH₁) antagonist and has been used successfully in animal models of anxiety (Webster, Lewis et al. 1996, Zorrilla, Valdez et al. 2002).

Behavior testing

Anxiety-like behavior

The behavior tests were conducted as described previously (Dimitrov, Tsuda et al. 2014). Standard LDB and O-maze for mice purchased from Stoelting Co, Wood Dale, IL. For the O-maze test, mice were placed in the center area between the arms and their behavior was monitored for 5 min with a camera placed on the top of the maze (Lorex Wireless Video Monitoring System, LW2750 series). The anxiety-like behavior was assessed based on percent of time spent in the open arms of the maze. The LDB apparatus consisted of two

equal acrylic compartments, one opaque and one open that were separated by a divider with an opening at the floor level. Each animal was placed in the center of the dark compartment and then was allowed to explore the entire box for 10 minutes. The time to exit from the dark to the light compartment and the percent of total time spent in the light compartment were measured. The natural propensity of mice to hide in the dark compartment is balanced by the animal exploratory behavior. An increased exploration of the brightly illuminated compartment is interpreted as reduced anxiety-like behavior.

Hippocampus-dependent memory

Spontaneous alternations test

The Y-maze test is a hippocampus-dependent spatial memory task based on the ability of rodents to navigate through a novel environment. Mice were tested in a symmetrical Y-maze apparatus (Stoelting Co, Wood Dale, IL). During a 5-minute trial, the mouse was allowed to roam freely through the maze and the behavior was video recorded. The percentage of “correct” spontaneous alternations was calculated from the video record, where an arm entry by the animal required that all four paws to be in a single arm of the maze.

Novel object recognition test

The NOR test assesses working, short term or long term memory depending on the elapsed testing time and is done without the presence of appetitive or aversive stimuli. The NOR task evaluates the ability to recognize a novel object that replaces an already familiar object in the rodent environment (Ennaceur 2010). The procedure consists of three phases: habituation, familiarization, and a test phase. In the habituation phase, each mouse was allowed to freely explore the experimental box in the absence of objects. The animal was then removed from the arena and returned to its cage. During the familiarization phase, a single mouse was placed for 3 to 4 minutes in the box containing two objects. The objects differed in color, texture, and form, were smaller than the mouse and odor neutral. After every animal the apparatus was cleaned with 70 % ethanol to avoid olfactory cues. During both the familiarization and the test phase, objects were located in center of the box with equal spacing from the opposite walls. The animals were placed in exactly the same location and away from the objects to avoid bias toward any particular object. The familiarization trial was repeated 3 times. The test trial was conducted after a retention interval of 60 min, which is considered a test for long-term memory. The mice were returned to the box where one of the two objects was replaced with a novel one. The animal was allowed to freely explore the two objects for five minutes. The mouse nose in less than 2 cm from the object was defined as object exploration and the total exploration time for each mouse was calculated from a video record. The Discrimination Index ($(N - F) / (N + F)$ where N is the time spent exploring the novel object and F is the time spent exploring the familiar object) was expressed as percent control and used to compare the performance of the experimental groups to their respective controls.

Tissue processing

Animals were euthanized with intraperitoneal injection of sodium pentobarbital (40 mg/kg) (Vedco Inc., Saint Joseph, MO) and then transcardially perfused with 0.01 M phosphate

buffer saline (0.01M PBS) followed by 4% paraformaldehyde in 0.01M PBS (PFA). Then the brains were dissected and post fixed in 4% PFA overnight. The brains were sectioned into 40 μ m thick sections with Leica VT1000S vibrotome.

