Contribution of NPY Y₅ Receptors to the Reversible Structural Remodeling of Basolateral Amygdala Dendrites in Male Rats Associated with NPY-Mediated Stress Resilience

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Endogenous neuropeptide Y (NPY) and corticotrophin-releasing factor (CRF) modulate the responses of the basolateral amygdala (BLA) to stress and are associated with the development of stress resilience and vulnerability, respectively. We characterized persistent effects of repeated NPY and CRF treatment on the structure and function of BLA principal neurons in a novel organotypic slice culture (OTC) model of male rat BLA, and examined the contributions of specific NPY receptor subtypes to these neural and behavioral effects. In BLA principal neurons within the OTCs, repeated NPY treatment caused persistent attenuation of excitatory input and induced dendritic hypotrophy via Y₅ receptor activation; conversely, CRF increased excitatory input and induced hypertrophy of BLA principal neurons. Repeated treatment of OTCs with NPY followed by an identical treatment with CRF, or vice versa, inhibited or reversed all structural changes in OTCs. These structural responses to NPY or CRF required calcineurin or CaMKII, respectively. Finally, repeated intra-BLA injections of NPY or a Y₅ receptor agonist increased social interaction, a validated behavior for anxiety, and recapitulated structural changes in BLA neurons seen in OTCs, while a Y₅ receptor antagonist prevented NPY's effects both on behavior and on structure. These results implicate the Y₅ receptor in the long-term, anxiolytic-like effects of NPY in the BLA, consistent with an intrinsic role in stress buffering, and highlight a remarkable mechanism by which BLA neurons may adapt to different levels of stress. Moreover, BLA OTCs offer a robust model to study mechanisms associated with resilience and vulnerability to stress in BLA.

Key words: basolateral amygdala; dendritic plasticity; NPY Y₅ receptor; organotypic slice cultures; social interaction; stress resilience

Significance Statement

Within the basolateral amygdala (BLA), neuropeptide Y (NPY) is associated with buffering the neural stress response induced by corticotropin releasing factor, and promoting stress resilience. We used a novel organotypic slice culture model of BLA, complemented with *in vivo* studies, to examine the cellular mechanisms associated with the actions of NPY. In organotypic slice cultures, repeated NPY treatment reduces the complexity of the dendritic extent of anxiogenic BLA principal neurons, making them less excitable. NPY, via activation of Y₅ receptors, additionally inhibits and reverses the increases in dendritic extent and excitability induced by the stress hormone, corticotropin releasing factor. This NPY-mediated neuroplasticity indicates that resilience or vulnerability to stress may thus involve neuropeptide-mediated dendritic remodeling in BLA principal neurons.

Introduction

The stress response represents a coordinated, adaptive response to perceived threats, terminated via neural and endocrine systems that buffer the stress and restore homeostasis. Inadequately

terminating this response can result in predisposition to affective- and anxiety-related disorders (McEwen, 2003). The

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neuronal network, particularly of the basolateral amygdala (BLA), integrates and processes stress-, emotion-, and memory-related input (Phelps and Ledoux, 2005). Chronic or extreme stress can unbalance this homeostatic control, increasing anxiogenic signaling via BLA projection neurons (Roozendaal et al., 2009), partly through dendritic hypertrophy and increased excitatory inputs (Vyas et al., 2002, 2006; Mitra et al., 2005; Hill et al., 2011; Adamec et al., 2012; Padival et al., 2013). These changes can persist (Vyas et al., 2004) and may underlie the development of stress-related psychiatric disease.

The endogenous neuropeptides, corticotropin-releasing factor (CRF) and neuropeptide Y (NPY), modulate BLA activity, respectively, increasing and inhibiting BLA output. CRF increases anxiety-like behavior (Rainnie et al., 2004; Sajdyk et al., 2004), whereas NPY is potently anxiolytic (Sajdyk et al., 2002a, 2004); and in humans, NPY is also linked to resilience (Yehuda et al., 2006; Zhou et al., 2008). In the BLA, CRF and NPY both contribute to the expression of various behavioral responses (Heilig et al., 1994; Sajdyk et al., 2004). Stress causes CRF release, inducing appropriate defensive behaviors (e.g., freezing, fleeing); thereafter, NPY buffers these responses, curtailing CRF actions and shortening the stress response (Heilig et al., 1994). Imbalances in NPY/CRF tone can induce anxiolysis and anxiogenesis, respectively (Heilig et al., 1994; Sajdyk et al., 2004). Repeated restraint stress (Padival et al., 2013) or daily injections of urocortin (UCN, a CRF-R1 and CRF-R2 agonist) induces anxiety that long outlasts the stimulus (Rainnie et al., 2004), whereas similar NPY treatment induces stress resilience persisting up to 2 months (Sajdyk et al., 2008), consistent with the ability of NPY to mitigate BLA-mediated behaviors induced by CRF or stress (Rainnie et al., 2004).

Acute cellular responses to NPY include hyperpolarization-induced inhibition of BLA principal neurons (PNs) via suppression of the tonically active, somatodendritic, hyperpolarization-activated, depolarizing H-current (I_h), a mixed (Na^+ , K^+) cation conductance important for regulating neuronal excitability and stress behaviors; CRF excites these same cells by activating their I_h (Giesbrecht et al., 2010). Moreover, NPY acutely decreases BLA activity by enhancing GABA_A-mediated IPSCs and reducing NMDA-mediated EPSCs in BLA PNs (Molosh et al., 2013). In addition to these acute effects, repeated injections of NPY into the BLA induce a long-term stress resilience assessed by increases in social interaction (SI) (Sajdyk et al., 2008), a validated measure of anxiety (File and Seth, 2003; Silveira Villarroel et al., 2018), which also permits repeated longitudinal measures in individual animals, unlike other paradigms (e.g., elevated plus maze, open field). Using this model of NPY-induced stress resilience, we recently showed that a reduction of I_h expression in BLA PNs contributes to, and mimics, long-term resilience (Silveira Villarroel et al., 2018), defined as the ability to rebound from trauma or severe stress (Yehuda et al., 2006). While multiple NPY receptors are present in the BLA, the acute anxiolytic actions of NPY are attributed to activation of the Y_1 receptor (Sajdyk et al., 2004; Giesbrecht et al., 2010).

Characterization of neural responses to NPY and CRF only in acute *ex vivo* BLA brain slices makes determining

mechanisms of persistent NPY and CRF effects challenging. However, organotypic slice cultures (OTCs) offer reliable models of structural, synaptic, and neurochemical phenotypes (Gähwiler, 1981; De Simoni et al., 2003; Lu et al., 2009) and can be validated against acute preparations (e.g., De Simoni et al., 2003; Lu et al., 2009). OTCs enable chronic manipulations in a well-defined system (Humpel, 2015). Here, we developed OTC preparations of the BLA. This novel model of stress-related circuitry predicted mechanisms of NPY and CRF actions *in vivo*, including bidirectional dendritic remodeling. Moreover, this action was unexpectedly mediated *in vivo* and *in vitro* by the NPY₅ receptor.

Materials and Methods

Animals

All animal procedures were approved by the University of Alberta Animal Care and Use Committee: Health Sciences, in accordance with the guidelines of the Canadian Council on Animal Care. Litters of postnatal day (P) 14 and 5-week-old male Sprague-Dawley rats from the University of Alberta colony were used. P14 pups were given complete access to the dams for maternal care, then were removed from the dam just before either preparation of OTCs or for a subset of acute slice studies. Five-week-old rats were grouped housed (2 or 3 animals per cage) with 12:12 h light:dark schedule and *ad libitum* access to food and water.

Development and validation of OTCs of BLA

Postnatal development of the rat BLA continues until P28 (Ehrlich et al., 2012, 2013). Generation of OTCs from late adolescent or early adult-stage animals would be ideal, but the viability of OTCs declines steeply with postnatal age of origin (Kim et al., 2013; Humpel, 2015). Our and others' previous work used larger (250–275 × g) Wistar or older (P42) Sprague-Dawley rats in studies of NPY and UCN effects on stress resilience/vulnerability, and acute and persistent NPY actions (Rainnie et al., 2004; Giesbrecht et al., 2010; Molosh et al., 2013; Silveira Villarroel et al., 2018).

OTC preparation

Organotypic cultures were prepared using the interface method described by Stoppini et al. (1991) with minor modifications. Briefly, P14 rats were decapitated, and their brains rapidly removed under sterile conditions and submerged in ice-cold slicing solution [Hanks balanced salt solution + D-glucose (0.6% final) + kynurenic acid (30 μM final)]. The brain was imbedded in agarose, and trimmed agarose-brain blocks were secured with cyanoacrylate glue to a custom slicing chamber, immersed in cold ($<4^\circ\text{C}$) slicing solution. Coronal slices (350 μm) were cut serially from rostral to caudal using a vibratome and trimmed to size. Four slices per hemisphere containing the BLA (as determined visually with a dissecting microscope) were allowed to rest in fresh slicing solution at 4°C for 30–45 min, and mounted on individual semiporous membrane inserts and placed in 24-well plates with 300 μl of culture media (50% MEM, 25% heat-inactivated horse serum, 25% Hanks balanced salt solution, supplemented with the following: 1 mM Glutamax, 1% D-glucose, 0.5 mM L-ascorbic acid, and 25 U/ml penicillin/streptomycin). After 48 h in culture, an antimetabolic solution of 1:1:1 cytosine- β -D-arabinofuranoside, uridine, and 5-fluoro-2'-deoxyuridine was added to the media (0.5 μM final concentration) for 24 h to prevent glial proliferation. Slices were maintained at 37°C in 5%/95% CO_2 /air. Media was changed three times per week for the duration of the experiment, except as indicated.

BLA OTCs prepared from rats at P21 and P28 were poorly viable, but those from P14 (2 week) rats remained robustly viable to at least 8 weeks in culture, the equivalent postnatal (EP) age of P70 (Fig. 1a), permitting comparison of the electrophysiological and morphologic properties of PNs from the OTCs (see below). Such OTCs were used for this entire study.

Figure 1. Experimental timelines and changes in PN synaptic properties with repeated NPY or CRF treatment in BLA OTCs. **a**, Comparative timelines for experiments with BLA OTCs (top timeline) and *in vivo* (bottom timeline) models. **b**, **c**, Representative electrophysiological recordings of spontaneous synaptic currents in BLA OTC PN treated repeatedly with vehicle (**b**) and (**c**) 100 nM NPY. **d**, Mean sIPSC amplitudes in neurons in vehicle control ($n = 25$) or 100 nM NPY ($n = 25$) (Mann-Whitney U test; $U = 171.0$, $p = 0.0054$). **e**, Scatter plots of mean sEPSC IEI from recordings of cells in **d**. Vehicle control ($n = 25$) or 100 nM NPY ($n = 25$) (Mann-Whitney U test; $U = 155.5$, $p = 0.0019$). **f**, **g**, Representative electrophysiological recordings of neurons treated repeatedly with vehicle or with 30 nM CRF. **h**, Mean sEPSC IEI in vehicle control ($n = 25$) or 100 nM 30 nM CRF ($n = 25$). (Mann-Whitney U test; $U = 194$, $p = 0.0208$). **d**, **e**, **h**, Circles represent neuron means. Black bars represent population means. Error bars indicate SEM. All statistical tests were two-sided. $^{**}p < 0.01$.

