Published in final edited form as:

J Comp Neurol. 2009 November 10; 517(2): 166–176. doi:10.1002/cne.22143.

Cell-Specific Expression of Neuropeptide Y Y1 Receptor Immunoreactivity in the Rat Basolateral Amygdala

AMANDA B. ROSTKOWSKI¹, TARA L. TEPPEN¹, DANIEL A. PETERSON^{1,2}, and JANICE H. URBAN^{1,3,*}

¹Department of Neuroscience, Chicago Medical School/Rosalind Franklin University of Medicine and Science, North Chicago, Illinois 60064

²Center for Stem Cell and Regenerative Medicine, Chicago Medical School/Rosalind Franklin University of Medicine and Science, North Chicago, Illinois 60064

³Physiology and Biophysics, Chicago Medical School/Rosalind Franklin University of Medicine and Science, North Chicago, Illinois 60064

Abstract

Activation of neuropeptide Y (NPY) Y1 receptors (Y1r) in the rat basolateral nuclear complex of the amygdala (BLA) produces anxiolysis and interferes with the generation of conditioned fear. NPY is important in regulating the output of the BLA, yet the cell types involved in mediating this response are currently unknown. The current studies employed multiple label immunocytochemistry to determine the distribution of Y1r-immunoreactivity (-ir) in glutamatergic pyramidal and GABAergic cell populations in the BLA using scanning laser confocal stereology. Pyramidal neurons were identified by expression of calcium-calmodulin dependent kinase II (CaMKII-ir) and functionally distinct interneuron subpopulations were distinguished by peptide (cholecystokinin, somatostatin) or calcium-binding protein (parvalbumin, calretinin) content. Throughout the BLA, Y1r-ir was predominately on soma with negligible fiber staining. The high degree of coexpression of Y1r-ir (99.9%) in CaMKII-ir cells suggests that these receptors colocalize on pyramidal cells and that NPY could influence BLA output by directly regulating the activity of these projection neurons. Additionally, Y1r-ir was also colocalized with the interneuronal markers studied. Parvalbumin-ir interneurons, which participate in feedforward inhibition of BLA pyramidal cells, represented the largest number of Y1r expressing interneurons in the BLA (≈4% of the total neuronal population). The anatomical localization of NPY receptors on different cell populations within the BLA provides a testable circuit whereby NPY could modulate the activity of the BLA via actions on both projection cells and interneuronal cell populations.

Keywords

calcium binding proteins; CaMKII; anxiety; interneuron; pyramidal neuron

The amygdala is an important brain structure involved in the acquisition, storage, and expression of emotional memory. In particular, the basolateral nucleus of the amygdala (BLA) is necessary for production of emotional behaviors and for consolidation of

emotional memories. Major cell types in the BLA include pyramidal ($\approx 85\%$) and nonpyramidal ($\approx 15\%$) neurons (Hall, 1972; McDonald, 1982). Pyramidal cells are spiny, glutamatergic, projection neurons (McDonald, 1992) that target various brain regions involved in fear learning and behavioral/ physiological stress responses including the hippocampus, prefrontal cortex, periaqueductal gray, hypothalamus, and brainstem (Pitkanen, 2000). Nonpyramidal neurons are aspiny, local interneurons that use γ -aminobutyric acid (GABA) as an inhibitory neurotransmitter (McDonald and Pearson, 1989). Upon exposure to stress, GABAergic disinhibition occurs in the BLA (Stork et al., 2002; Martijena et al., 2002; Rodriguez Manzanares et al., 2005; Isoardi et al., 2007). This change in GABAergic tone presumably leads to the increased pyramidal neuron activity and BLA output seen in stressful or anxiety-provoking states (Pare and Collins, 2000). Changes in GABAergic tone have direct consequences on pyramidal neuron activity and on the formation of fear memory/anxiety behaviors resulting from changes in BLA output.

Pyramidal neurons and interneurons can be distinguished by their preferential expression of phenotypic markers. In the rat BLA, expression of calcium-calmodulin dependent kinase II (CaMKII) is limited to pyramidal neurons (McDonald et al., 2002). Additionally, functionally distinct interneuronal subpopulations can be characterized based on colocalization of GABA with different calcium-binding proteins (CaBP; calretinin, CR; parvalbumin, PV; calbindin, CB) and neuropeptides (cholecystokinin, CCK; somatostatin, SOM; neuropeptide Y, NPY) (McDonald, 1985, 1997; Kemppainen and Pitkanen, 2000; McDonald and Mascagni, 2001a). These BLA interneuron subpopulations exhibit distinctive firing properties (Rainnie et al., 2006) and synaptologies (Sorvari et al., 1998; Muller et al., 2003, 2007a; McDonald et al., 2005; Chung and Moore, 2007) and are important for regulating pyramidal neuron activity and the resultant BLA output (McDonald and Pearson, 1989; McDonald and Mascagni, 2001a; McDonald et al., 2002; Muller et al., 2003).

