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Neuropeptide Y2 receptors in anteroventral BNST control remote fear memory depending on extinction training

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ABSTRACT

The anterior bed nucleus of stria terminalis (BNST) is involved in reinstatement of extinguished fear, and neuropeptide Y2 receptors influence local synaptic signaling. Therefore, we hypothesized that Y2 receptors in anteroventral BNST (BNSTav) interfere with remote fear memory and that previous fear extinction is an important variable. C57BL/6NCrl mice were fear-conditioned, and a Y2 receptor-specific agonist (NPY_{3-36}) or antagonist (JNJ-5207787) was applied in BNSTav before fear retrieval at the following day. Remote fear memory was tested on day 16 in two groups of mice, which had (experiment 1) or had not (experiment 2) undergone extinction training after conditioning. In the group with extinction training, tests of remote fear memory revealed partial retrieval of extinction, which was prevented after blockade of Y2 receptors in BNSTav. No such effect was observed in the group with no extinction training, but stimulation of Y2 receptors in BNSTav mimicked the influence of extinction during tests of remote fear memory. Pharmacological manipulation of Y2 receptors in BNSTav before fear acquisition (experiment 3) had no effect on fear memory retrieval, extinction or remote fear memory. Furthermore, partial retrieval of extinction during tests of remote fear memory was associated with changes in number of c-Fos expressing neurons in BNSTav, which was prevented or mimicked upon Y2 blockade or stimulation in BNSTav. These results indicate that Y2 receptor manipulation in BNSTav interferes with fear memory and extinction retrieval at remote stages, likely through controlling neuronal activity in BNSTav during extinction training.

1. Introduction

Relapse of extinguished fear is a major problem in the treatment of fear-like anxiety disorders and poses a serious challenge to the long-term outcome of existing extinction-based therapies. Return of fear can affect up to 62% of treated patients (Mystkowski, Craske, Echiverri, & Labus, 2006). For instance, approximately 27% panic disorder patients have been reported with relapse following exposure-based therapies (Brown and Barlow, 1995; Fava et al., 2001). Thus, a profound pharmacological support is prerequisite to regulate remote fear memory retrieval to achieve long-term and stable suppression of fear.

NPY, a 36-amino-acid peptide known for its anxiolytic properties is involved in fear and extinction learning (Gutman, Yang, Ressler, & Davis, 2008; Tasan, Verma, Wood, & Lach, 2016; Verma, Tasan, Herzog, & Sperk, 2012; Verma et al., 2015). Pavlovian fear conditioning and fear extinction are valid clinically relevant models to explore the behavior and brain mechanisms of fear acquisition, extinction and relapse (Davis, 2002; Goode and Maren, 2014; Pape and Paré, 2010; Verma et al., 2012). Of particular interest being that over-expression of a Y2 receptor-specific agonist (NPY_{3-36}) in the central amygdala (CEA) resulted in a reduction in spontaneous recovery and reinstatement of fear suggesting that Y2 receptor activation supports a permanent suppression of fear (Verma et al., 2015). In contrast, local deletion of Y2 receptors in the CEA increased the expression of conditioned stimulus (CS)-induced freezing during fear recall and fear extinction (Verma et al., 2015). Recently, Wood et al. (2015) provided evidence that GABAergic, NPY-expressing neurons of the medial nucleus of the central amygdala (CEm) reciprocally connect to the bed nucleus of the stria terminalis (BNST).

The BNST is part of the extended amygdala and considered a center of valence, integrating information with negative valence or anxiety-like states, and has gained recent attention as a relevant region for human stress-related psychiatric diseases (Alheid and Heimer, 1988; Lebow and Chen, 2016; Walker, Miles, & Davis, 2009). The BNST is divided into anterior/posterior and dorsal/ventral sections through fiber bundles of the stria terminalis and anterior commissure, respectively (Hammack, Mania, & Rainnie, 2007). The anterior BNST is the main termination zone of axonal inputs from the CEA. Both regions

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robustly innervate each other, and reportedly play a crucial role to mediate behavioral processes related to fear and anxiety (Fendt, Endres, & Apfelbach, 2003; Tovote, Fadok, & Luthi, 2015; Walker, Toufexis, & Davis, 2003). The role of these regions is highlighted in an experimental rat model of posttraumatic stress disorder (PTSD), suggesting diminished CEA and enhanced BNST influences during alterations in fear/anxiety characterizing PTSD-like states (Rodriguez-Sierra, Goswami, Turesson, & Paré, 2016). In particular, the ventral sector of the anterior BNST (BNSTav) seems well positioned to control anxiety-like responses, given its projections via the paraventricular hypothalamic nucleus that regulate the HPA axis, and its axonal connections with the ventral tegmental area (VTA) (Gungor and Paré, 2016; Herman, Ostrander, Mueller, & Figueiredo, 2005). BNSTav also receives inputs from various brain regions involved in fear behavior, including infralimbic prefrontal cortex, basomedial and central amygdala, various thalamic and brainstem nuclei (Shin, Geerling, & Loewy, 2008). In fact, optogenetic activation of glutamatergic or GABAergic neurons in BNSTav produced anxiogenic- or anxiolytic-like effects, respectively, likely through projections to VTA (Jennings et al., 2013). It was reported recently that inactivation of the anterior BNST prevents reinstatement but not renewal of conditioned fear to an extinguished CS suggesting that the BNST is critical for fear recovery following stress-exposure, but not for contextual retrieval processes that mediate fear renewal (Goode et al., 2015).

While relatively few studies have investigated the effects of NPY in the BNST, there is some evidence indicating that NPY can modulate BNST neurons depending on whether pre- or postsynaptic receptors are stimulated (Tasan et al., 2010, 2016; Wood et al., 2015). Of interest here is that Y2 receptors exist on fibers of NPY-negative neurons in BNST (Tasan et al., 2010; Tasan et al., 2016; Wood et al., 2015), and that stimulation of presynaptic Y2 receptors reduces GABAergic transmission to neurons in the ventral sector of the anterior BNST (Kash and Winder, 2006; McCall et al., 2013; Pleil et al., 2012). Chronic stress impairs this ability of NPY to suppress inhibitory postsynaptic currents in DBA/2J mice, but not in C57BL/6J mice, suggesting that stress can alter NPY signaling in BNST depending on genetic background (Pleil et al., 2012).

Therefore, we hypothesized that Y2 receptors in BNSTav interfere with fear extinction and remote fear memory. C57BL/6NCrl adult mice were exposed to a fear conditioning and extinction paradigm where a Y2 receptor-specific agonist (NPY_{3-36}) or antagonist (JNJ-5207787) was applied locally in BNSTav at 20 min before fear conditioning or 20 min before fear retrieval on the next day. Remote fear memory retrieval was assessed 16 days after fear conditioning in groups of animals that had or had not received fear extinction training after Y2 receptor manipulation. Furthermore, neuronal activation patterns were assessed through immediate early gene (c-Fos) mapping in BNSTav.

2. Material and methods

2.1. Animals

All experiments were carried out in accordance with the European Committees Council Directive (86/609/EEC) and were approved by local authorities LANUV NRW (AZ 84-02.04.2014.A414). Experiments were performed in adult male C57BL/6NCrl mice [n = 65 (n = 60 for behavioral studies, n = 5 for histological verification of drug diffusion sites) 10–12 weeks old, weighing 25–32 g, Charles River, Sulzfeld, Germany]. Each experimental animal was housed under standard laboratory conditions (12 h/12 h light/dark cycle, lights being on at 07:00, food and water *ad libitum*). Animals were group housed (3–5 per cage) in transparent standard Macrolon cages type III, provided with sawdust, plastic tube and nesting material. A week before stereotaxic surgery and for subsequent experiments, animals were single housed.

2.2. Stereotaxic surgery for guide cannula implantation

Adult male C57BL/6NCrl (8–10 weeks of age) mice were bilaterally implanted with guide cannulae using a stereotaxic frame (Model 962, David Kopf Instruments, Tujunga, California, USA) with blunt ear bars. For BNSTav, coordinates were taken according to Paxinos and Franklin (2008) (in mm, from bregma): BNSTav: A, 0.02; L, ± 0.7; V, −4.5. All surgical procedures were performed under deep anesthesia by intraperitoneal injection of pentobarbital (Narkoren; 50 mg/kg), supplemented by subcutaneous injection of carprofen (Rimadyl; 5 mg/kg). Injected volume was varied between 0.15 and 0.25 ml, according to the weight of the individual animal. Depth of the anesthesia was ascertained by lack of the ocular and hind limb withdrawal reflexes. The scalp was incised to level bregma and lambda in the same dorsoventral plane. After setting the coordinates, small holes were drilled into the skull and a guide cannula (Polymicro tech., fused silica capillary, ID: 250 µm, OD: 355 µm) was lowered into the BNSTav. The entire skull surface was covered with dental cement to secure the cannula to the skull. A dummy cannula (Polymicro tech., fused silica capillary, ID: 73.5 µm, OD: 150.8 µm) was inserted into each guide cannula following the surgery. Mice were allowed to recover from surgery for at least one week prior to drug treatment and behavioral training.