OTC drug incubations

To mimic EP 6 weeks (4 weeks in culture), slices were treated with fresh media containing indicated concentrations of reagents, changed daily for 5 consecutive days; the last change remained until the normal day 7

media change. When cultures were serially treated as above first with NPY and then with CRF (or vice versa), incubations proceeded with one peptide as above, followed with an identical 1 week daily protocol using the opposing peptide starting with the day 7 media change. A control

Table 1. Comparison of electrophysiological and morphologic properties of acute P14, acute 10W, and EP 10W OTC pyramidal neurons in the BLA

	Acute P14	Acute 10W	OTC EP 10W	ANOVA
RMP (mV)	−76.8 ± 0.5***, <i>n</i> = 56	−81.3 ± 0.7, <i>n</i> = 44	−77.9 ± 1.0**, <i>n</i> = 54	$F_{(2,151)} = 9.05, p = 0.0002$
Rheobase (pA)	126.3 ± 5.2****, <i>n</i> = 56	309.2 ± 13.8, <i>n</i> = 44	248.2 ± 24.8*, ****, <i>n</i> = 54	$F_{(2,151)} = 29.84, p = 0.00019$
R_{in} (MΩ)	176.8 ± 6.4****, <i>n</i> = 56	71.5 ± 5.2, <i>n</i> = 22	103.4 ± 6.3*, ****, <i>n</i> = 49	$F_{(2,124)} = 64.16, p = 7.43e-20$
I_h max (pA)	−175.2 ± 7.3***, <i>n</i> = 52	−362.5 ± 25.9, <i>n</i> = 48	−290.4 ± 20.1*, ****, <i>n</i> = 49	$F_{(2,146)} = 25.09, p = 4.31e-10$
I_h density (pA/pF)	−0.96 ± 0.03*, <i>n</i> = 52	−1.36 ± 0.10, <i>n</i> = 22	−1.64 ± 0.13****, <i>n</i> = 49	$F_{(2,120)} = 14.38, p = 2.52e-6$
C_m (pF)	180.2 ± 3.7****, <i>n</i> = 56	230.6 ± 7.3, <i>n</i> = 44	150.0 ± 5.8****, ****, <i>n</i> = 58	$F_{(2,155)} = 49.98, p = 1.75e-18$
sIPSC AMP (pA)	25.5 ± 1.1, <i>n</i> = 25	24.13 ± 1.8, <i>n</i> = 25	49.8 ± 4.9****, ****, <i>n</i> = 25	$F_{(2,72)} = 21.59, p = 4.51e-8$
sIPSC IEI (ms)	2249 ± 417****, <i>n</i> = 25	213.9 ± 22.1, <i>n</i> = 25	155.6 ± 19.3****, ****, <i>n</i> = 25	$F_{(2,72)} = 24.41, p = 8.07e-9$
sEPSC AMP (pA)	25.9 ± 0.9, <i>n</i> = 25	26.87 ± 2.3, <i>n</i> = 25	44.8 ± 2.1****, ****, <i>n</i> = 25	$F_{(2,72)} = 32.15, p = 1.05e-10$
sEPSC IEI (ms)	1241 ± 347.2**, <i>n</i> = 25	171.1 ± 26.4, <i>n</i> = 25	221.5 ± 32.2*, <i>n</i> = 25	$F_{(2,72)} = 8.95, p = 0.0003$
Total dendritic length (μm)	5986 ± 337****, <i>n</i> = 17	8644 ± 284, <i>n</i> = 36	3625 ± 187.4****, ****, <i>n</i> = 39	$F_{(2,89)} = 113.6, p = 3.16e-25$
No. of branches	37.5 ± 2.3*, <i>n</i> = 17	46.3 ± 1.9, <i>n</i> = 36	20.5 ± 1.5****, ****, <i>n</i> = 39	$F_{(2,89)} = 60.17, p = 2.95e-17$

Data are mean ± SEM. One-way ANOVA with Tukey's *post hoc* test for multiple comparisons were used for all analyses: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 versus Acute 10W; #*p* < 0.01, ###*p* < 0.001, *****p* < 0.0001 versus Acute P14 for multiple comparisons.

subset of cultures was treated with the inactive desamido-NPY analog (da-NPY, 100 nM). After incubations, media changes were again performed three times weekly (Fig. 1a).

Acute slice preparation

P14 or P70 rats were decapitated, and their brains were rapidly submerged in cold (4°C) aCSF slicing solution, containing the following (in mM): 118 NaCl, 3 KCl, 1.3 MgSO₄, 1.4 NaH₂PO₄, 5.0 MgCl₂, 10 D-glucose, 26 NaHCO₃, 2.5 CaCl₂, and 1.0 kynurenic acid and bubbled with carbogen (95% O₂, 5% CO₂) (Silveira Villarroel et al., 2018). Coronal sections (300 μm) containing the BLA were prepared using a vibrating slicer (HR2; Sigmund Elektronik). Brain slices were then transferred to a room temperature (22°C), carbogenated aCSF containing the following (in mM): 124 NaCl, 3 KCl, 1.3 MgSO₄, 1.4 NaH₂PO₄, 10 D-glucose, 26 NaHCO₃, and 2.5 CaCl₂. Osmolality was adjusted to 300 mOsm/kg.

Comparison with PNs from acute slice preparations

PNs from BLA OTCs were morphologically complex and appeared to mature with respect to their electrophysiological properties while in culture compared with PNs in acute slices from P14 and P70 rats. While many electrophysiological measures from BLA OTC PNs were significantly different compared with those of PNs in acute slices from P14 rats, some parameters in OTC PNs were more closely aligned with those of PNs from acute P70 slices. However, despite culturing to the equivalent postnatal age of 70 d, some differences to acute P70 neurons remained. PNs in OTCs were considerably smaller than those from either acutely *ex vivo* group (Table 1). Moreover, repeated NPY (100 nM) treatment of OTCs *in vitro*, as illustrated in Figure 1a, increased the mean amplitude of sIPSCs while increasing the mean interevent interval (IEI) of sEPSCs (Fig. 1b,c), with no effects on sIPSC (Control: 165.5 ± 16.4 ms; 100 nM NPY: 155.0 ± 14.8 ms; Mann-Whitney *U* test; *U* = 273.5, *p* = 0.46) IEI or sEPSC amplitude (Control: 41.8 ± 2.5 pA; 100 nM NPY: 41.63 ± 1.6 pA; Mann-Whitney *U* test; *U* = 288.0, *p* = 0.64; data not shown). In all, repeated NPY treatment (Fig. 1b–e) resulted in a similar net increase in synaptic inhibition and reduction of synaptic excitation onto BLA PNs in OTCs, as previously reported from *ex vivo* slices prepared from animals treated with NPY *in vivo* (Silveira Villarroel et al., 2018).

Stereotaxic surgery and intracranial injections

Stereotaxic surgery was conducted as described in detail previously (Silveira Villarroel et al., 2018). Briefly, 5-week-old animals were acclimatized to the animal facility and handled daily for 1 week before experimental manipulation. Rats were anesthetized with ketamine/xylazine (90:10 mg/kg) or with isoflurane using a SomnoSuite low flow vaporizer (Kent Scientific) and placed in a stereotaxic apparatus (Kopf Instruments). Anesthesia was monitored carefully to maintain

surgical plane throughout, and animals were kept warm with a temperature-controlled blanket (Harvard Apparatus). Bilateral guide cannulas (26 gauge; Plastics One) were implanted to just above (2 mm) the BLA (AP: −2.3; ML: ±5.0; DV: −6.4; incisor bar: −3.2 mm) (Paxinos and Watson, 1986). The cannulas were secured to the skull with four stainless-steel screws (2.8 mm; Plastics One) and self-curing acrylic resin (Lang Dental Manufacturing). After completion of surgery, all animals received meloxicam (Boehringer Ingelheim; 1 mg/kg, s.c.), 1 ml of saline 0.9% s.c., and were placed in a warm environment until they had fully recovered from the anesthetic. Rats remained singly housed until they fully recovered from surgery, then were housed in pairs. Pairs were separated and animals housed singly 1 d before behavioral testing (below).

Intracranial injections

As reported previously (Silveira Villarroel et al., 2018), all compounds were delivered bilaterally via 33 gauge injection cannulas (Plastics One) that extended 2 mm beyond the guide cannulas, using a dual-channel infusion pump (PHD ULTRA, Harvard Apparatus). Vehicle (sterile saline) alone or containing drug(s) as described was delivered at 100 nl/30 s per side; cannulae were left in place for 1 min afterward to prevent backflow. All injections were performed once daily between 8:00 A.M. and 10:00 A.M. for successive 5 d. Animals were returned to their home cage for 30 min following injections, then tested (if in their protocol) for SI.

Behavioral testing

The SI test was performed according to Sajdyk et al. (2008), with minor modifications. Briefly, an experimental animal was placed into the open-field SI box (96 cm long × 96 cm wide × 30 cm high) simultaneously with a partner rat of the same sex, age, and weight, housed under identical conditions but which had not previously encountered the experimental animal. Seventy-two hours after implantation surgery, rats received a sham intra-BLA injection, then were placed alone in the behavioral testing arena for 10 min to acclimate to the testing arena. Twenty-four hours later, they received another mock injection, and SI was observed for 10 min to establish baseline levels before any experimental manipulations. Two days later, rats received a bilateral intra-BLA injection (vehicle or drug) and were tested for SI 30 min after injection (day 1). Injections continued daily at the same time, and the SI test was repeated 30 min after the fifth injection (day 5), then at 2 weeks and 4 weeks from the first injection. Behavior was video recorded and later analyzed by individuals blinded to treatment. SI time is reported as a percent of baseline for each animal using the formula: SI Time (%) = (SI Time_{Test}/SI Time_{Baseline}) × 100.

Figure 2. NPY treatment of BLA OTC causes hypotrophy of pyramidal neurons. **a**, Scatter plot of mean capacitance of OTC pyramidal neurons treated with vehicle (Control; $n = 42$), NPY at 100 pM ($n = 31$), 1 nM ($n = 43$), 10 nM ($n = 47$), 100 nM ($n = 49$), and the inactive analog, da-NPY at 100 nM ($n = 30$) ($H_{(5)} = 40.72$, $p = 1.07 \times 10^{-7}$). Inset, Representative capacitive transients for control- or 100 nM NPY-treated BLA OTC neurons. **b**, Scatter plot of mean total dendritic length for OTC neurons treated as in **a**. Control ($n = 26$), NPY at 100 pM ($n = 26$), 1 nM ($n = 25$), 10 nM ($n = 21$), 100 nM ($n = 24$), and da-NPY 100 nM ($n = 20$) ($H_{(5)} = 19.04$, $p = 0.0019$). **c**, Scatter plot of mean number of branch points in neurons in **b** ($H_{(5)} = 17.02$, $p = 0.0045$). **d**, Sholl analysis of neurons in **b** and **c** treated as indicated (Treatment: $F_{(2,72)} = 9.96$, $p = 0.00015$; Distance: $F_{(40,2880)} = 185.4$, $p \sim 0$; Interaction: $F_{(80,2880)} = 2.09$, $p = 8.57 \times 10^{-8}$). **e–j**, Representative reconstructions of neurons treated as indicated above. N values for **c** and **d** are as in **b**. **a–c**, Kruskal-Wallis H tests with Dunn's *post hoc* tests. **d**, Two-way repeated-measures ANOVA with Tukey's *post hoc* test. **a–c**, Circles represent neurons; black bars represent population means. **d**, Symbols represent population means. Error bars indicate SEM. All tests were two-sided. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, *post hoc* multiple comparisons. Scale bars: **e–j**, 100 μm .

