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NPY Y_1 Receptors Differentially Modulate $GABA_A$ and NMDA Receptors via Divergent Signal-Transduction Pathways to Reduce Excitability of Amygdala Neurons

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Neuropeptide Y (NPY) administration into the basolateral amygdala (BLA) decreases anxiety-like behavior, mediated in part through the Y_1 receptor (Y_1R) isoform. Activation of Y_1Rs results in G-protein-mediated reduction of cAMP levels, which results in reduced excitability of amygdala projection neurons. Understanding the mechanisms linking decreased cAMP levels to reduced excitability in amygdala neurons is important for identifying novel anxiolytic targets. We studied the intracellular mechanisms of activation of Y_1Rs on synaptic transmission in the BLA. Activating Y_1Rs by [Leu^{31},Pro^{34}]-NPY (L-P NPY) reduced the amplitude of evoked NMDA-mediated excitatory postsynaptic currents (eEPSCs), without affecting AMPA-mediated eEPSCs, but conversely increased the amplitude of $GABA_A$-mediated evoked inhibitory postsynaptic currents (eIPSCs). Both effects were abolished by the Y_1R antagonist, PD160170. Intracellular GDP-β-S, or pre-treatment with either forskolin or 8Br-cAMP, eliminated the effects of L-P NPY on both NMDA- and $GABA_A$-mediated currents. Thus, both the NMDA and $GABA_A$ effects of Y_1R activation in the BLA are G-protein-mediated and cAMP-dependent. Pipette inclusion of protein kinase A (PKA) catalytic subunit blocked the effect of L-P NPY on $GABA_A$-mediated eIPSCs, but not on NMDA-mediated eEPSCs. Conversely, activating the exchange protein activated by cAMP (Epac) with 8CPT-2Me-cAMP blocked the effect of L-P NPY on NMDA-mediated eEPSCs, but not on $GABA_A$-mediated eIPSCs. Thus, NPY regulates amygdala excitability via two signal-transduction events, with reduced PKA activity enhancing $GABA_A$-mediated eIPSCs and Epac deactivation reducing NMDA-mediated eEPSCs. This multipathway regulation of NMDA- and $GABA_A$-mediated currents may be important for NPY plasticity and stress resilience in the amygdala.

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INTRODUCTION

Neuropeptide Y (NPY) is widely distributed in the central and peripheral nervous system (Herzog *et al*, 1992; Tatemoto *et al*, 1982), where it binds to a family of G-coupled receptors. To date, five NPY receptor subtypes have been cloned (Y_1, Y_2, Y_4, Y_5, and Y_6) from different species (Michel *et al*, 1998). NPY has been reported to influence and regulate brain circuits involved in a number of behaviors, such as food intake, water consumption, emotion, learning, memory, and locomotion (Bertocchi *et al*, 2011; Edelsbrunner *et al*, 2009; Gehlert, 1999; Heilig, 2004; Heilig and Murison, 1987; Kask *et al*, 2002). NPY is also suggested to play a key role in many neuropsychiatric disorders, including post-traumatic stress disorder, anxiety, depression, eating disorders, and epilepsy (Heilig, 2004; Heilig and Widerlov, 1995; Lin *et al*, 2006; Sah and Geracioti, 2012; Thorsell, 2010; Wahlestedt *et al*, 1993a).

The amygdala is a crucial region in the brain circuitry implicated in many of these psychiatric syndromes and is a well-known component of fear, anxiety, and memory circuits (Fendt and Fanselow, 1999; LeDoux, 2000; Maren and Quirk, 2004; Pare *et al*, 2004; Shekhar *et al*, 2005). NPY and its receptors are present in the amygdala (Holmes *et al*, 2003; Kask *et al*, 2002; Kishi *et al*, 2005; Wolak *et al*, 2003), leading to the hypothesis that the anxiolytic-like effects of NPY are mediated in part by the amygdala. Furthermore, NPY injection into the basolateral amygdala (BLA) produces resilience to restraint stress, as measured in the social interaction test (Sajdyk *et al*, 2008). Among the many NPY receptors, the Y_1 subtype has been implicated in mediating anxiolytic behaviors (Karl *et al*, 2006; Kask *et al*, 2002;

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Sajdyk *et al*, 1999, 2002; Sorensen *et al*, 2004). *In situ* hybridization reveals the distribution of Y_1 receptor (Y_1R) mRNA in the brain of several mammalian species, with the highest levels of expression consistently seen in forebrain regions, including the cerebral cortex, the hippocampal formation, and several amygdaloid, thalamic, and hypothalamic nuclei (Kopp *et al*, 2002; Mikkelsen and Larsen, 1992; Parker and Herzog, 1999; Wolak *et al*, 2003). Central administration of Y_1R agonists elicits a potent anxiolytic effect in rodents, whereas mice lacking the *Y_1* gene display anxiety-like behavior in certain animal models (Karl *et al*, 2006). In addition, the inhibition of Y_1R expression with injection of antisense oligonucleotides into the amygdala prevented the anxyiolytic action of NPY in rats (Heilig, 1995). Furthermore, preadministration of the Y_1R antagonist BIBO 3304 blocks the anxiolytic effect of NPY injection into the BLA (Sajdyk *et al*, 1999).

Major cell types in the BLA include pyramidal (glutamatergic) and non-pyramidal (GABAergic) neurons (Hall, 1972; McDonald, 1982; McDonald and Pearson, 1989). Y_1Rs are expressed on both BLA pyramidal and non-pyramidal neurons, where they are postsynaptically localized (Rostkowski *et al*, 2009; Stanic *et al*, 2011). A functional interaction between GABAergic and Y_1Rs mediating transmission was first demonstrated in the cortical region (Kask *et al*, 1996) and, subsequently, in other brain regions, such as posterior hypothalamus (Naveilhan *et al*, 2001) and central and medial amygdala (Oberto *et al*, 2000, 2001) of mice. Treatment with positive (diazepam and abecarnil) or negative (FG7142) modulators of $GABA_A$ receptor function induces, respectively, significant increases or decreases of Y_1R gene expression in the medial amygdala (Oberto *et al*, 2000). However, the mechanisms by which Y_1Rs modulate GABAergic function, however, remain to be unknown.

Recently, Giesbrecht *et al* (2010) suggested that NPY, acting through the Y_1R subtype, also inhibits pyramidal neurons in the BLA by suppressing a hyperpolarization-activated, depolarizing current (I_h). The intracellular mechanisms by which NPY receptor activation in the BLA produces this additional postsynaptic membrane effect are also not well understood.

All NPY receptors have been shown to mediate their responses through $G_{i/o}$ proteins, which inhibit the accumulation of cAMP (Bard *et al*, 1995; Gerald *et al*, 1995, 1996; Herzog *et al*, 1992; Larhammar *et al*, 1992; Lundell *et al*, 1995; Mullins *et al*, 2000). Several additional intracellular signaling pathways of NPY have been reported in peripheral tissues or cell lines. For example, a mitogen-activated protein kinase pathway is involved in Y_1R signaling in gut epithelial cells (Mannon and Mele, 2000), whereas a protein kinase C-dependent pathway is involved in Y_1, Y_2, Y_4, and Y_5 receptor signaling in Chinese hamster ovary cells (Mullins *et al*, 2002; Zhang *et al*, 2011a). To investigate the intracellular mechanisms by which activation of Y_1Rs and subsequent reduction of cAMP levels modulate the inhibitory and excitatory ionotropic systems in the BLA, we combined whole-cell patch-clamp techniques with selective pharmacological interventions and biochemistry. Our results reveal distinct and novel Y_1R-mediated mechanisms utilizing divergent signaling cascades to regulate selectively distinct postsynaptic receptor populations.

MATERIALS AND METHODS

Animals

All animals used for this study were male Wistar rats (100–150 g) obtained from Harlan Laboratories (Indianapolis, IN). Animals were housed in a temperature-controlled room (21–22 °C) with a 12-h light/dark cycle schedule and given food and water *ad libitum*. All the procedures used were approved by the Institutional Animal Care and Use Committee (IACUC) of Indiana University–Purdue University Indianapolis and were in compliance with National Institutes of Health (NIH) *Guidelines for the Care and Use of Laboratory Animals*.