2.3. Drugs

The Y2 receptor agonist NPY_{3-36} (PolyPeptide Laboratories, France) and the Y2 receptor antagonist JNJ-5207787 (Tocris Bioscience, Germany) were prepared at the day of the experiment from stock solutions at concentrations of 200 nM in 0.9% NaCl (pH 7.4) and infused into the BNSTav. Fluorescently labelled NPY_{3-36} (Cy3-NPY_{3-36}; Phoenix Pharmaceuticals, Inc., 330 Beach Road, Burlingame, CA 94,010, USA) was similarly prepared as NPY_{3-36}.

2.4. Drugs administration procedure

In two separate groups of animals, twenty minutes before fear conditioning or fear extinction training, respectively, the dummy cannula was removed and replaced with infusion cannula that protruded an additional 1 mm from the tip of the guide cannula. NPY_{3-36}, JNJ-5207787 or saline was infused bilaterally into the BNSTav at a rate of 100 nl/min and a total volume of 300 nl. Before retracting the infusion cannula additional 5 min were given for diffusion of the drug. Animals were kept under light inhalation anesthesia (Isoflurane, 2.5%; CP Pharma, Germany) during these procedures.

2.5. Experimental design

Behavioral experiments were conducted with single housed, 10–12 weeks old male C57BL/6NCrl mice (n = 60). Mice were divided into three groups according to the time point of drug injection (20 min before acquisition; 20 min before retrieval) and fear extinction procedure (with extinction (Ext); without extinction (no Ext)), according to the following scheme:

1. Fear acquisition—24 h—Drug—20 min—Fear extinction—2 weeks— Remote fear memory retrieval. (n = 21; n = 7/drug treatment categories saline, NPY_{3-36} and JNJ-5207787; with extinction (Ext)).
2. Fear acquisition—24 h—Drug———————— No extinction ————————2 weeks— Remote fear memory retrieval. (n = 21; n = 7/drug treatment categories saline, NPY_{3-36} and JNJ-5207787; without extinction (no Ext)).
3. Drug—20 min—Fear acquisition—24hr—Fear retrieval—2 weeks—Remote fear memory retrieval. (n = 18; n = 6/drug treatment categories saline, NPY_{3-36} and JNJ-5207787).

Due to inappropriate guide cannula placement 2–3 animals were excluded from each experimental group.

2.6. Fear conditioning paradigm

Fear acquisition was performed in context A consisting of a transparent acrylic rodent conditioning chamber with a metal grid floor that was enclosed by a sound attenuating chamber (TSE, Bad Homburg, Germany). Illumination was 80 lx and chambers were cleaned with 70% ethanol after each session. Fear extinction and remote fear memory retrieval was performed in a different context B consisting of a standard Macrolon cage type III horizontally separated into two parts, providing an open field-like arena and arena walls (Daldrup et al., 2015).

2.6.1. Fear acquisition

After post-surgical recovery on day 1 (context A), mice were subjected to a differential fear conditioning paradigm in which one auditory stimulus served as a CS (CS+, 30 s white noise, 80 dB) because it was explicitly paired with a US, whereas the second auditory stimulus was not paired (CS−, 30 s, 3.5 kHz, 80 dB). All animals were exposed to the context A for 2 min habituation (pre CS) followed by 5 CS+ and 5 CS− in an alternating order (starting with a CS+), the protocol ended with 2 min post CS. The unconditioned stimulus co-terminating with each CS+ consisted of a scrambled mild electric foot shock (0.5 mA, 2 s). The inter-trial interval (ITI) between each CS+ and CS− varied between 60 and 100 s. To check the drug effect on fear acquisition the respective group of mice were drug infused in the BNSTav 20 min before fear acquisition.

2.6.2. Fear extinction

On day 2, intra BNSTav infusion of the Y2 receptor agonist, antagonist or vehicle, NPY_{3-36}, JNJ-5207787 or saline, respectively was performed 20 min prior to cued-fear recall, followed by extinction training. CS induced fear retrieval and CS induced extinction training was performed in context B. After a 2 min habituation period, 5 CS− (30 s, inter-stimulus interval 5 s) were presented followed by 25 presentations of CS+ (30 s, inter-stimulus interval 5 s). For evaluation of discriminative fear, freezing to CS− and CS+ was compared during initial presentation of the stimuli (5 consecutive CS−, 3 consecutive CS+).

2.6.3. Remote fear memory retrieval

Fear expression was assessed at remote stages 16 days after conditioning in groups of animals that had or had not received fear extinction training after Y2 receptor manipulation. Remote fear memory retrieval was tested by re-exposing the animals to 10 CS+ in the extinction context (context B).

2.6.4. Scoring and presentation of freezing behavior

During the whole experiment freezing behavior was recorded by using two oppositely located, horizontal cameras (equipped with the MATLAB based software MOVE) providing synchronous recordings at any point of time required for scoring the precise freezing behaviors (Meuth et al., 2013). Freezing analysis was done as previously shown (Daldrup et al., 2015). Briefly, during the experiment, freezing was scored manually by using key-logger. Subsequent offline analysis involved interval sampling method where freezing response was calculated as percentage of 5 s bins across experimental period using a customized MATLAB routine (MATLAB 12 (R2012b), The MathWorks, Natick, MA, USA) and averaged (each 5 s bin) for the respective duration of stimulus pre CS (2 min), CS− (30 s) and CS+ (30 s).

2.7. Histochemistry

2.7.1. Tissue preparation

After the final behavioral experiment (remote fear memory retrieval) mice were sacrificed with an overdose of barbiturate (Narkoren 100 mg/kg, ip), the brains were removed and incubated in a 4% formaldehyde solution. For localization of the guide cannula and infusion site (Fig. 1), 40 µm thick frozen sections were prepared and stained with cresyl violet. For c-Fos immunohistochemistry, brains were perfused with 4% paraformaldehyde (PFA) 90 min after remote fear testing and snap frozen (isopentane, −70C°, 3 min) for further processing.

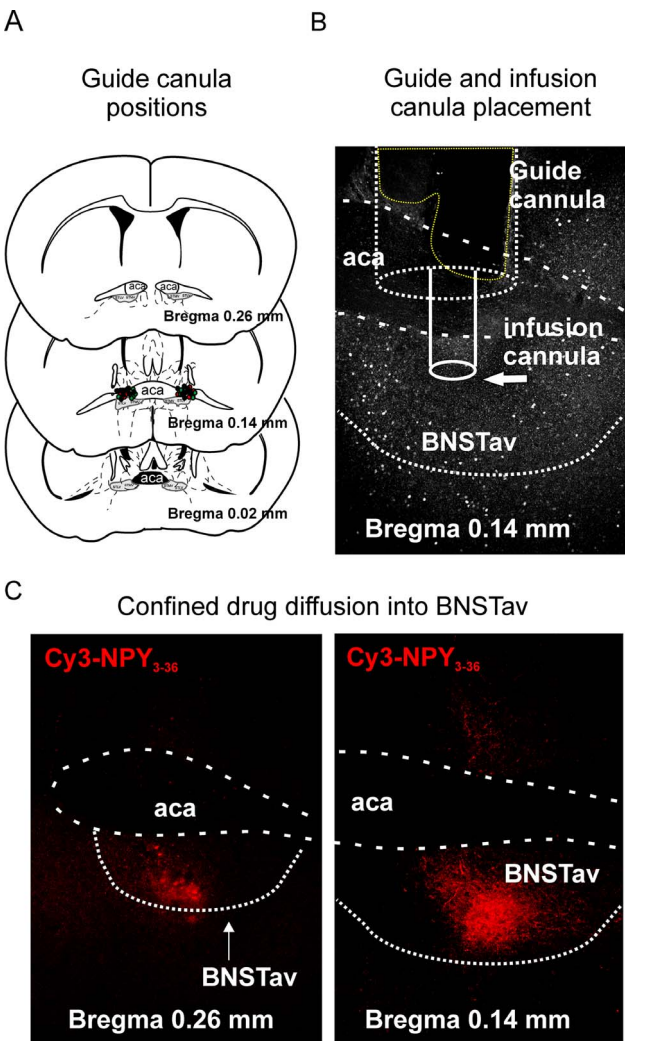

Fig. 1. Verification of injection sites in BNSTav. (A) Schematic representation of guide cannula placement in BNSTav, at level 0.14 mm rostral to bregma, based on Paxinos and Franklin (2008). Black, green and red circles represent the position of the guide cannula tip for the different treatment groups: saline, Y2 receptor agonist (NPY_{3-36}) and the Y2 receptor antagonist (JNJ-5207787), respectively. Of note, substances were applied bilaterally, and only injection sites in the right hemisphere are illustrated further. (B) Representative coronal section of c-Fos immunoreactivity, exemplifying the position of the guide and infusion cannula. (C) Representative photomicrograph of a coronal brain section obtained after local injection of Cy3-NPY_{3-36}, revealing diffusion restricted to BNSTav. aca: anterior commissure, anterior part, STLV: bed nucleus of the stria terminalis, lateral division, ventral part, STMV: bed nucleus of the stria terminalis, medial division, ventral part. STLV and STMV defined as BNSTav (Gungor and Paré, 2016). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7.2. Immunohistochemistry