Whole-cell patch-clamp electrophysiology

Acutely prepared slices or EP 9–10 week OTC slices (with culture insert membrane still attached) were transferred to a fixed recording chamber beneath a movable upright microscope (Axioskop FS2; Carl Zeiss) (Giesbrecht et al., 2010; Silveira Villarroel et al., 2018). Slices were perfused at 2–3 ml/min for at least 10 min before recording, with warmed (32°C–34°C), carbogenated aCSF (300 mOsm/L acute slices and 320 mOsm/L for OTCs, adjusted with NaCl). Patch pipettes were pulled from borosilicate glass (TW150F; World Precision Instruments) with a two-stage puller (PP-83; Narishige) and had a resistance of 4–6 M Ω with an internal solution (mM) as follows: 126 K-gluconate, 4 KCl, 10 HEPES, 5 MgATP, 0.3 NaGTP, 1 EGTA, 0.3 CaCl₂, and 0.2% neurobiotin (pH 7.27, 275 mOsm/L for acute slices and 300 mOsm/L for OTCs).

The BLA was identified under a 5 $\times$ objective, and PNPs were identified visually with infrared-differential interference contrast (60 $\times$) optics. Neurons were selected as previously reported (Giesbrecht et al., 2010; Silveira Villarroel et al., 2018) based on morphologic and electrophysiological criteria. PNPs chosen for study were in random locations throughout the BLA; 1–3 neurons per slice were studied. Either a Multiclamp 700B or AxoClamp2A amplifier was used together with a DigiData 1322 or Digidata 1440 interface and pCLAMP version 10.4 software (all Molecular Devices). Gigohm seal, whole-cell recordings were low-pass filtered at 3 kHz and digitized at 10 kHz. Access resistance was measured throughout the experiment, and only those cells with changes <20% were kept for further analysis. The membrane potential reported was corrected offline for the calculated 15 mV liquid junction potential (Chee et al., 2010; Silveira Villarroel et al., 2018). Recordings (>20 min) were terminated by gradually withdrawing the pipette until the membrane resealed.

Capacitance was calculated offline with pClamp, from the area under the curve of the current amplitude from a 200 ms, 10 mV hyperpolarizing voltage step (Taylor, 2012). No capacitance compensation was applied during these measurements. Spontaneous synaptic activity was assessed in voltage clamp at a (liquid junction potential-corrected) holding potential of –55 mV and sampled at 20 kHz. Continuous, 2 min recordings of spontaneous synaptic events, filtered at 3 kHz and digitized at 20 kHz, were made with the neuron held at –55 mV. Spontaneous event detection was performed using MiniAnalysis software (Synaptosoft) using default thresholding parameters for IPSCs and EPSCs, then manually inspected and curated as needed. The first 400 synaptic events of each polarity were analyzed from each such recording. Although synaptic frequencies were low, a small possibility exists that a few synaptic events of opposite polarity occurred simultaneously and thus could not be resolved under these conditions.

Cell processing and labeling

Neurons from *ex vivo* and OTC slices were all processed, stained, imaged, reconstructed, and subjected to the same morphologic analyses. Following patch-clamp recordings, OTCs and acute slices were fixed with 10% formalin (Thermo Fisher Scientific) for 24–72 h at 4°C, and then transferred to 0.2% sodium azide in PBS for subsequent storage at 4°C. Slices were processed within 4 weeks following patch-clamp recordings. Free-floating slices were washed for 3 $\times$ 10 min periods in PBS before blocking and permeabilization for 2 h with 0.3%

Figure 3. Treatment with NPY, CRF, or the Y₂R agonist cPP affects the estimated total number of spines per cell, but not spine density of PNPs in BLA OTCs. **a–c**, Representative images of spines on pyramidal neurons treated with vehicle control, 100 nM NPY, 100 nM cPP, and 30 nM CRF, as indicated. **d**, Scatter plot of mean spine density of pyramidal neurons treated as in **a**. Vehicle control ($n = 15$), 100 nM NPY ($n = 15$), 100 nM cPP ($n = 15$), and 30 nM CRF ($n = 15$); one-way ANOVA with Dunnett's multiple comparisons test: $F_{(3,56)} = 1.437$, $p = 0.24$. **e**, Scatter plot of mean estimated total number of spines for neurons in **d** (one-way ANOVA with Welch's correction and Dunnett's T3 multiple comparisons test, $W_{(3, 29.15)} = 10.61$, $p = 7.09 \times 10^{-5}$). **d**, **e**, Circles represent neuron means; black bars represent population means. Error bars indicate SEM. All tests were two-sided. * $p < 0.05$, **** $p < 0.0001$.

Triton X-100 in PBS and 4% normal goat serum (Sigma-Aldrich). Slices were incubated with streptavidin conjugated with AlexaFluor-555 or -546 (1:1000; Invitrogen) in 0.3% Triton X-100 in PBS with 4% normal goat serum for 3 h at room temperature. Slices were washed 4 $\times$ 10 min each in PBS, mounted on Superfrost Plus slides, and coverslips were applied with Prolong Gold mounting media (Thermo Fisher Scientific). Slides were allowed to air dry in the dark at room temperature before being imaged via confocal microscopy.

Imaging and neuronal reconstruction

For morphologic analysis of filled neurons, z-stack images were obtained with a 20 $\times$ objective, 1024 $\times$ 1024 resolution, 100 Hz, excitation wavelength 543 nm, for a series of 0.8 μ m steps with a laser scanning confocal microscope (TCS SP5; Leica Microsystems). To determine the position of each neuron within the slice, multiple single-plane images were taken at 10 $\times$ magnification, 512 $\times$ 512 resolution and 100 Hz, and stitched together using Leica LAS AF software. Only those neurons whose dendritic arbor was clearly and completely filled and cell body was clearly within the

Figure 4. CRF treatment of BLA OTC causes pyramidal neuron hypertrophy. **a**, Scatter plot of mean capacitance of OTC pyramidal neurons treated with vehicle control ($n = 39$) and CRF at 300 pM ($n = 40$), 3 nM ($n = 36$), and 30 nM ($n = 38$) ($H_{(3)} = 22.67$, $p = 4.72 \times 10^{-5}$). Inset, Representative capacitance transients from control- or 30 nM CRF-treated BLA OTC neurons. **b**, Scatter plot of mean total dendritic length for OTC neurons treated as in **a**. Vehicle control ($n = 33$) and CRF at 300 pM ($n = 31$), 3 nM ($n = 26$), and 30 nM ($n = 28$) ($H_{(3)} = 22.71$, $p = 4.63 \times 10^{-5}$). **c**, Scatter plot of mean number of branch points in neurons in **b** ($H_{(3)} = 19.90$, $p = 0.0002$). **d**, Sholl analysis of neurons in **b** and **c** treated as indicated (Treatment: $F_{(2,84)} = 11.43$, $p = 4.05 \times 10^{-5}$; Distance: $F_{(40,3360)} = 137.4$, $p \sim 0$; Interaction: $F_{(80,3360)} = 2.58$, $p = 1.45 \times 10^{-12}$). **e–g**, Representative reconstructions of neurons treated as indicated above. **a–c**, Kruskal–Wallis H tests with Dunn’s *post hoc* tests. **d**, Two-way repeated-measures ANOVA with Tukey’s *post hoc* test. **a–c**, Circles represent neurons; black bars represent population means. **d**, Symbols represent population means. Error bars indicate SEM. All tests were two-sided. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, *post hoc* multiple comparisons. Scale bars: **e–g**, 100 μm.

boundaries of the BLA were used for analysis. Neuronal reconstruction was performed using the simple neurite tracer function in FIJI (National Institutes of Health). Following tracing, Sholl analysis (Sholl, 1953) of the entire 3D dendritic arbor was performed with another FIJI software module using concentric circles with increasing radii of 10 μm to determine dendritic intersections versus distance from the soma. Total dendritic length and dendritic branching were calculated using FIJI and Excel software. Quantification of dendritic spine density was achieved by manually counting dendritic spines in z-stack images taken at 100× magnification, 1024 × 1024 resolution and 100 Hz for a series of 0.5 μm steps. Spines were counted on three separate, randomly selected, 100 μm dendrite segments for each neuron analyzed in a treatment group. All protrusions that were connected to the dendrite segment were considered as spines but were not categorized by spine morphologies. Total spine number was estimated from the average spine density and total dendritic length for an individual neuron. Experimenters were blinded to culture treatment at the time of reconstructions.

Reagents and drugs

NPY (human, rat) was purchased from the Polypeptide Group, whereas CRF was obtained from Phoenix Pharmaceuticals. The Y_1 -agonist (F^7, P^{34} -NPY), (Söll et al., 2001) the Y_5 -agonist ([cPP $^{1-7}$, NPY $^{19-32}$, Ala 31 , Aib 32 , Gln 34]hPP), (Cabrele et al., 2001) and the Y_2 agonist ([ahx $^{5-24}$] NPY) (Cabrele and Beck-Sickinger, 2000) were generous

gifts from Dr A.G. Beck-Sickinger, whereas the Y_5 -antagonist, CGP71683, was purchased from Tocris Bioscience. Cyclosporine A (CsA) and okadaic acid were gifts from Drs. Shairaz Baksh and Charles Holmes (University of Alberta), respectively. The cell-permeable CaMKII inhibitor, myristoylated-autocamtide-2-related inhibitory peptide, was obtained from Enzo Life Sciences. Culture reagents were all obtained from Invitrogen, except for cytosine-β-D-arabinofuranoside, uridine, and 5′fluro-2′deoxyuridine, which were from Sigma-Aldrich. Pipette solution reagents were all from Sigma-Aldrich, except for Na-GTP (Roche Diagnostics).