Immunohistochemistry

The free-floating brain sections were washed with 0.01M PBS and incubated in 1% hydrogen peroxidase for 15 minutes, again washed with PBS, and then incubated in a blocking solution (0.01M PBS, 0.05% Triton X-100, 3% normal donkey serum) for 2 hours at room temperature. The forebrain sections were immunostained for c-Fos with rabbit anti-c-Fos antibody in dilution 1:10,000 (Cat. No ABE457; EMD Millipore, CA) and for mCherry, which is a tag for the DREADD receptor, with chicken anti-mCherry antibody in dilution 1:1000 (CPCA-mCherry, EnCor Biotechnology Inc., Gainesville, FL). The brainstem sections were incubated with c-Fos rabbit antibody followed by incubations with sheep anti-tyrosine hydroxylase (TH) polyclonal antibody in dilution 1:5000 (Cat. No SO25000, Neuromics, Edina, MN) and in chicken anti-GFP in dilution 1:2000 (Cat. No A10262, Invitrogen, Carlsbad, CA). The incubations with primary antibodies were followed by incubations with secondary antibodies for development of fluorescence. c-Fos was visualized by incubation with biotinylated anti-rabbit polyclonal secondary antibody (Jackson ImmunoResearch Inc., West Grove, PA) diluted 1: 2000 for 2 hours, followed by incubation with avidin-biotin complex (Vectastain Elite ABC kit PK-6100, Vector Laboratories, Burlingame, CA) for 1 hour at room temperature and then incubation with tyramide conjugated Alexa Fluor 405, at 20 nmol concentration, for 12 minutes. Direct secondary antibodies conjugated to Alexa 594 or Alexa 488 (Jackson ImmunoResearch Inc., West Grove, PA) and diluted 1:400 were used to develop mCherry, TH and GFP immunoreactivity. Sections were cover-slipped with 2.5% polyvinyl alcohol-1, 4-diazabicyclo [2.2.2] octane (DABCO) antifade mounting medium.

Microscopy

Imaging and subsequent analyses were carried out without knowledge of the group assignment. Leica DM 5500B epifluorescence microscope was used to acquire 16-bit images of amygdala for c-Fos-ir counts, while Zeiss 510 confocal microscope was used to obtain 16-bit images of the LC and CeA for the expression of the viral vectors. c-Fos-ir was evaluated on three to four sections per animal. The atlas matched brain sections covered the CeA between bregma level - 1.1 mm and - 1.8 mm to bregma. For c-Fos-ir in the LC and peri-LC zone, the sections were analyzed between bregma levels - 5.3 and - 5.5 mm. The number of c-Fos positive neurons and TH, GFP or mCherry expressing cells was assessed on separate single channels independently and after that the colocalization of the different markers was assessed after merging the channels with already marked neurons. The neurons were manually identified and marked with ImageJ software (ImageJ, Bethesda, MD).

Statistical analysis

All statistical analyses were performed using Prism 7 (GraphPad Software, La Jolla, CA). Data are reported as mean $\pm$ SEM. Data were analyzed using Student's t-test for comparisons of groups with normal distributions and Mann-Whitney U-test for comparisons of non-parametric data. The results were considered significantly different when $P < 0.05$.

Results

Activation of CeA CRH neurons increases the anxiety-like behavior and negatively affects the hippocampus-dependent memory.

CRH-cre transgenic mice were bilaterally injected into the CeA with cre activated pAAV-hSyn-DIO-rM3D(Gs)-mCherry and the mice were tested for anxiety-like behavior after three weeks of recuperation. The control and experimental groups were tested on standard mouse elevated O-maze and LDB 30 to 40 minutes after intraperitoneal injection of vehicle or CNO. The control group spent on an average 25.8 ± 8.8 % of their time in the open arms of the elevated O-maze while the CNO group spent 0 % of their time in the open arms of the elevated O-maze (Mann-Whitney test, $P = 0.009$, $n = 5/5$) (Figure 1A). In the LDB test, the control mice spent 11.4 ± 0.7 % in the light field while the CNO treated mice spent only 4.5 ± 2.2 % in the light compartment (Mann-Whitney test, $P = 0.016$, $n = 5/5$) (Figure 1B). Also, the time to exit from the dark compartment to the light compartment was 92 ± 21.6 seconds for vehicle treated mice but it increased to 183.8 ± 29 seconds for the CNO injected mice (Mann-Whitney test, $P = 0.032$, $n = 5/5$) (Figure 1C).

