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NPY Y2 receptors in the central amygdala reduce cued but not contextual fear

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ABSTRACT

The amygdala is fundamental for associative fear and extinction learning. Recently, also the central nucleus of the amygdala (CEA) has emerged as a site of plasticity actively controlling efferent connections to downstream effector brain areas. Although synaptic transmission is primarily mediated by glutamate and GABA, neuropeptides critically influence the overall response. While neuropeptide Y (NPY) acting via postsynaptic Y1 receptors exerts an important anxiolytic and fear-reducing action, the role of the predominantly presynaptic Y2 receptors is less defined.

To investigate the role of Y2 receptors in the CEA we employed viral-vector mediated over-expression of the Y2 selective agonist NPY_{3-36} in fear conditioning and extinction experiments. NPY_{3-36} over-expression in the CEA resulted in reduced fear expression during fear acquisition and recall. Interestingly, this effect was blocked by intraperitoneal injection of a brain-penetrant Y2 receptor antagonist. Furthermore, over-expression of NPY_{3-36} in the CEA also reduced fear expression during fear extinction of CS-induced but not context-related fear. Again, fear extinction appeared delayed by peripheral injection of a Y2 receptor antagonist JNJ-31020028. Importantly, mice with over-expression of NPY_{3-36} in the CEA also displayed reduced spontaneous recovery and reinstatement, suggesting that Y2 receptor activation supports a permanent suppression of fear. Local deletion of Y2 receptors in the CEA, on the other hand, increased the expression of CS-induced freezing during fear recall and fear extinction. Thus, NPY inhibits fear learning and promotes cued extinction by reducing fear expression also via activation of presynaptic Y2 receptors on CEA neurons.

1. Introduction

The amygdala controls emotional processing including fear and anxiety (Pape and Pare, 2010; Sah et al., 2003). Fear, an emotional arousal provoked by distinct learning processes is generally investigated by *Pavlovian* fear conditioning, where an initially neutral stimulus, such as a tone (conditioned stimulus, CS) is repetitively paired with an aversive stimulus (unconditioned stimulus, US). After a few pairings the CS elicits a fear reaction even in the absence of a US (conditioned response, CR). Repetitive presentation of the CS alone, however, triggers the formation of a new memory, called extinction memory (LeDoux, 2000; Maren, 2005; Quirk and Mueller, 2008). In contrast to fear, extinction memory is rather fragile, and consequently passage of time, changing of context or re-experience of the initial traumatic event may result in rapid recovery of the initial conditioned fear response (Ji and Maren, 2007). The central nucleus of the amygdala (CEA), a pivotal component for fear expression, also harbors a highly elaborated micro-network capable of diverse types of plasticity (Ciocchi et al., 2010; Haubensak et al., 2010; Li et al., 2013; Wilensky et al., 2006). CEA output is mediated by GABAergic neurons projecting to different brain regions, including hypothalamus, brainstem and bed nucleus of the stria terminalis (BNST) (Dong et al., 2001; LeDoux et al., 1988). However, several neuropeptides significantly

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modify the overall response (Bowers et al., 2012; Heilig, 2004). Recent evidence suggests that NPY, a 36-amino-acid peptide known for its anxiolytic properties, is also involved in fear and extinction learning (Broqua et al., 1995; Fendt et al., 2009; Gutman et al., 2008; Lach and de Lima, 2013; Verma et al., 2012). NPY acts through at least 5 different G protein-coupled receptors (Y1, Y2, Y4, Y5 and y6) generally exerting a prolonged inhibitory action (Michel et al., 1998).

Gutman et al. (2008) demonstrated that, NPY in the basolateral amygdala reduces fear expression and facilitates extinction of a fear potentiated startle response. However, NPY reduces fear expression also in Y1KO mice, indicating that different NPY receptor subtypes are contributing to the modulation of learned fear (Fendt et al., 2009). Recently, we demonstrated that NPYKO mice displayed faster acquisition, increased expression and impaired extinction of conditioned fear. Interestingly, while Y1 and Y2 receptor single KO mice exhibited only moderate changes in fear processing, the phenotype of NPYKO mice was fully recapitulated in Y1Y2 receptor double KO mice (Verma et al., 2012).

Therefore we propose that also Y2 receptors significantly modulate fear conditioning and extinction. To test this hypothesis we combined viral vector mediated over-expression of the Y2 preferring agonist $NPY_{3\text{-}36}$ and local deletion of Y2 receptors in conditional knockout mice with fear conditioning and extinction experiments. We demonstrate that $NPY_{3\text{-}36}$ over-expression in the CEA reduces the expression and facilitates the extinction of conditioned fear, while local deletion of Y2 receptors from CEA neurons results in the opposite effect.

2. Material and methods

2.1. Animals

All procedures involving animals and animal care were conducted in accordance with international laws and policies (Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 2011) and were approved by the Austrian Ministry of Science. All effort was taken to minimize the number of animals used and their suffering.

Experiments were performed in adult male mice (10–16 weeks old, weighing 25–30 g) maintained on a pure C57BL/6N background (Charles River, Sulzfeld, Germany). Conditional knockout mice for the Y2 receptor ($Y2^{lox/lox}$) as well as NPYKO mice were backcrossed to a C57BL/6N background for at least 10 generations. Mice were housed in groups of 3–5 animals under standard laboratory conditions (12 h/12 h light/dark cycle, lights being on at 07:00, food and water *ad libitum*). Generation of NPYKO mice and $Y2^{lox/lox}$ mice has been described in detail previously (Sainsbury et al., 2002; Verma et al., 2012).

2.2. Production of viral vectors

For recombinant adeno-associated-virus (rAAV)-mediated expression of Cre-recombinase-Green fluorescent fusion protein (Cre-GFP, or GFP alone as a control), rAAV serotype 2-based vectors pseudotyped with rAAV serotype 2 capsid proteins (rAAV-2/2) were prepared by a two-plasmid co-transfection procedure using HEK293T cells (Grimm et al., 2003). The rAAV-Cre vector was kindly supplied by Dr. Fred H. Gage, LaJolla, USA (Kaspar et al., 2002). For production of rAAV-$NPY_{3\text{-}36}$, a respective plasmid (Genescript, USA) encoding the human prepro-NPY but lacking the first 2 amino acids of the resulting NPY was custom synthesized and packaged into an rAAV backbone as described below (codon optimized DNA sequence of $NPY_{3\text{-}36}$: 5′ GGTACCGCGGCCGCGCCACCATGCTGGGCAACAAGCGGCTGGGCCTGAGCGGCCTGACCCTGGCCCTGAGCCTGCTGGTCTGCCTGGGAGCACTGGCAGAGGCCAGCAAGCCCGACAACCCCGGCGAGGACGCCCCTGCCGAGGACATGGCCCGGTACTACAGCGCCCTGCGGCACTACATCAACCTGATCACCCGGCAGAGATACGGCAAGCGGAGCAGCCCCGAGACACTGATCAGCGACCTGCTGATGCGGGAGAGCACCGAGAACGTGCCCCGGACCCGGCTGGAAGATCCCGCCATGTGGTGATGAGTCGACGAGCTC-3′). After processing in neurons, the amino acid sequence of the resulting neuropeptide $NPY_{3\text{-}36}$ is identical to the corresponding mouse sequence.

The rAAV vector backbone was flanked by rAAV serotype 2 ITRs and encoded either Cre-GFP or GFP alone under the control of the human cytomegalovirus early enhancer/promoter. rAAV vectors expressing prepro-$NPY_{3\text{-}36}$ were under the control of a truncated version of the cytomegalovirus (CMV)-chicken β-actin (CBA) hybrid promoter. This hybrid promoter contained the enhancer element (−585 to −287) of the CMV promoter fused upstream to the CBA promoter with its first exon and intron. For the truncated version, 754 bp of the intron were deleted keeping the 111 bp at the 5′ and 51 bp at the 3′ end of the intron. The helper plasmid pDP2rs (Plasmid factory, Germany) carried the rAAV-2 rep gene and the rAAV-2 cap gene together with essential adenoviral helper functions. Harvest and purification of rAAVs was performed by DOC-lysis, iodixanol density gradient centrifugation and ion-exchange chromatography, as previously described (Zolotukhin et al., 2002). Purified vectors were stored in aliquots at −80 °C. Physical rAAV titers were determined by quantitative real-time PCR analysis of the recombinant genome. Final titers were 6×10^{10} genomic particles (GP)/ml and 3×10^{10} GP/ml for rAAV 2/2-GFP/Cre and rAAV 2/2-GFP, respectively.