Experimental design and statistical analysis

Electrophysiological recordings were viewed offline using pCLAMP. Traces from electrophysiological recordings, statistical analysis, and graphs were prepared using versions 5–8 of Prism software (GraphPad) or SPSS (version 20, IBM). Neuronal reconstruction and Sholl analysis were performed using modules of the FIJI software suite (National Institutes of Health). Unless otherwise stated, all data are presented as mean ± SEM. The D’Agostino–Pearson omnibus normality test was applied to determine data distributions. One-way ANOVA with Tukey’s *post hoc* test or Kruskal–Wallis H tests with Dunn’s *post hoc* test were used as indicated, to compare cell capacitance, total dendritic lengths, branch points, and spine density/total spine estimates between treatments. For Sholl analysis, two-way repeated measures ANOVA were used with Tukey’s *post hoc* test for multiple comparisons. A linear mixed-model was used

Figure 5. Sequential treatment with either NPY or CRF reverses or prevents the effects of the other neuropeptide alone on BLA OTC pyramidal neuron morphology. **a**, Scatter plot of mean capacitance of OTC pyramidal neurons treated with media control ($n=42$), 30 nM CRF followed by 100 nM NPY ($n=31$) and 100 nM NPY followed by 30 nM CRF ($n=32$), with the mean capacitance for 30 nM CRF and 100 nM NPY alone (data from **Figures 1** and **2**) indicated by dashed lines ($H_{(2)} = 0.73$, $p=0.69$). Inset, Representative capacitance transients from control, CRF, NPY- and NPY, CRF-treated BLA OTC neurons. **b**, Scatter plot of mean total dendritic length for OTC neurons treated with control ($n=26$), CRF, NPY, and NPY, CRF ($n=23$) as in **a**. Data are shown along with the mean total length for 30 nM CRF and 100 nM NPY alone (from **Figures 1** and **2**) as dashed lines ($H_{(2)} = 0.80$, $p=0.67$). **c**, Scatter plot of mean number of branch points in neurons in **b** with the mean values for 30 nM CRF and 100 nM NPY (from **Figures 1** and **2**) as dashed lines ($H_{(2)} = 0.36$, $p=0.84$). **d**, Sholl analysis of neurons in **b** and **c** with the Sholl analysis for 30 nM CRF and 100 nM NPY (from **Figures 1** and **2**) as dashed lines (Treatment: $F_{(2,69)} = 0.21$, $p=0.81$; Distance: $F_{(40,2760)} = 157.3$, $p \sim 0$; Interaction: $F_{(80,2760)} = 0.70$, $p=0.98$). **e-g**, Representative traces from neurons treated as indicated. **h**, Mean sEPSC IEP in neurons treated with 30 nM CRF followed by 100 nM NPY ($n=25$), 100 nM NPY followed by 30 nM CRF ($n=25$), 30 nM CRF alone ($n=25$), and 100 nM NPY alone ($n=25$) ($H_{(3)} = 28.18$, $p=3.33 \times 10^{-6}$). **i-k**, Representative recordings from neurons treated as indicated. **a-c**, Kruskal-Wallis H test with Dunn's *post hoc* tests. **d**, Two-way repeated-measures ANOVA with Tukey's *post hoc* tests. **h**, Kruskal-Wallis H test with Dunn's *post hoc* test. **a-c**, Circles represent individual neurons; black bars represent population means. Error bars indicate SEM. All statistical tests were two-sided. $**p < 0.01$, $****p < 0.0001$. Scale bars: **e-j**, 100 μm .

for analysis of SI studies. Treatment and time (with interaction term) were used as fixed effects while we used intercepts for subjects as random effects. For data in **Table 1**, one-way ANOVA with Tukey's *post hoc* test for multiple comparisons was used. For sEPSC

and sIPSC analyses, two-sided Student's *t* test or Mann-Whitney *U* tests were performed when applicable. OTC cultures for any given experiment were obtained from at least 3 animals (typically more), with data acquired from 1 to 3 neurons per OTC. With the extent of

Figure 6. Treatment with the selective Y_1 receptor agonist F^7P^{34} -NPY does not affect BLA OTC pyramidal neuron morphology. **a**, Scatter plot of mean capacitance of OTC pyramidal neurons treated with vehicle control ($n=30$) and F^7P^{34} -NPY at 1 nM ($n=28$), 10 nM ($n=34$), and 100 nM ($n=34$) ($H_{(3)} = 0.58$, $p=0.90$). Inset, Representative capacitance transients from control- and F^7P^{34} -NPY-treated BLA OTC neurons. **b**, Scatter plot of mean total dendritic length for neurons treated as in **a**. Vehicle control ($n=27$) and F^7P^{34} -NPY at 1 nM ($n=19$), 10 nM ($n=18$), and 100 nM ($n=22$) ($H_{(3)} = 1.04$, $p=0.79$). **c**, Scatter plot of mean number of branch points for neurons in **b** ($H_{(3)} = 4.25$, $p=0.24$). **d**, Sholl analysis for neurons in **b** and **c** treated as indicated (Treatment: $F_{(1,47)} = 1.82$, $p=0.18$; Distance: $F_{(40,1920)} = 109.3$, $p \sim 0$; Interaction: $F_{(40,1920)} = 0.33$, $p=0.99$). **a–c**, Kruskal-Wallis H tests with Dunn's *post hoc* tests. **d**, Two-way repeated-measures ANOVA with Tukey's *post hoc* test. **a–c**, Circles represent neurons; black bars represent population means. **d**, Symbols represent population means. Error bars indicate SEM. All tests were two-sided.

time in culture and minimal number of neurons recorded from per slice, we opted to treat each neuron as an independent observation for statistical analysis. The number of animals, cultures, and neurons per experimental group are indicated in Table 2. No statistical test was used to predetermine sample sizes, but our sample sizes are similar to those previously reported in the field (De Simoni et al., 2003; Adamec et al., 2012; Silveira Villarroel et al., 2018). All analyses were two-sided.

Results

Repeated NPY and CRF treatments cause dendritic hypertrophy and hypertrophy, respectively, in OTC PNs

Repeated restraint stress induces lasting dendritic hypertrophy in BLA neurons (Padival et al., 2013). Whereas *in vivo* administration of the CRFR1 agonist UCN into the BLA elicits long-term stress vulnerability, NPY administration persistently decreases the animals' vulnerability to stress (Rainnie et al., 2004; Sajdyk et al., 2008; Silveira Villarroel et al., 2018). The enduring effects of NPY may parallel the persistence of the stress-induced dendritic hypertrophy in BLA neurons (Padival et al., 2013), but mechanisms underlying the effects of either endogenous neuropeptide remain unclear. To test our hypothesis that repeated application of either NPY or CRF to BLA OTCs will alter electrophysiological properties of PNs and result in their respective hypertrophy or

hypertrophy, we monitored PN properties with electrophysiology and postrecording morphologic analysis. Initial validation of the OTC preparation of BLA (see Materials and Methods) included an extensive comparison of PN neuronal properties with neurons from acute *ex vivo* brain slices of P14 and P70 animals (Table 1); the PNs from OTCs matured, electrically, from P14 acute slices, however, did not develop equivalent electrophysiological properties of acute slices from P70 PNs and were more compact compared with those from either acute slice preparation.

Repeated application of NPY (1–100 nM) reduced PN whole-cell capacitance relative to vehicle controls (Fig. 2a). Consistent with this, NPY (≥ 1 nM) reduced and simplified the dendritic arbor (Fig. 2b–j), decreasing the total dendritic length (Fig. 2b) and complexity of dendritic branching (Fig. 2c,d). The effects of NPY on dendritic morphology were already significant at 1 nM ($p < 0.0001$ vs control). Although dendritic spine density was not altered by 100 nM NPY treatment, estimates of total spine numbers showed a trend ($p = 0.095$) toward reduction compared with controls (Fig. 3), consistent with the significant reduction in EPSC frequencies observed (Fig. 1b,c,e). Neurons treated with the inactive da-NPY (100 nM) (Wahlestedt et al., 1986) did not differ in any aspect from untreated controls (Fig. 2a–c,f).

Repeated treatment of OTCs with CRF induced a diametrically opposite set of changes in BLA PN physiology and structure. Thus, application of 30 nM CRF increased sEPSC frequency compared with vehicle-treated PNs (Fig. 1f–h), without affecting sEPSC amplitude (Control: 44.8 ± 2.1 pA; 30 nM CRF: 50.68 ± 3.9 pA; Mann-Whitney *U* test; $U = 246.0$, $p = 0.20$) or sIPSC amplitude (Control: 75.5 ± 8.7 pA; 30 nM CRF: 70.24 ± 6.8 pA; Mann-Whitney *U* test; $U = 297.0$, $p = 0.77$) or sIPSC frequency (Control: 155.6 ± 19.3 ms; 30 nM CRF: 157.6 ± 13.5 ms; Mann-Whitney *U* test; $U = 294.5$, $p = 0.73$; data not shown). Furthermore, compared with controls, CRF increased whole-cell capacitance (Fig. 4a) and caused hypertrophy of the dendritic arbor (Fig. 4b), including increased branching (Fig. 4c) and dendritic complexity (Fig. 4d–g). The effects of CRF on dendritic morphology occurred in a more clearly concentration-dependent manner than did those of NPY. Although CRF treatment did not alter spine density, estimates of total spine numbers were significantly increased compared with controls (Fig. 3).

Bidirectional PN dendritic remodeling after successive treatments with NPY and CRF in BLA OTCs

BLA output and thus the physiological and behavioral responses to stress are governed in part by the opposing actions of NPY and CRF neuropeptide systems. Disruption of this homeostatic mechanism may in part underlie some persistent anxiety-related

Figure 7. Treatment with the Y₁R agonist F⁷P³⁴-NPY does not affect the sEPSC frequency, while the Y₅R agonist cPP reduces sEPSC frequency onto BLA OTC pyramidal neurons. *a, b, d, e*, Representative electrophysiological recordings of spontaneous synaptic currents in BLA OTC pyramidal neurons treated with vehicle (*a, d*), F⁷P³⁴-NPY (*b*), and cPP (*e*). *c*, Scatter plot of mean interevent sEPSC intervals onto pyramidal neurons treated with vehicle ($n = 25$) and 100 nM F⁷P³⁴-NPY ($n = 25$) (Mann-Whitney U test; $U = 299.0$, $p = 0.80$). *f*, Scatter plot of mean IEI of sIPSCs (t test with Welch's correction: $t_{(36,20)} = 2.92$, $p = 0.0060$) and sEPSCs (Mann-Whitney U test; $U = 111.0$, $p < 0.0001$) from vehicle control ($n = 25$) and cPP-treated neurons ($n = 25$). *g*, Scatter plot of mean amplitudes of sIPSCs (Mann-Whitney U test; $U = 259.0$, $p = 0.30$) and sEPSCs (Mann-Whitney U test; $U = 269.0$, $p = 0.40$) from vehicle control ($n = 25$) and cPP (100 nM)-treated ($n = 25$) neurons. *c, f, g*, Circles represent neuron means; black bars represent population means. Error bars indicate SEM. All statistical tests were two-sided. n.s., Not significant ($p > 0.05$). ** $p < 0.01$, *** $p < 0.001$.

disorders (Schmeltzer et al., 2016). We therefore next determined whether NPY and CRF can also act as counterregulatory signals in BLA OTCs. BLA OTCs were incubated for 1 week with either CRF or NPY; then some were incubated the following week, respectively, with either NPY or CRF, and maintained in control media for 1 additional week before electrophysiological and morphologic analyses were performed on both single- and double-treated populations. Specifically, when PNs were reciprocally treated with the two peptides in either order, there were no differences either in cell capacitance (Fig. 5*a*) or dendritic size or complexity (Fig. 5*b–g*) from vehicle controls. As in the previous experiments, application of NPY or CRF alone either decreased or increased the frequency of sEPSCs observed in the OTCs, whereas sEPSC frequency in the CRF,NPY- or NPY,CRF-treated groups was not significantly different from vehicle controls (Fig. 5*h–k*). There were no effects observed either on sEPSC amplitude (Control: 44.7 ± 2.0 pA; NPY,CRF: 49.1 ± 1.9 pA; CRF,NPY: 45.6 ± 2.6 pA; one-way ANOVA with Tukey's multiple comparisons test: $F_{(2,2)} = 1.02$, $p = 0.37$; data not shown) or sIPSC amplitude (Control: 76.0 ± 8.8 pA; NPY,CRF: 85.4 ± 9.3 pA; CRF,

NPY: 75.9 ± 7.0 pA; one-way ANOVA with Tukey's multiple comparisons test: $F_{(2,72)} = 0.45$, $p = 0.64$; data not shown) or sIPSC IEI (Control: 158.0 ± 19.2 ms; NPY,CRF: 155.8 ± 18.5 ms; CRF,NPY: 149.0 ± 17.5 ms; one-way ANOVA with Tukey's multiple comparisons test: $F_{(2,72)} = 0.065$, $p = 0.94$; data not shown). Assessment of BLA PN morphology in the CRF,NPY and NPY, CRF groups was consistent with the neurons undergoing a bidirectional structural plasticity with such reversed, repeated treatments.