Immunostaining was performed on free-floating, PFA-fixed, 40 µm thick coronal sections. Brain sections containing anterior BNST were screened microscopically and BNSTav brain region was identified by comparison with a mouse brain atlas (Paxinos and Franklin 3th edition, 2008). In brief, sections were first treated with 0.3% H_2O_2 in methanol

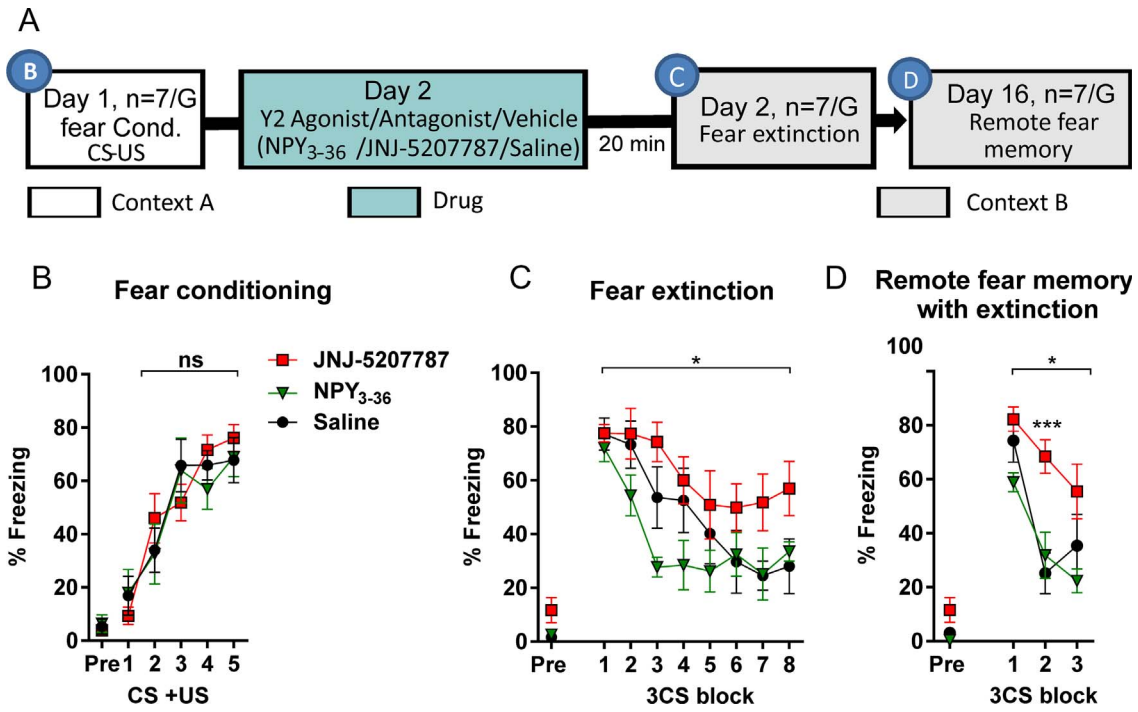

Fig. 2. Pharmacological manipulation of Y2 receptors in BNSTav before extinction: Effect on extinction and remote fear memory. (A) Experimental design. At day 1, mice underwent fear conditioning (B) and were divided into three equally performing groups for further pharmacological treatment, according to the conditioned response (CS+ evoked freezing; n = 7 per group). The black, green and red data points represent the different treatment groups: saline, Y2 receptor agonist (NPY_{3-36}) and Y2 receptor antagonist (JNJ-5207787), respectively. 20 min after pharmacological treatment at day 2, mice underwent extinction training (C). Remote fear memory retrieval was tested at day 16 in each group (D). Statistical significance tested against saline. Data represent means ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, ns; not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for 20 min to inactivate endogenous peroxidases and then incubated in blocking solution (10% normal goat serum, 3% BSA, 0.3% Triton-X100 in PBS) for 120 min to minimize nonspecific labeling, followed by overnight incubation at 4 °C in primary antiserum c-Fos (1:1000, in blocking solution, SC-52; Santa Cruz Biotechnology, Santa Cruz, CA). The resulting complex was visualized by incubation with horseradish peroxidase (HRP)-coupled secondary antibody (1:250 P0448; Dako, Vienna, Austria) at room temperature for 90 min. For immunofluorescence sections were incubated in a tyramide signal amplification solution (1:100, TSA-CY3, in-house) for 6 min. Sections were mounted on slides and covered using Vectashield mounting medium + DAPI (4,6-Diamidino-2-phenylindole, dihydrochloride) (Vector laboratories, Inc., Burlingame, USA).

2.7.3. Quantification of immunohistochemical labeling

For quantification of c-Fos labelled neurons, three coronal brain sections per mouse containing different rostrocaudal levels of the BNSTav were used, and the numbers of c-Fos positive nuclei were counted. In brief, stained slices were analyzed with a laser scanning confocal microscope (Nikon eC1 plus) using an HC PL FLUOTAR 10×/0.30 objective (Leica) to obtain 10× and 10 × 4 times digitally magnified images. c-Fos fluorescence images were acquired blindly, without information on treatment. Captured images of c-Fos staining were thresholded to clearly identify c-Fos-immunoreactive neurons based on staining density, cell size and shape. The quantification of the c-Fos immunoreactive cells was performed by image J software (NIH) as automated cell counts in regions of interest (ROIs) within the BNSTav, according to the mouse brain atlas (Paxinos and Franklin 3th edition, 2008) (Figs. 5, S2, S3). All sections that were quantitatively compared to each other (drug only or drug + extinction) were stained in the same batch and cells were counted using the same thresholding parameters and microscope settings.

2.8. Statistical analysis

Data are presented as means ± SEM. They were analyzed for normal distribution and equal variances using GraphPad Prism software (Prism 6 for Windows, GraphPad Software Inc., San Diego, CA, USA). The mixed model two-way ANOVA (within-subject factors) for repeated measurements was used to analyze overall changes in percentage freezing for time and treatment (drug only or drug + extinction), during acquisition (including Pre CS and 1st CS-US as internal control for the baseline), extinction and remote fear memory retrieval (including pre CS as baseline). To evaluate treatment by time interaction Bonferroni post hoc test (between/within groups) was employed to compare individual CS blocks. During extinction and remote fear memory retrieval (except pre-CS), each data point represents a mean of 3 CS (3 CS block). Mixed model two-way ANOVA followed by Bonferroni post hoc test was also used to analyze the differences between absolute numbers of c-Fos immunoreactive cells during remote fear memory retrieval across the treatment groups. Significance was set at $p < 0.05$.

3. Results

3.1. Histological verification of injection sites

The placement of the guide and injection cannulae was histologically verified in c-Fos immunoreacted coronal brain sections (Fig. 1). Guide cannulae were typically located immediately above the BNSTav (Fig. 1A). The infusion cannula projected an additional 1 mm from the tip of the guide cannula (Fig. 1B; see Methods for details), corresponding to the tip of the infusion cannula located 1 mm ventral within the BNSTav. In fact, application of fluorescently labelled NPY_{3-36} (Cy3-NPY_{3-36}) revealed sites of injection and regional rostro-caudal diffusion within the BNSTav (Fig. 1C, left and right images; n = 5 animals). Only animals showing guide cannula placement immediately above and/or

injection site in the BNSTav were included in the behavioral analysis.