Next, the effects of the amygdala CRH neurons activation on hippocampus-dependent memory was assessed using Y-maze and NOR tests. The vehicle treated mice demonstrated 59.5 ± 4.4 % correct alternations, whereas the mice that received CNO demonstrated just 49.5 ± 3.7 % correct alternations in the Y-maze test (Mann-Whitney test, $P = 0.015$, $n = 5/5$) (Figure 2A). In NOR test, the mean discrimination index decreased by 132 % in the CNO injected mice when compared to the vehicle injected group (Mann-Whitney test, $P = 0.031$, $n = 5/5$) (Figure 2B).

Inhibition of the CeA CRH neurons decreases the anxiety-like behavior and positively affects the hippocampus-dependent memory.

We hypothesized that if activation of the amygdala CRH neurons is anxiogenic, then the inhibition of the same CeA CRH neurons very likely will have an opposite effect on mice behavior that is it will be anxiolytic. With this in mind, we set forward experiments to test the effects of CRH inhibition on mice behavior and memory. In the first experiment, we injected pAAV-hSyn-DIO-rM4D(Gi)-mCherry bilaterally into the CeA of CRH-cre mice and tested the mice for anxiety-like behavior after the administration of CNO or vehicle respectively. The inhibition of the CeA CRH neurons decreased the anxiety-like behavior in the O-maze and improved the memory performance in NOR test. The vehicle treated mice spent 11.9 ± 2.6 % of their time in the open arms of the elevated O-maze while the CNO treated mice spent 34.8 ± 9.1 % of their time in the open arms of the elevated O-maze (T-test, $t_{15} = 2.4$, $P < 0.05$, $n = 9/8$). Performance in NOR was also improved in CNO treated mice with an increase of their mean discrimination index by 170% (T-test, $t_{15} = 2.89$, $P < 0.05$, $n = 9/8$). However, there was no significant difference between the groups when tested for anxiety-like behavior with the LDB and for hippocampus-dependent memory with Y-maze. Briefly the results were as follows: LDB, percent of time spent in the light compartment was 21.5 ± 1.3 % for vehicle group and 16.5 ± 2.2 % of time spent in the light compartment for CNO group, (T-test, $t_{14} = 1.9$, $P > 0.05$, $n = 8/8$). LDB, time to exit was 39 ± 12 seconds for vehicle group and 47 ± 13.5 seconds time to exit for CNO group, (T-test,

$t_{14} = 0.4$, $P > 0.05$, $n = 8/8$). For Y- maze test, the vehicle group demonstrated 58.3 ± 2.7 % correct alternations, whereas mice that received CNO demonstrated 61.5 ± 3.2 % correct alternations in the Y-maze test (T-test, $t_{14} = 0.75$, $P > 0.05$, $n = 8/8$).

Verification of the AAV-DREADD expression in CeA and evaluation of CeA neuronal activation.

After completion of the behavioral experiments, the injection site and viral expression was examined for both viral constructs, pAAV-hSyn-DIO-rM3D(Gs)-mCherry and pAAV-hSynhM4D(Gi)-mCherry. The animals were perfused and brain sections were analyzed for viral expression. Immunostaining for mCherry, the fluorescent reporter for DREADD receptor, was used to visualize the expression of both viral constructs. The injections yielded limited expression outside the borders of CeA (Figure 3A). This mCherry expression pattern matches the previously described pattern of CRH neuronal distribution in the CeA of CRH transgenic mice (Peng, Long et al. 2017). We also performed immunostaining for c-Fos, a marker for neuronal activity. CNO treatment produced robust excitation of CeA neurons in mice injected with the excitatory pAAV-hSyn-DIO-rM3D(Gs)-mCherry construct. Vehicle treated mice showed on average 6 ± 1 c-Fos-ir cells (Figure 3 B, C, D), while mice treated with CNO showed on average 28 ± 7 c-Fos-ir cells in the CeA, (T-test, $t_8 = 2.66$, $p < 0.05$, $n = 5/5$) (Figure 3 E, F, G). The expression of c-Fos was reversed in mice injected with the inhibitory pAAV-hSyn-hM4D(Gi)-mCherry. Vehicle treated mice showed on average 17 ± 3 c-Fos-ir cells, while the CNO group showed on average 3 ± 1 c-Fos-ir cells (T-test, $t_{12} = 6.7$, $P < 0.001$, $n = 7/7$), indicating robust inhibition of CeA neurons in the CNO group.