2.3. Stereotaxic microinjections of adeno-associated viral vectors

Adult (8–12 weeks of age), male $Y2^{lox/lox}$ mice from the same litter were injected with either rAAV-Cre (6×10^7 GP/μl), or rAAV-GFP (3×10^7 GP/μl) using a stereotaxic frame (Model 962, David Kopf Instruments, California, USA) with blunt ear bars. Male C57BL/6NCrl littermates (8–12 weeks) were used for rAAV-$NPY_{3\text{-}36}$ injections and rAAV-GFP in controls. Coordinates according to Paxinos and Franklin (2001) were (in mm, from bregma): CEA: A, −1.0; L, ±2.8; V, −4.9. Anesthesia was initiated by injection of ketamine (160 mg/kg, i.p.; Gräub AG, Bern, Switzerland) and maintained throughout the entire procedure by Sevoflurane (Sevorane, Abbott, Austria) inhalation (2–3%, mixed with oxygen/air). Injection cannulas prepared from a stainless steel tube (guide cannula gauge 23, injection cannula gauge 30, Coopers needle works LTD., Birmingham, UK) were inserted bilaterally through small holes drilled into the skull. The cannulas were connected by polyethylene micro-tubes (0.28 × 0.61 mm, NeoLab, Heidelberg, Germany) to 5 μl Hamilton syringes. 1 μl of rAAV vector was infused on each side using micro-pumps (50 nl/min; Nexus3000, Chemyx, Science-products, Germany). At the end of the infusion, cannulas were kept in place for 5 min, and were then slowly withdrawn within 2 min. After closing the wound, mice were single housed for optimal recovery. Behavioral testing started 2 and 3 weeks after injection enabling local over-expression of the Y2 receptor agonist $NPY_{3\text{-}36}$ (rAAV-$NPY_{3\text{-}36}$) or local deletion of Y2 receptors (rAAV-Cre), respectively.

2.4. Characterization of rAAV-$NPY_{3\text{-}36}$

NPYKO mice were injected with 1 μl of rAAV-$NPY_{3\text{-}36}$ vector into the central amygdala as described above. The amygdala was dissected after 3 weeks and peptides were extracted with 2 M acetic acid. Homogenates were separated by HPLC and

radioimmunoassay and compared to concomitantly run synthetic peptides as described in detail previously (Bellmann et al., 1991).

2.5. Chemicals

The Y2 receptor antagonist JNJ-31020028 was synthesized at Janssen Research & Development LLC (San Diego, USA). JNJ-31020028 injection solution was always prepared on the day of the experiment by dissolving it in 20% of 2-hydroxypropyl-beta-cyclodextrin in 0.9% NaCl (pH 7.4) and injected i.p. at a concentration of 20 mg/kg bodyweight.

2.6. Behavioral experiments

2.6.1. Fear conditioning paradigm

Fear conditioning 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. Illumination was 80 lux and chambers were cleaned with 70% ethanol after each session. Fear recall as well as fear extinction and extinction recall were performed in a different context consisting of a dimly illuminated (10 lux) chamber with black, smooth walls and the floor cleaned with 1% acetic acid (*context B*).

2.6.1.1. Fear acquisition. On day 15 (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 received 5 CS− and 5 CS+ in an alternating order, starting with a CS+. The unconditioned stimulus co-terminating with each CS+ consisted of a scrambled mild electric foot shock (0.5 mA, 2 s). A short-term memory test was performed 2 h after acquisition to divide both groups (rAAV-NPY$_{3\text{-}36}$ and rAAV-GFP) into two equally performing subgroups for cued fear testing and extinction. Thus, 50% of the rAAV-NPY$_{3\text{-}36}$ and 50% of the rAAV-GFP injected mice were injected i.p. with a brain-penetrating Y2 receptor antagonist or vehicle before fear testing, respectively (for details see below and Results section).

2.6.1.2. Contextual fear testing and extinction. On day 16, all mice were exposed to the conditioning context (context A) without auditory stimulus presentation for 15 min to determine context dependent freezing (first 3 min) as well as context fear extinction (time-course of freezing during 15 min analyzed in 1 min bins).

2.6.1.3. Cued fear testing and extinction. On day 17, CS-induced fear recall 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 15 presentations of CS+ (30 s, inter-stimulus interval 5 s). Two additional extinction sessions (morning and evening, context B) were performed on day 18, each consisting of 15 CS+. Extinction recall was tested on day 19 by presenting 5 CS+ in context B. Intraperitoneal (i.p.) injection of the brain penetrant Y2 receptor antagonist JNJ-31020028 or vehicle was performed 30 min before cued-fear recall followed by extinction training. Freezing to the 1st CS was taken as a measure for fear recall/expression and the time-course of freezing to all 15 consecutive CS presentations as a measure for fear extinction.

2.6.1.4. Spontaneous recovery and reinstatement. To investigate the stability and permanence of the extinction memory mice were subjected to spontaneous recovery and reinstatement tests. *Spontaneous recovery* was tested 7 days after extinction recall (day 26) by re-exposing the animals to 5 CS+ in the extinction context (context B). *Reinstatement* was performed 7 days later (day 33) by exposing the mice to two un-signaled foot shocks (2 s, 0.5 mA, inter-stimulus interval 60 s) in the conditioning context (context A) and *reinstatement* testing was performed 24 h later (day 34) by presenting 5 CS+ in context B (extinction context).

Behavior was recorded by a video camera and scored offline by a pixel based analysis software (http://topowatch.sourceforge.net/, TopoWatch v0.3). The parameters of the program (detection threshold −246, freezing threshold −4) were validated previously by comparison to the manual analysis of two experienced observers, as described in detail previously (Verma et al., 2012).

2.7. Histochemistry

2.7.1. Tissue preparation

After the final behavioral experiment mice were killed by carbon dioxide gas inhalation or by injecting an overdose of thiopental (Thiopental, Sandoz, Austria) for *in situ* hybridization and immunohistochemistry, respectively. Brains were either snap frozen (isopentane, −70 °C, 3 min) for *in situ* hybridization experiments or perfused with 4% paraformaldehyde (PFA) for immunohistochemistry (Tasan et al., 2010).

2.7.2. In situ hybridization

In situ hybridization was performed as described previously in detail (Hortnagl et al., 2013; Tasan et al., 2010). Oligonucleotides used as probes were custom synthesized (Y2 mRNA, 5′ GAC AGT CAT TGC TCT GGA CCG CCA TCG TTG CAT TGT CTA CCA CCT GGA GAG C 3′ and 5′ CGG AGG CTA CCA ATG TGT AAG GAC ACA GGT GTG AAA GCA CAT GG 3′; Cre-recombinase mRNA, 5′ CCG TCT CTG GTG TAG CTG ATG ATC CGA ATA ACT ACC TGT TTT GCC 3′; Microsynth, Balgach, Switzerland, purified by HPLC). Oligonucleotides (2.5 pmol) were 3′ end-labeled by incubation with [^{35}S]α-dATP (50 μCi; 1300 Ci/mmol, Hartmann Analytic GmbH, Braunschweig, Germany) and terminal transferase (Roche Diagnostics, Basel, Switzerland), as described previously in detail (Tasan et al., 2010, 2011). Hybridization was performed in 50% formamide, 4× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.2), 500 μg/ml salmon sperm DNA, 250 μg/ml yeast tRNA, 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 10% dextran sulfate, and 20 mM dithiothreitol (all from Sigma) at 42 °C for 18 h. The slides were washed at high stringency conditions (50% formamide in 2× SSC, 42 °C) and briefly rinsed in water followed by 70% ethanol, and dried. Slides were exposed to BioMax MR films (Amersham Pharmacia Biotech, Buckinghamshire, UK) together with [^{14}C]-microscales for 7–14 days. For evaluation of the hybridization signal at the cellular level, some slides were dipped in Kodak NTB-2 photosensitive emulsion (Kodak, Rochester, NY; diluted 1:1 with distilled water) at 42 °C, air dried overnight, and then exposed for 4–6 weeks at 4 °C. The BioMax MR films and the dipped slides were developed with Kodak D19 developer. Sections were counterstained with cresyl violet, dehydrated, cleared in butyl acetate, and covered with a coverslip using Eukitt (Merck, Darmstadt, Germany).

2.7.3. Immunohistochemistry

Immunohistochemical analysis was performed on free-floating, PFA-fixed, 40 μm thick coronal sections using indirect peroxidase labeling, as described previously (Tasan et al., 2011). In brief, coronal sections were incubated free floating in 10% normal horse or goat serum (Biomedica, Vienna, Austria) in Tris–HCl buffered saline (TBS; 50 mM, pH 7.2) for 90 min, followed by incubation with primary antiserum (rabbit anti-NPY 1:2000, Bellmann et al., 1991). The resulting complex was visualized by incubation with horseradish peroxidase (HRP)-coupled secondary antibody (1:250

P0448; Dako, Vienna, Austria) at room temperature for 150 min. For immunofluorescence sections were incubated in a tyramide signal amplification solution (1:100, TSA fluorescein, in-house) for 3–8 min. Sections were mounted on slides and covered using Vectashield mounting medium (Vector laboratories, Inc., Burlingame, USA).