Slice Preparation and Patch-Clamp Recording

Rats were quickly decapitated and coronal slices (350 μm) containing the BLA were obtained using standard procedures (Brittain *et al*, 2011; Rainnie, 1999). Slices were incubated in oxygenated ACSF with the following composition (in mM): 130 NaCl, 3.5 KCl, 1.1 KH_2PO_4, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 10 glucose, and 30 $NaHCO_3$ at room temperature for at least 1 h before recording. Slices were then transferred to a submersion-type chamber mounted on the stage of a Nikon E600FN Eclipse microscope (Nikon Instruments, Melville, NY) and perfused at a rate of 2–3 ml/min with ACSF heated to 30 °C. Whole-cell patch-clamp recordings were obtained using standard techniques with borosilicate glass electrodes (resistance 3–6 MΩ; WPI, Sarasota, FL) filled with an intracellular solution with the following composition (in mM: 140 K-gluconate, 2 KCl, 3 $MgCl_2$, 10 HEPES, 5 phosphocreatine, 2 K-ATP, and 0.2 Na-GTP) adjusted to pH 7.3 with KOH, and having an osmolarity of 280–290 mOsm. The internal pipette solution for the recording of miniature inhibitory postsynaptic current (mIPSC) had the following ionic composition (in mM): 140 KCl, 3 $MgCl_2$, 10 HEPES, 5 phosphocreatine, 2 K-ATP, and 0.2 Na-GTP. Whole-cell access resistance measured in voltage clamp ranged from 5 to 20 MΩ and was monitored throughout each experiment; a change of $\leqslant$15% was deemed acceptable.

The basolateral complex of the amygdala, including the lateral and basolateral nuclei, was visualized in the coronal slice as it was outlined laterally by the white matter tract of the external capsule (corpus callosum) and medially by the white matter tract of the longitudinal association bundle (Rainnie, 1999). Neuronal responses reported in this study were obtained only from pyramidal neurons located in the basolateral subdivision of this basolateral complex. Pyramidal neurons were identified according to their characteristic size and pyramidal shape. Whole-cell recordings were made with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) using the pClamp 10.2 software and a Digidata 1322A interface (Molecular Devices, Sunnyvale, CA).

Experimental Protocol

Evoked postsynaptic currents or potentials were generated using a concentric stimulating electrode (FHC, Bowdoinham, ME) placed on the fiber tract of the external capsule, and $\sim$500 μm from the recorded neuron. A paired-pulse paradigm with two stimuli of half-maximal intensity

separated by ~75 ms was used to evaluate the locus of action of Y_1R activation. Paired stimuli were repeated five times at a frequency of 0.2 Hz, and then averaged for subsequent data analysis. The paired pulses were delivered once every 2 min. Once a stable baseline of responses was obtained for at least 4 min, we applied a drug and continued recording throughout the course of drug application and washout. Paired-pulse ratio (PPR) was determined as the peak amplitude of the second paired stimulus current divided by the peak amplitude of first paired stimulus current (PSC1). For comparison between control, drug, and washout states, peak current amplitudes within the group were normalized to the mean PSC1 of two control peak amplitude values acquired immediately before the onset of drug application (approximately 4 min of control data). In these experiments, *N*-(2,6-dimethylphenylcarbamoyl methyl) triethylammonium bromide (QX-314, 2 mM) was included in the internal solution to block regenerative sodium spikes. NMDA-mediated excitatory postsynaptic currents (eEPSCs) were elicited from a holding potential of −40 mV, whereas AMPA-mediated eEPSCs were elicited from a holding potential of −60 mV. To isolate NMDA-mediated eEPSCs, the GABA receptor antagonists SR95531 (5 μM) and 3-(3,4-dichlorophenylmethylaminopropyl) diethoxymethyl phosphinic acid (CGP 52432, 1 μM) were applied to block $GABA_A$ and $GABA_B$ receptors, respectively. AMPA/kainate receptors were blocked using 6,7-dinitroquinoxaline-2,3-(1*H*,4*H*)-dione (DNQX, 20 μM). The NMDA-mediated eEPSCs were further confirmed by the application of the NMDA receptor-specific blocker CPP (1 μM) at the end of the experiment. The holding potential for measuring evoked inhibitory postsynaptic current (eIPSCs) was −50 mV. To isolate $GABA_A$ receptor-mediated eIPSCs, the AMPA/kainate and NMDA receptors were blocked using DNQX (20 μM) and CPP (1 μM), respectively. In addition, CGP 52432 (1 μM) was added to block $GABA_B$ receptors. At the end of the experiments, the $GABA_A$ antagonist SR95531 (1 μM) was applied to confirm that recorded currents were $GABA_A$-mediated eIPSCs. Access resistance was continuously monitored by injection of a voltage step (−3 mV, 50 ms) immediately before the beginning of stimulation pulse.

mIPSCs in BLA pyramidal cells were recorded at −60 mV in the whole-cell configuration in the presence of 500 nM tetrodotoxin (TTX), DNQX (20 μM), CPP (1 μM), and CGP 52432 (1 μM).

Drug Applications

Various drugs, diluted to the final concentration in ACSF, were delivered by gravity to the recording chamber containing the brain slice using a VC-6 control perfusion system (Harvard Apparatus, Holliston, MA), unless stated otherwise. During experiments with GDP-β-S, forskolin, 8Br-cAMP, cPKA, or 8CPT-2Me-cAMP, we preincubated cells for at least 30 min before adding [Leu^{31},Pro^{34}]-NPY (L-P NPY) to the recording chamber. The following drugs were obtained from (1) Sigma-Aldrich (St Louis, MO): K-gluconate, KCl, $MgCl_2$, HEPES, NaCl, KH_2PO_4, $CaCl_2$, glucose, $NaHCO_3$, KOH, phosphocreatine, K-ATP, Na-GTP, DNQX, 8Br-cAMP, GDP-β-S; (2) Tocris Cookson (Ellisville, MO): [Leu^{31}-Pro^{34}]-NPY, PD160170, (*RS*)-CPP, CGP 52432, SR95531, 8CPT-2Me-cAMP, forskolin, QX-314; and (3) EMD Chemicals (Gibbstown, NJ): protein kinase A (PKA) catalytic subunit.

Western Blot

BLA, hippocampus, and PFC tissue punches from rats, or CHOK1 and HEK293 cells, were lysed with buffer (30 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 10 mM EDTA, 1 mM Na_2VO_3, and 160 mM NaF) freshly supplemented with proteinase inhibitors, and protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Proteins (100–150 μg) were separated in 10% SDS-PAGE, and transferred to PVDF membranes. After a 30 min block with milk, the membranes were incubated with primary antibody (1 : 500 mouse anti-exchange protein activated by cAMP (Epac)1 or Epac2 (Cell Signaling Technology, Danvers, MA), 1 : 250 rabbit anti-Epac and Epac2 (Santa Cruz Biotechnology, Santa Cruz, CA); and 1 : 3 000 mouse GAPDH antibody (Millipore, Billerica, MA)) for overnight at 4 °C. After three TBS-T washes, the membranes were incubated with 1 : 10 000 goat anti-rabbit antibody conjugated with Alexa Fluor 680 or 1 : 10 000 goat anti-mouse antibody conjugated with IR dye 800 (Invitrogen, Carslbad, CA) for 1 h at room temperature. After three TBS-T washes, membranes were scanned on an Odyssey plate reader (Li-Cor Biosciences, Lincoln, NE) at channel 700 or 800.

Data Analysis and Statistics

eIPSC data were analyzed using pClamp 10.2 (Molecular Devices, Sunnyvale, CA). Spontaneous mIPSCs were analyzed using the MiniAnalysis program (Synaptosoft, Decatur, GA). All events were identified visually to avoid errors in detection by automation. The threshold for detection of currents was set at three times the root mean square baseline noise.

Statistics and graphs were produced using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). All data are presented as the mean ± SEM. Differences among multiple groups were evaluated either by repeated measures (RM) two-way ANOVA or one-way ANOVA when warranted. In the presence of significant main effects, *post hoc* pairwise comparisons were conducted using Dunnett's to compare back to baseline values and Tukey's or Fisher's LSD tests between groups. The confidence level for significance in all tests was set at $P < 0.05$.