Activation of the CeA projections to LC increases the anxiety-like behavior and positively affects the hippocampus-dependent memory.

CRH neuronal population in the CeA may be divided into subgroups of projection neurons that target different hypothalamic and brain stem structures. One of these subgroups in the CeA provides the main CRH innervation to the brain stem including LC and the surrounding peri-LC dendritic zone (Tjouwakaris, Rudoy et al. 2003). Keeping this fact in mind, we used viral vectors to label and express DREADD receptors only in the CeA neurons that project to LC and therefore to label a majority of the CRH projections to LC with excitatory DREADD. We injected rAAV-CAG-eGFP-F2A-Cre bilaterally into the LC and cre-dependent pAAV-hSyn-DIO-rM3D(Gs)-mCherry into the CeA. The mice were tested for anxiety-like behavior and hippocampus-dependent memory three to four weeks after the viral injections. In the O-maze, the vehicle injected mice spent on an average 29.5 ± 8.7 % in the open arms; whereas the mice that were injected with CNO spent an average of 7.6 ± 2 % in the open arms of the maze (Mann-Whitney test, $P = 0.007$, $n = 5/5$) (Figure 4A). In LDB, the time to exit from dark to light compartment was 40.2 ± 2.4 seconds for mice that received vehicle and 32.2 ± 10.6 seconds for CNO injected mice, (Mann-Whitney test, $P = 0.38$, $n = 5/5$). Time spent in light compartment was 28.6 ± 4.3 % for vehicle and decreased to only 12.2 ± 2.7 %, for CNO injected mice (Mann-Whitney test, $P = 0.031$, $n = 5/5$) (Figure 4 B and C).

Activation of the CeA projections to LC improved the performance of the mice when tested for hippocampus-dependent memory. The vehicle injected mice had on an average 51.4

± 3.3 % of correct alternations whereas mice that received CNO had 65 ± 2.1 % of correct alternations (Mann-Whitney test, $P = 0.008$, $n = 5/5$) in the Y-maze (Figure 4D). The activation of the CeA projections to LC also increased the mean discrimination index in the NOR test by 100 %, (Mann-Whitney test, $P = 0.007$, $n = 5/5$) (Figure 4E).

Activation of CeA projections to LC increases the expression of cFos-ir in LC proper and peri-LC zone.

Evaluation of c-Fos expression in the LC proper after CNO/vehicle injection revealed that the c-Fos expression in LC proper was significantly higher in the CNO injected group when compared to the vehicle injected group: LC proper, CNO injected mice with an average of 12 ± 2 cells versus vehicle injected mice with an average of 6 ± 1 cells (Mann-Whitney test, $P = 0.028$, $n = 5/4$). The count of c-Fos-ir positive neurons in the rostromedial pericerular zone, the region that contains the majority of CRH projection fibers from the CeA, revealed similar results. The c-Fos expression in was significantly higher in the CNO injected group when compared to the vehicle injected group: peri-LC zone, CNO injected mice with an average of 47 ± 5 cells versus vehicle injected mice with an average of 29 ± 1 cells (Mann-Whitney test, $P = 0.028$, $n = 5/4$) (Figure 5).

Expression of pAAV-hSyn-DIO-rM3D(Gs)-mCherry by CeA projections to LC.

The animals were sacrificed after the completion of the behavioral experiments and immunohistochemistry for mCherry and GFP was performed using standard protocols. The rAAVCAG-eGFP-F2A-Cre expression at the injections site was found in the LC but mostly in the surrounding peri-LC dendritic zone (Figure 6). While retrogradely labeled cells expressing GFP were found in all major brain regions that project to LC including the CeA, the posterior lateral hypothalamus, PVN and the sensory cortex, the expression of mCherry as a marker for the excitatory DREADD was restricted to a population of CeA neurons (Figure 7).