2.8. Statistical analysis

Data are presented as means ± SEM. They were analyzed for normal distribution and equal variances using GraphPad Prism software (Prism 5 for Macintosh, GraphPad Software Inc., San Diego, CA). Two-way ANOVA for repeated measurements with Bonferroni *post hoc* test was used to analyze overall changes in percentage freezing for time and treatment in acquisition and extinction experiments. Statistical analysis of behavior comparing two groups was done by Student's test or Mann Whitney U test as a non-parametric test. Analysis of multiple groups was done by one-way ANOVA followed by Bonferroni *post hoc* test.

3. Results

3.1. Over-expression of NPY_{3-36} in the CEA

To investigate the role of exogenous Y2 receptor stimulation in the central extended amygdala in fear conditioning we aimed for a long-term activation of Y2 receptors within the central extended amygdala, including the stria terminalis. Thus we performed local injection of an rAAV-NPY_{3-36} vector resulting in over-expression of the Y2 receptor specific ligand NPY_{3-36} in central amygdala neurons of C57BL/6N mice (Fig. 1). rAAV-NPY_{3-36} encodes the respective analogue of prepro-NPY in the target neurons. Prepro-NPY_{3-36} is processed to NPY_{3-36} and released by exocytosis (Noe et al., 2008). The identity of NPY_{3-36} was confirmed by subjecting amygdala samples form rAAV-NPY_{3-36} and rAAV-GFP injected NPYKO mice to HPLC and subsequent radio-immuno assay using synthetic NPY and NPY_{3-36} as standards (Fig. 1A and B). Thus we achieved a permanent, locally restricted over-expression of a Y2 receptor agonist in the CEA (Fig. 1E) compared to endogenous NPY expression in an rAAV-GFP injected control mouse (Fig. 1D).

3.2. Fear conditioning after NPY_{3-36} over-expression in the CEA

We next explored the role of NPY and Y2 receptors in associative fear learning (Fig. 2A). Compared to rAAV-GFP injected controls local over-expression of NPY_{3-36} in CEA neurons reduced acquisition (Fig. 2B, two-way ANOVA for repeated measurements: time: $F_{(4/84)} = 40.52$, $P < 0.0001$, treatment: $F_{(1/21)} = 4.55$, $P < 0.05$ but no interaction: $F_{(4/84)} = 0.9$, $P > 0.05$) and expression of conditioned fear (Fig. 2C, E). While context freezing (pool of the first 3 min for fear expression, day 16) was similar in rAAV-NPY_{3-36} and rAAV-GFP injected mice (Fig. 2D), freezing to the CS, when tested on day 17, was reduced in mice over-expressing NPY_{3-36} in CEA neurons ($t_{(9)} = 2.25$, $P = 0.05$, Fig. 2E).

To investigate the specificity and selectivity of NPY_{3-36} over-expression on fear recall we injected rAAV-NPY_{3-36} or rAAV-GFP into the CEA and subsequently performed fear conditioning experiments (Fig. 2A). Thirty minutes before cued fear recall half of the animals were injected i.p. with a Y2 antagonist (JNJ-31020028, 20 mg/kg) and the other half with vehicle. Fear expression, measured by % freezing to the first CS, was analyzed in mice over-expressing NPY_{3-36} in the CEA compared to rAAV-GFP injected

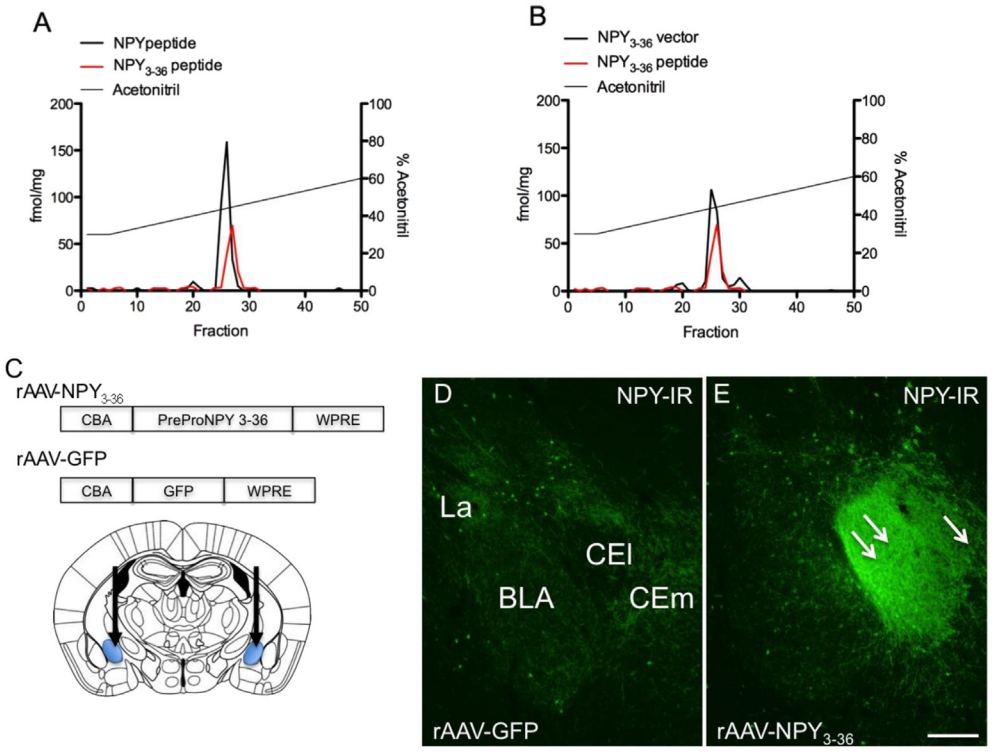

Fig. 1. Characterization and over-expression of viral vector derived NPY_{3-36} in the CEA. (A) Characterization of NPY and NPY_{3-36} in HPLC and RIA, (B) the peptide NPY_{3-36} and viral-vector derived NPY_{3-36} from an rAAV-Prepro-NPY_{3-36} injected NPYKO mouse (n = 3) are found in the same fraction after HPLC and RIA, suggesting correct processing to the active form, (C) schematic of rAAV vectors and illustration of the injection site in the CEA and (E) immunohistochemistry demonstrating site restricted over-expression of NPY after rAAV-NPY_{3-36} injection into the CEA of a male C57Bl/6N mouse compared to (D) NPY IR of a rAAV-GFP injected control (Scale bar 200 μm).

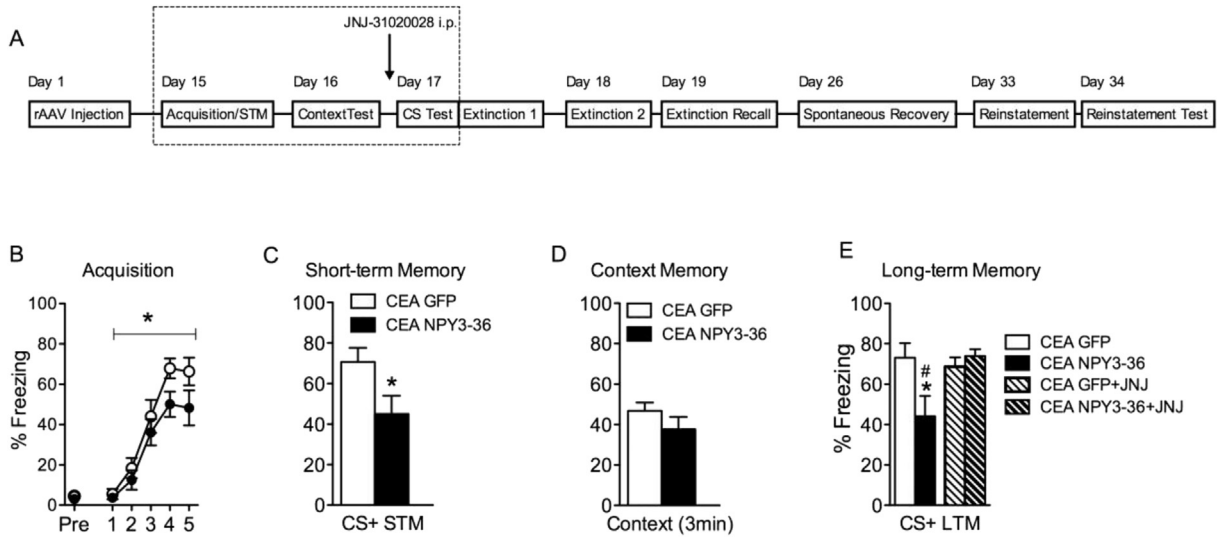

Fig. 2. Local over-expression of the Y2 receptor agonist $NPY_{3\text{-}36}$ in the CEA results in reduced acquisition and expression of fear. (A) Timeline of the experiment: C57BL/6N mice were injected bilaterally with an rAAV-$NPY_{3\text{-}36}$ vector or an rAAV-GFP vector in controls into the CEA (day 1) followed by fear conditioning and extinction experiments two weeks later. Thirty min before fear testing half of the mice received an i.p. injection of the specific Y2 receptor antagonist JNJ31020028 while the other half was injected with vehicle (day 17), followed by additional extinction sessions under drug-free conditions (day 18), extinction recall (day 19) and spontaneous recovery and reinstatement procedures (day 26, 33, and 34). The dashed square indicates the experiments depicted in Fig. 2. (B) Reduced acquisition and (C) reduced expression of CS induced freezing (2 h-short-term memory) but (D) no change during the first 3 min of context freezing after $NPY_{3\text{-}36}$ over-expression in the CEA. (E) Reduced long-term memory (day 17, freezing to the first CS) in rAAV-$NPY_{3\text{-}36}$ injected mice compared to rAAV-GFP injected controls and peripheral injection of a Y2 receptor antagonist blocks the reduced fear expression of rAAV-$NPY_{3\text{-}36}$ injected mice. Data are presented as means ± SEM, $^{*}P < 0.05$ rAAV-GFP-vehicle compared to rAAV-$NPY_{3\text{-}36}$-vehicle, $\#P < 0.05$ rAAV-$NPY_{3\text{-}36}$-vehicle compared to rAAV-$NPY_{3\text{-}36}$-JNJ.