RESULTS

All recordings were performed on pyramidal neurons, which were identified based upon their location, the pyramidal-shaped soma. Only those neurons ($n = 240$) that were visually identified as BLA pyramidal neurons were included in this study (Rainnie *et al*, 1993). A typical response to a step current injection is shown in Figure 1a. In response to depolarizing current injection of increasing amplitude, neurons initially fired either a single action potential or a doublet/triplet burst, after which a slower, more rhythmic firing pattern was observed (Figure1a, top). These neurons also showed a depolarizing sag in the voltage

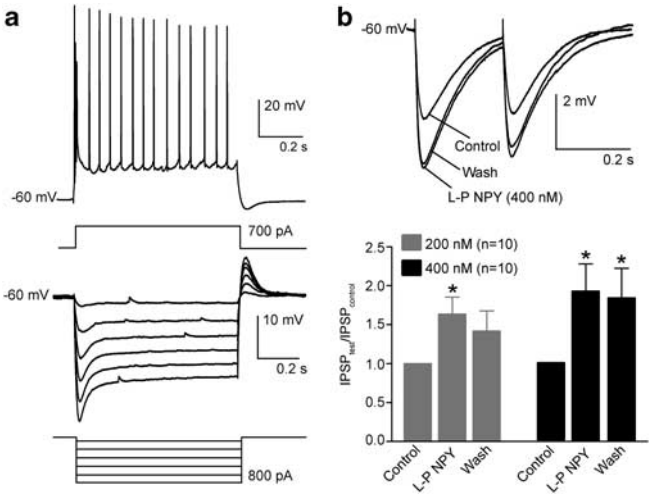


Figure 1 Application of Y_1 receptor (Y_1R) agonist [Leu^{31},Pro^{34}]-neuropeptide Y (L-P NPY) increases the amplitude of evoked inhibitory postsynaptic currents (eIPSPs). (a) Current-clamp recordings showing the response of a typical basolateral amygdala (BLA) pyramidal neuron to transient depolarizing (upper trace) and hyperpolarizing (lower traces) current injection. (b) At a holding potential of −60 mV, L-P NPY induced an increase of amplitude of eIPSPs during application (6 min) and wash periods (15 min) (upper traces). Summary graph showing the effect of L-P NPY in concentrations of 200 nm (gray) and 400 nM (black) on eIPSPs amplitude (bottom). *Significantly different compared with control ($P<0.05$).

excursion in response to hyperpolarizing current pulses (Figure1a, bottom) (Womble and Moises, 1993). Initially, using current-clamp mode, we showed that bath pre-treatment with L-P NPY (200 or 400 nM) induced a significant increase of evoked inhibitory postsynaptic potentials (eIPSPs) (RM-ANOVA 200 nM L-P NPY: $F_{(2,18)}=5.13$, $P=0.017$; 400 nM L-P NPY: $F_{(2,18)}=5.8$, $P=0.011$; Figure 1b), which differed significantly from baseline during both 200 and 400 nM drug application ($P=0.01$ and 0.01, respectively, Dunnett's) and remained significantly increased from baseline during wash for 400 nM dose ($P=0.02$, Dunnett's; Figure 1b). Furthermore, no significant change in input resistance (MΩ) was observed after the application of L-P NPY (400 nM) (baseline: 70.6 ± 7.7; L-P NPY (400 nM): 66.6 ± 5.3; wash: 66.1 ± 4.7; $F_{(2,18)}=0.69$, $P=0.513$; data not shown). Finally, we observed no consistent responses of the resting membrane potential to L-P NPY application (baseline (mean ± SEM, mV): −60.4 ± 0.16; L-P NPY: −60.1 ± 0.56; wash: −61.2 ± 0.6; $F_{(2,18)}=1.5$, $P=0.242$) that would suggest modulation of HCN channels underlying I_h as reported previously (Giesbrecht *et al*, 2010).

Activating Y_1Rs Increases $GABA_A$, but Decreases NMDA-Mediated Postsynaptic Currents

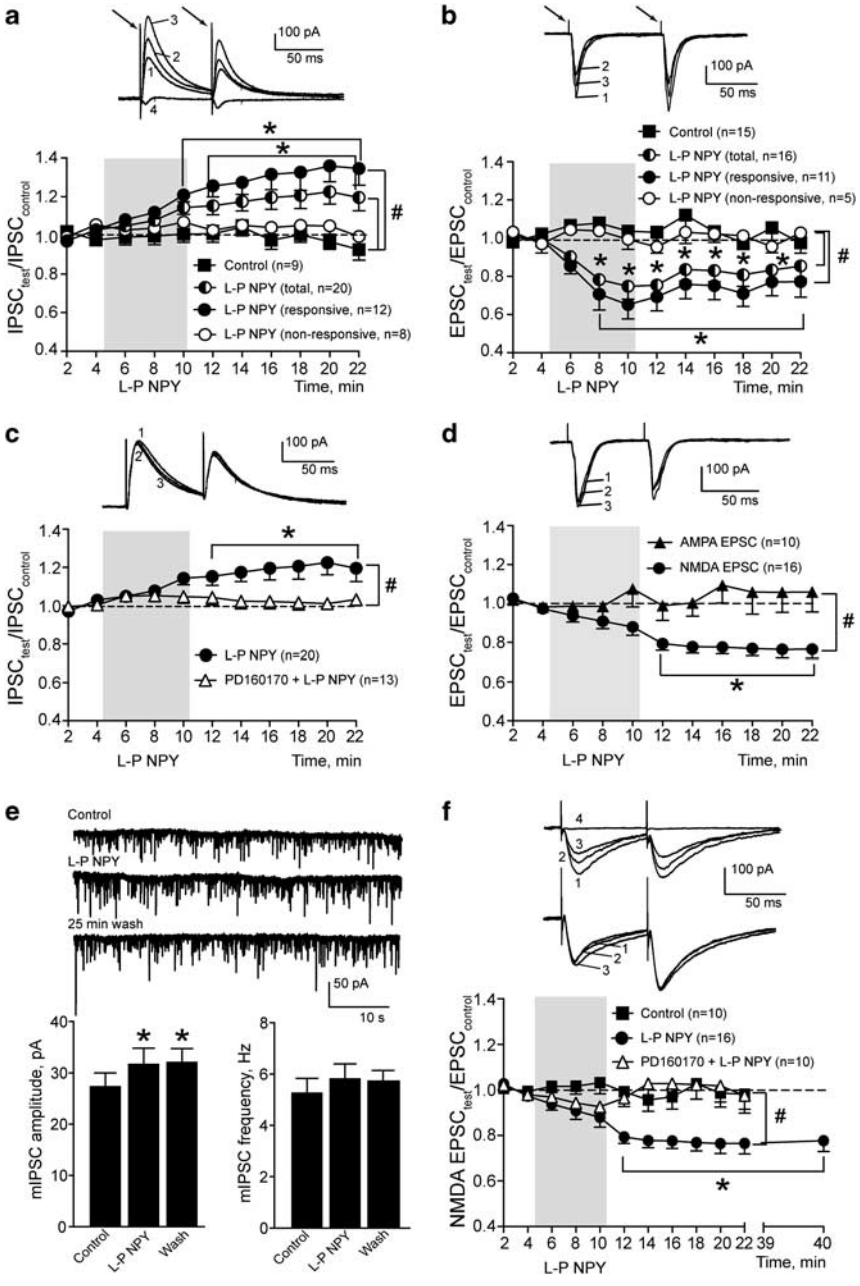
All further experiments were performed in voltage-clamp mode to record postsynaptic currents. The chloride equilibrium potential for our recording conditions was −75 mV; therefore, to further study GABA-mediated eIPSCs, we used a holding potential of −50 mV to enhance the currents. We also included QX-314 (2 mM) in the internal solution to block regenerative sodium spikes. Because addition of QX-314 blocks voltage-activated sodium channels obscuring assessment of firing properties, for these experiments pyramidal BLA cells were selected based on their large pyramidal-shaped soma, the presence of a large dendrite, and low input resistance. To enable a direct comparison between the drug-treated and time-control groups, data were normalized to the peak eIPSC amplitude evoked immediately before L-P NPY application (see Materials and methods). Data from this experiment resulted in significant effects of time and a significant drug × time interaction when comparing L-P NPY (total) and control group (two-way RM-ANOVA, effect of time: $F_{(10,270)}=2.48$, $P=0.007$; treatment × time interaction: $F_{(10,270)}=2.49$, $P=0.007$; Figure 2a). The application of L-P NPY (400 nM, 6 min) induced a slowly developing and persistent increase of eIPSC amplitude (baseline: 1.03 ± 0.01; L-P NPY: 1.14 ± 0.04; wash: 1.20 ± 0.07), which, compared with baseline (4 min), reached significance at 12 min ($P<0.05$, 12–22 min, Dunnett's; Figure 2a). In some experiments, even after a 1 h wash period eIPSC amplitudes did not return to baseline levels (data not shown). Of the 20 BLA neurons examined, addition of L-P NPY to ACSF induced an increase of eIPSC amplitude in 12 neurons (60% of neurons; baseline: 0.97 ± 0.01; L-P NPY: 1.21 ± 0.04; wash: 1.35 ± 0.09; $P<0.05$, 10–22 min, Dunnett's; Figure 2a), whereas the remaining eight neurons (40%) showed no increase of eIPSC amplitude (baseline: 0.99 ± 0.02; L-P NPY: 1.07 ± 0.05; wash: 0.99 ± 0.06; $P>0.05$, Dunnett's; Figure 2a). The L-P NPY-induced increase is likely a postsynaptic effect as the PPR remained unchanged following L-P NPY (baseline: 0.8 ± 0.02; L-P NPY: 0.8 ± 0.02; wash: 0.9 ± 0.02; $F_{(10,219)}=0.86$, $P=0.57$). Addition of the $GABA_A$ antagonist SR95531 (1 μM) to the bath at the end of the experiment eliminated the eIPSCs (Figure 2a, inset trace 4). Moreover, an overall two-way RM-ANOVA revealed a significant treatment effect ($F_{(1,31)}=4.45$, $P=0.043$) and treatment *vs* time interaction ($F_{(10,310)}=3.56$, $P=0.0002$) when comparing eIPSC amplitudes of L-P NPY and PD160170 + L-P NPY groups (Figure 2c). Pre-treatment with the Y_1R antagonist PD160170 (1 μM) blocked the effect of L-P NPY on eIPSC amplitude (baseline: 1.004 ± 0.01; PD160170 + L-P NPY: 1.05 ± 0.04; wash: 1.03 ± 0.03; $P>0.05$, Dunnett's) without changing the PPR (baseline: 0.8 ± 0.07; PD160170 + L-P NPY: 0.8 ± 0.06; wash: 0.8 ± 0.06; $P>0.05$, Dunnett's; Figure 2c). Significant differences in amplitude of eIPSCs between PD160170 + L-P NPY and L-P NPY groups were also observed ($P<0.05$, 14–22 min, Fisher's LSD; Figure 2c).