The Y₅R, but not the Y₁R, mediates dendritic hypertrophy in BLA OTCs

While NPY plays a key role in behavioral stress resilience, the receptor subtype(s) mediating the long-term effect on behavior is unknown. The BLA expresses Y₁, Y₂, and Y₅ NPY receptors (Kopp et al., 2002; Wolak et al., 2003; Stanić et al., 2011; Mackay et al., 2019) and while the acute anxiolytic effects of NPY in the BLA are mediated predominantly via the Y₁R (Sajdyk et al., 2002a), evidence also suggests a role for the Y₅R (Sajdyk et al., 2002a). Unexpectedly, selective activation of Y₂ receptors in the

Figure 8. Treatment of BLA OTCs with the Y_2 R agonist ahx causes pyramidal neuron hypertrophy. **a**, Scatter plot of mean capacitance of OTC pyramidal neurons treated with vehicle ($n = 26$), ahx at 1 nM ($n = 23$), 10 nM ($n = 21$), and 100 nM ($n = 25$) ($H_{(3)} = 11.31$, $p = 0.010$). **b**, Scatter plot of mean total dendritic length for OTC neurons treated as in **a** ($H_{(3)} = 13.25$, $p = 0.0041$). Vehicle ($n = 23$), ahx at 1 nM ($n = 19$), 10 nM ($n = 23$), and 100 nM ($n = 23$). **c**, Scatter plot of mean number of branch points in neurons in **b** ($H_{(3)} = 17.94$, $p = 0.0005$). **d**, Sholl analysis of neurons in **b** and **c** treated as indicated (Treatment: $F_{(2,66)} = 4.06$, $p = 0.022$; Distance: $F_{(40,2640)} = 119.5$, $p \sim 0$; Interaction: $F_{(80,2640)} = 2.27$, $p = 1.92 \times 10^{-9}$). Light purple represents control versus 10 nM ahx. Purple represents control versus 100 nM ahx. **e–g**, Representative reconstructions of neurons treated as indicated above. N values for **c** and **d** are as in **b**. **a–c**, Kruskal-Wallis H tests with Dunn's *post hoc* tests. **d**, Two-way repeated-measures ANOVA with Tukey's *post hoc* test. **a–c**, Circles represent neurons; black bars represent population means. **d**, Symbols represent population means. Error bars indicate SEM. All tests were two-sided. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *post hoc* multiple comparisons. Scale bars: **e–g**, 100 μm .

BLA acutely increases PN excitability (Mackay et al., 2019) and facilitates the anomalous expression of anxiety-like behaviors (Sajdyk et al., 2002b). We thus examined the roles of different NPY receptors in a pharmacological experiment using BLA OTCs.

In a repeated incubation experiment as with NPY above, the Y_1 R-selective agonist $F^{7,34}$ -NPY (1–100 nM) had no measurable long-lasting effects on the properties of BLA OTC PNs compared with controls at any concentration tested. Thus, whole-cell capacitance (Fig. 6a) or any of the morphologic parameters previously affected by NPY itself in this system were unchanged (Fig. 6b–d). Treatment with the Y_1 R-selective agonist also had no significant effect on sEPSC frequency (Fig. 7a–c) or any other synaptic properties measured (data not shown). The Y_2 R-selective agonist, $[\text{ahx}^{5-24}]$ NPY (1–100 nM) (Beck-Sickinger et al., 1992), when tested at 1 and 10 nM, had no significant effects on the same parameters as for the Y_1 R agonist as above. However when applied at 100 nM, $[\text{ahx}^{5-24}]$ NPY induced a significant increase, both in whole-cell capacitance and in dendritic arborization (total dendritic length and number of branches; Fig. 8a–g), which was similar to that seen with CRF. These results are consistent with previously reported anxiogenic actions of Y_2 receptor agonists (Sajdyk et al., 2002a,b) and indicate that the Y_2 receptor is unlikely to mediate NPY-induced hypotrophy.

Finally, we tested the Y_5 R-selective agonist, $[\text{cPP}^{1-7}, \text{NPY}^{19-23}, \text{Aib}^{32}, \text{Gln}^{34}]$ hPP (cPP, 1–100 nM) (Cabrele et al., 2001). At 100

nM, cPP caused significant reductions in PN sEPSC frequency and increased sIPSC frequency compared with controls, with no changes in sIPSC or sEPSC amplitude (Fig. 7d–g). Higher concentrations of cPP (≥ 10 nM) also caused robust decreases in PN whole-cell capacitance (Fig. 9a), total dendritic length, and numbers of branch points (Fig. 9b–g). While spine density was not altered by 100 nM cPP, estimates of total spine numbers per neuron were significantly reduced compared with vehicle controls (Fig. 3). Furthermore, coincubation of the Y_5 R antagonist CGP71683A (CGP, 30 nM) with a maximally effective concentration (30 nM) of NPY not only prevented NPY effects on whole-cell capacitance and dendritic extent, but actually caused dendritic hypertrophy (Fig. 9a–g). This result is consistent with the Y_5 antagonist unmasking the Y_2 -mediated actions of NPY reported above.

***In vivo* treatment of BLA with NPY or cPP results both in long-term behavioral stress resilience and dendritic hypotrophy**

Based on the *in vitro* results, we hypothesized that NPY will cause similar effects on PN structure in BLA *in vivo*, and that the structural changes would also correlate with persistent increases in SI, a validated measure of anxiety (File and Seth, 2003; Sajdyk et al., 2008; Silveira Villarroel et al., 2018). Five sequential daily injections of NPY (10 pmol/100 nl), the Y_1 -agonist $F^{7,34}$ NPY (10 pmol/100 nl), the Y_5 -agonist, cPP (10 pmol/100 nl), or NPY

Figure 9. Treatment of BLA OTC with the Y₅R agonist cPP results in pyramidal neuron hypotrophy, which is blocked by the Y₅R antagonist CGP71683A (Y₅A). **a**, Scatter plot of mean capacitance of OTC pyramidal neurons treated with vehicle control ($n = 30$), cPP at 1 nM ($n = 32$), 10 nM ($n = 47$), and 100 nM ($n = 34$) and 10 nM NPY with 30 nM CGP ($n = 45$) ($H_{(4)} = 33.61$, $p = 8.97 \times 10^{-7}$). Inset, Representative capacitance transients from Control-, cPP-, and NPY + Y₅A-antagonist-treated BLA OTC neurons. **b**, Scatter plot of mean total dendritic length of neurons treated as in **a**. Vehicle control ($n = 26$), cPP at 1 nM ($n = 23$), 10 nM ($n = 31$), and 100 nM ($n = 32$), and 10 nM NPY with 30 nM CGP ($n = 34$) ($H_{(4)} = 49.88$, $p = 3.83 \times 10^{-10}$). **c**, Mean number of branch points in neurons in **b** ($H_{(4)} = 36.99$, $p = 1.81 \times 10^{-7}$). **d**, Sholl analysis for neurons in **b** and **c** treated as indicated (Treatment: $F_{(2,89)} = 23.98$, $p = 4.49 \times 10^{-9}$; Distance: $F_{(40,3600)} = 175.1$, $p \sim 0$; Interaction: $F_{(80,3600)} = 3.91$, $p = 2.68 \times 10^{-28}$). Black represents control versus 100 nM cPP. Green represents control versus NPY + Y₅A. Blue represents 100 nM cPP versus NPY + Y₅A. **e–g**, Representative traces from neurons treated as indicated. **a–c**, Kruskal–Wallis H tests with Dunn’s *post hoc* tests. **d**, Two-way repeated-measures ANOVA with Tukey’s *post hoc* test. **a–c**, Circles represent neurons; black bars represent population means. **d**, Symbols represent population means. Error bars indicate SEM. All tests were two-sided. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *post hoc* multiple comparisons. Scale bars: **e–g**, 100 μm.

coapplied with the Y₅-antagonist CGP (each 10 pmol/100 nl) were administered bilaterally into BLA of 8-week-old male rats. All NPY agonist treatments in this experiment acutely increased SI on injection days 1 and 5 relative to both pretreatment baseline and vehicle controls (Fig. 10a). However, only animals treated with NPY or the Y₅-agonist retained this increase in SI at 2 and 4 weeks (Fig. 10a). In recordings from PNs in acute *ex vivo* BLA slices from these same animals at 4 weeks after treatment, prior treatment with either NPY or the Y₅R agonist decreased whole-cell capacitance (Fig. 10b) and significantly reduced dendritic extent and complexity relative to PNs from the vehicle-treated animals (Fig. 10c–j). Coadministration of the Y₅R antagonist with NPY prevented the long-term effects of NPY both on behavior and PN morphology (Fig. 10a–e,j); no anxiogenic behavior was observed at any time point, nor were increases in

PN capacitance or arborization as seen in the OTCs. Therefore, the Y₅R is both necessary and sufficient to mediate both the NPY-induced long-term structural plasticity and decreased behavioral stress responses *in vivo*.

Dendritic remodeling by NPY or CRF requires calcineurin or CaMKII, respectively

Because the long-term stress-vulnerability and -resilience effects of CRF- and NPY-receptors are mediated via CaMKII and calcineurin, respectively, in the BLA *in vivo* (Rainnie et al., 2004; Sajdyk et al., 2008), we hypothesized that the effects of NPY or CRF on BLA PN dendritic structure require the actions of calcineurin or CaMKII activity, respectively. We first repeatedly incubated BLA OTCs with NPY (10 nM) together with CsA (2 μM), a protein phosphatase (PP) inhibitor, which blocks

Figure 10. Repeated *in vivo* injection of NPY or the Y_5 receptor agonist, but not the Y_1 receptor agonist, results in increased SI times and dendritic hypotrophy. **a**, Scatter plot depicting SI time shown as percent of individual animals' baselines for treatment with vehicle control (100 nl) at day 1 ($n = 50$), day 5 ($n = 49$), week 2 ($n = 43$), and week 4 ($n = 19$); NPY (10 pmol/100 nl) at day 1 ($n = 42$), day 5 ($n = 40$), week 2 ($n = 29$), and week 4 ($n = 11$); the Y_1 agonist F^{7P34} -NPY (10 pmol/100 nl) at day 1 ($n = 15$), day 5 ($n = 14$), week 2 ($n = 14$), and week 4 ($n = 10$); the Y_5 agonist cPP (10 pmol/100 nl) at day 1 ($n = 7$), day 5 ($n = 7$), week 2 ($n = 7$), and week 4 ($n = 7$); and NPY (10 pmol/100 nl) immediately preceded by the Y_5 receptor antagonist CGP71683A (Y_5 A, 10 pmol/100 nl) at day 1 ($n = 5$), day 5 ($n = 5$), week 2 ($n = 5$), and week 4 ($n = 5$) (linear mixed-model; Treatment: $F_{(4,102.30)} = 14.11$, $p = 3.33e^{-9}$; Time: $F_{(3,93.88)} = 1.66$, $p = 0.18$; Treatment $\times$ Time: $F_{(12,91.12)} = 1.87$, $p = 0.049$). **b**, Scatter plot of mean capacitance of BLA pyramidal neurons in acute slices treated with vehicle control ($n = 64$), NPY

calcineurin (Liu et al., 1991). Addition of CsA inhibited NPY-mediated dendritic hypotrophy and decreases in capacitance (Fig. 11*a–d*), resulting instead in PN dendritic hypertrophy, possibly by unmasking a Y_2R -mediated effect (Fig. 11*b–d*). While these results are consistent with a role for calcineurin, CsA is a general PP inhibitor and is not selective for calcineurin (PP2b). Okadaic acid (OA) selectively inhibits both PP1 and PP2a but not calcineurin (Cohen et al., 1990). Treatment of the BLA OTCs with the Y_5 -agonist cPP (100 nM) in the presence of OA (10 nM) did not alter the effects of the Y_5R agonist on either capacitance or dendritic morphology, consistent with a specific role for calcineurin not only in NPY-mediated anxiolysis (Sajdyk et al., 2008) but also in NPY-mediated PN dendritic hypotrophy (Fig. 11*a–d*).