3.2. Y2 receptor manipulation in BNSTav: Effects on fear extinction and retrieval of remote fear memory

The effect of Y2 receptor stimulation or blockade in BNSTav was tested on fear extinction and retrieval of remote fear memory (experiment 1). The time line of the experiment, training and pharmacological treatment schemes, is illustrated in Fig. 2A. After fear conditioning on day 1, mice were divided into three equally performing (behavioral freezing) groups (n = 7/group), statistically insignificant for further respective drug treatment (Fig. 2B, Table S1; mixed model, two-way ANOVA for repeated measurements; time: $F_{(5,90)} = 50.78$, $P < 0.0001$, treatment: $F_{(2,18)} = 0.06$, $P = 0.9419$, but no interaction (time × treatment): $F_{(10,90)} = 0.906$, $P = 0.5314$, followed by Bonferroni's post hoc multiple comparisons test, see Table S1). On day 2, one group was infused with the Y2 receptor agonist NPY_{3-36}, a second group with the Y2 receptor antagonist JNJ-5207787, and a third group was injected with saline and served as a vehicle control. Substances were bilaterally injected into the BNSTav, and all injection sites were histologically verified, as described in methods. Extinction training commenced 20 min after substance application. In all groups expression of remote fear was assessed at day 16 after conditioning. During fear extinction training, freezing in NPY_{3-36} infused mice readily declined upon consecutive CS presentations compared to saline infused control mice. Infusion of the Y2 antagonist JNJ-5207787 resulted in a delayed decline of freezing during extinction training (Fig. 2C, mixed model, two-way ANOVA for repeated measurements: time: $F_{(8,144)} = 24.27$, $P < 0.0001$, treatment (drug + Ext): $F_{(2,18)} = 5.500$, $P = 0.0137$; interaction (time × treatment (drug + Ext)): $F_{(16,144)} = 1.292$, $P = 0.2095$). In order to further characterize the rate of extinction in the different groups, we compared the first time point of significantly different CS+ freezing as compared to initial CS+ freezing. Mice treated with saline displayed significantly decreased freezing at the 5th CS block. Freezing in NPY_{3-36} injected animals was significantly reduced at the 3rd CS block, and freezing in JNJ-5207787 injected mice was at no time point significantly different from initial values. Remote fear memory retrieval at day 16 (2 weeks after drug treatment) revealed similar levels of freezing to the first three CS presentations in the saline-treated controls, and a fast decline of freezing during consecutive CS presentations. In JNJ-5207787-treated mice after extinction training, remote fear remained at a significantly higher level compared to saline controls, whereas NPY_{3-36}-treated mice showed no difference compared to saline controls (Fig. 2D, mixed model, two-way ANOVA for repeated measurements: time: $F_{(3,54)} = 76.63$, $P < 0.0001$, treatment (drug + Ext): $F_{(2,18)} = 7.871$, $P = 0.0035$ and interaction (time × treatment (drug + Ext)): $F_{(6,54)} = 2.876$, $P = 0.0166$, followed by Bonferroni's post hoc multiple comparisons test).

Regarding baseline freezing, the two-way ANOVA for repeated measurements did not show significant differences between treatment groups during fear acquisition, fear extinction and remote fear memory retrieval at Pre CS time points.

3.3. Y2 receptor manipulation in BNSTav: Effects on retrieval of remote fear memory

Next (experiment 2), we investigated the possible influence of Y2 receptor manipulation in BNSTav on retrieval of remote fear memory with no preceding extinction training. The time line of the experiment, training and pharmacological treatment schemes, is illustrated in Fig. 3A. After fear conditioning on day 1, mice were divided into 3 equally performing (behavioral freezing) groups (n = 7/group), statistically insignificant for further respective drug treatment (Fig. 3B, Table S1, mixed model, two-way ANOVA for repeated measurements: time: $F_{(5,90)} = 69.56$, $P < 0.0001$, treatment: $F_{(2,18)} = 0.096$, $P = 0.908$, interaction (time × treatment): $F_{(10,90)} = 0.613$, $P = 0.798$, followed by Bonferroni's post hoc multiple comparisons test). On day 2, one group was infused with the Y2 receptor agonist NPY_{3-36}, a second group with the Y2 receptor antagonist JNJ-5207787, and a third group was injected with saline and served as a vehicle control. Substances were bilaterally injected into the BNSTav, and all injection sites were histologically verified, as described in methods. After drug infusion, animals were returned to the home cage. Expression of remote fear was assessed at day 16 (2 weeks after drug treatment). Analyzing the effect of drug alone with no extinction training, revealed that NPY_{3-36} treated mice displayed significantly reduced expression of remote fear compared to saline controls, whereas freezing levels in the JNJ-5207787 treated group were similar to saline controls (Fig. 3C, Table S1, mixed model, two-way ANOVA for repeated measurements: time: $F_{(3,54)} = 103.0$, $P < 0.0001$, treatment (drug): $F_{(2,18)} = 6.500$, $P = 0.0075$, interaction (time × treatment (drug)): $F_{(6,54)} = 1.199$, $P = 0.321$, followed by Bonferroni's post hoc multiple comparisons test, see Table S1).

3.4. Comparison of remote fear memory retrieval with and without preceding extinction training

The two groups of saline-treated animals that had or had not received extinction training (Fig. 2D and 3C) displayed similar levels of freezing to the first 3CS presentations during remote fear memory retrieval. During consecutive CS presentations, the group that had received extinction training displayed a fast decline of freezing compared with the group that had not received extinction training (Fig. 2D and 3C; Table S2, mixed model, two-way ANOVA for repeated measurements: time: $F_{(3,36)} = 77.03$, $P < 0.0001$, treatment: $F_{(1,12)} = 6.330$, $P = 0.0271$ and interaction (time × treatment (extinction)): $F_{(3,36)} = 6.143$, $P = 0.0017$, followed by Bonferroni's post hoc multiple comparisons test, see Table S2).

Overall, these data indicate that fear extinction facilitates the decline of fear at remote stages (see Table S2), which is impaired following Y2 blockade before extinction training in BNSTav, while high levels of fear prevailing at remote stages with no extinction training are reduced following Y2 stimulation in BNSTav.

3.5. Y2 receptor manipulation in BNSTav before fear acquisition

In a final set of pharmacological experiments (experiment 3), we investigated a possible influence of Y2 receptor manipulation in BNSTav before fear acquisition, and subsequent effect on fear extinction and remote memory. NPY_{3-36} or JNJ-5207787 was bilaterally injected into BNSTav 20 min before fear training commenced, and effects were compared with those in saline-injected animals. The time line of the experiment, training and pharmacological treatment schemes, is illustrated in Fig. 4A. On day 1, there was no significant main effect of drug treatment at any time point of fear acquisition (Fig. 4B, Table S1, mixed model, two-way ANOVA for repeated measurements: time: $F_{(5,75)} = 41.09$, $P < 0.0001$, treatment: $F_{(2,15)} = 1.140$, $P = 0.3459$, but no interaction (time × treatment): $F_{(10,75)} = 1.075$, $P = 0.3921$, followed by Bonferroni's post hoc multiple comparisons test, see Table S1). On day 2 fear extinction training was performed, and no significant treatment effect was noticed between groups injected with Y2 receptor agonist, antagonist or vehicle prior to fear acquisition (Fig. 4C, Table S1, Mixed model, two-way ANOVA for repeated measurements: time: $F_{(8,120)} = 16.24$, $P < 0.0001$, treatment: $F_{(2,15)} = 2.451$, $P = 0.1199$, but no interaction (time × treatment): $F_{(16,120)} = 0.945$, $P = 0.5206$, followed by Bonferroni's post hoc multiple comparisons test, see Table S1). Remote fear memory retrieval was tested on day 16, and no significant treatment effect was noticed between groups injected with Y2 receptor agonist, antagonist or vehicle prior to fear acquisition (Fig. 4D, Table S1, Mixed model, two-way ANOVA for repeated measurements: time: $F_{(3,45)} = 66.52$, $P < 0.0001$, treatment: $F_{(2,15)} = 1.527$, $P = 0.2492$, but interaction (time × treatment): $F_{(6,45)} = 1.225$, $P = 0.3114$, followed by Bonferroni's post hoc multiple comparisons test, see Table S1).