The L-P NPY-induced facilitation of $GABA_A$ eIPSCs might be a result of a presynaptic increase of GABA release or a postsynaptic increase in response to GABA. To assess the functional locus of the Y_1R agonist, we analyzed the effect of L-P NPY on frequency and amplitude of mIPSCs. Under our experimental conditions in the presence of TTX (500 nM), we only observed increases in amplitude (RM-ANOVA baseline: 27.4 ± 2.6 pA; L-P NPY: 31.8 ± 3.04 pA; wash: 32.2 ± 2.6; treatment effect: $F_{(2,22)}=9.13$, $n=12$, $P=0.001$), but not frequency (RM-ANOVA baseline: 5.3 ± 0.6 Hz; L-P NPY: 5.8 ± 0.6 Hz; wash: 5.7 ± 0.4 Hz; treatment effect: $F_{(2.22)}=1.66$, $n=12$, $P=0.21$) of mIPSCs (Figure 2e). This effect of L-P NPY on the amplitude of mIPSCs again persisted during application of L-P NPY ($P=0.004$, Dunnett's) and through the 30 min wash period ($P=0.002$, Dunnett's). Taken together, these data support

the view that L-P NPY modulates postsynaptic GABA responses in pyramidal BLA neurons.

Next, we tested the effect of L-P NPY on glutamatergic eEPSCs. In this experiment, overall two-way RM-ANOVA revealed significant time ($F_{(10,290)} = 2.27$, $P < 0.015$) and treatment effects ($F_{(1,29)} = 11.44$, $P = 0.002$), as well as treatment *vs* time interaction ($F_{(10,290)} = 3.6$, $P = 0.0002$) between control and L-P NPY(total) groups (Figure 2b). At a holding potential of -60 mV, incubation with L-P NPY (400 nM) in the presence of $GABA_A$ and $GABA_B$ antagonists compared with baseline (4 min) caused a reduction of eEPSC amplitude (baseline: 1 ± 0.03; L-P NPY: 0.75 ± 0.07; wash: 0.85 ± 0.07), reaching significance at 8 min ($P < 0.05$, 8–20 min, Dunnett's) in BLA projection neurons (Figure 2b). There was a trend toward a gradual recovery in the eEPSC amplitude with time and at 22 min the amplitude of eEPSCs were not significantly different ($P = 0.082$, Dunnett's) from baseline values (Figure 2b). Interestingly, of 16 neurons included in the study, 11 neurons (68.8%) demonstrated a significant reduction of eEPSC amplitude (baseline: 1.01 ± 0.04, L-P NPY: 0.65 ± 0.08, wash: 0.77 ± 0.08; $P < 0.05$, 8–22 min, Dunnett's), whereas the other five neurons (31.2%) showed no significant changes in eEPSC amplitude (baseline: 1.03 ± 0.05; L-P NPY: 0.99 ± 0.05; wash: 1.03 ± 0.04; $P > 0.05$, Dunnett's; Figure 2b). Moreover, there were no changes in PPR (baseline: 1.03 ± 0.07; L-P NPY: 1.1 ± 0.07; wash: 1.1 ± 0.04; $F_{(10,150)} = 0.66$, $P = 0.76$), suggesting that this effect on excitatory neurotransmission was also postsynaptic.

To determine whether L-P NPY inhibits AMPA- or NMDA-mediated eEPSCs, or both, we repeated the experi-



ments in the presence of specific antagonists of AMPA (DNQX, 20 μM) or NMDA ((*RS*)-CPP, 5 μM) receptors, respectively. Since at −60 mV, the majority of the evoked synaptic current is carried by AMPA receptors, we shifted the holding potential to −40 mV to study the effect of L-P NPY on NMDA-mediated eEPSCs. Our results demonstrated a significant effect of treatment and time, as well as treatment *vs* time interaction (two-way RM-ANOVA, time effect: $F_{(10,240)} = 2.96$, $P = 0.002$; effect of treatment: $F_{(1,24)} = 9.66$, $P = 0.005$; treatment × time interaction: $F_{(10,240)} = 5.35$, $P < 0.0001$) between L-P NPY + AMPA- and L-P NPY + NMDA-mediated eEPSCs groups (Figure 2d). Interestingly, application of L-P NPY induced a significant reduction of NMDA-mediated eEPSCs compared with baseline (baseline: 0.97 ± 0.007; L-P NPY: 0.88 ± 0.04; wash: 0.78 ± 0.05; $P < 0.05$, 12–22 min, Dunnett's; Figure 2d and f), but had no effect on AMPA-mediated eEPSCs (baseline: 0.98 ± 0.02; L-P NPY: 1.08 ± 0.09; wash: 1.06 ± 0.11; $P > 0.05$, Dunnett's; Figure 2d). As was the case for eIPSCs before, the reduction in eEPSC amplitude persisted beyond 30 min of agonist washout (Figure 2f). In addition, compared with the AMPA-mediated eEPSC L-P NPY group, the amplitude of the NMDA-mediated eEPSC L-P NPY group was significantly lower ($P < 0.05$, 10–22 min, Fisher's LSD; Figure 2d). Finally, no significant changes in PPR were detected, suggesting that this effect is again postsynaptic (NMDA, baseline: 1.2 ± 0.06; L-P NPY: 1.2 ± 0.06; wash: 1.2 ± 0.06; $F_{(10,150)} = 1.32$, $P = 0.22$; AMPA, baseline: 0.96 ± 0.09; L-P NPY: 0.9 ± 0.05; wash: 1.1 ± 0.11; $F_{(8,72)} = 1.75$, $P = 0.1$).

The inhibitory effect of L-P NPY on NMDA-mediated eEPSCs was blocked by 10 min pretreatment with the Y_1R antagonist PD160170 (1 μM) (two-way RM-ANOVA, effect of treatment: $F_{(2,33)} = 11.13$, $P = 0.0002$; treatment × time interaction: $F_{(20,330)} = 4.77$, $P < 0.0001$; Figure 2f). Where L-P NPY, but not PD160170 + L-P NPY, significantly reduced NMDA-mediated eEPSCs compared with baseline (4 min) (baseline: 1.02 ± 0.01; PD160170 + L-P NPY: 0.93 ± 0.03; wash: 0.98 ± 0.08; $P > 0.05$, Dunnett's; Figure 2f) without affecting PPR (baseline: 1.4 ± 0.03; L-P NPY: 1.5 ± 0.03; wash: 1.5 ± 0.06; $F_{(10,99)} = 0.71$, $P = 0.71$). In addition, a significant difference in amplitudes of NMDA-mediated eEPSCs between L-P NPY, PD160170 + L-P NPY ($P < 0.05$, 12–22 min, Tukey's; Figure 2f) and control groups was observed ($P < 0.05$, 10–22 min, Tukey's; Figure 2f). Taken together, these data suggest that in the majority of BLA neurons, activation of postsynaptic Y_1Rs caused a preferential attenuation of the NMDA receptor-mediated component of evoked EPSCs.