We then tested the role of CaMKII in mediating the hypertrophic actions of CRF in BLA OTC PNs by using a cell-permeant CaMKII inhibitor, the myristoylated version of autocamtide-2-related inhibitory peptide (AIP) (Ishida et al., 1995). Inhibiting CaMKII activity with AIP (40 nM) prevented the CRF-mediated (30 nM) increase in cell capacitance and dendritic hypertrophy (Fig. 11*e–h*). This is consistent with a role for CaMKII in CRF-mediated dendritic hypertrophy and the resultant stress vulnerability generated by repeated stress and CRF-receptor activation (Rainnie et al., 2004).

Discussion

Unraveling mechanisms mediating adaptive and pathophysiological responses to stress is key to developing treatments for stress-related disorders. The countervailing actions of CRF and NPY on BLA PNs represent mechanisms supporting adaptive stress responses in the amygdala. Although both neuropeptides induce long-term neural changes linked to the initiation or mitigation of stress responses, the mechanisms underlying the effects of these neuropeptides are unclear. Here, we identify three novel and important insights into the function of NPY and CRF in amygdala physiology. First, NPY-induced stress resilience ultimately involves a novel, persistent form of homeostatic plasticity, specifically, the reversible remodeling of dendritic structure in BLA pyramidal output neurons mediated by the Y_5R , which counterbalances the persistent hypertrophy seen with stress or repeated CRF-receptor activation. Second, different NPY receptors play nuanced, complex roles in the short- and long-term regulation of BLA excitability, structure, and behavior. Finally, the BLA OTC preparation is a robust *in vitro* model, which mimics and predicts long-term *in vivo* responses of BLA neurons.

If the bidirectional changes in dendritic structure seen here with repeated neuropeptide treatments *in vitro* or *in vivo* are indeed biologically relevant, then intermittent but prolonged periods of stress should result in similar changes that can be reversed when conditions improve. Indeed, chronic stress or UCN treatment causes dendritic hypertrophy and increases excitatory inputs to BLA PNs (Rainnie et al., 2004; Padival et al., 2013); these increases in dendritic length and spine number are associated with enhanced excitatory drive onto BLA PNs, which increases anxiety (Vyas et al., 2002, 2006; Mitra et al., 2005; Hill et al., 2011; Adamec et al., 2012; Padival et al., 2013). While there is no simple behavioral manipulation to reverse the effects of prolonged stress, repeated intra-BLA administration of NPY *in vivo* decreases the overall excitability of BLA PNs (Silveira Villarroel et al., 2018), and here we demonstrate similar effects of NPY on PNs both in BLA OTCs and *in vivo*, consistent with such a role for NPY. Moreover, the ability of NPY to constrain or reverse CRF's actions, and vice versa, suggests a symmetrical regulation of PN dendritic properties. Following repeated NPY or CRF treatment, PNs in BLA OTCs also demonstrated respective decreases and increases in sEPSC frequencies, consistent with their respective behavioral effects. NPY and CRF thus appear to act via multiple, parallel mechanisms to mediate their opposing forms of plasticity, which when combined, drive BLA PN structure toward a resting state similar to that of naive BLA. Consistent with a key role in stress homeostasis, NPY is thus poised to restore balance to the organism once a threat has dissipated. Future experiments using either optogenetically or chemogenetically induced activation of NPY neurons innervating BLA, such as the amygdalostratial transition area, entorhinal cortex, or amygdalopiriform transition area (McDonald and Zaric, 2015; Leitermann et al., 2016), could test this hypothesis unambiguously.

The predominant role of the Y_5R in driving the long-term effects on BLA physiology and behavior was unanticipated. The Y_1R , which to date has received considerable attention in stress mitigation, contributes only to the acute actions of NPY on SI behavior and fear extinction (Gutman et al., 2008; Giesbrecht et al., 2010). Because all NPY receptors couple to $G_{i/o}$ proteins (Michel et al., 1998), the different roles played by these two receptors might result from independent trafficking into separate signaling compartments within BLA neurons (Marley et al., 2013). Moreover, while the Y_1R desensitizes in somatic cell lines (Berglund et al., 2003) and intact systems (Holliday et al., 2005), evidence indicates that the Y_5R does not desensitize (Böhme et al., 2008). This potentially significant difference suggests that the Y_5R is more likely to be available for lasting mitigation of stress responses.

The long-term effects of NPY or CRF require the respective activation of the Ca^{2+} -dependent enzymes calcineurin and CaMKII, indicating a critical role for alterations in intracellular Ca^{2+} levels in BLA PNs as demonstrated both *in vivo* (Rainnie et al., 2004; Sajdyk et al., 2008) and now here in BLA OTCs. The acute and long-term increases in excitability caused by CRF would result in robust elevations in intracellular Ca^{2+} ($[Ca^{2+}]_i$), consistent with requirements for CaMKII activation (Rainnie et al., 2004). However, how NPY achieves the more modest $[Ca^{2+}]_i$ elevation needed by calcineurin is less obvious. In this context, acute activation of BLA Y_2Rs paradoxically results in anxiogenic behavioral responses (Sajdyk et al., 2004) and resulted in CRF-like changes in BLA OTC neurons here. In rat and mouse BLA, activation of Y_2R reduces tonic GABA_AR activation in BLA PNs by a presynaptically mediated reduction in dendritic GABA

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($n = 53$), F^{P34} -NPY ($n = 57$), cPP ($n = 57$), and NPY with CGP ($n = 51$) (Kruskal-Wallis H test with Dunn's *post hoc* test; $H_{(4)} = 47.74$, $p = 1.07e^{-9}$). Inset, Representative capacitance transients from Vehicle, NPY, F^{P34} -NPY, cPP, and NPY + Y_5 antagonist-injected acute slices. **c**, Scatter plot of mean total dendritic length for cells treated as in **b** (ANOVA with Tukey's *post hoc*; $F_{(4,206)} = 17.70$, $p = 1.70e^{-12}$). Vehicle ($n = 52$), NPY ($n = 38$), F^{P34} -NPY ($n = 32$), cPP ($n = 50$), and NPY with CGP ($n = 39$). **d**, Scatter plot of mean number of branch points for neurons in **c** (Kruskal-Wallis H test with Dunn's *post hoc* test; $H_{(4)} = 56.53$, $p = 1.55e^{-11}$). **e**, Sholl analysis for neurons in **c** and **d** as indicated (two-way repeated-measures ANOVA with Tukey's *post hoc* test; Treatment: $F_{(4,181)} = 13.09$, $p = 2.18e^{-9}$; Distance: $F_{(49,8869)} = 815.7$, $p \sim 0$; Interaction: $F_{(196,8869)} = 4.81$, $p = 1.23e^{-91}$). Blue represents vehicle versus NPY. Green represents vehicle versus cPP. **f–j**, Representative traces from neurons treated as indicated above. **a**, Circles represent animals; black bars represent population means. **b–d**, Circles represent neurons; black bars represent population means. **e**, Symbols represent population means. Error bars indicate SEM. All tests were two-sided. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *post hoc* multiple comparisons. Scale bars: **f–j**, 100 μm .

Figure 11. Inhibitors of calcineurin (CsA) and of CaMKII (AIP), respectively, block NPY- and CRF-mediated dendritic remodeling in BLA OTCs. **a**, Scatter plot of mean capacitance of BLA OTC pyramidal neurons treated with vehicle control ($n = 28$) compared with CsA (2 nM) alone ($n = 35$), CsA (2 nM) + NPY (10 nM) ($n = 31$), OA (10 nM) alone ($n = 39$), and OA (10 nM) + cPP (10 nM) ($n = 38$) ($H_{(4)} = 22.80$, $p = 0.0001$). Dashed lines indicate mean for neurons treated with NPY (10 nM) (blue) and cPP (100 nM) (green). **b**, Scatter plot of mean total dendritic length of pyramidal neurons treated as in **a**, vehicle ($n = 26$), CsA ($n = 26$), CsA + NPY ($n = 30$), OA ($n = 26$), and OA + cPP ($n = 24$) ($H_{(4)} = 27.88$, $p = 1.32 \times 10^{-5}$). **c**, Scatter plot of mean number of branch points for neurons in **b** and **c**. Dashed lines indicate the mean for neurons treated with NPY (blue) or cPP (green) ($H_{(4)} = 28.93$, $p = 8.10 \times 10^{-6}$). **d**, Sholl analysis for neurons in **b** treated

Figure 12. Proposed model for the mechanism of action of NPY and CRF leading to dendritic hypotrophy or hypertrophy, respectively, in BLA pyramidal cells. **a**, NPY treatment results in net activation of the Y_5 R and PP2B (calcineurin), resulting in a reduction in dendritic arborization (right). **b**, CRF treatment results in net activation of CaMKII and hypertrophy of the dendritic arbor (right).

release. This causes an increase in dendritic excitability and a disinhibition of dendritic Ca^{2+} channels, which in turn permits a moderately elevated Ca^{2+} influx in approximately half of BLA PNs (Mackay et al., 2019). These effects suggest that NPY release

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as indicated; data for neurons treated with NPY (blue) or cPP (green). Black represents control versus OA + cPP; gray represents control versus CSA + NPY (Treatment: $F_{(2,78)} = 11.86$, $p = 3.18 \times 10^{-5}$; Distance: $F_{(40,3120)} = 150.0$, $p \sim 0$; Interaction: $F_{(80,3120)} = 2.39$, $p = 1.15 \times 10^{-10}$). **e**, Scatter plot of mean capacitance values for BLA OTC pyramidal cells treated with vehicle ($n = 28$), AIP (40 nm) ($n = 27$), and AIP (40 nm) + CRF (30 nm) ($n = 29$). Dashed line indicates neurons treated with CRF ($H_{(2)} = 0.77$, $p = 0.68$). **f**, Scatter plot of mean total dendritic length for pyramidal cells treated as in **e** with neurons treated with CRF (dashed line) ($H_{(2)} = 1.23$, $p = 0.54$). Vehicle ($n = 26$), AIP ($n = 21$), and AIP + CRF ($n = 21$). **g**, Scatter plot of mean number of branch points in neurons in **f** with the mean for neurons treated with CRF (dashed line) ($H_{(2)} = 0.81$, $p = 0.67$). **h**, Sholl analysis for neurons in **f** and **g** treated as indicated compared with neurons treated with CRF (dashed line) (Treatment: $F_{(2,65)} = 0.50$, $p = 0.61$; Distance: $F_{(40,2600)} = 165.3$, $p \sim 0$; Interaction: $F_{(80,2600)} = 0.48$, $p = 0.99$). **a–c**, **e–g**, Kruskal–Wallis H tests with Dunn's *post hoc* tests. **d**, **h**, Two-way repeated-measures ANOVA with Tukey's *post hoc* tests. **a–c**, **e–g**, Circles represent neurons; black bars represent population means. **d**, **h**, Symbols represent population means. Error bars indicate SEM. All tests were two-sided. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *post hoc* multiple comparisons.