In addition to GABA and glutamate, various peptides have been shown to either decrease (neuropeptide Y [Heilig et al., 1989, 1992], substance P [Gadd et al., 2003]) or increase (corticotropin releasing factor, CRF [Radulovic et al., 1999; Sajdyk et al., 1999a; Smagin and Dunn, 2000]) anxiety behaviors via actions in the BLA. NPY, a highly conserved 36 amino acid peptide widely expressed throughout the mammalian brain, has a well-established role in decreasing anxiety behaviors. Central administration of NPY decreases stress-related behaviors in various animal models of anxiety (Heilig et al., 1992; Karlsson et al., 2005). In particular, direct intra-BLA delivery of NPY impedes the formation of fear memories and expression of anxiety behaviors (Flood et al., 1989; Heilig et al., 1993; Sajdyk et al., 1999b; Gutman et al., 2008). The mechanisms by which NPY exerts its effects, i.e., modulating GABA tone and/or pyramidal neuron activity, are currently unknown. A number of studies have demonstrated that NPY Y1 receptors (Y1r), and to a lesser extent Y5 receptors (Y5r), play an important role in mediating the anxiolytic actions of NPY (Heilig, 1995; Sajdyk et al., 1999b, 2002; Sorensen et al., 2004; Karl et al., 2006). However, while the receptor subtypes mediating NPY anxiolytic effects are clear, the neuronal circuitry in the BLA underlying these responses is not well understood.

We and others have previously shown both Y1r-immunoreactivity (ir), binding sites, and mRNA in the rat BLA (Larsen et al., 1993; Dumont et al., 1993; Parker and Herzog, 1999; Durkin et al., 2000; Kopp et al., 2002; Wolak et al., 2003; Kishi et al., 2005; Oberto et al., 2007). The present studies used stereological techniques to examine the distribution of Y1r-ir on pyramidal neurons as well as assess the expression of Y1r on different subtypes of interneurons (SOM, PV, CR, and CCK) in the BLA. The current results identify neuroanatomical substrates, i.e., the cell types expressing NPY receptors in the BLA, through which NPY may mediate its anxiolytic actions.

MATERIALS AND METHODS

Animals

Rats—Adult male (250–350 g) Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were housed three to a conventional cage, with free access to standard lab chow and water, located in a temperature (20–22°C), humidity (50–55%), and illumination (14:10-hour light:dark cycle) controlled, AAALAC-approved facility. Five days of acclimatization to our facilities were allowed before the animals were included in any experimental procedure. All procedures were approved by the Rosalind Franklin University of Medicine and Science (RFUMS) Institutional Animal Care and Use Committee (IACUC).

Tissue preparation—Animals were deeply anesthetized with sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, 100 mg/kg, i.p.) and transcardially perfused with 30 mL of phosphate-buffered saline (PBS: 10 mM Na₂HPO₄, 150 mM NaCl, pH 7.5) containing 0.1% procaine and 100 U/mL heparin at 37°C followed by 60 mL fixative solution consisting of 4% paraformaldehyde (PFA) in PBS at 4°C. Animals used in GABA/CaMKII multiple-label experiments were perfused with 120 mL 4% PFA containing 0.5% glutaraldehyde to better preserve GABA immunoreactivity. Brains were rapidly dissected out, postfixed overnight in 4% PFA solution, followed by an hour-long PBS wash at 4°C. Coronal brain sections were cut in a bath of ice-cold PBS at 40 μm thickness using a vibratome (Vibratome 1000, Ted Pella, Redding, CA).