controls but also between mice over-expressing $NPY_{3\text{-}36}$ in the CEA and pre-treated with a Y2 antagonist 30 min before testing (day 17, Fig. 2E, two-way ANOVA: AAV injections: $F_{(1/19)} = 6.25$, $P < 0.05$, i.p. injections: $F_{(1/19)} = 3.45$, $P > 0.05$ and interaction: $F_{(1/19)} = 3.03$, $P > 0.05$). *Bonferroni post hoc test* revealed that mice injected into the CEA with rAAV-$NPY_{3\text{-}36}$ displayed significantly reduced freezing to the 1st CS when compared to rAAV-GFP injected control mice, both treated with vehicle 30 min before testing ($t_{(3/19)} = 2.93$, $P < 0.05$) while injections of the Y2 antagonist JNJ-31020028 before testing reversed the effects of $NPY_{3\text{-}36}$ over-expression in the CEA ($t_{(3/19)} = 2.61$, $P < 0.05$). This suggests that during fear recall the acute effects of Y2 stimulation by $NPY_{3\text{-}36}$ over-expression are blocked by systemic application of the Y2 receptor antagonist.

3.3. *Fear extinction after $NPY_{3\text{-}36}$ over-expression in the CEA*

Mice with over-expression of $NPY_{3\text{-}36}$ in the CEA and rAAV-GFP injected controls were also subjected to extinction of context fear and CS-induced fear on day 16 and 17, respectively (Fig. 3A). Both groups displayed successful extinction of context fear, there was, however, no effect of treatment (Fig. 3B, two-way ANOVA for repeated measurements: time: $F_{(14/308)} = 11.96$, $P < 0.0001$, treatment: $F_{(1/22)} = 0.15$, $P > 0.05$ and interaction: $F_{(14/308)} = 1.23$, $P > 0.05$). According to the results of the STM test (Fig. 2C), both rAAV-GFP and rAAV-$NPY_{3\text{-}36}$ injected mice were divided into 2 equally performing groups for consecutive peripheral injection of the brain-penetrant Y2 antagonist JNJ-31020028 or vehicle, respectively. As shown in Fig. 3A, mice were injected i.p. with JNJ-31020028 or vehicle 30 min before cued fear extinction (Fig. 3A). Interestingly, while fear expression tested by comparing freezing to the 1st CS+ was similar (Fig. 2E) cued fear extinction, measured by comparing the freezing levels upon 15 consecutive CS, appeared delayed in rAAV-GFP injected controls that were injected i.p. with the Y2 antagonist JNJ-31020028, 30 min before the behavioral experiment (Fig. 3C, two-way ANOVA for repeated measurements: time: $F_{(14/140)} = 4.35$, $P < 0.0001$, treatment: $F_{(1/10)} = 8.31$, $P < 0.05$ and interaction: $F_{(14/140)} = 1.36$, $P > 0.05$). Note also the increased freezing to the CS− in mice pre-treated with JNJ-31020028, suggesting a generalization of fear (Fig. 3C, $t_{(1/10)} = 2.28$, $P < 0.05$). Furthermore, a difference in freezing levels during cued fear extinction was observed also in those mice that were injected with rAAV-$NPY_{3\text{-}36}$ into the CEA and treated with the Y2 antagonist before extinction training (Fig. 3D, two-way ANOVA for repeated measurements: time: $F_{(14/140)} = 0.52$, $P > 0.05$, treatment: $F_{(1/10)} = 11.29$, $P < 0.01$ and interaction: $F_{(14/140)} = 0.93$, $P > 0.05$). On the other hand, fear extinction appeared to be more prominent in mice over-expressing $NPY_{3\text{-}36}$ in the CEA (and i.p. vehicle) compared to those with $NPY_{3\text{-}36}$ over-expression in the CEA and pretreatment with a peripheral injection of the Y2 antagonist. This effect was probably depending on reduced fear expression. Thus, we analyzed fear expression on extinction 2 (day 18, Fig. 3E) by comparing the freezing level to the 1st CS+ (Fig. 3E, two-way ANOVA: AAV injections: $F_{(1/19)} = 2.16$, $P > 0.05$, i.p. injections: $F_{(1/19)} = 16.56$, $P < 0.001$ and interaction: $F_{(1/19)} = 0.21$, $P > 0.05$). *Bonferroni post-hoc* test demonstrated that fear expression was significantly increased in rAAV-GFP ($t_{(3/19)} = 2.61$, $P < 0.05$) as well as in rAAV-$NPY_{3\text{-}36}$ ($t_{(3/19)} = 3.13$, $P < 0.05$) injected mice that had been pre-treated with the Y2 antagonist before extinction 1 (day 17), indicating that in addition to altered fear expression also extinction may have been affected. To demonstrate the specificity of the Y2 antagonist, all groups were subjected to two additional extinction trainings in a drug free state 24 and 48 h later (Fig. 4A). Importantly, both groups that had been pre-treated with the Y2 antagonist during extinction 1 and consequently displayed higher freezing levels, now successfully performed fear extinction, demonstrating that the effect of Y2 receptor blockade was reversible (two-way ANOVA for repeated measurements: time: $F_{(2/40)} = 15.03$, $P < 0.0001$, treatment: $F_{(3/20)} = 3.98$, $P < 0.05$ and interaction: $F_{(6/40)} = 0.73$, $P > 0.05$). Thus, during fear recall, all four groups displayed equally low freezing levels, indicating successful fear extinction (Fig. 4C).