The Effects of Y_1R Activation on $GABA_A$- and NMDA-Mediated Currents are Both G-Protein-Coupled and cAMP-Dependent

NPY receptors, including Y_1Rs, are coupled through $G_{i/o}$ proteins to the inhibition of adenylyl cyclase (Michel *et al*, 1998), and subsequently reduce the intracellular concentration of cAMP (Gerald *et al*, 1995, 1996; Larhammar *et al*, 1992; Wahlestedt *et al*, 1990). To confirm a role of G-proteins in L-P NPY actions in the BLA, we examined the effect of including GDP-β-S, a stable analog of GDP, which competitively inhibits GTP binding to G-proteins, in the recording pipette. Consistent with a requirement for G-protein activation, inclusion of GDP-β-S (500 nM) abrogated the effect of L-P NPY on both $GABA_A$- and NMDA-mediated currents (two-way RM-ANOVA, $GABA_A$, treatment effect: $F_{(1,27)} = 6.92$, $P = 0.01$, treatment × time interaction: $F_{(10,270)} = 3.58$, $P = 0.0002$; NMDA, treatment effect: $F_{(1,25)} = 11.86$, $P = 0.002$, treatment × time interaction: $F_{(10,250)} = 6.2$, $P < 0.0001$; Figure 3a and b).

Figure 2 Stimulation of Y_1 receptors (Y_1Rs) induces significant elevation of γ-aminobutyric acid A ($GABA_A$)-mediated inhibitory postsynaptic currents (eIPSCs) and reduction of *N*-methyl-D-aspartate (NMDA)-mediated excitatory postsynaptic currents (eEPSCs) in basolateral amygdala (BLA) projection neurons. (a) Summarized grouped data show the effect of [Leu^{31},Pro^{34}]-neuropeptide Y (L-P NPY) (400 nm) on normalized $GABA_A$-mediated eIPSCs as a function of time. Of 20 neurons (black–white circle), in 12 neurons (black circle) application of L-P NPY induced a significant increase of amplitude of eIPSCs, whereas 8 cells (white circle) showed no response. (Inset) Voltage-clamp recordings traces of eIPSCs from BLA projection neuron at holding potential −50 mV in control (trace 1), during the application of L-P NPY (400 nM) (6 min) (trace 2) and wash (trace 3) periods. At the end of the experiment, addition of the γ-aminobutyric acid A ($GABA_A$) antagonist 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl) pyridazinium (SR95531) (1 μM) fully blocked eIPSCs (trace 4). Arrows indicate stimulation artifact. (b) Summarized grouped data show the effect of L-P NPY (400 nm) on normalized eEPSCs as a function of time. Of 16 neurons (black–white circle), in 11 neurons (black circle) the application of L-P NPY induced a significant decrease of amplitude of eEPSCs, whereas 5 cells (white circle) did not respond. (Inset) Voltage-clamp recordings of eEPSCs from BLA projection neuron (−60 mV) during baseline (1), application of L-P NPY (400 nM, 6 min) (2) and wash (3) conditions. (c) Summarized grouped data show the effects of L-P NPY (400 nM) (black circle) and pre-treatment with 6-(2-(1-methylethyl)phenyl))sulfonyl-5-nitro-8-quinolinamine (PD160170) (1 μM) (white triangle) on normalized eIPSCs as a function of time. *Significantly different compared with baseline within group ($P < 0.05$); #significantly different between groups ($P < 0.05$). (Inset) Typical voltage-clamp current traces show that pre-treatment with the Y_1R antagonist PD160170 (1 μM) blocks the effect of L-P NPY (400 nM) on eIPSCs. Trace 1 min—baseline (with PD160170 present in artificial cerebrospinal fluid (ACSF)), trace 2–6 min of perfusion with PD160170 (1 μM) + L-P NPY (400 nm), and trace 3–12 min of wash. (d) Exogenous L-P NPY (400 nM) has no effect on the amplitude of pharmacologically isolated AMPA EPSCs evoked from a holding potential of −60 mV. Summary graph shows the effect of L-P NPY (400 nM) on AMPA- (black triangle) and NMDA-mediated (black circle) eEPSCs as a function of time. (Inset) Typical eEPSCs recordings are illustrated before (1), during (2), and after L-P NPY application (3). (e) Application of L-P NPY induced significant increase of amplitude, but not frequency of miniature inhibitory postsynaptic current (mIPSC). Summary plot of amplitude (left) and frequency (right) of mIPSCs before, during L-P NPY application, and after 25 min of wash periods ($P < 0.05$). (Inset) Sample traces of mIPSC from slices, containing BLA neurons, before (Control), during L-P NPY application (L-P NPY), and after 25 min wash period were recorded in the presence of 6,7-dinitroquinoxaline-2,3-(1*H*,4*H*)-dione (DNQX) (20 μM), 3-((*R*)-2-carboxypiperazin-4yl)-propyl-1-phosphonic acid (CPP) (1 μM), 3-(3,4-dichlorophenylmethylaminopropyl) diethoxymethyl phosphinic acid (CGP 52432) (1 μM) and tetrodotoxin (TTX) (500 nM). (f) Perfusion with L-P NPY (400 nM) decreases the amplitude of isolated NMDA-mediated eEPSCs (−40 mV). Grouped data show the effect of L-P NPY (400 nM) (black circle) and the effect of L-P NPY in the presence of Y_1 antagonist PD160170 (1 μM) (white triangle) on eEPSCs over time. *Significantly different compared with baseline within group ($P < 0.05$); #significantly different between groups ($P < 0.05$). (Insets, top) eEPSC recordings that are represent before (1), 6 min during (2), after L-P NPY application (3), and after incubation with the NMDA receptor blocker CPP (1 μM) (4). (bottom) Preincubation with the Y_1 agonist PD160170 (1 μM) abolished the inhibitory effect of L-P NPY on NMDA-mediated eEPSCs. eEPSC recordings are shown before (1), during PD160170 (1 μM) + L-P NPY (400 nM) application (2), and during wash period (3).