We also evaluated c-Fos-ir in CeA after CNO/vehicle injection in order to assess CeA activation due with the DREADDs; the number of c-Fos-ir positive cells in vehicle treated mice was an average of 26 ± 4 cells versus CNO treated mice with a significantly higher average of 53 ± 7 cells (Mann-Whitney test, $P = 0.015$, $n = 5/4$).

Application of CRH1 receptor antagonist blocked the effects of the activation of CeA projections on anxiety-like behavior and hippocampus-dependent memory.

CeA is the main source of CRH innervation to the LC but CRH is a co-transmitter with GABA and dynorphin in these projection fibers (Reyes, Carvalho et al. 2011). Pretreatment of the mice with CRH1receptor antagonist antalarmin hydrochloride in dose of 30 mg/kg suppressed the effects of DREADD expressing projection neurons that connect the CeA with LC. The group pretreated with CRH1 antagonist spent on an average 51.7 ± 7.5 % of their time in the open arms whereas mice that received CNO spent only 22.4 ± 3.6 % of their time in the open arms of the elevated plus-maze (Mann-Whitney test, $P = 0.031$, $n = 5/5$). In LDB test, time spent in light compartment was 46.4 ± 3.6 % for CNO and antalarmin treated mice and 20.8 ± 2.7 % for the CNO treated mice (Mann-Whitney test, $P = 0.008$, $n = 5/5$) The time to exit was 88.2 ± 10.7 seconds for the group that received CNO alone and 33.8 ± 3.3

seconds for the antalarmin pretreated group (Mann-Whitney test, $P = 0.007$, $n = 5/5$). The hippocampus-dependent memory was also significantly affected by the antalarmin treatment. The mean discrimination index for mice that were pretreated with antalarmin was decreased by 169.4 % (Mann-Whitney test, $P = 0.007$, $n = 5/5$).

Discussion

We describe the involvement of CeA CRH neurons in modulation of anxiety-like behavior and hippocampus-dependent working and long-term memory. Chemogenetic technique was used to modulate the activity of the CRH neurons in the CeA and the CeA projection neurons to LC. The CeA is the main output nucleus of the amygdala that projects to various hypothalamic and brainstem effector regions, which are known to modulate the physiological endocrine and autonomic response to stress but which also, may contribute to the development of anxiety and anxiety-related comorbidities. In our experiments, the activation of CeA CRH neurons using excitatory DREADDs as well as the activation of the CeA neurons that project to the LC increased the anxiety-like behavior in mice. Also, while the activation of the majority of CeA CRH neurons impaired spatial and long-term memory, the activation of CeA projections to the LC improved performance on the tests for spatial and long-term memory.

Numerous studies have shown that development of anxiety depends on a complex circuitry that involves multiple brain structures and includes but is not restricted to the extended amygdala, hippocampus and various brainstem nuclei (Davis and Whalen 2001, Adhikari 2014, Tovote, Fadok et al. 2015). Previous studies in monkeys and rodents demonstrate that the CeA is in a position to modulate not only some of the key signs of anxiety, but also animal arousal (Penzo, Robert et al. 2014). CeA modulates arousal via dense monosynaptic and polysynaptic projections to brainstem effector regions such as LC (Van Bockstaele, Colago et al. 1998, Kravets, Reyes et al. 2015). The CeA modulatory output is generated by neuronal subgroups with different neuropeptide content and the CRH is a transmitter that dominates the CeA projections to the brainstem (Gray 1993). In particular, tract-tracing studies have identified robust CRH projections from the CeA to the LC, parabrachial nuclei, the midbrain central gray, the mesencephalic trigeminal nucleus and the BNST (Moga and Gray 1985, Sakanaka, Shibasaki et al. 1986, Gray and Magnuson 1992). In our studies, the increased anxiety-like behavior after activation of the entire CeA CRH population very likely includes activation of CRH projections to LC, BNST and other downstream targets of CeA CRH neurons. The opposite effect, a reduction of anxiety-like behavior, was observed after inhibition of the CeA CRH neuronal population. This finding is consistent with the anxiogenic role of amygdala CRH (Davis and Whalen 2001, Tovote, Fadok et al. 2015). Our experimental design allowed for comparison between the activation of the entire CRH population in the CeA with the activation of only CeA projections to LC. The anatomical correlate between the two CRH populations in CeA is clearly discernible from the expression of the DREADDs in the CeA. While the expression of cre-activated DREADDs that were injected directly into the CeA of CRH-cre mice included both centrolateral and centromedial subdivisions of the CeA (Figure 3 A), the expression of the DREADDs in the CeA after retrograde transfection from LC was restricted to the centromedial (or output) subdivision of the CeA (Figure 7 B and C) in a pattern already described in rats (Van