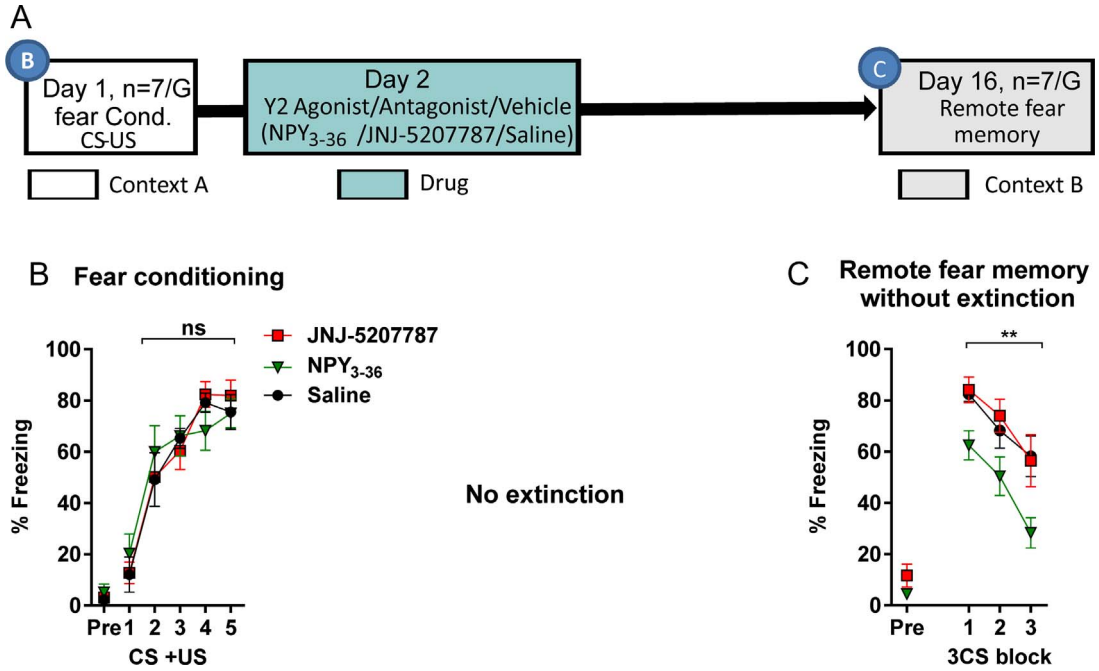

Fig. 3. Pharmacological manipulation of Y2 receptors in BNSTav before extinction: Effect on remote fear memory without extinction training. (A) Experimental design. At day 1, mice underwent fear conditioning (B) and were divided into three equally performing groups for further pharmacological treatment, according to the conditioned response (CS-evoked freezing; n = 7 per group). The black, green and red data points represent the different treatment groups: saline, Y2 receptor agonist (NPY_{3-36}) and Y2 receptor antagonist (JNJ-5207787), respectively. Remote fear memory retrieval was tested at day 16 in each group (C). Statistical significance tested against saline. Data represent means ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, ns; not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

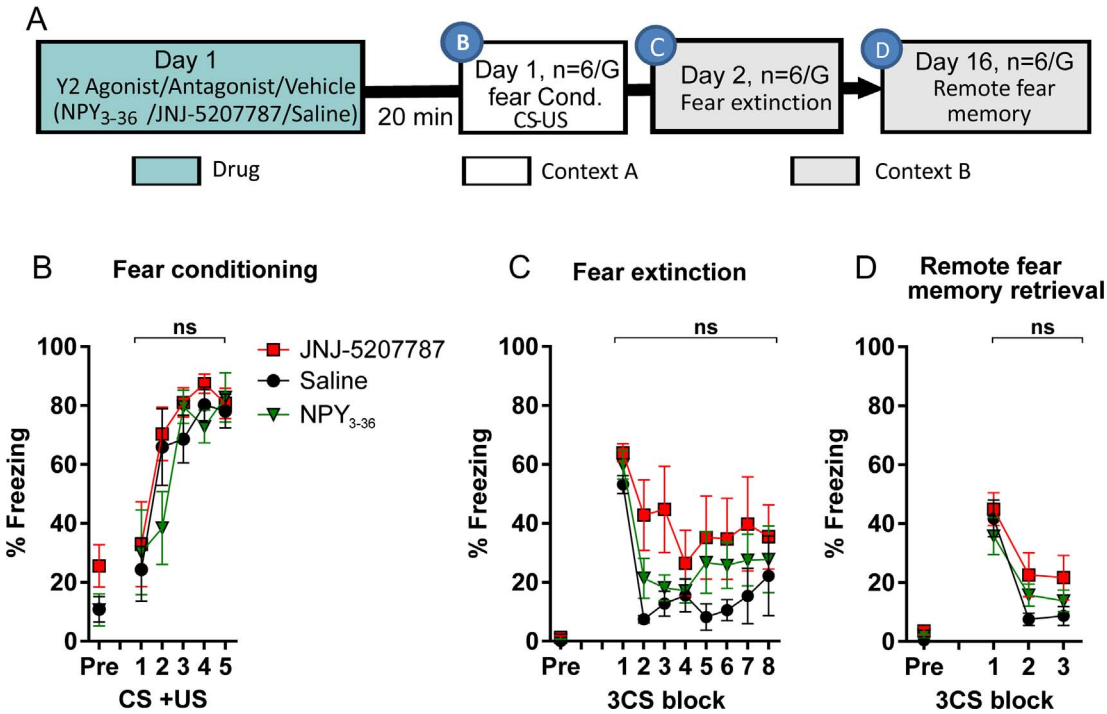

Fig. 4. Pharmacological manipulation of Y2 receptors in BNSTav before acquisition: Lack of treatment effect on fear acquisition, extinction and remote memory: (A) Experimental design. At day 1, substances were injected into BNSTav and 20 min later animals underwent fear conditioning (B). Subsequent division of groups, extinction training (day 2; C) and tests of remote memory (day 16; D) were performed as in Fig. 2. The black, green and red data points represent the different treatment groups (n = 6 per group): saline, Y2 receptor agonist (NPY_{3-36}) and Y2 receptor antagonist (JNJ-5207787), respectively. Statistical significance was tested against saline. Data represent means ± SEM. ns: not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Discrimination of CS− with CS+ during fear retrieval

Next, we evaluated whether animals can discriminate between predictive (CS+) and non-predictive (CS−) stimuli. In experiment 1 and 3, freezing to CS− and CS+ was compared during retrieval of conditioned fear at day 2. Baseline freezing was at 2–10%, and freezing to CS− and CS+ was between 25 and 37 % and 72–77%, respectively. Freezing to CS+ and CS− was significantly different in all groups, and

there were no differences between groups (Fig. S1A, Table S3 - experiment 1, mixed model, two-way ANOVA for repeated measurements: time: $F_{(2,36)} = 122.4$, $P < 0.0001$, treatment (drug): $F_{(2,18)} = 2.452$, $P = 0.1143$ and interaction (time × treatment (drug)): $F_{(4,36)} = 0.4389$, $P = 0.7797$; B - experiment 3, time: $F_{(2,30)} = 206.5$, $P < 0.0001$, treatment (drug): $F_{(2,15)} = 2.644$, $P = 0.1039$ and interaction (time × treatment (drug)): $F_{(4,30)} = 2.617$, $P = 0.0547$, followed by Bonferroni's post hoc multiple comparisons test, see Table S3).

3.7. Immediate early gene (c-Fos) expression after remote fear

In a next step, in order to assess neuronal activation patterns in the BNSTav during remote fear memory retrieval, we employed immediate early gene mapping (using c-Fos immunohistochemistry) in the groups of mice, which had or had not undergone fear extinction training, and had been treated with Y2 receptor agonist or antagonist 20 min before fear memory retrieval (experiment 1, 2). The absolute numbers of c-Fos immunoreactive cells were quantified from regions of interest (ROIs) in 3 coronal sections (40 µm thick) at different rostrocaudal levels in the BNSTav (Figs. S2, S3). Results are illustrated in Fig. 5 (Fig. 5A, Table S4, Ext vs. no Ext; Mixed model, two-way ANOVA revealed a significant interaction between extinction and drug treatment on c-Fos expression; interaction (extinction × drug): $F_{(2,15)} = 5.32$, $P = 0.0179$, drug treatment: $F_{(2,15)} = 23.7$, $P < 0.0001$; extinction: $F_{(1,15)} = 1.78$, $P = 0.2015$, followed by Bonferroni's post hoc multiple comparisons test, see Table S4). In detail, the Bonferroni's multiple comparisons test aimed to analyze the effects of extinction and of drug applications between and within groups. The absolute numbers of c-Fos immunoreactive cells were significantly reduced in BNSTav after extinction (Ext) compared to mice not having undergone extinction training (no Ext) (Fig. 5A–C, Table S4). Similarly, the drug effects were compared within each group (Ext, no Ext). In the group with extinction training (Ext), the Y2 receptor antagonist (JNJ-5207787) significantly interfered with the extinction effect on c-Fos expression compared to saline (Fig. 5A, Table S4). In the group without extinction training (no Ext), Y2 receptor agonist (NPY_{3-36}) treatment was associated with a significant decrease in c-Fos activated cells compared to saline (Fig. 5A, Table S4).