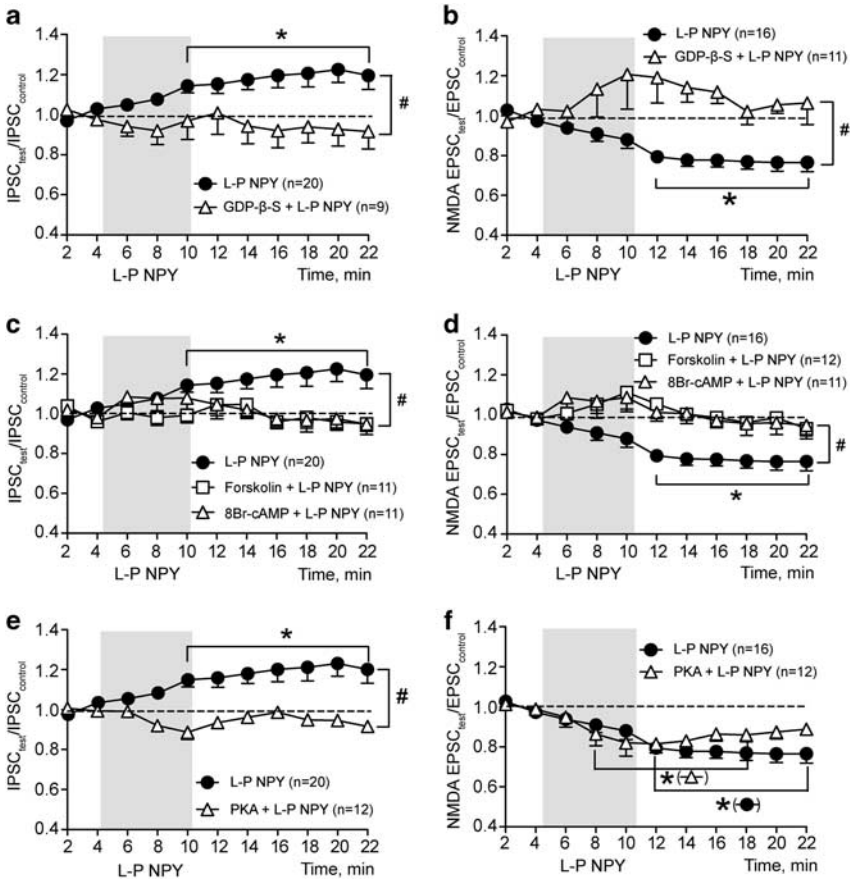


Figure 3 The effects of [Leu^{31},Pro^{34}]-neuropeptide Y (L-P NPY) on γ-aminobutyric acid A ($GABA_A$)-mediated evoked inhibitory postsynaptic currents (eIPSCs) and *N*-methyl-D-aspartate (NMDA)-mediated excitatory postsynaptic currents (eEPSCs) are G-protein-related and cAMP-dependent. (a, b) Inclusion of guanosine 5′-o-(2-thiodiphosphate) (GDP-βS) (500 nM) in the recording pipette blocked the effects of L-P NPY on $GABA_A$-mediated eIPSCs and NMDA-mediated eEPSCs. Summarized grouped data show the effects of L-P NPY (400 nM) (black circle), GDP-βS (500 nM) + L-P NPY (white triangle) on normalized eIPSCs (a) and eEPSCs (b) as a function of time. (c, d) Inclusion of forskolin (10 μM) and 8 Br-cAMP (500 μM) 30 min before L-P NPY application blocked the effects of L-P NPY on $GABA_A$-mediated eIPSCs and NMDA-mediated eEPSCs. Summary graphs comparing the effects of L-P NPY (400 nM) (black circle), forskolin + L-P NPY (white box), and 8 Br-cAMP (white triangle) on normalized eIPSCs (c) and eEPSCs (d) as a function of time. (e, f) The effect of L-P NPY on $GABA_A$-mediated eIPSCs, but not NMDA-mediated eEPSCs, involves activation of PKA. Graphs show the effects of L-P NPY (black circle) and with added intracellular PKA catalytic subunit (100 U/ml) (white triangle) on normalized eIPSCs (e) and eEPSCs (f) as a function of time. *Significantly different compared with baseline (4 min) within group ($P<0.05$); #significantly different between groups ($P<0.05$).

The addition of GDP-β-S to L-P NPY treatment blocked both the increases of GABA-mediated eIPSCs and reductions in NMDA-mediated eEPSCs compared with baseline ($GABA_A$, baseline: 1.03 ± 0.02; GDP-β-S + L-P NPY: 0.97 ± 0.09; wash: 0.92 ± 0.09; $P>0.05$, Dunnett's; NMDA, baseline: 0.97 ± 0.03; GDP-β-S + L-P NPY: 1.21 ± 0.17; wash: 1.06 ± 0.11; $P>0.05$, Dunnett's; Figure 3a and b), observed following L-P NPY treatment alone. In addition, compared with the GDP-β-S + L-P NPY group, the L-P NPY group exhibited significantly greater GABA-mediated eIPSCs ($P<0.05$, 10 and 14–22 min, Fisher's LSD; Figure 3a), and significantly lower NMDA-mediated eEPSCs ($P<0.05$, 12–22 min, Fisher's LSD; Figure 3b).

Next, we used the adenylyl cyclase activator forskolin and 8Br-cAMP, to test the role of cAMP in mediating the effect of L-P NPY. The rationale is that direct elevation of cAMP would overwhelm $G_{i/o}$-mediated inhibition of adenylyl cyclase and occlude the actions of Y_1R activation. Consistent with this expectation, 30 min preincubation with either forskolin (10 μM) or 8Br-cAMP (500 μM) occluded the effect of L-P NPY on $GABA_A$-mediated eIPSCs or NMDA-mediated eEPSCs (two-way RM-ANOVA, $GABA_A$, treatment effect: $F_{(2,39)}=6.47$, $P=0.004$; treatment × time interaction: $F_{(20,390)}=4.75$, $P<0.0001$; NMDA, treatment effect: $F_{(2,36)}=8.75$, $P=0.0008$; treatment × time interaction: $F_{(20,360)}=1.71$, $P=0.031$; Figure 3c and d). Moreover, in the presence of forskolin, the amplitudes of $GABA_A$-mediated eIPSCs and NMDA-mediated eEPSCs did not change in response to Y_1Rs activation by L-P NPY compared with baseline ($GABA_A$, baseline: 1.04 ± 0.02; forskolin + L-P NPY: 0.99 ± 0.04; wash: 0.95 ± 0.04; $P>0.05$, Dunnett's; NMDA, baseline: 1.02 ± 0.01; forskolin + L-P NPY: 1.11 ± 0.1; wash: 0.93 ± 0.05; $P>0.05$, Dunnett's; Figure 3c and d). The inclusion of 8Br-cAMP also diminished the effect of L-P NPY on isolated $GABA_A$-mediated eIPSCs and NMDA-mediated eEPSCs ($GABA_A$, baseline: 1.02 ± 0.01; 8Br-cAMP + L-P NPY: 1.08 ± 0.06; wash: 0.95 ± 0.05; $P>0.05$, Dunnett's; NMDA, baseline: 1.01 ± 0.02; 8Br-cAMP + L-P NPY: 1.09 ± 0.06; wash: 0.95 ± 0.05; $P>0.05$, Dunnett's; Figure 3c and d). Moreover, compared with L-P NPY group, the amplitude of $GABA_A$-mediated eIPSCs was significantly lower in forskolin + L-P

NPY group ($P<0.05$, 16–22 min, Tukey's; Figure 3c) and 8Br-cAMP + L-P NPY group ($P<0.05$, 14–22 min, Tukey's; Figure 3c). Furthermore, the amplitude of NMDA-mediated eEPSC in forskolin + L-P NPY group ($P<0.05$, 8–20 min, Tukey's; Figure 3d) and 8Br-cAMP + L-P NPY group ($P<0.05$, 10–14 min, Tukey's; Figure 3d) was significantly higher compared with L-P NPY group.

Y_1Rs in the BLA Modulate $GABA_A$-Mediated Currents via a PKA Pathway

To further dissect the second messenger pathways involved in Y_1R-based synaptic modulation, we examined whether PKA was involved downstream of cAMP reduction in mediating the effects of L-P NPY. We hypothesized that addition of the active catalytic subunit of PKA in the intracellular solution would bypass any receptor-mediated reduction in PKA activation through lowered cAMP levels. Accordingly, inclusion of the PKA catalytic subunit (100 U/ml) in the recording electrode occluded the Y_1R effect on $GABA_A$-mediated current (two-way RM-ANOVA, treatment effect: $F_{(1,30)}=13.13$, $P=0.001$; treatment × time interaction: $F_{(10,300)}=5.88$, $P<0.0001$; Figure 3e). Instead of the increase of eIPSC amplitude we observed previously in the L-P NPY group (eg Figure 2a), in the presence of PKA we witnessed a small reduction of amplitude of $GABA_A$-mediated eIPSCs compared with baseline (baseline: 1.01 ± 0.01; PKA + L-P NPY: 0.89 ± 0.04; wash: 0.92 ± 0.03; $P>0.05$, Dunnett's; Figure 3e). There was also a significant difference in amplitudes of $GABA_A$-mediated eIPSCs between PKA + L-P NPY and L-P NPY groups ($P<0.05$, 8–22 min, Fisher's LSD; Figure 3e).

In contrast, inclusion of PKA in the electrode did not alter the inhibition of NMDA-mediated eEPSCs by L-P NPY (two-way RM-ANOVA, treatment effect $F_{(1,26)}=0.99$, $P=0.33$; Figure 3f). In the presence of PKA there was a trend toward a gradual recovery in the eEPSC amplitude (baseline: 1.01 ± 0.01; PKA + L-P NPY: 0.82 ± 0.07; wash: 0.89 ± 0.03; $P<0.05$, 8–18 min, Dunnet's; Figure 3f). Moreover, the amplitudes of NMDA-mediated eEPSCs were not significantly different between the L-P NPY and PKA + L-P NPY groups ($P>0.05$; Figure 3f).

Y_1Rs in the BLA Modulate NMDA-Mediated Currents via an Epac Pathway

Another possible target of cAMP is the 'Epac', also known as cAMP-GEF (de Rooij *et al*, 1998; Gloerich and Bos, 2010). Using *in situ* and northern hybridization, it has been shown that mRNAs of Epac1 and Epac2 are expressed in the amygdala, as well as in the hippocampus and cortex (Kawasaki *et al*, 1998). To determine whether Epac1 and/or Epac2 protein is expressed in the BLA, we used western blot analysis of BLA tissue punches. We found that Epac1 and Epac2 are both expressed in the basal amygdala, although Epac2 protein is more abundant than that of Epac1 (Figure 4a). We also detected Epac1 and Epac2 protein in the hippocampus and prefrontal cortical regions. Interestingly, CHOK1 and HEK293 cells used as controls show expression of only Epac1, but not Epac2 (Figure 4a).