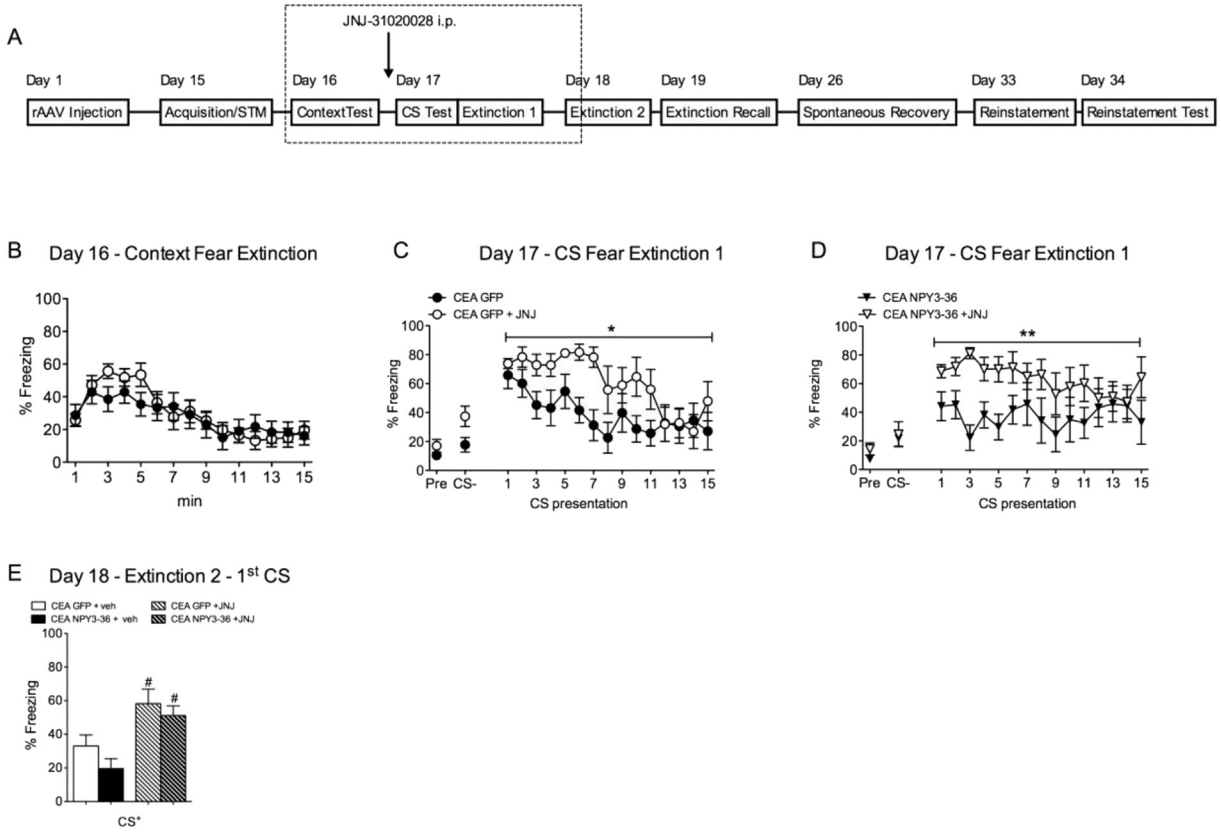

Fig. 3. Local over-expression of a Y2 receptor agonist in the CEA reduced fear expression during extinction of CS-induced fear. (A) Timeline of the experiment: dashed square indicates experiments depicted in Fig. 3. Mice with local-overexpression of NPY_{3-36} in the CEA were subjected to context extinction 24 h after fear acquisition (day16). Day 17: Mice with local over-expression of NPY_{3-36} (or GFP in controls) in the CEA were injected i.p. with the Y2 receptor antagonist JNJ31020028 30 min before cued fear extinction. (B) There was no difference in context extinction of rAAV-NPY_{3-36} injected mice compared to rAAV-GFP injected controls (same group as in Fig. 2D, however, analyzed was the whole time-course for fear extinction). (C) Reduced fear expression during cued fear extinction after i.p. injection of a Y2 antagonist in rAAV-GFP injected controls and (D) peripheral injection of a Y2 antagonist blocked the reduced fear expression of cued fear during extinction in those mice that were injected with an rAAV-NPY_{3-36} vector into the CEA (same groups as in Fig. 2E analyzing, however, the time-course of freezing behavior during all consecutive CS presentations of the extinction session). (E) Increased fear expression during the 1st CS presentation on extinction 2 (day18) under drug-free conditions of previously JNJ-31020028 treated mice. Data are presented as means ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $\#P < 0.05$ rAAV-NPY_{3-36}-vehicle compared to rAAV-NPY_{3-36}-JNJ and rAAV-GFP-vehicle compared to rAAV-GFP-JNJ.

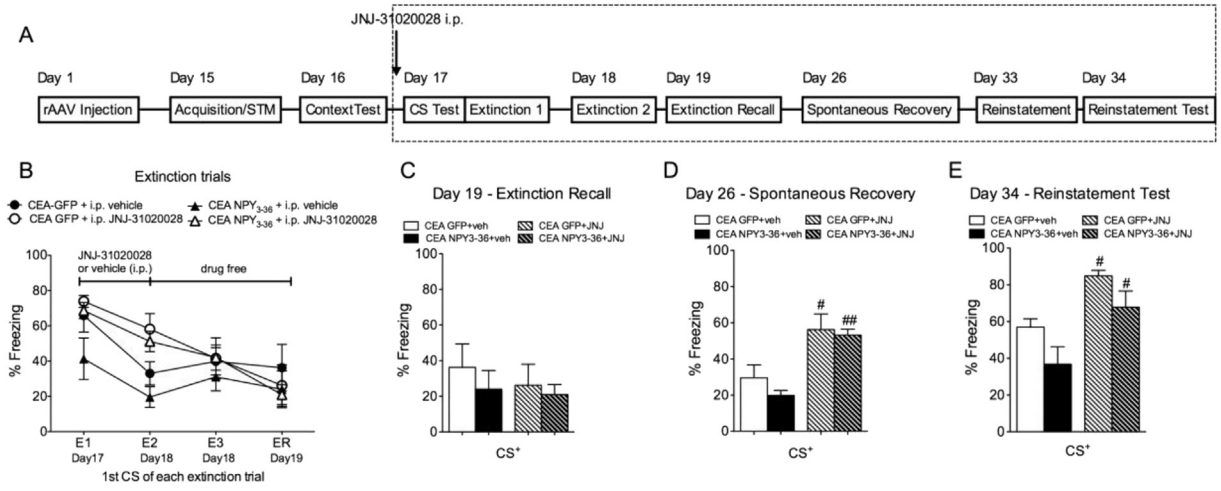

Fig. 4. Local over-expression of the Y2 receptor agonist NPY_{3-36} in the CEA promotes long-term suppression of fear. (A) Timeline of the experiment: Following 2 additional sessions of fear extinction under drug free conditions on day 18, mice were subjected to fear recall in context B on day 19, spontaneous recovery in context B on day 26, re-instatement in context A on day 33 and re-instatement testing in context B on day 34 (dashed square indicates experiments depicted in Fig. 4). (B) All 4 groups of mice displayed equal freezing levels during fear recall suggesting successful extinction learning. (C) Freezing levels during fear recall (same as last data points in (B) depicted again for better comparison to spontaneous recovery and reinstatement data). Reduced freezing in mice over-expressing NPY_{3-36} in the CEA during spontaneous recovery while mice that were treated with a Y2 receptor antagonist during the 1st session of extinction (Fig. 3C and D) displayed increased freezing, (E) a tendency towards reduced freezing during reinstatement of NPY_{3-36} over-expressing mice and significantly increased freezing levels of mice that were treated with i.p. injections of a Y2 receptor antagonist during extinction training. Data are presented as means ± SEM, $\#P < 0.05$, $\#\#P < 0.01$ rAAV-NPY_{3-36}-vehicle compared to rAAV-NPY_{3-36}-JNJ and rAAV-GFP-vehicle compared to rAAV-GFP-JNJ.

3.4. Permanence of fear extinction and recovery of fear memory

To investigate the stability of fear suppression after extinction training, we explored two paradigms generally used to model relapse of fear after successful extinction training: spontaneous recovery and reinstatement (Fig. 4A). Interestingly, although both groups, rAAV-GFP and rAAV-NPY_{3-36}, treated with vehicle during extinction day 1 did not display spontaneous recovery, those treated with the Y2 antagonist displayed increased freezing (day 26, Fig. 4D, two-way ANOVA: AAV injections: $F_{(1/19)} = 2.30$, $P > 0.05$, i.p. injections: $F_{(1/19)} = 21.15$, $P < 0.001$ and interaction: $F_{(1/19)} = 1.06$, $P > 0.05$). *Bonferroni post-hoc* test revealed that injection of the Y2 receptor antagonist JNJ-31020028 facilitated the development of spontaneous recovery in rAAV-GFP injected ($t_{(3/19)} = 2.58$, $P < 0.05$) and rAAV-NPY_{3-36} injected mice ($t_{(3/19)} = 3.90$, $P < 0.01$). Reinstatement tested 8 days after spontaneous recovery, was evident in CEA-GFP mice, while there was a trend towards a reduction in CEA-NPY_{3-36} mice (Fig. 4E, $t_{(10)} = 2.71$, $P = 0.061$). Interestingly, again both groups injected i.p. with the Y2 antagonist before extinction session 1 showed increased freezing levels during reinstatement testing (day 34, Fig. 4E, two-way ANOVA: AAV injections: $F_{(1/19)} = 7.01$, $P < 0.05$, i.p. injections: $F_{(1/19)} = 17.60$, $P < 0.001$ and interaction: $F_{(1/19)} = 0.05$, $P > 0.05$). *Bonferroni post hoc test* revealed that pre-extinction i.p. injection of the brain-penetrating Y2 antagonist JNJ-31020028 increased freezing during re-instatement in rAAV-NPY_{3-36} ($t_{(3/19)} = 3.12$, $P < 0.05$) and rAAV-GFP injected mice ($t_{(3/19)} = 2.81$, $P < 0.05$). These data indicate, that Y2 receptor activation during fear extinction promotes the long-term suppression of fear, while a Y2 receptor antagonist generates the opposite effect.

3.5. Local deletion of Y2 receptors in the central amygdala

To investigate the role of endogenous NPY on Y2 receptors in the CEA in fear conditioning and extinction we performed local, site-restricted deletion of Y2 receptors. An rAAV-Cre vector was injected bilaterally into the CEA of conditional $Y2^{lox/lox}$ mice resulting in local deletion of Y2 receptors from CEA neurons and fear conditioning and extinction experiments were performed 3 weeks later (Fig. 5A). Compared to rAAV-GFP injected controls there was a marked reduction in Y2 mRNA (Fig. 5D and E) in the CEA.