Single-label immunocytochemistry in Y1R knockout (KO) and wildtype (WT) mice

To address the specificity of the Y1r antibody used in these studies, Y1r-ir was assessed in PFA-fixed brains from adult male WT and Y1r KO mice obtained from Eli Lilly (Indianapolis, IN). A biotinylated tyramide amplification immunofluorescence protocol adapted from Adams (1992) was used. Briefly, free-floating sections were rinsed through three changes of PBS over 10 minutes, followed by a 15-minute wash in 1% H₂O₂ in PBS to diminish endogenous peroxidase activity. Next, tissues were blocked for 3 hours in immunocytochemistry (ICC) buffer (0.1 M PBS containing 0.2% gelatin, 0.01% thimerosal and 0.002% neomycin, pH 7.5) with 5% normal donkey serum (NDS; Equitech-Bio, Kerrville, TX) to block non-specific binding. Sections were then incubated at 4°C for 72 hours with primary Y1r antibody (rabbit, described in Wolak et al. [2003]) diluted 1:2,000 in ICC with 2% NDS. Following incubation with primary antibody, sections were washed through five changes of ICC buffer over 50 minutes and then incubated with biotinylated, affinity-purified donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:2,000) for 1 hour at room temperature. After rinses in ICC buffer, sections were incubated in Vectastain Elite ABC (Vector Laboratories, Burlingame, CA; 2 μL/mL) for 30 minutes. Next, sections were rinsed with PBS and incubated in biotinylated tyramide solution (3 μg/mL biotinylated tyramide and 0.01% H₂O₂ in PBS) for 10 minutes. Tissues were then rinsed in ICC buffer and immersed in ICC buffer containing fluorescein isothiocyanate conjugated streptavidin (FITC-SA; Jackson ImmunoResearch Laboratories; 1:250) for 3 hours. Following washes in four changes of Tris-buffered saline (TBS: 100 mM Tris base, 150 mM NaCl, pH 7.5) over 20 minutes, sections were mounted on gelatin subbed slides and air-dried; coverslips were applied with 2.5% polyvinyl alcohol-1,4-diazabicyclo[2.2.2]octane (PVA-DABCO) antifade mounting medium.

Multiple-label immunocytochemistry

CaMKII/GABA—To assess the specificity of CaMKII as a specific marker for BLA glutamatergic projection neurons, we performed double label immunocytochemistry for CaMKII (mouse monoclonal, clone 6G9, Millipore, Billerica, MA) and GABA (mouse monoclonal, clone 5A9, MP Biomedicals, Solon, OH). A potential concern when using two

primary antibodies raised in the same species is crossreactivity of the secondary fluorophores. To address this issue we employed a variation of the biotinylated tyramide amplification immunofluorescence protocol described above for visualization of GABA combined with standard secondary antibody immunofluorescence for visualization of CaMKII. The use of biotinylated tyramide amplification allows for visualization of one primary antibody, in this case GABA, at a concentration otherwise not detectable using traditional indirect immunofluorescence methods. Omission of the CaMKII antibody produced no signal in the appropriate channel, indicating that there was no crossreactivity of secondary antibodies.

Free-floating sections were treated as above except for the following changes: blocking was done in 10% NDS for 1 hour followed by incubation with GABA antibody (1:2,500) and treatment with biotinylated, affinity-purified donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) was at a higher concentration (1:1,000) and for 1 hour at room temperature followed by 72 hours incubation at 4°C with FITC-SA. Following staining for GABA, sections were incubated at 4°C for 72 hours in primary antibody directed against CaMKII (1:6,000) in ICC buffer with 2% NDS. Sections were subsequently washed through five changes of ICC buffer over 50 minutes followed by a 3-hour incubation in ICC buffer containing Cy3 donkey antimouse secondary antibody (Jackson ImmunoResearch Laboratories; 1:250) for visualization of CaMKII. Following washes in four changes of TBS over 20 minutes, sections were mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and coverslips were rapidly applied with PVA-DABCO antifade mounting medium.

Y1r/CaMKII—To assess the colocalization of NPY receptors on BLA projection neurons we used the protocol described above for amplification of Y1r-ir followed by standard secondary antibody immunofluorescence. Following staining for Y1r-ir (1:1,000, rabbit [Wolak et al., 2003]), sections were incubated at 4°C for 72 hours in primary antibody directed against CaMKII in ICC buffer with 2% NDS and visualized with Cy3 donkey anti-mouse.

Y1r/GAD—Initial studies assessing Y1r-ir on GABAergic interneurons were performed using double label immunocytochemistry for colocalization of Y1r-ir and glutamic acid decarboxylase (GAD), the enzyme that synthesizes GABA from glutamate. Sections of the BLA were coincubated for 48 hours at 4°C with anti-GAD (1:1,000; Millipore) and anti-Y1r (1:1,500) antibodies. Tissue was processed with standard indirect immunofluorescence techniques as described above. GAD65- and Y1r-ir were detected with Cy3 donkey anti-mouse and FITC donkey anti-rabbit antisera, respectively (Jackson ImmunoResearch Laboratories; 1:250).