4. Discussion

Our results identify NPY and Y2 receptors in the BNSTav as crucial components for remote fear memory retrieval. We specifically demonstrate that: (1) In the group with extinction training, tests of remote fear memory revealed partial retrieval of extinction, which was prevented after blockade of Y2 receptors in BNSTav. (2) No such effect was observed in the group with no extinction training, but stimulation of Y2 receptors in BNSTav mimicked the influence of extinction during tests of remote fear memory. (3) Pharmacological manipulation of Y2 receptors in BNSTav 20 min before fear acquisition had no effect on fear memory retrieval, extinction or remote fear memory. (4) Partial retrieval of extinction during tests of remote fear memory was associated with changes in number of c-Fos expressing neurons in BNSTav, which was prevented or mimicked upon Y2 blockade or stimulation in BNSTav.

The BNST is divided into anterior/posterior and dorsal/ventral sections through fiber bundles of the stria terminalis and anterior commissure, respectively (Hammack et al., 2007). The BNSTav, situated ventral to the anterior commissure, participates in reciprocal connections to amygdala, hypothalamus and ventral tegmental area and also receives projections from dorsal raphe, frontal cortex, locus coeruleus, ventral subiculum and nucleus of solitary tract (Lebow and Chen, 2016). The BNSTav contains mostly GABAergic but also glutamatergic neurons, which locally connect BNSTav to anterodorsal lateral (BNSTadl) and anterodorsal medial (BNSTadm) sector of the BNST (Turesson, Rodríguez-Sierra, & Paré, 2013). While the BNSTadl has been proposed to control activity patterns to and within BNSTadm and BNSTav synaptic networks (Turesson et al., 2013), the BNSTav seems to be particularly positioned to control anxiety-like responses, given its projections via the paraventricular hypothalamic nucleus that regulate the HPA axis (Herman et al., 2005), and its axonal connections with the ventral tegmental area (VTA; see Gungor and Paré, 2016). Neuronal activity in the BNSTav has indeed been found to correlate with behavioral functions, in that exposure to aversive stimuli or associated cues alone enhances glutamatergic cell activity, whereas activity of GABAergic cells is inhibited (Tovote et al., 2015). Jennings et al. (2013) further demonstrated that optogenetic activation/inhibition of glutamatergic/GABAergic projections of the BNSTav to the ventral tegmental area (VTA) produced anxiogenic/anxiolytic like behavior.

A vast literature supports the view that NPY is a major neurochemical component of the stress response, coordinating neuronal, vascular, immune and metabolic functions (Heilig, 2004; Rasmusson, Schnurr, Zukowska, Scioli, & Forman, 2010; Tasan et al., 2016). Overall, the NPY system is considered to adapt the organism to stressful, potentially life-threatening conditions and to maintain physiological integrity, in both rodents (Cohen et al., 2011) and humans (Wu et al., 2013). While relatively few studies have investigated the effects of NPY in the BNST, there is evidence indicating that NPY can modulate inhibitory GABAergic input or directly hyperpolarize BNST neurons depending on whether pre- or postsynaptic receptors are stimulated (Heilig, 2004; Rasmusson et al., 2010; Tasan et al., 2016). For instance, stimulation of postsynaptic NPY receptors of Y1 or Y5 subtype induces a negative shift in resting membrane potential in a subset of BNSTal neurons by blocking the I_h current (Ide et al., 2013). Stimulation of presynaptic Y2 receptors has been found to reduce GABAergic transmission to BNSTav neurons (Kash and Winder, 2006; McCall et al., 2013; Pleil et al., 2012). Chronic stress impairs this ability of NPY to suppress inhibitory postsynaptic currents in DBA/2J mice, but not in C57BL/6J mice, suggesting that stress can alter NPY signaling in BNST depending on genetic background (Pleil et al., 2012).

It is noteworthy that compared to other amygdala nuclei, the highest levels of Y2 receptors are found in the CEA and BNST (Tasan et al., 2010). These Y2 receptors seem to be present not only on local circuit neurons, but dense Y2 receptor labeling has also been found within the stria terminalis in association with GABAergic, NPY-negative projection neurons between CEA and BNST (Tasan et al., 2016). In keeping with this, local deletion of Y2 receptors in the CEA resulted in a reduction of $[^{125}I]PYY_{3-36}$ receptor binding in target areas, such as the BNST (Tasan et al., 2010). Overall these findings indicate that projections along the stria terminals are modulated via Y2 activity, thereby adding to the classical scenario, where Y2 receptors in CEA control conditioned fear expression and extinction (Tasan et al., 2016; Verma et al., 2015). Recently, Dum et al., 2016 provided evidence that functionally G-protein coupled Y2 receptors are not only located at nerve terminals but also along fiber tracts like fimbria, stria terminalis and Schaffer collaterals (Dum et al., 2016). The performance of mice lacking Y2 receptors differs depending on their genetic background. While the 129SvJ-C57Bl/6 mice lacking Y2 receptors displayed anxiolytic-like behavior (Tschenett et al., 2003), back-crossing with pure C57BL/6 genetic background failed to confirm an anxiolytic-like phenotype (Zambello et al., 2010). Furthermore, local deletion of Y2 receptors in BLA and CEA in 129SvJ-C57Bl/6 mice resulted in an anxiolytic-like phenotype, whereas a similar manipulation in BNST had no such effect (Tasan et al., 2010). The present study extends these findings by showing that pharmacological manipulation of Y2 receptors in BNSTav prior to extinction learning modulates retrieval of remote fear memory. Extinction training has previously been shown to facilitate the decline of fear expression at remote stages likely reflecting retrieval of extinction (Myers et al., 2006; Quirk and Mueller, 2007), although evidence for spontaneous recovery and reinstatement following immediate extinction has also been reported (Schiller et al., 2008). In the present study, these effects of extinction training on remote fear expression