While local deletion of Y2 receptors in the CEA did not alter the acquisition of conditioned fear (Fig. 5F), freezing to the CS during cued fear testing (day 23) was significantly increased compared to rAAV-GFP injected control mice (Fig. 5G, $t_{(2)} = 2.75$, $P < 0.05$). Interestingly context dependent freezing, tested 24 h after fear acquisition (day 22) was not changed (Fig. 5H). Extinction of conditioned fear appeared delayed in mice with local deletion of Y2 receptors in CEA neurons, an effect that was presumably due to the increased fear expression (Fig. 5I). This was mainly due to delayed within session extinction or reduced fear expression, whereas between-session extinction was similar as in rAAV-GFP injected controls (two-way ANOVA for repeated measurements: time: $F_{(3/39)} = 8.81$, $P < 0.0001$, treatment: $F_{(1/13)} = 4.71$, $P < 0.05$ and interaction: $F_{(3/39)} = 0.74$, $P > 0.05$). These data indicate that endogenous NPY acting on Y2 receptors in the CEA predominantly reduces cued fear recall.

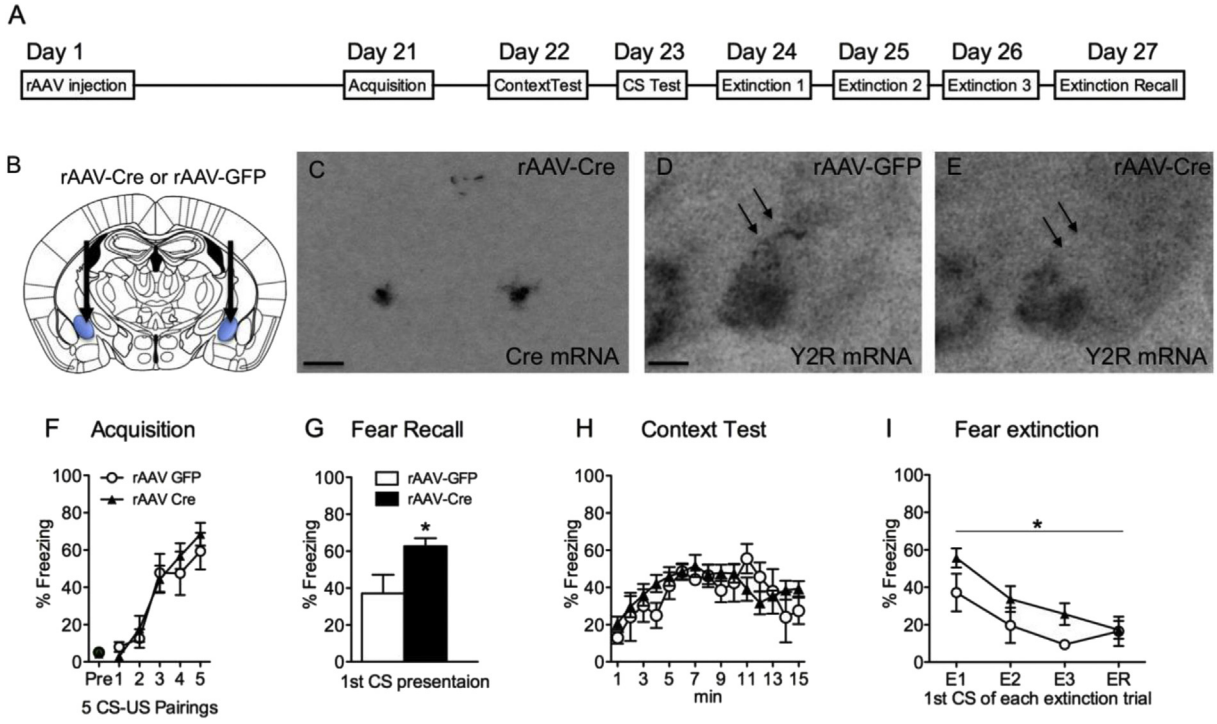

Fig. 5. Local deletion of Y2 receptors in the CEA reduces the expression and impairs the extinction of conditioned fear. (A) Following the bilateral injection of an rAAV-Cre vector into the CEA, $Y2^{lox/lox}$ mice were subjected to a fear conditioning paradigm consisting of acquisition, context and cued fear testing and 3 sessions of extinction followed by extinction recall. (B) Bilateral targeting of the CEA with an rAAV-Cre vector results in (C) strong expression of Cre recombinase mRNA in the CEA on an autoradiograph of a coronal brain section and (E) absence of Y2 receptor mRNA locally restricted to the CEA compared to (D) an rAAV-GFP injected control mouse on a representative autoradiograph of a coronal brain section depicting the right amygdala. Behavioral testing revealed that local deletion of Y2 receptors did not change (F) acquisition of conditioned fear but (G) increased expression of cued fear. (H) Unchanged expression of context fear and context fear extinction, but (I) reduced fear expression during extinction of cued fear after local deletion of Y2 receptors in the CEA compared to rAAV-GFP injected control mice (Scale bars in C 1 mm, D-E 500 μm). Data are presented as means ± SEM, $^{*}P < 0.05$.

4. Discussion

Our results identify NPY and presynaptic Y2 receptors in the CEA as crucial components for the modulation of conditioned fear. We demonstrate that chronic stimulation of Y2 receptors in the CEA delays the acquisition and reduces the expression of fear. In contrast, local deletion of Y2 receptors in CEA neurons increases the expression of cued fear during recall.

As shown previously, Y2 receptors on CEA neurons are also located on axon terminals in the BNST, hypothalamus and brainstem arising from CEA projection neurons (Tasan et al., 2010). This suggests that in our experiments the rAAV-mediated over-expression of $NPY_{3\text{-}36}$ reduced GABA release not only locally in the CEA, but also from CEm projections and thus increased the activity of e.g. BNST neurons. In particular the lateral part of the anterior BNST that harbors predominantly GABAergic neurons (Poulin et al., 2009) and receives dense projections from the CEA (Dong et al., 2001) is involved in the mediation of anxiolytic-like behavior (Gungor and Pare, 2014; Jennings et al., 2013; Sink et al., 2011). On the other hand, GABAergic terminals in the CEA may originate from local interneurons, but also from projection neurons of BNST or hypothalamus. Interestingly, we and others did not observe immunohistochemical co-labeling of Y2 receptors and NPY in the CEA (Stanic et al., 2006; Wood et al., 2015), suggesting that the concept of Y2 receptors as auto-receptors (Broberger et al., 1997) regulating and limiting NPY release does not hold true for the CEA (Stanic et al., 2011). Thus, we rather propose Y2 receptors as heteroreceptors that are expressed on afferent and efferent projections of the CEm, while NPY is provided by multiple sources within this system. Y2 containing projections are surrounded by NPY expressing interneurons, providing a source of significant, long-term suppression of synaptic activity. During fearful stimuli concomitant activation of these neurons with Y2 receptor-containing fibers may result in a time-lagged but prolonged action of NPY limiting an otherwise excessive fear response (Heilig et al., 1994; Heilig, 2004).

Recent evidence demonstrated a Y2 receptor dependent reduction of mIPSC frequency in the BNST (Kash and Winder, 2006; Pleil et al., 2012). Neuropeptides are released at relatively low concentrations and during periods of high frequency firing, but display high receptor affinity and prolonged duration of action. NPY generally acts on different Y receptors (Y1, Y2, Y5) and is converted to the Y2 receptor preferring agonist $NPY_{3\text{-}36}$ by the action of dipeptidylpeptidase IV (Mentlein et al., 1993; Pedrazzini et al., 2003). Thus, $NPY_{3\text{-}36}$ is a metabolite of endogenous NPY and may diffuse within the central extended amygdala and along the stria terminalis by volume transmission acting there on Y2 receptors generating a prolonged inhibitory action by reducing the activity of long-distance fiber tracts.