We next explored whether Epac might also have a functional role in L-P NPY modulation of BLA synaptic transmission. To accomplish this, we needed a mechanism to manipulate PKA and Epac independently. While PKA and Epac can both be activated by cAMP, Epac is selectively activated by 8CPT-2Me-cAMP (Enserink *et al*, 2002; Kwon *et al*, 2004; Novara *et al*, 2004); thus, we used this reagent to activate preferentially Epac in BLA neurons. These experiments demonstrated a significant treatment effect, as well as treatment × time interaction comparing 8CPT-2Me-cAMP(5) + L-P NPY and L-P NPY groups (two-way RM-ANOVA, treatment effect: $F_{(1,27)}=11.42$, $P=0.002$; treatment × time interaction: $F_{(10,270)}=3.73$, $P=0.0001$ Figure 4b). Furthermore, addition of 8CPT-2Me-cAMP (5 μM) to the recording pipette completely occluded the effect of L-P NPY on NMDA-mediated eEPSCs (baseline: 1.01 ± 0.07; 8CPT-2Me-cAMP(5) + L-P NPY: 0.96 ± 0.05; wash: 0.94 ± 0.03; $P>0.05$, Dunnett's; Figure 4b). Moreover, the amplitudes of NMDA-mediated eEPSCs were significantly different between 8CPT-2Me-cAMP(5) + L-P NPY and L-P NPY groups ($P<0.05$, 6 and 12–22 min, Fisher's LSD Figure 4b).

Inclusion of a lower concentration (1 μM) of 8CPT-2Me-cAMP in the intracellular recording solution reduced, but did not entirely eliminate the effect of L-P NPY on NMDA-mediated eEPSCs (two-way RM-ANOVA, treatment effect: $F_{(1,22)}=3.27$, $P=0.08$; treatment × time interaction: $F_{(10,220)}=1.82$, $P=0.06$; Figure 4c). However, in the presence of 1 μM of 8CPT-2Me-cAMP, L-P NPY did not significantly reduce the amplitude of NMDA-mediated eEPSCs (baseline: 1.01 ± 0.02; 8CPT-2Me-cAMP(1 μM) + L-P NPY: 0.87 ± 0.06; wash: 0.9 ± 0.05; $P>0.05$, Dunnett's; Figure 4c). Moreover, starting from 18 min, there was significant difference in the amplitude of NMDA-mediated eEPSCs between 8CPT-2Me-cAMP(1) + L-P NPY and L-P NPY groups ($P>0.05$, 18–22 min, Fisher's LSD; Figure 4c).

In contrast to eEPSCs, eIPSCs were unaffected by the addition of 8CPT-2Me-cAMP to neurons. In Figure 4d, a 30 min pre-treatment with 8CPT-2Me-cAMP (5 μM) does not change the amplitude of eIPSCs compared with control before the application of L-P NPY. Furthermore, the enhancement of eIPSCs by L-P NPY was likewise unaffected by 8CPT-2Me-cAMP pre-treatment as there was no significant treatment effect or significant time *vs* treatment interaction between 8CPT-2Me-cAMP(5) + L-P NPY and L-P NPY groups (two-way RM-ANOVA, treatment effect: $F_{(1,27)}=0.76$, $P=0.39$; time × treatment interaction: $F_{(10,270)}=1.61$, $P=0.104$; Figure 4d). Pre-treatment failed to inhibit the L-P NPY-induced increase of $GABA_A$-mediated eIPSC (baseline: 0.99 ± 0.01; 8CPT-2Me-cAMP(5) + L-P NPY: 1.12 ± 0.06; wash: 1.33 ± 0.13; $P<0.05$, 14–22 min, Dunnett's; Figure 4d). Moreover, there were no significant differences in the amplitude of $GABA_A$-mediated eIPSC between 8CPT-2Me-cAMP(5) + L-P NPY and L-P NPY groups ($P>0.05$, Fisher's LSD; Figure 4d).

DISCUSSION

Implications of Y_1R-Mediated Increases of $GABA_A$-Mediated Currents and Reduction of NMDA-Mediated Currents

We report here for the first time that application of the Y_1R agonist L-P NPY in the BLA not only enhances

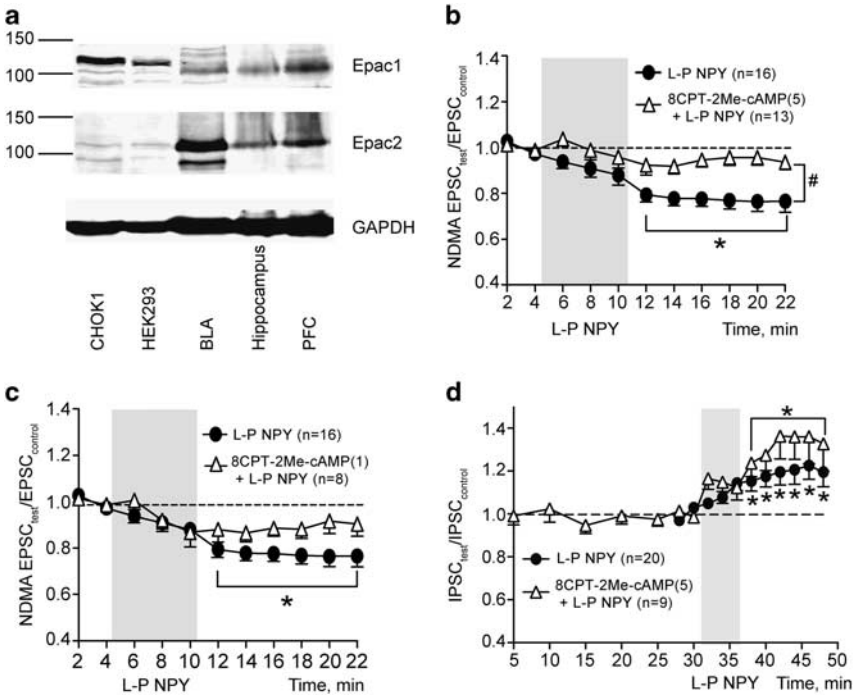


Figure 4 [Leu^{31},Pro^{34}]-neuropeptide Y (L-P NPY) reduction of *N*-methyl-D-aspartate (NMDA)-mediated excitatory postsynaptic currents (eEPSCs) but not γ-aminobutyric acid A ($GABA_A$)-mediated evoked inhibitory postsynaptic currents (eIPSCs) requires activation of exchange protein activated by cAMP (Epac). (a) Western blot analysis shows Epac expression in basolateral amygdala (BLA), hippocampus and prefrontal cortex (PFC) tissue. Moderate Epac1 expression (~100 kDa) was detected in BLA and hippocampal tissue, Epac1 expression is higher in CHOK1 and HEK293 cells with bands shifted to higher molecular weight, indicating a difference in glycosylation between brain tissue and cell lines. Epac2 expression (~120 kDa) was detected strongly in BLA, weakly in hippocampus and PFC tissue, but barely in CHOK1 and HEK293 cells. (b–d) Electrophysiological experiments revealed that the addition of the Epac activator 8CPT-2Me-cAMP to the recording pippette dose-dependently attenuated the effect of L-P NPY on NMDA-mediated eEPSCs and increase $GABA_A$-mediated eIPSCs. (b, c) Summary graphs comparing the group with L-P NPY (400 nM) (black circle) and the group where slices were preincubated with 8CPT-2Me-cAMP in concentrations of 5 μM (b) and 1 μM (c). (d) Summarized grouped data show the effects of L-P NPY (400 nM) (black circle) and 8CPT-2Me-cAMP (5 μM) + L-P NPY (white triangle) on normalized eIPSCs as a function of time. *Significantly different compared with control within group ($P<0.05$); #significantly different between groups ($P<0.05$).