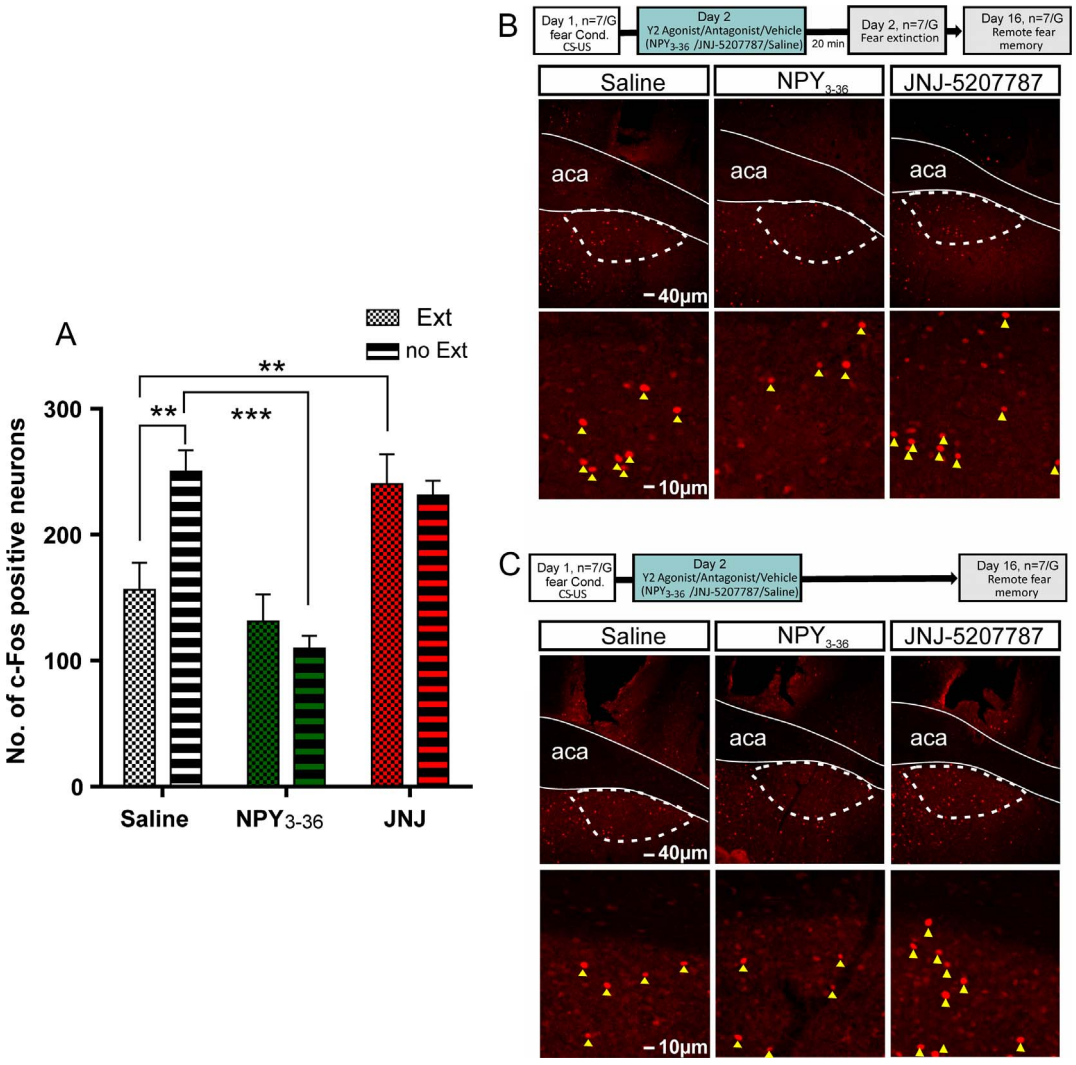

Fig. 5. Neuronal activation patterns assessed through early gene (c-Fos) mapping in BNSTav after remote fear memory retrieval. Data are from two experiments of mice which had undergone fear extinction training (A, B; Ext) or had not undergone fear extinction training (A, C; no Ext), prior to remote fear memory retrieval. Bar graphs display absolute numbers of c-Fos immunoreactive cells, from regions of interest (ROI) in 3 coronal sections (40 µm thick) at different rostro-caudal levels in the BNSTav (see Fig. S2 & S3). A black, green and red bar represents the different treatment groups, saline, Y2 receptor agonist (NPY_{3-36}) and Y2 receptor antagonist (JNJ-5207787), respectively. Photomicrographs show higher magnification of c-Fos immunostainings in the BNSTav of different treatment groups, saline (left column), NPY_{3-36} (middle) and JNJ-5207787 (right column). Of note, cells were counted bilaterally, and only cells in the right hemisphere are illustrated. Arrow heads indicate c-Fos positive neurons. Scale bars are 40 µm and 10 µm, respectively. Data represent means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

seem to reflect the sustained influence of the NPY system on Y2 receptors in BNSTav, as is indicated by the following line of evidence. Preventing the recruitment of Y2 receptors during extinction learning through pharmacological blockade in BNSTav prevented the retrieval of extinction during tests of remote fear. Along the same line, additional stimulation of Y2 receptors through agonist treatment in BNSTav could substitute an extinction session in supporting the decline of remote fear, indicative of functional compensation in cases with no extinction training. Our results from c-Fos mapping support these conclusions, and indicate an influence on fear-active neurons in BNSTav. The rapid decline of remote fear after extinction training was associated with a decrease in number of c-Fos expressing neurons in BNSTav, suggesting a reduced population of fear-active neurons.

Pharmacological Y2 blockade in BNSTav during extinction learning prevented this change in c-Fos activation pattern, associated with a maintained high level of remote fear. By comparison, c-Fos expression remained at a high level in BNSTav at remote stages with no extinction training and was reduced after previous Y2 stimulation in BNSTav, thereby paralleling the changes in conditioned fear expression. The molecular mechanisms of this sustained Y2 influence remain to be delineated, and may relate to various effectors associated with $G_{i/o}$ protein, such as e.g. adenylyl cyclase, cAMP, phospholipase C (PLC), phosphatidylinositol 3-kinase activity (PI3K), and ion channels, which may coordinate and sustain physiological responses (Sah, Ekhator, Jefferson-Wilson, Horn, & Geracioti, 2013). Along this line, an early study is noteworthy reporting that administration of NPY and the Y2 receptor-preferring fragment NPY_{20-36} resulted in memory impairment in well-trained mice (Flood and Morley, 1989), supporting the view that Y2 receptors may reduce remote fear also by interfering with memory retention. Future studies using conditional deletion of Y2 receptors or interfering with intracellular pathways of Y2 receptors will be needed to provide a mechanistic view on recruitment of these receptors during extinction and their influence on remote fear memory.

Y2 receptors typically do not co-localize with NPY neurons in networks of the amygdala and extended amygdala (Stani'c, Mulder, Watanabe, & Hokfelt, 2011; Tasan et al., 2016; Wood et al., 2015), precluding a direct release-controlling effect on NPY neurons. Rather, Y2 receptors may function as hetero-receptors on long-fiber tracts, like stria terminalis, and NPY may be released from adjacent local interneurons upon strong and repetitive stimulations (Tasan et al., 2016).

While the NPY-releasing neurons in BNSTav remain to be characterized in detail, it is interesting to note that a considerable portion of NPY-GFP-positive neurons in CEA co-express somatostatin (SST) (Tasan et al., 2016). SST-positive neurons in the lateral section of the CEA (CeL) receive major afferent input from the paraventricular thalamic nucleus (PVT), and the PVT-CeL pathway is considered essential for linking stress detection to both fear memory and behavioral expression of fear (Penzo et al., 2015). Furthermore, in ventrolateral BNST neurons in vitro, Y2 receptor stimulation reduced the amplitude of evoked inhibitory postsynaptic currents (IPSCs) (Kash and Winder, 2006), and chronic stress was found to impair this NPY-mediated suppression of IPSCs (Pleil et al., 2012). While the neuronal populations that release NPY in the BNST and the axonal inputs that are regulated via Y2 receptor stimulation remain to be identified, it seems reasonable to conclude that stress can alter NPY signaling via Y2 receptors in BNST, and that the ventral sector of the anterior BNST is critically involved. In addition, extinction training after threat exposure seems a relevant stimulus for recruitment of the NPY-Y2 system in BNSTav, enabling control of behavioral fear also at remote stages after the stressful event. Overall these conclusions add to the notion that NPY can act as a stress buffer in response to traumatic events in both rodents and humans (Cohen et al., 2011). In fact, NPY plasma levels loosely parallel the disease course in PTSD patients (reviewed in Tasan et al., 2016). Based upon the present results it is tempting to propose that providing Y2 receptor stimulation as an addition or substitute to extinction therapies may function as an improved approach to treat trauma and anxiety-related disorders in a more efficient and persistent way at remote stages.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2018.01.011.

References

Alheid, G. F., & Heimer, L. (1988). New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: The striatopallidal, amygdaloid, and corticopetal components of substantia innominata. *Neuroscience, 27*, 1.

Brown, T. A., & Barlow, D. H. (1995). Long-term outcome in cognitive-behavioral treatment of panic disorder: Clinical predictors and alternative strategies for assessment. *Journal of Consulting and Clinical Psychology, 63*, 754.

Cohen, H., Liu, T., Kozlovsky, N., Kaplan, Z., Zohar, J., & Math'e, A. A. (2011). The neuropeptide Y (NPY)-ergic system is associated with behavioral resilience to stress exposure in an animal model of post-traumatic stress disorder. *Neuropsychopharmacology, 37*, 350–363.

Daldrup, T., Remmes, J., Lesting, J., Gaburro, S., Fendt, M., Meuth, P., et al. (2015). Expression of freezing and fear-potentiated startle during sustained fear in mice. *Genes, Brain and Behavior, 14*, 281–291.

Davis, M. (2002). Role of NMDA receptors and MAP kinase in the amygdala in extinction of fear: Clinical implications for exposure therapy*. *European Journal of Neuroscience, 16*, 395.

Dum, E., Furtinger, S., Gasser, E., Bukovac, A., Drexel, M., Tasan, R., et al. (2016). Effective G-protein coupling of Y2 receptors along axonal fiber tracts and its relevance for epilepsy. *Neuropeptides, 61*, 49–55.

Fava, G. A., Rafanelli, C., Grandi, S., & Conti, S. (2001). Long-term outcome of panic disorder with agoraphobia treated by exposure. *Psychological Medicine, 31*.

Fendt, M., Endres, T., & Apfelbach, R. (2003). Temporary inactivation of the bed nucleus of the stria terminalis but not of the amygdala blocks freezing induced by trimethylthiazoline, a component of fox feces. *The Journal of Neuroscience, 23*, 23–28.

Flood, J. F., & Morley, J. E. (1989). Dissociation of the effects of neuropeptide Y on feeding and memory: Evidence for pre- and postsynaptic mediation. *Peptides, 10*, 963.