Compared to other amygdala nuclei, the highest levels of Y2 receptors are found in the CEA (Stanic et al., 2006; Tasan et al., 2010; Wood et al., 2015). NPY that ultimately acts on these receptors may originate from multiple sources, such as local interneurons but potentially also from afferent projections. Thus, we aimed to recapitulate increased and prolonged expression of NPY that specifically activates Y2 receptors by viral-vector-mediated over-expression of the Y2 preferring agonist $NPY_{3\text{-}36}$. This presumably resulted in increased $NPY_{3\text{-}36}$ release not only locally in the CEm but potentially also along the stria terminalis and at the respective terminals, such as in the BNST or brain stem. The use of AAV vectors to overexpress a Y2 receptor specific agonist or to delete Y2 receptors in the CEA has several advantages but also limitations. First, while permanent cannula implantation often results in inflammatory responses (Holguin et al., 2007) a one-time injection of an rAAV vector is generally well tolerated (Tasan et al., 2010). Second, controlling the localization and the resulting diffusion of an injected pharmacological compound in a small brain area, such as the CEA is impossible. On the other hand, the use of viral vectors allows a one-time injection and a precise *post-hoc* visualization of the affected brain area including the identification of projection targets and a confirmation of the expected neurochemical results, such as local deletion of Y2 receptors in the CEA. The viral vectors for $NPY_{3\text{-}36}$ express the pre-pro-peptide allowing its processing to the mature peptide within synaptic vesicles and release from axon terminals even remote from the injection site. However, a limitation of the viral vector approach is its permanent over-expression and a reduced temporal resolution. Thus, it is reasonable to assume that an altered fear acquisition in our experiments had also an influence on the behavioral response during fear recall and fear extinction. To reduce this potential pitfall we combined AAV vector-mediated over-expression of $NPY_{3\text{-}36}$ with peripheral injection of a Y2 antagonist before fear recall and fear extinction. Indeed, acute antagonism of Y2 receptors compensates the reduced fear expression of CEA-$NPY_{3\text{-}36}$ over-expressing mice to the level of rAAV-GFP injected controls. This suggests that Y2 receptor stimulation in the CEA also affects fear expression during fear recall and thus potentially also fear extinction. In particular, the increased freezing of previously (extinction 1) JNJ-31020028 injected mice to the 1st CS on extinction 2 (day18, Fig. 3E) suggests that also fear extinction may have been compromised by peripheral injection of a brain-penetrant Y2 antagonist. Interestingly, mice that were injected with the brain-penetrant Y2 receptor antagonist JNJ-31020028 displayed increased freezing to the CS−, suggesting a generalization of fear, similar as previously reported for Y2KO mice (Verma et al., 2012). On the other hand, since increased freezing was only seen in response to auditory cues, but not to the context, it may also represent a sensitization to auditory stimuli, independent of fear conditioning. However, further studies are needed to clearly distinguish Y2 receptor-mediated effects on fear expression from those on fear extinction. Furthermore, the fact that the systemic application of the Y2 receptor antagonist reduced fear expression also in control mice suggests that Y2 receptors in other brain areas, such as hypothalamus or hippocampus could also contribute to fear extinction. Interestingly, mice over-expressing $NPY_{3\text{-}36}$ in CEm neurons and mice, which received rAAV-GFP injections, both failed to exhibit spontaneous recovery or reinstatement of fear. On the other hand, mice treated with a Y2 receptor antagonist during extinction training displayed increased re-emergence of conditioned fear. Because all mice received two additional extinction trainings under drug-free conditions, the presence of the Y2 receptor antagonist during extinction training 1 may have even consolidated fear memory. Long-term suppression of fear is, however, important considering the labile and transient nature of extinction memories obtained during exposure therapy in humans contesting with strong and permanent fear memories (Ji and Maren, 2007).

To investigate the role of endogenous NPY acting on Y2 receptors in the central extended amygdala we performed local deletion of Y2 receptors in CEA neurons. These data suggest a Y2 receptor-dependent reduction of fear expression while an apparent delay of fear extinction may be due to the recued fear expression. The difference between local over-expression of a Y2 receptor selective ligand and local deletion of Y2 receptors could have several reasons. Release of endogenous NPY is considerably lower than over-expression of a Y2 specific agonist. Furthermore, local deletion of Y2 receptors in CEm neurons will delete Y2 receptors from CEm interneurons and from axon terminals in CEm projection areas, such as the BNST, hypothalamus or brain stem (Tasan et al., 2010). Local over-expression of a Y2 receptor preferring agonist will, however, also target Y2 receptors located in other brain areas (e.g.

interneurons of the BNST, brain stem or projections to the CEm). Thus, further experiments are needed to elucidate the role of Y2 receptors in CEm target areas (e.g. BNST, hypothalamus).

It is important to mention that NPY_{3-36} does not exclusively act on Y2 receptors, but may have a similar efficacy at Y5 receptors (Durkin et al., 2000; Gerald et al., 1996). However, in the present study we demonstrated that the behavioral effects of NPY_{3-36} overexpression in the CEA were fully reversed by peripheral injection of the brain-penetrant Y2 receptor antagonist JNJ-31020028, suggesting that Y2 receptors in the CEA are most relevant for processing of fear.

Previous evidence suggests that Y2 receptor stimulation promotes anxiety (Bacchi et al., 2006; Nakajima et al., 1998; Redrobe et al., 2003; Tasan et al., 2010; Tschenett et al., 2003) but see also (Kask et al., 1998). Here, we demonstrated that Y2 receptor activation reduces fear expression and facilitates fear extinction. Different brain circuits are involved in fear and anxiety (Davis et al., 2010; Walker et al., 2003; Walker and Davis, 1997) and Y2 receptors may play different roles in the respective circuitries. However, a similar effect is known for glucocorticoids and the alpha2-adrenoreceptor antagonist yohimbine. Both provoke anxiety-like symptoms but on the other hand are successfully used to promote fear extinction (Barrett and Gonzalez-Lima, 2004; Blundell et al., 2011; Holmes and Quirk, 2010; Yang et al., 2006). In contrast, benzodiazepines reduce anxiety-like behavior but inhibit fear extinction (Graham et al., 2011; Milad and Quirk, 2012). Furthermore, the behavioral effects of our interventions, involving long-term stimulation of Y2 receptors, may have triggered counteracting downstream adaptations. On the other hand, Fendt et al. (Fendt et al., 2009) demonstrated recently that NPY produces a significant reduction in fear expression also in Y1KO mice, suggesting that other Y receptors are equally involved. Y2 receptors that are considerably expressed in limbic brain areas may represent a reasonable candidate. This is in line with our recent finding in Y receptor KO mice indicating a synergistic action of Y1 and Y2 receptors to reduce fear and promote extinction learning (Verma et al., 2012). Fear reducing and possibly also extinction-promoting effects of Y2 receptors may be mediated largely in the central extended amygdala. It is well known that activation of CEA neurons is necessary for fear conditioning (Nader et al., 2001). Thus, inhibition of GABA-release from CEm projection neurons may be accomplished by presynaptic Y2 receptors, disinhibiting downstream brain areas. At the same time, Y2 receptors on CEm afferents, may promote fear extinction and a permanent suppression of fear. Thus, we propose a concept, in which NPY in the CEA is a fear-reducing neuromodulator that mediates its action in a timed manner through multiple receptors by first, acutely inhibiting postsynaptic neurons predominantly via Y1 receptors and subsequently promoting a long-lasting inhibitory action on CEA projection neurons by presynaptic Y2 receptors.

Conflict of interest

PB is an employee of Janssen Research & Development, LLC. All other authors declare no competing financial interests.

Acknowledgments

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References

Bacchi, F., Mathe, A.A., Jimenez, P., Stasi, L., Arban, R., Gerrard, P., et al., 2006. Anxiolytic-like effect of the selective neuropeptide Y Y2 receptor antagonist BIIE0246 in the elevated plus-maze. Peptides 27, 3202–3207.
Barrett, D., Gonzalez-Lima, F., 2004. Behavioral effects of metyrapone on Pavlovian extinction. Neurosci. Lett. 371, 91–96.
Bellmann, R., Widmann, R., Olenik, C., Meyer, D.K., Maas, D., Marksteiner, J., et al., 1991. Enhanced rate of expression and biosynthesis of neuropeptide Y after kainic acid-induced seizures. J. Neurochem. 56, 525–530.
Blundell, J., Blaiss, C.A., Lagace, D.C., Eisch, A.J., Powell, C.M., 2011. Block of glucocorticoid synthesis during re-activation inhibits extinction of an established fear memory. Neurobiol. Learn. Mem. 95, 453–460.
Bowers, M.E., Choi, D.C., Ressler, K.J., 2012. Neuropeptide regulation of fear and anxiety: implications of cholecystokinin, endogenous opioids, and neuropeptide Y. Physiol. Behav. 107, 699–710.
Broberger, C., Landry, M., Wong, H., Walsh, J.N., Hokfelt, T., 1997. Subtypes Y1 and Y2 of the neuropeptide Y receptor are respectively expressed in pro-opiomelanocortin- and neuropeptide-Y-containing neurons of the rat hypothalamic arcuate nucleus. Neuroendocrinology 66, 393–408.
Broqua, P., Wettstein, J.G., Rocher, M.N., Gauthier-Martin, B., Junien, J.L., 1995. Behavioral effects of neuropeptide Y receptor agonists in the elevated plus-maze and fear-potentiated startle procedures. Behav. Pharmacol. 6, 215–222.
Ciocchi, S., Herry, C., Grenier, F., Wolff, S.B., Letzkus, J.J., Vlachos, I., et al., 2010. Encoding of conditioned fear in central amygdala inhibitory circuits. Nature 468, 277–282.
Davis, M., Walker, D.L., Miles, L., Grillon, C., 2010. Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. Neuropsychopharmacology 35, 105–135.
Dong, H.W., Petrovich, G.D., Swanson, L.W., 2001. Topography of projections from amygdala to bed nuclei of the stria terminalis. Brain Res. Brain Res. Rev. 38, 192–246.
Durkin, M.M., Walker, M.W., Smith, K.E., Gustafson, E.L., Gerald, C., Branchek, T.A., 2000. Expression of a novel neuropeptide Y receptor subtype involved in food intake: an in situ hybridization study of Y5 mRNA distribution in rat brain. Exp. Neurol. 165, 90–100.
Fendt, M., Burki, H., Imobersteg, S., Lingenhohl, K., McAllister, K.H., Orain, D., et al., 2009. Fear-reducing effects of intra-amygdala neuropeptide Y infusion in animal models of conditioned fear: an NPY Y1 receptor independent effect. Psychopharmacology (Berl) 206, 291–301.
Gerald, C., Walker, M.W., Criscione, L., Gustafson, E.L., Batzl-Hartmann, C., Smith, K.E., et al., 1996. A receptor subtype involved in neuropeptide-Y-induced food intake. Nature 382, 168–171.
Graham, B.M., Langton, J.M., Richardson, R., 2011. Pharmacological enhancement of fear reduction: preclinical models. Br. J. Pharmacol. 164, 1230–1247.
Grimm, D., Kay, M.A., Kleinschmidt, J.A., 2003. Helper virus-free, optically controllable, and two-plasmid-based production of adeno-associated virus vectors of serotypes 1 to 6. Mol. Ther. 7, 839–850.
Gungor, N.Z., Pare, D., 2014. CGRP inhibits neurons of the bed nucleus of the stria terminalis: implications for the regulation of fear and anxiety. J. Neurosci. 34, 60–65.
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. J. Neurosci. 28, 12682–12690.
Haubensak, W., Kunwar, P.S., Cai, H., Ciocchi, S., Wall, N.R., Ponnusamy, R., et al., 2010. Genetic dissection of an amygdala microcircuit that gates conditioned fear. Nature 468, 270–276.
Heilig, M., 2004. The NPY system in stress, anxiety and depression. Neuropeptides 38, 213–224.
Heilig, M., Koob, G.F., Ekman, R., Britton, K.T., 1994. Corticotropin-releasing factor and neuropeptide Y: role in emotional integration. Trends Neurosci. 17, 80–85.
Holguin, A., Frank, M.G., Biedenkapp, J.C., Nelson, K., Lippert, D., Watkins, L.R., et al., 2007. Characterization of the temporo-spatial effects of chronic bilateral intrahippocampal cannulae on interleukin-1beta. J. Neurosci. Methods 161, 265–272.
Holmes, A., Quirk, G.J., 2010. Pharmacological facilitation of fear extinction and the search for adjunct treatments for anxiety disorders – the case of yohimbine. Trends Pharmacol. Sci. 31, 2–7.
Hortnagl, H., Tasan, R.O., Wieselthaler, A., Kirchmair, E., Sieghart, W., Sperk, G., 2013. Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain. Neuroscience 236, 345–372.
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.
Ji, J., Maren, S., 2007. Hippocampal involvement in contextual modulation of fear extinction. Hippocampus 17, 749–758.
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.
Kask, A., Rago, L., Harro, J., 1998. Anxiolytic-like effect of neuropeptide Y (NPY) and NPY13-36 microinjected into vicinity of locus coeruleus in rats. Brain Res. 788, 345–348.
Kaspar, B.K., Vissel, B., Bengoechea, T., Crone, S., Randolph-Moore, L., Muller, R., et al., 2002. Adeno-associated virus effectively mediates conditional gene