Figure 13. Correlation between whole-cell capacitance and dendritic length. Whole-cell capacitance plotted against total dendritic length for 259 BLA OTC neurons from 100 different OTCs taken from all experimental groups.

Table 2. Numbers of neurons, OTCs, and animals used for capacitance and morphologic analysis, indexed by figure

Electrophysiological figures					Morphology figures				
Figure	Treatment	Neurons	Cultures	Animals	Figure	Treatment	Neurons	Cultures	Animals
1c,d	Control	25	11	5	2b–d	Control	26	19	16
1c,d	NPY (10^{-7} M)	25	13	7	2b–d	NPY (10^{-10} M)	26	14	3
1g	Control	25	14	8	2b–d	NPY (10^{-9} M)	25	16	15
1g	CRF (3×10^{-8} M)	25	16	9	2b–d	NPY (10^{-8} M)	21	16	14
2a	Control	42	20	18	2b–d	NPY (10^{-7} M)	24	16	13
2a	NPY (10^{-10} M)	31	15	3	2b–d	da-NPY (10^{-7} M)	20	12	11
2a	NPY (10^{-9} M)	43	25	23	3d,e	Control	15	11	6
2a	NPY (10^{-8} M)	47	27	20	3d,e	NPY (10^{-7} M)	15	10	6
2a	NPY (10^{-7} M)	49	26	21	3d,e	CRF (3×10^{-8} M)	15	10	5
2a	da-NPY (10^{-7} M)	30	14	13	3d,e	cPP (10^{-7} M)	15	8	5
4a	Control	39	19	14	4b–d	Control	33	19	14
4a	CRF (3×10^{-10} M)	40	21	11	4b–d	CRF (3×10^{-10} M)	31	19	11
4a	CRF (3×10^{-9} M)	36	18	11	4b–d	CRF (3×10^{-9} M)	26	15	11
4a	CRF (3×10^{-8} M)	38	20	11	4b–d	CRF (3×10^{-8} M)	28	18	10
5a	Control	42	24	21	5b–d	Control	26	21	16
5a	CRF–NPY	31	15	6	5b–d	CRF–NPY	23	13	6
5a	NPY–CRF	32	15	7	5b–d	NPY–CRF	23	12	6
5h	CRF–NPY	25	12	5	6b–d	Control	27	15	13
5h	NPY–CRF	25	10	4	6b–d	F ⁷ p ³⁴ –NPY (10^{-9} M)	19	14	12
6a	Control	30	22	19	6b–d	F ⁷ p ³⁴ –NPY (10^{-8} M)	18	15	10
6a	F ⁷ p ³⁴ –NPY (10^{-9} M)	28	19	15	6b–d	F ⁷ p ³⁴ –NPY (10^{-7} M)	22	14	13
6a	F ⁷ p ³⁴ –NPY (10^{-8} M)	34	20	14	8b–d	Control	23	11	9
6a	F ⁷ p ³⁴ –NPY (10^{-7} M)	34	22	18	8b–d	ahx (10^{-9} M)	19	11	10
7c	Control	25	15	9	8b–d	ahx (10^{-8} M)	23	15	13
7c	F ⁷ p ³⁴ –NPY (10^{-7} M)	25	18	13	8b–d	ahx (10^{-7} M)	23	13	10
7f,g	Control	25	15	9	9b–d	Control	26	17	13
7f,g	cPP (10^{-7} M)	25	14	8	9b–d	cPP (10^{-9} M)	23	17	11
8a	Control	26	12	9	9b–d	cPP (10^{-8} M)	31	17	12
8a	ahx (10^{-9} M)	23	10	8	9b–d	cPP (10^{-7} M)	32	17	13
8a	ahx (10^{-8} M)	21	12	8	9b–d	NPY (10^{-8} M) + CGP (3×10^{-8} M)	34	17	5
8a	ahx (10^{-7} M)	25	14	10	11b–d	Control	26	17	4
9a	Control	30	20	13	11b–d	CsA (2×10^{-9} M)	26	14	4
9a	cPP (10^{-9} M)	32	18	12	11b–d	CsA (2×10^{-9} M) + NPY (10^{-8} M)	30	17	4
9a	cPP (10^{-8} M)	47	26	16	11b–d	OA (10^{-8} M)	26	12	4
9a	cPP (10^{-7} M)	34	23	15	11b–d	OA (10^{-8} M) + cPP (10^{-7} M)	24	13	4
9a	NPY (10^{-8} M) + CGP (3×10^{-8} M)	45	25	6	11f,g	Control	26	13	10
11a	Control	28	17	4	11f,g	AIP (4×10^{-8} M)	21	10	4
11a	CsA (2×10^{-9} M)	35	16	4	11f,g	AIP (4×10^{-8} M) + CRF (3×10^{-8} M)	21	11	4
11a	CsA (2×10^{-9} M) + NPY (10^{-8} M)	31	18	4					
11a	OA (10^{-8} M)	39	15	4					
11a	OA (10^{-8} M) + cPP (10^{-7} M)	38	15	4					
11e	Control	28	15	11					
11e	AIP (4×10^{-8} M)	27	13	4					
11e	AIP (4×10^{-8} M) + CRF (3×10^{-8} M)	29	14	4					

in BLA activates all three NPY receptors: Y₁Rs, which acutely hyperpolarize BLA PNs; Y₂Rs, which reduce dendritic GABA_BR activation; and Y₅Rs, whose long-term effects rely on the preferential activation of calcineurin. In this scenario, the actions of the three separate NPY receptors would synergize to facilitate the Y₅R effect by modestly elevating Ca²⁺ influx into BLA PNs (Fig. 12a) while preventing the larger increases in [Ca²⁺]_i that would preferentially activate the CaMKII pathway favored by the CRF system (Fig. 12b). This model could in part also explain the greater potency of the pan-agonist NPY to affect dendritic structure. Nonetheless, further work is needed to more completely understand the pathways and mechanisms involved in this long-term structural plasticity.

Regulation of cytosolic protein phosphorylation plays a key role in the structural plasticity seen here, consistent with evidence that calcineurin and CaMKII act as downstream targets of

anxiolytic and anxiogenic molecules in the BLA (Lin et al., 2003; Rainnie et al., 2004; Mineur et al., 2014). Both enzymes are inherently associated with synaptic plasticity in the BLA, so CRF- and NPY-mediated alterations in dendritic structure might also engage mechanisms that underlie LTP and LTD, respectively (Pape and Pare, 2010). As LTP and LTD each require different levels of [Ca²⁺]_i, studies on intradendritic Ca²⁺ dynamics could address the contributions made by acute or chronic changes in Ca²⁺ influx, either via NMDARs (Rainnie et al., 2004) or voltage-dependent Ca²⁺ channels, to dendritic remodeling (Pape and Pare, 2010).

Total dendritic length and whole-cell capacitance were tightly correlated in BLA OTC PNs over a variety of treatments (Fig. 13), were seen in BLA both *in vitro* and *in vivo*, and corresponded with long-term behavioral changes. Despite the potent acute behavioral effects of Y₁R agonists, they altered

neither dendritic structure nor long-term behavior. It appears reasonable to speculate that structural changes mediated by repeated peptide treatments result in the long-term changes in behavioral responses to stress. In any case, this structural plasticity could provide a novel *in vitro* bioassay predicting efficacy of drug candidates.

OTCs have proved useful in the study of longer-term nervous system changes, such as synaptic plasticity (Debanne et al., 1999; Selcher et al., 2012), and those associated with pathologies, such as chronic pain (Lu et al., 2006), or even prion disorders (Campeau et al., 2013). Since such changes can involve alterations not only in synaptic microanatomy but also more robust changes in cellular architecture, the preservation of anatomic relations within the OTC is a distinct advantage. PNs in BLA OTCs mature in culture to develop electrophysiological attributes that are distinct from those measured in BLA slices from P14 rats yet are not completely similar to those in age-matched (10W) *ex vivo* BLA; additionally, they show increased compactness compared with neurons from either *ex vivo* preparation. Also, in BLA OTCs, capacitance values were smaller, I_h activation kinetics (τ_{fast} and τ_{slow}) were faster, and sEPSC and sIPSC amplitudes were larger compared with the acute *ex vivo* neurons (Table 1). However, the properties of BLA OTC PNs, as indicated by action potential properties, firing rates, and AHPs, suggest that growth within OTCs constrains the physical extent of otherwise physiologically mature OTC neurons.

In conclusion, we report a novel, entirely reversible alteration in PN dendritic structure that accompanies the long-term increase in behavioral stress resilience caused by NPY in the BLA, the mirror opposite of equivalent CRF actions. This action is unexpectedly mediated by the Y_5R , considered up to now a relatively minor player in mediating NPY's anxiolytic actions. These robust changes are readily observed in BLA OTCs, and are reflected nearly perfectly *in vivo*. Interestingly, the short- and longer-term actions of NPY are mediated by three different receptors, acting at three different targets, to induce complex and comprehensive changes in postsynaptic properties and presynaptic connections in BLA PNs. The acute and long-term actions of both NPY and CRF involve regulation of the same elements of the PN (I_h , dendrites, synaptic inputs), changes that correlate with, and in some cases (I_h) determine, alterations in both excitability and behavior. The coordinated, countervailing regulation of these PN properties by NPY and CRF suggests that other signals could access this machinery to regulate stress responses mediated by the BLA.