Bockstaele, Colago et al. 1998). The excitation of specific CeA projections to LC was similar to the activation of the entire CRH neuronal population in that it led to anxiety-like behavior and was accompanied by increased c-Fos expression in the LC and peri-LC zone, a fact that suggests activation of the norepinephrine neurons. The fact that pretreating the mice with CRH1 antagonist abolished the effect of CeA neuronal activation on anxiety supports the view that CRH is the main neurotransmitter in the CeA to LC projections. Therefore, exciting the CeA CRH projections to the LC was sufficient to simulate effects of acute stress.

The last finding replicates a phenomenon already described in a recent paper by McCall et al. (McCall, Al-Hasani et al. 2015). In their studies, McCall et al. used an elaborate set of chemogenetic and optogenetic experiments to support the notion that the amygdala CRH projections to LC play an important role in stress-induced anxiety. From these experiments they concluded that the dysregulation of this circuitry could lead to affective disorders. However, the role of CRH - LC projections in the normal stress response is not investigated or discussed in details. Our study supports all of the findings made by McCall et al. that are pertinent to anxiety-like behavior and further expands on the known CRH functions by describing the participation of the CRH in optimizing memory processes during a normal stress response. It is very likely that this function is part of the eustress response and it is essential for the survival of the organism.

Our speculation that LC activation by CRH projections is necessary to maximize memory performance during normal stress responses is well supported by the published literature. The noradrenergic LC has an essential role in processing of sensory information, working memory, long-term synaptic plasticity (Sara and Bouret 2012). Noradrenergic neurons in the LC have been implicated in emotional memory formation of stressful stimuli via a neural circuit that involves the basolateral amygdala (BLA) (McGaugh and Roozendaal 2002, Roozendaal and McGaugh 2011). Norepinephrine and glucocorticoids released during and immediately after the emotionally arousing event act synergistically in BLA to mediate the synaptic plasticity necessary for memory consolidation and storage of the emotionally arousing event (Roozendaal, Okuda et al. 2006). It has been shown that under emotional arousal there is not only a better recollection of the experience but the temporal and spatial context in which the event occurred is also remembered better (Sharot, Delgado et al. 2004, Schwabe, Dalm et al. 2008). This fact indicates possible interactions of LC not only with the BLA but also with other brain regions namely hippocampus and prefrontal cortex, which are involved in the formation of spatial memory and object recognition memory. The effects of emotional arousal on memory are complex. Previous studies demonstrated that high emotional arousal created after exposure to behavioral or psychological stressors either enhances or impairs memory depending on the type of the stressor, stressor duration, stressor intensity, stressor timing regarding memory phase and the learning task (Leong and Packard 2014, Leong, Goodman et al. 2015). Sandi et al, 2007 notes that the presence of acute stressor can interfere with information processing and impact spatial memory in an inverted U manner (Sandi and Pinelo-Nava 2007). The relationship between strong emotionality and memory performance has long been established in the form of the Yerkes-Dodson law that describes how stress affects memory performance (Diamond, Campbell et al. 2007). The Yerkes-Dodson law states that moderate arousal can enhance performance on