$GABA_A$-mediated eIPSCs consistent with previous literature (Eva *et al*, 2006a, b) but also selectively reduces NMDA-mediated but not AMPA-mediated eEPSCs (Figure 5). Regulation of excitability in BLA neurons by Y_1Rs has been suggested to involve a variety of mechanisms, including enhancing GABA inhibition (Kask *et al*, 1996), activating Kir3 channels (Sosulina *et al*, 2008), and increasing I_h current (Giesbrecht *et al*, 2010). However, to our knowledge, the modulation of postsynaptic $GABA_A$ receptors by NPY receptor activation has never been directly and systematically studied. Here we report that L-P NPY produces long-lasting enhancement of $GABA_A$-mediated eIPSCs that is mediated through G-protein coupling, and subsequently through cAMP and preferentially through PKA. Conversely, we demonstrated that while L-P NPY inhibition of NMDA-mediated eEPSCs is also G-protein- and cAMP-dependent, it is not mediated by PKA. These dual effects of a Y_1R agonist in combination would increase overall inhibition of BLA glutamatergic pyramidal neurons and would be consistent with anxiolytic behavior. Previous findings in our lab and others support the hypothesis that NPY in the BLA exerts an anxiolytic effect (Karlsson *et al*, 2008; Sajdyk *et al*, 2004, 2006; Wahlestedt and Reis, 1993b). Furthermore, for the first time we demonstrate that the activation of Y_1Rs decreases NMDA-mediated currents, an important new finding with implications for the role of NPY in modulating not only excitability but also synaptic plasticity within the BLA. We have previously shown that CRF- and stress-induced synaptic plasticity within the BLA

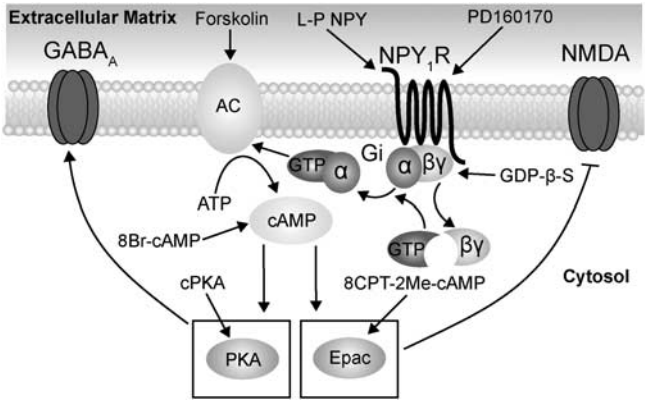


Figure 5 The proposed mechanisms that contribute to anxiolytic action of neuropeptide Y (NPY) in the basolateral amygdala (BLA) during stress or anxiety. After NPY is released, it activates postsynaptic, $G_{i/o}$-coupled Y_1 receptors (Y_1Rs) in the BLA, which leads to a reduction of cAMP levels and decrease of PKA and exchange protein activated by cAMP (Epac) activities. The reduction of PKA activity causes an increase of $GABA_A$-mediated currents, while reduction of Epac activity decreases *N*-methyl-D-aspartate (NMDA)-mediated currents. Both of these mechanisms increase the inhibition and reduce the excitation in the BLA.

may be a key component of the pathophysiology of several chronic anxiety disorders, including panic and post-traumatic stress disorder (Rainnie *et al*, 2004; Shekhar *et al*, 2005). Stress-induced plasticity within the BLA is mediated by the activation of NMDA receptors and pre-treatment with an NMDA receptor antagonist blocks such plasticity (Rainnie *et al*, 2004). As reported here, since Y_1R agonists reduce NMDA currents, NPY would be ideally positioned to block or reduce such stress-related plasticity. This is consistent with our previous finding that repeated activation of NPY receptors in the BLA induces persistent reductions in anxiety and induces stress resilience that lasts several weeks (Sajdyk *et al*, 2008). Thus, this newly described action of NPY on NMDA currents provides a plausible cellular mechanism for these long-term protective effects of NPY on stress-induced conditions, such as post-traumatic stress disorder and depression (Cohen *et al*, 2012; McGuire *et al*, 2011; Sah *et al*, 2009; Sajdyk *et al*, 2008).

Implications of Epac as a Novel Intracellular Signal-Transduction Pathway of Y_1 Effects

In addition to demonstrating the opposing but complimentary modulation of postsynaptic receptor currents (enhanced $GABA_A$- and reduced NMDA-mediated currents) by L-P NPY, we have also found that the effects of Y_1R activation on the $GABA_A$ and NMDA receptors occur through predominantly different signal-transduction pathways. The effect of NPY to reduce cAMP levels is well known (Hsieh *et al*, 2007; Misra *et al*, 2004; Sheriff *et al*, 2003; Zhang and Pandey, 2003). A reduction of cAMP has previously been implicated in enhancing $GABA_A$ currents (Kapur and Macdonald, 1996) and in reducing NMDA currents (Fourcaudot *et al*, 2008; Huang *et al*, 1993; Huang and Kandel, 1998; Zhang *et al*, 2011b) in various brain regions. The new finding here is that the reduction in NMDA currents elicited by NPY is preferentially mediated through a reduction in Epac activation, a signal-transduction pathway not previously implicated in mediating any NPY effects. Recent studies of Epac-related signaling have revealed that these novel cAMP sensors regulate many neuronal processes, including calcium dynamics, learning and memory, cell proliferation and differentiation, apoptosis, and axonal growth (Gloerich *et al*, 2010; Grandoch *et al*, 2010). Our finding of distinct signaling pathways provides a novel molecular and pharmacological target to dissociate the GABAergic and NMDA effects of NPY action within the amygdala (Figure 5).

There is increasing evidence that Epac is a mediator of cAMP-dependent changes in hippocampal synaptic plasticity (Gelinas *et al*, 2008; Whitaker and Wei, 2009; Woolfrey *et al*, 2009) and memory retrieval (Ostroveanu *et al*, 2010; Ouyang *et al*, 2008). Epac also appears to be implicated in the regulation of neurotransmitter release (Gekel and Neher, 2008; Kaneko and Takahashi, 2004) as well as synaptic and spine modulation that are critical for neuronal plasticity (Woolfrey *et al*, 2009). Epac is also emerging as a potential candidate molecule associated with a number of neuropsychiatric disorders. For example, changes in Epac levels have been reported in post-mortem brains of depressed, suicide victims (Dwivedi *et al*, 2006) and Epac 1 gene variants are associated with anxiety and depression in twin studies (Middeldorp *et al*, 2010). An Epac-null mutation impairs long-term potentiation (LTP) that is paralleled with the severe deficits in spatial learning and social interactions (Yang *et al*, 2012). Epac is thought to be a key mediator in the effects of PACAP (Ster *et al*, 2009), a peptide that has been recently implicated in the pathophysiology of post-traumatic stress disorder (PTSD) and fear memory formation (Ressler *et al*, 2011). Thus, NPY may modulate a variety of behaviors, including conditioned fear (Gutman *et al*, 2008) and human stress reactivity (Mickey *et al*, 2011; Witt *et al*, 2011) via Epac pathways and serve as a critical element in the pathophysiology of depression, PTSD, and other neuropsychiatric disorders. Indeed, several studies implicate changes in NPY levels in depression (Domschke *et al*, 2010; Nikisch and Mathe, 2008), anxiety disorders (Amstadter *et al*, 2010; Wu *et al*, 2011), and PTSD (Rasmusson *et al*, 2010; Sah *et al*, 2009).

Technical Comments

In previous studies in a variety of neuronal types, including the amygdala (Fourcaudot *et al*, 2008; Huang *et al*, 1993, 1998) and hippocampus (Zhang *et al*, 2011b), enhancing PKA activity was associated with increased NMDA-mediated LTP. In these cases, however, it appears that presynaptic modulation of glutamate release, rather than postsynaptic NMDA receptor properties as seen here is the target of this form of cAMP signaling. An increase rather than a decrease of cAMP levels was previously shown to be involved in enhancing $GABA_A$ currents in cerebellar granule cells (Kapur *et al*, 1996), most likely through PKA-mediated phosphorylation. However, direct effects of GABA rather than evoked GABAergic synaptic responses were measured; thus, a distinction between extrasynaptic and synaptic receptor populations could explain this dichotomy as scaffolding and anchoring proteins (eg AKAPs) would be expected to regulate the nature of signaling between receptors and effectors in synaptic compartments. Giesbrecht *et al* (2010) previously reported a significant hyperpolarization of membrane potential caused by NPY or the Y_1R agonist $F^7P^{34}NPY$. Our data are not necessarily inconsistent with these findings, as their study selected cells based on their membrane potential response, while we included all neurons irrespective of the effect of NPY on membrane potential. Nonetheless, we failed to observe consistent and robust changes in membrane potential with L-P NPY application throughout the neuron population we examined.

CONCLUSIONS

In conclusion, we report here for the first time a reduction of NMDA receptor-mediated and an enhancement of $GABA_A$ receptor-mediated postsynaptic currents by activating Y_1Rs in the BLA. Also, we report here for the first time that Y_1R activation preferentially utilizes an Epac signaling pathway to modulate the NMDA effects but a PKA signaling pathway to modulate the $GABA_A$ effects, thus dissociating the NPY effector pathways to postsynaptic NMDA and $GABA_A$ receptors (Figure 5). The novel finding of modulation of NMDA receptors has implications for the role of

NPY in regulating a variety of behaviors related to synaptic plasticity in the amygdala. The finding of a critical role for Epac signaling suggests a novel avenue for selectively targeting the NMDA-modulating effects of NPY in the amygdala without impacting other effects, and in developing novel therapeutics for a variety of chronic neuropsychiatric disorders that result in part from amygdala plasticity.

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DISCLOSURE

The authors declare no conflict of interest.

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