References

- Adamec R, Hebert M, Blundell J, Mervis RF (2012) Dendritic morphology of amygdala and hippocampal neurons in more and less predator stress responsive rats and more and less spontaneously anxious handled controls. *Behav Brain Res* 226:133–146.
- Beck-Sickinger AG, Grouzmann E, Hoffmann E, Gaida W, van Meir EG, Waerber B, Jung G (1992) A novel cyclic analog of neuropeptide Y specific for the Y_2 receptor. *Eur J Biochem* 206:957–964.
- Berglund MM, Schober DA, Statnick MA, McDonald PH, Gehlert DR (2003) The use of bioluminescence resonance energy transfer 2 to study neuropeptide Y receptor agonist-induced beta-arrestin 2 interaction. *J Pharmacol Exp Ther* 306:147–156.
- Böhme I, Stichel J, Walther C, Mörl K, Beck-Sickinger AG (2008) Agonist induced receptor internalization of neuropeptide Y receptor subtypes depends on third intracellular loop and C-terminus. *Cell Signal* 20:1740–1749.
- Cabrele C, Beck-Sickinger AG (2000) Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J Pept Sci* 6:97–122.
- Cabrele C, Wieland HA, Langer M, Stidsen CE, Beck-Sickinger AG (2001) Y-receptor affinity modulation by the design of pancreatic polypeptide/neuropeptide Y chimera led to $Y(5)$ -receptor ligands with picomolar affinity. *Peptides* 22:365–378.
- Campeau JL, Wu G, Bell JR, Rasmussen J, Sim VL (2013) Early increase and late decrease of Purkinje cell dendritic spine density in prion-infected organotypic mouse cerebellar cultures. *PLoS One* 8:e81776.
- Chee MJ, Myers MG Jr, Price CJ, Colmers WF (2010) Neuropeptide Y suppresses anorexigenic output from the ventromedial nucleus of the hypothalamus. *J Neurosci* 30:3380–3390.
- Cohen P, Holmes CF, Tsukitani Y (1990) Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem Sci* 15:98–102.
- De Simoni A, Griesinger CB, Edwards FA (2003) Development of rat CA1 neurones in acute versus organotypic slices: role of experience in synaptic morphology and activity. *J Physiol* 550:135–147.
- Debanne D, Gähwiler BH, Thompson SM (1999) Heterogeneity of synaptic plasticity at unitary CA3-CA1 and CA3-CA3 connections in rat hippocampal slice cultures. *J Neurosci* 19:10664–10671.
- Ehrlich DE, Ryan SJ, Rainnie DG (2012) Postnatal development of electrophysiological properties of principal neurons in the rat basolateral amygdala. *J Physiol* 590:4819–4838.
- Ehrlich DE, Ryan SJ, Hazra R, Guo JD, Rainnie DG (2013) Postnatal maturation of GABAergic transmission in the rat basolateral amygdala. *J Neurophysiol* 110:926–941.
- File SE, Seth P (2003) A review of 25 years of the social interaction test. *Eur J Pharmacol* 463:35–53.
- Gähwiler BH (1981) Morphological differentiation of nerve cells in thin organotypic cultures derived from rat hippocampus and cerebellum. *Proc R Soc Lond B Biol Sci* 211:287–290.
- Giesbrecht CJ, Mackay JP, Silveira HB, Urban JH, Colmers WF (2010) Countervailing modulation of I_h by neuropeptide Y and corticotrophin-releasing factor in basolateral amygdala as a possible mechanism for their effects on stress-related behaviors. *J Neurosci* 30:16970–16982.
- Gutman AR, Yang Y, Ressler KJ, Davis M (2008) The role of neuropeptide Y in the expression and extinction of fear-potentiated startle. *J Neurosci* 28:12682–12690.
- Heilig M, Koob GF, Ekman R, Britton KT (1994) Corticotropin-releasing factor and neuropeptide Y: role in emotional integration. *Trends Neurosci* 17:80–85.
- Hill MN, Hillard CJ, McEwen BS (2011) Alterations in corticolimbic dendritic morphology and emotional behavior in cannabinoid CB1 receptor-deficient mice parallel the effects of chronic stress. *Cereb Cortex* 21:2056–2064.
- Holliday ND, Lam CW, Tough IR, Cox HM (2005) Role of the C terminus in neuropeptide Y Y_1 receptor desensitization and internalization. *Mol Pharmacol* 67:655–664.
- Humpel C (2015) Organotypic brain slice cultures: a review. *Neuroscience* 305:86–98.
- Ishida A, Kameshita I, Okuno S, Kitani T, Fujisawa H (1995) A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. *Biochem Biophys Res Commun* 212:806–812.
- Kim H, Kim E, Park M, Lee E, Namkoong K (2013) Organotypic hippocampal slice culture from the adult mouse brain: a versatile tool for translational neuropsychopharmacology. *Prog Neuropsychopharmacol Biol Psychiatry* 41:36–43.
- Kopp J, Xu ZQ, Zhang X, Pedrazzini T, Herzog H, Kresse A, Wong H, Walsh JH, Hökfelt T (2002) Expression of the neuropeptide Y Y_1 receptor in the CNS of rat and of wild-type and Y_1 receptor knock-out mice. Focus on immunohistochemical localization. *Neuroscience* 111:443–532.
- Leitermann RJ, Rostkowski AB, Urban JH (2016) Neuropeptide Y input to the rat basolateral amygdala complex and modulation by conditioned fear. *J Comp Neurol* 524:2418–2439.
- Lin CH, Yeh SH, Leu TH, Chang WC, Wang ST, Gean PW (2003) Identification of calcineurin as a key signal in the extinction of fear memory. *J Neurosci* 23:1574–1579.
- Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 66:807–815.
- Lu VB, Colmers WF, Smith PA (2009) Long-term effects of brain-derived neurotrophic factor on the frequency of inhibitory synaptic events in the rat superficial dorsal horn. *Neuroscience* 161:1135–1143.

- Lu VB, Moran TD, Balasubramanyan S, Alier KA, Dryden WF, Colmers WF, Smith PA (2006) Substantia gelatinosa neurons in defined-medium organotypic slice culture are similar to those in acute slices from young adult rats. *Pain* 121:261–275.
- Mackay JP, Bompalaki M, DeJoseph MR, Michaelson SD, Urban JH, Colmers WF (2019) NPY2 receptors reduce tonic action potential-independent GABAB currents in the basolateral amygdala. *J Neurosci* 39:4909–4930.
- Marley A, Choy RW, von Zastrow M (2013) GPR88 reveals a discrete function of primary cilia as selective insulators of GPCR cross-talk. *PLoS One* 8:e70857.
- McDonald AJ, Zaric V (2015) Extrinsic origins of the somatostatin and neuropeptide Y innervation of the rat basolateral amygdala. *Neuroscience* 294:82–100.
- McEwen BS (2003) Mood disorders and allostatic load. *Biol Psychiatry* 54:200–207.
- Michel MC, Beck-Sickinger A, Cox H, Doods HN, Herzog H, Larhammar D, Quirion R, Schwartz T, Westfall T (1998) XVI: International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol Rev* 50:143–150.
- Mineur YS, Taylor SR, Picciotto MR (2014) Calcineurin downregulation in the amygdala is sufficient to induce anxiety-like and depression-like behaviors in C57BL/6J male mice. *Biol Psychiatry* 75:991–998.
- Mitra R, Jadhav S, McEwen BS, Vyas A, Chattarji S (2005) Stress duration modulates the spatiotemporal patterns of spine formation in the basolateral amygdala. *Proc Natl Acad Sci USA* 102:9371–9376.
- Molosh AI, Sajdyk TJ, Truitt WA, Zhu W, Oxford GS, Shekhar A (2013) NPY Y1 receptors differentially modulate GABAA and NMDA receptors via divergent signal-transduction pathways to reduce excitability of amygdala neurons. *Neuropsychopharmacology* 38:1352–1364.
- Padival MA, Blume SR, Rosenkranz JA (2013) Repeated restraint stress exerts different impact on structure of neurons in the lateral and basal nuclei of the amygdala. *Neuroscience* 246:230–242.
- Pape HC, 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, Watson C (1986) The rat brain in stereotaxic coordinates. Sydney: Academic.
- Phelps EA, LeDoux JE (2005) Contributions of the amygdala to emotion processing: from animal models to human behavior. *Neuron* 48:175–187.
- Rainnie DG, Bergeron R, Sajdyk T, Patil M, Gehlert D, Shekhar A (2004) Corticotrophin releasing factor-induced synaptic plasticity in the amygdala translates stress into emotional disorders. *J Neurosci* 24:3471–3479.
- Roosendaal B, McEwen BS, Chattarji S (2009) Stress, memory and the amygdala. *Nat Rev Neurosci* 10:423–433.
- Sajdyk TJ, Johnson PL, Leitermann RJ, Fitz SD, Dietrich A, Morin M, Gehlert DR, Urban JH, Shekhar A (2008) Neuropeptide Y in the amygdala induces long-term resilience to stress-induced reductions in social responses but not hypothalamic-adrenal-pituitary axis activity or hyperthermia. *J Neurosci* 28:893–903.
- Sajdyk TJ, Schober DA, Gehlert DR (2002a) Neuropeptide Y receptor subtypes in the basolateral nucleus of the amygdala modulate anxiogenic responses in rats. *Neuropharmacology* 43:1165–1172.
- Sajdyk TJ, Schober DA, Smiley DL, Gehlert DR (2002b) Neuropeptide Y-Y2 receptors mediate anxiety in the amygdala. *Pharmacol Biochem Behav* 71:419–423.
- Sajdyk TJ, Shekhar A, Gehlert DR (2004) Interactions between NPY and CRF in the amygdala to regulate emotionality. *Neuropeptides* 38:225–234.
- Schmeltzer SN, Herman JP, Sah R (2016) Neuropeptide Y (NPY) and post-traumatic stress disorder (PTSD): a translational update. *Exp Neurol* 284:196–210.
- Selcher JC, Xu W, Hanson JE, Malenka RC, Madison DV (2012) Glutamate receptor subunit GluA1 is necessary for long-term potentiation and synapse unsilencing, but not long-term depression in mouse hippocampus. *Brain Res* 1435:8–14.
- Sholl DA (1953) Dendritic organization in the neurons of the visual and motor cortices of the cat. *J Anat* 87:387–406.
- Silveira Villarroel H, Bompalaki M, Mackay JP, Miranda Tapia AP, Michaelson SD, Leitermann RJ, Marr RA, Urban JH, Colmers WF (2018) NPY induces stress resilience via downregulation of I_h in principal neurons of rat basolateral amygdala. *J Neurosci* 38:4505–4520.
- Söll RM, Dinger MC, Lundell I, Larhammer D, Beck-Sickinger AG (2001) Novel analogues of neuropeptide Y with a preference for the Y1-receptor. *Eur J Biochem* 268:2828–2837.
- Stanić D, Mulder J, Watanabe M, Hökfelt 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.
- Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* 37:173–182.
- Taylor AL (2012) What we talk about when we talk about capacitance measured with the voltage-clamp step method. *J Comput Neurosci* 32:167–175.
- Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002) Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci* 22:6810–6818.
- Vyas A, Pillai AG, Chattarji S (2004) Recovery after chronic stress fails to reverse amygdaloid neuronal hypertrophy and enhanced anxiety-like behavior. *Neuroscience* 128:667–673.
- Vyas A, Jadhav S, Chattarji S (2006) Prolonged behavioral stress enhances synaptic connectivity in the basolateral amygdala. *Neuroscience* 143:387–393.
- Wahlestedt C, Yanaihara N, Håkanson R (1986) Evidence for different pre- and post-junctional receptors for neuropeptide Y and related peptides. *Regul Pept* 13:307–318.
- Wolak ML, DeJoseph MR, Cator AD, Mokashi AS, Brownfield MS, Urban JH (2003) Comparative distribution of neuropeptide Y Y1 and Y5 receptors in the rat brain by using immunohistochemistry. *J Comp Neurol* 464:285–311.
- Yehuda R, Brand S, Yang RK (2006) Plasma neuropeptide Y concentrations in combat exposed veterans: relationship to trauma exposure, recovery from PTSD, and coping. *Biol Psychiatry* 59:660–663.
- Zhou Z, Zhu G, Hariri AR, Enoch MA, Scott D, Sinha R, Virkkunen M, Mash DC, Lipsky RH, Hu XZ, Hodgkinson CA, Xu K, Buzas B, Yuan Q, Shen PH, Ferrell RE, Manuck SB, Brown SM, Hauger RL, Stohler CS, et al. (2008) Genetic variation in human NPY expression affects stress response and emotion. *Nature* 452:997–1001.