memory tests, but high levels of arousal can decrease performance by affecting memory consolidation. In our studies, we examined the effects of CeA neuronal excitation in the modulation of hippocampus-dependent spatial and long-term memory. The two memory test that we used, Y-maze and NOR, both assess the hippocampus-dependent type of explicit memory (Roberts, Dember et al. 1962, Antunes and Biala 2012). Studies show that stress affects the spontaneous alternations on the Y-maze (Hughes 2004). The NOR is a non-appetitive hippocampus-dependent working, short term or long-term memory test depending on the elapsed testing time. We first showed that the activation of CeA CRH neurons impairs Y-maze spatial memory and NOR long-term memory and that memory deficit can be reversed by the inhibition of the entire CeA CRH neuronal population during the memory task. That memory impairment was contrasted with our next experiment, in which the excitation of CeA projections to LC, likely only a part of the total CRH neuronal population in CeA, resulted in improvement of the spatial and long-term memory. Based on the well-established correlation between level of stress and memory, our findings show that while the activation of the entire CeA CRH population by a strong stimulus impairs working and long-term memory, the activation only of the CeA projections to LC improves memory performance. The memory improvement very likely depends on the activation of LC neurons with subsequent release of norepinephrine in the forebrain and from here, to increased levels of arousal.

All together we showed that the activation of CeA CRH neurons increases anxiety-like behavior and negatively affects hippocampus-dependent spatial and long-term memory. We hypothesize that the over activity of CeA CRH neurons maybe the underlying mechanism for emergence of memory deficits associated with anxiety disorders. However, a part of the CeA CRH neuronal population that projects to LC, very likely is responsible for the maintaining of arousal that is needed for normal memory processing by the brain. In conclusion, our findings highlight important directions for future research underlying memory disorders, especially those associated with anxiety disorders.

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Highlights

- Activation of the CeA CRH neurons increased anxiety-like behavior and impaired hippocampus-dependent memory.
- Inhibition of the CeA CRH neurons reversed the anxiety-like behavior and memory impairment.
- Activation of the CeA - LC projections was anxiogenic but improved the performance on hippocampus-dependent memory tests.
- A novel insight to the role of the CeA CRH neurons in the modulation of hippocampus-dependent memory is proposed.

Figure 1: The chemogenetic activation of CeA CRH neurons increased anxiety-like behavior. Panel **A** shows that the mice injected with CNO spent less time in the open arms when compared to the mice that were injected with vehicle. Mann-Whitney test, $P = 0.009$, * - significant difference for time spent in open arms of the O-maze, vehicle versus CNO, $n = 5/5$. Panels **B** and **C** show that the CNO injected mice spent less time in the light field (**B**) Mann-Whitney test, $P = 0.016$, * - significant difference for time spent in the light field of the LDB, vehicle versus CNO, $n = 5/5$ and took longer time to exit from the dark compartment (**C**), Mann-Whitney test, $P = 0.032$, * -significant difference for time to exit, vehicle versus CNO, $n = 5/5$.

Figure 2: The chemogenetic activation of CeA CRH neurons negatively affected hippocampus-dependent memory

. Panel **A** shows that mice injected with CNO made fewer correct spontaneous alternations in the Y-maze as compared to the vehicle injected mice, Mann-Whitney test, $P = 0.015$, * - significant difference for percent correct alternations, vehicle versus CNO, $n = 5/5$. Panel **B** shows that mice injected with CNO spent less time with the novel object when compared to the vehicle injected controls, Mann-Whitney test, $P = 0.031$, * - significant difference for vehicle versus CNO, $n = 5/5$.