Goode, T. D., Kim, J. J., & Maren, S. (2015). Reversible inactivation of the bed nucleus of the stria terminalis prevents reinstatement but not renewal of extinguished fear. *eNeuro, 2* ENEURO.0037–15.2015.

Goode, T. D., & Maren, S. (2014). Animal models of fear relapse. *ILAR Journal, 55*, 246–258.

Gungor, N. Z., & Paré, D. (2016). Functional heterogeneity in the bed nucleus of the stria terminalis. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 36*, 8038–8049.

Gutman, A. R., Yang, Y., Ressler, K. J., & Davis, M. (2008). The role of neuropeptide Y in the expression and extinction of fear-potentiated startle. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 28*, 12682–12690.

Hammack, S. E., Mania, I., & Rainnie, D. G. (2007). Differential expression of intrinsic membrane currents in defined cell types of the anterolateral bed nucleus of the stria terminalis. *Journal of Neurophysiology, 98*, 638–656.

Heilig, M. (2004). The NPY system in stress, anxiety and depression. *Neuropeptides, 38*, 213–224.

Herman, J. P., Ostrander, M. M., Mueller, N. K., & Figueiredo, H. (2005). Limbic system mechanisms of stress regulation: Hypothalamo-pituitary-adrenocortical axis. *Progress in Neuropsychopharmacology and Biological Psychiatry, 29*, 1201–1213.

Ide, S., Hara, T., Ohno, A., Tamano, R., Koseki, K., Naka, T., et al. (2013). Opposing roles of corticotropin-releasing factor and neuropeptide Y within the dorsolateral bed nucleus of the stria terminalis in the negative affective component of pain in rats. *Journal of Neuroscience, 33*, 5881–5894.

Jennings, J. H., Sparta, D. R., Stamatakis, A. M., Ung, R. L., Pleil, K. E., Kash, T. L., et al. (2013). Distinct extended amygdala circuits for divergent motivational states. *Nature, 496*, 224–228.

Kash, T. L., & Winder, D. G. (2006). Neuropeptide Y and corticotropin-releasing factor bi-directionally modulate inhibitory synaptic transmission in the bed nucleus of the stria terminalis. *Neuropharmacology, 51*, 1013–1022.

Lebow, M. A., & Chen, A. (2016). Overshadowed by the amygdala: The bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Molecular Psychiatry, 21*, 450–463.

McCall, N. M., Sprow, G. M., Delpire, E., Thiele, T. E., Kash, T. L., & Pleil, K. E. (2013). Effects of sex and deletion of neuropeptide Y2 receptors from GABAergic neurons on affective and alcohol drinking behaviors in mice. *Frontiers in Integrative Neuroscience, 7*, 100.

Meuth, P., Gaburro, S., Lesting, J., Legler, A., Herty, M., Budde, T., et al. (2013). Standardizing the analysis of conditioned fear in rodents: A multidimensional software approach. *Genes, Brain and Behavior, 12*, 583–592.

Myers, K. M., Ressler, K. J., & Davis, M. (2006). Different mechanisms of fear extinction dependent on length of time since fear acquisition. *Learning & Memory (Cold Spring Harbor, NY), 13*, 216–223.

Mystkowski, J. L., Craske, M. G., Echiverri, A. M., & Labus, J. S. (2006). Mental reinstatement of context and return of fear in spider-fearful participants. *Behavior Therapy, 37*, 49–60.

Pape, H. C., & Paré, D. (2010). Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiological Reviews, 90*, 419–463.

Paxinos, G., & Franklin, K. B. J. (2008). *Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates* (3rd ed.). Academic Press.

Penzo, M. A., Robert, V., Tucciarone, J., De Bundel, D., Wang, M., Van Aelst, L., et al. (2015). The paraventricular thalamus controls a central amygdala fear circuit. *Nature, 519*, 455–459.

Pleil, K. E., Lopez, A., McCall, N., Jijon, A. M., Bravo, J. P., & Kash, T. L. (2012). Chronic stress alters neuropeptide y signaling in the bed nucleus of the stria terminalis in DBA/2J but not C57BL/6J mice. *Neuropharmacology, 62*, 1777–1786.

Quirk, G. J., & Mueller, D. (2007). Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology, 33*, 56–72.

Rasmusson, A. M., Schnurr, P. P., Zukowska, Z., Scioli, E., & Forman, D. E. (2010). Adaptation to extreme stress: Post-traumatic stress disorder, neuropeptide Y and metabolic syndrome. *Experimental Biology and Medicine, 235*, 1150–1162.

Rodriguez-Sierra, O. E., Goswami, S., Turesson, H. K., & Paré, D. (2016). Altered responsiveness of BNST and amygdala neurons in trauma-induced anxiety. *Translational Psychiatry, 6*, e857.

Sah, R., Ekhator, N. N., Jefferson-Wilson, L., Horn, P. S., & Geracioti, T. D. (2013). Cerebrospinal fluid neuropeptide Y in combat veterans with and without posttraumatic stress disorder. *Psychoneuroendocrinology, 40*, 277–283.

Schiller, D., Cain, C. K., Curley, N. G., Schwartz, J. S., Stern, S. A., Ledoux, J. E., et al. (2008). Evidence for recovery of fear following immediate extinction in rats and humans. *Learning & Memory (Cold Spring Harbor, NY), 15*, 394–402.

Shin, J. W., Geerling, J. C., & Loewy, A. D. (2008). Inputs to the ventrolateral bed nucleus of the stria terminalis. *The Journal of Comparative Neurology, 511*, 628.

Stani'c, D., Mulder, J., Watanabe, M., & Hokfelt, T. (2011). Characterization of NPY Y2 receptor protein expression in the mouse brain. II. Coexistence with NPY, the Y1 receptor, and other neurotransmitter-related molecules. *The Journal of Comparative Neurology, 519*, 1219–1257.

Tasan, R. O., Nguyen, N. K., Weger, S., Sartori, S. B., Singewald, N., Heilbronn, R., et al. (2010). The central and basolateral amygdala are critical sites of neuropeptide Y/Y2 receptor-mediated regulation of anxiety and depression. *The Journal of Neuroscience:*

The Official Journal of the Society for Neuroscience, 30, 6282–6290.
Tasan, R. O., Verma, D., Wood, J., & Lach, G. (2016). The role of Neuropeptide Y in fear conditioning and extinction. *Neuropeptides*.
Tovote, P., Fadok, J. P., & Luthi, A. (2015). Neuronal circuits for fear and anxiety. *Nature Reviews Neuroscience, 16*, 317–331.
Tschenett, A., Singewald, N., Carli, M., Balducci, C., Salchner, P., Vezzani, A., et al. (2003). Reduced anxiety and improved stress coping ability in mice lacking NPY-Y2 receptors. *European Journal of Neuroscience, 18*, 143.
Turesson, H. K., Rodríguez-Sierra, O. E., & Paré, D. (2013). Intrinsic connections in the anterior part of the bed nucleus of the stria terminalis. *Journal of Neurophysiology, 109*, 2438–2450.
Verma, D., Tasan, R. O., Herzog, H., & Sperk, G. (2012). NPY controls fear conditioning and fear extinction by combined action on Y_1 and Y_2 receptors. *British Journal of Pharmacology, 166*, 1461–1473.
Verma, D., Wood, J., Lach, G., Mietzsch, M., Weger, S., Heilbronn, R., et al. (2015). NPY Y2 receptors in the central amygdala reduce cued but not contextual fear. *Neuropharmacology, 99*, 665–674.
Walker, D. L., Miles, L. A., & Davis, M. (2009). Selective participation of the bed nucleus of the stria terminalis and CRF in sustained anxiety-like versus phasic fear-like responses. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 33*, 1291–1308.
Walker, D. L., Toufexis, D. J., & Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *European Journal of Pharmacology, 463*, 199–216.
Wood, J., Verma, D., Lach, G., Bonaventure, P., Herzog, H., Sperk, G., et al. (2015). Structure and function of the amygdaloid NPY system: NPY Y2 receptors regulate excitatory and inhibitory synaptic transmission in the centromedial amygdala. *Brain Structure and Function, 221*, 3373–3391.
Wu, G., Feder, A., Cohen, H., Kim, J. J., Calderon, S., & Charney, D. S. (2013). Understanding resilience. *Frontiers in Behavioral Neuroscience, 7*, 10.
Zambello, E., Zanetti, L., Hédou, G. F., Angelici, O., Arban, R., Tasan, R. O., et al. (2010). Neuropeptide Y-Y2 receptor knockout mice: Influence of genetic background on anxiety-related behaviors. *Neuroscience, 176*, 420–430.