modification in the brain. Proc. Natl. Acad. Sci. U. S. A. 99, 2320–2325.
Lach, G., de Lima, T.C., 2013. Role of NPY Y1 receptor on acquisition, consolidation and extinction on contextual fear conditioning: dissociation between anxiety, locomotion and non-emotional memory behavior. Neurobiol. Learn. Mem. 103, 26–33.
LeDoux, J.E., 2000. Emotion circuits in the brain. Annu. Rev. Neurosci. 23, 155–184.
LeDoux, J.E., Iwata, J., Cicchetti, P., Reis, D.J., 1988. Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. J. Neurosci. 8, 2517–2529.
Li, H., Penzo, M.A., Taniguchi, H., Kopec, C.D., Huang, Z.J., Li, B., 2013. Experience-dependent modification of a central amygdala fear circuit. Nat. Neurosci. 16, 332–339.
Maren, S., 2005. Synaptic mechanisms of associative memory in the amygdala. Neuron 47, 783–786.
Mentlein, R., Dahms, P., Grandt, D., Kruger, R., 1993. Proteolytic processing of neuropeptide Y and peptide YY by dipeptidyl peptidase IV. Regul. Pept. 49, 133–144.
Michel, M.C., Beck-Sickinger, A., Cox, H., Doods, H.N., Herzog, H., Larhammar, D., et al., 1998. XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. Pharmacol. Rev. 50, 143–150.
Milad, M.R., Quirk, G.J., 2012. Fear extinction as a model for translational neuroscience: ten years of progress. Annu. Rev. Psychol. 63, 129–151.
Nader, K., Majidishad, P., Amorapanth, P., LeDoux, J.E., 2001. Damage to the lateral and central, but not other, amygdaloid nuclei prevents the acquisition of auditory fear conditioning. Learn. Mem. 8, 156–163.
Nakajima, M., Inui, A., Asakawa, A., Momose, K., Ueno, N., Teranishi, A., et al., 1998. Neuropeptide Y produces anxiety via Y2-type receptors. Peptides 19, 359–363.
Noe, F., Pool, A.H., Nissinen, J., Gobbi, M., Bland, R., Rizzi, M., et al., 2008. Neuropeptide Y gene therapy decreases chronic spontaneous seizures in a rat model of temporal lobe epilepsy. Brain 131, 1506–1515.
Pape, H.C., Pare, D., 2010. Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. Physiol. Rev. 90, 419–463.
Paxinos, G., Franklin, K.B.J., 2012. Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, fourth ed., p. 360
Pedrazzini, T., Pralong, F., Grouzmann, E., 2003. Neuropeptide Y: the universal soldier. Cell. Mol. Life Sci. 60, 350–377.
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.
Poulin, J.F., Arbour, D., Laforest, S., Drolet, G., 2009. Neuroanatomical characterization of endogenous opioids in the bed nucleus of the stria terminalis. Prog. Neuropsychopharmacol. Biol. Psychiatry 33, 1356–1365.
Quirk, G.J., Mueller, D., 2008. Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology 33, 56–72.
Redrobe, J.P., Dumont, Y., Herzog, H., Quirion, R., 2003. Neuropeptide Y (NPY) Y2 receptors mediate behaviour in two animal models of anxiety: evidence from Y2 receptor knockout mice. Behav. Brain Res. 141, 251–255.
Sah, P., Faber, E.S., Lopez De Armentia, M., Power, J., 2003. The amygdaloid complex: anatomy and physiology. Physiol. Rev. 83, 803–834.
Sainsbury, A., Schwarzer, C., Couzens, M., Fetissov, S., Furtinger, S., Jenkins, A., et al., 2002. Important role of hypothalamic Y2 receptors in body weight regulation revealed in conditional knockout mice. Proc. Natl. Acad. Sci. U. S. A. 99, 8938–8943.
Sink, K.S., Walker, D.L., Yang, Y., Davis, M., 2011. Calcitonin gene-related peptide in the bed nucleus of the stria terminalis produces an anxiety-like pattern of behavior and increases neural activation in anxiety-related structures. J. Neurosci. 31, 1802–1810.
Stanic, D., Brumovsky, P., Fetissov, S., Shuster, S., Herzog, H., Hokfelt, T., 2006. Characterization of neuropeptide Y2 receptor protein expression in the mouse brain. I. Distribution in cell bodies and nerve terminals. J. Comp. Neurol. 499, 357–390.
Stanic, 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. J. Comp. Neurol. 519, 1219–1257.
Tasan, R.O., Bukovac, A., Peterschmitt, Y.N., Sartori, S.B., Landgraf, R., Singewald, N., et al., 2011. Altered GABA transmission in a mouse model of increased trait anxiety. Neuroscience 183, 71–80.
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. J. Neurosci. 30, 6282–6290.
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. Eur. J. Neurosci. 18, 143–148.
U.S. National Research Council, 2011. Guide for the Care and Use of Laboratory Animals, xxv, p. 220.
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. Br. J. Pharmacol. 166, 1461–1473.
Walker, D.L., Davis, M., 1997. Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. J. Neurosci. 17, 9375–9383.
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. Eur. J. Pharmacol. 463, 199–216.
Wilensky, A.E., Schafe, G.E., Kristensen, M.P., LeDoux, J.E., 2006. Rethinking the fear circuit: the central nucleus of the amygdala is required for the acquisition, consolidation, and expression of Pavlovian fear conditioning. J. Neurosci. 26, 12387–12396.
Wood, J., Verma, Lach, G., Bonaventure, P., Herzog, H., Sperk, G., et al., 2015. NPY Y2 receptor reduces excitatory and inhibitory synaptic transmission in the centromeidal amygdala. Brain Struct. Funct. (in press).
Yang, Y.L., Chao, P.K., Lu, K.T., 2006. Systemic and intra-amygdala administration of glucocorticoid agonist and antagonist modulate extinction of conditioned fear. Neuropsychopharmacology 31, 912–924.
Zolotukhin, S., Potter, M., Zolotukhin, I., Sakai, Y., Loiler, S., Fraites, T.J.J., et al., 2002. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 28, 158–167.