Figure 3: Expression of Cre-dependent pAAV-hSyn-DIO-rM3D(Gs)-mCherry in the CeA of CRH-cre mice and increased expression of c-Fos-ir in the CeA after CNO treatment. Panel **A** shows that the expression of pAAV-hSyn-DIO-rM3D(Gs)-mCherry neurons was restricted to the lateral and the medial subdivisions of the CeA and that the neighboring striatum and BLA were completely devoid of viral expression. This distribution of the DREADD virus matches the distribution of CRH mRNA and CRH-ir shown in the literature (Cassell, Freedman et al. 1999). High magnification images (**B** to **G**) show mCherry-ir (**B** and **E**), c-Fos-ir (**C** and **F**) and mCherry/c-Fos-ir (**D** and **G**) in CeA of vehicle treated (**B**, **C** and **D**) and CNO treated mice (**E**, **F** and **G**). Arrowheads point to colocalization between c-Fos-ir (green) and mCherry-ir neurons (red). Abbreviations: BLA - basolateral amygdala, CeM – centromedial amygdala, CeL– centrolateral amygdala. Scale bar = 200 μ m in A and scale bar = 20 μ m in G.

Figure 4: Chemogenetic activation of the CeA projections to LC increases anxiety-like behavior but positively affects hippocampus-dependent memory

We injected rAAV-CAG-eGFP-F2A-Cre bilaterally into the LC and cre-activated pAAV-hSyn-DIO-rM3D(Gs)-mCherry into the CeA and three weeks later tested the mice for anxiety-like behavior and hippocampus-dependent memory after injections of CNO, the ligand for the DREADD(Gs). Panel **A** shows that the experimental group spent less time in the open arms of the O-maze when compared to the controls, Mann-Whitney test, $P = 0.007$, * - significant difference for vehicle versus CNO, $n = 5/5$. Panel **B** shows that the CNO injected mice spent less time in the light field of the LDB, Mann-Whitney test, $P = 0.031$, * - significant difference for time spent in the light field, vehicle versus CNO, $n = 5/5$. CNO administration also led to an increased percent of correct alternations in the Y-maze (**C**), Mann-Whitney test, $P = 0.008$, * - significant difference for vehicle versus CNO, $n = 5/5$ and (**D**) a better discrimination index in the NOR, Mann-Whitney test, $P = 0.007$, * - significant difference for vehicle versus CNO, $n = 5/5$.

Figure 5: Chemogenetic activation of CeA projections to LC increases cFos-ir in LC and peri-LC zone

. The CNO treatment increased c-Fos-ir expression in LC and peri-LC zone in mice with bilateral injections of retrograde cre virus into LC and excitatory cre-activated DREADD into CeA. Panels **A, B, C** show the brain sections from vehicle and panels **D, E, F** show brain sections from CNO treated mice. Panels **A** and **D** show TH-ir (red), panels **B** and **E** show c-Fos-ir (green) and panels **C** and **F** show merged images of TH/c-Fos-ir. Scale bar = 100 μ m

Figure 6: Expression of the retrograde rAAV-CAG-eGFP-F2A-Cre viral vector in LC
. The figure shows the distribution of GFP-ir, a marker for rAAV-CAG-eGFP-F2A-Cre in LC and the dendritic peri-LC region, which is the target for the projection fibers from the CeA. Panel A shows TH-ir (red), panel B shows GFP-ir (green) and panel C shows a merged image of GFP/TH-ir labeled neurons distributed evenly throughout the entire LC and peri-LC region. Scale bar = 100 μ m.

Figure 7: Expression of retrogradely expressed GFP and locally expressed mCherry as a marker for DREADDs in the CeA

. Panel **A** shows GFP-ir (green) neurons in CeA after rAAV-CAG-eGFP-F2A-Cre injection in the LC. Panel **B** shows mCherry-ir (red) labeled neurons in the CeA after injection of cre dependent pAAV-hSyn-DIO-rM3D(Gs)-mCherry in the CeA. Panel **C** shows the merged image of the GFP/mCherry **A** and **B**. Panels **D** to **F** are high magnification images of LC injected rAAV-CAG- eGFP-F2A-Cre and CeA injected cre-dependent pAAV-hSyn-DIO-rM3D(Gs)-mCherry-ir neurons. Arrowheads indicate colocalization between GFP-ir and mCherry-ir in high power image of CeA (**F**). Abbreviations: CeM – centromedial amygdala, CeL – centrolateral amygdala, BLA – basolateral amygdala. Scale bar = 200 μm in **C**; Scale bar = 20 μm in **F**