Y1r/CaBP—To further determine colocalization of Y1r within specific interneuronal populations in the BLA, Y1r was again visualized using biotinylated tyramide amplification immunofluorescence with FITC-SA. Sections were subsequently incubated with either primary antibodies for PV (polyclonal goat, Swant, Bellinzona, Switzerland; 1:10,000) and SOM (monoclonal rat AB354, Millipore; 1:250) or CCK (mouse monoclonal AB 9303, generously provided by Dr. G.V. Ohning, CURE Center, UCLA; 1:250) and CR (polyclonal goat, Swant; 1:10,000) diluted in ICC buffer with 2% NDS and 0.1% Triton X-100. Interneuronal markers were visualized using the following fluorophore conjugated secondary antibodies (Jackson ImmunoResearch Laboratories; 1:250), Cy5 donkey anti-goat (PV or CR), and Cy3 donkey anti-rat (SOM) or Cy3 donkey anti-mouse (CCK).

Antibody specificity

The complete listing of antibodies used in these studies is presented in Table 1.

GABA—This mouse monoclonal antibody was raised against GABA conjugated to BSA. This antibody does not crossreact with other amino acids as determined by enzyme-linked immunoassays, and its binding can be blocked by preadsorption with GABA (Szabat et al., 1992).

GAD (GAD 65)—This antibody was raised against purified GAD from rat brain. On Western blot this antibody recognizes the lower molecular weight form of GAD (GAD65) from rat brain.

CaMKII—This mouse monoclonal antibody was raised against the α -subunit of CaMKII and recognizes both phosphorylated and nonphosphorylated forms of CaMKII. Its specificity has been well documented in previous studies (Erondy and Kennedy, 1985) where it recognizes a single band at the expected weight of at 50 kDa on Western blots.

CR—The CR antibody was raised in goat (polyclonal) against human recombinant calretinin. It has been extensively characterized by Schwaller et al. (1999). On Western blot this antibody detects a clear and specific band in rat brain tissue corresponding to the expected size (30 kDa) of the calretinin protein.

CCK—The CCK antibody was raised against gastrin but recognizes CCK due to homologies in the terminal pentapeptide shared by these peptides. Gastrin is not found in the amygdala; therefore, the signal detected in the BLA is not due to the presence of the immunogenic peptide (gastrin) in the tissue (Marley et al., 1984). This antibody has been used in numerous studies examining the distribution of CCK in the BLA (Mascagni and McDonald, 2003; Muller et al., 2007b). Preadsorption of this antibody with CCK peptide (25 $\mu\text{g/mL}$) abolished all specific signal in the amygdala and other forebrain regions (McDonald and Mascagni, 2001b).

SOM—This is a rat monoclonal antibody, the specificity of which was determined by us with preadsorption of the primary antibody with saturating amounts (10–15 $\mu\text{g/mL}$) of the immunogenic peptide, which abolished all signal.

Y1r—This polyclonal antibody has been previously characterized by our laboratory (Wolak et al., 2003). The antisera is affinity-purified and Western analysis identified a band at 42 kDa within brain homogenates and also at ≈ 95 kDa, which likely represents a dimerized form of the receptor. Preadsorption of the primary antibody with saturating amounts (10–15 $\mu\text{g/mL}$) of the immunogenic peptide (C-terminal amino acids 363–382) eliminated signal in both immunohistochemistry and Western blot analysis of rat brain. We also confirmed the absence of immunoreactivity for the Y1r in tissue obtained from Y1r knockout mice (see Fig. 1). Similar staining patterns were also obtained when using another Y1r antiserum (Kopp et al., 2002). Additionally, the CaBP and CaMKII antibodies used here are all markers for specific neuronal subpopulations in the BLA and each produced a characteristic pattern of immunostaining seen in numerous previous studies of the rat BLA (Kempainen and Pitkanen, 2000; McDonald and Betette, 2001; McDonald and Mascagni, 2001a, 2002; Mascagni and McDonald, 2003).

Confocal microscopy and stereological procedures

Tissues were labeled with multiple fluorescent markers for experiments that required quantification of colocalized immunostained cells. Accordingly, sections were analyzed

using scanning laser confocal stereology. After selecting a starting section using a random number generator, every sixth section (240 μm apart) was taken for analysis to ensure that cells were not represented in more than one section and, therefore, were not counted twice. This selection sequence resulted in a sampling of 10 sections per animal. Selected sections of the BLA throughout the rostral-caudal extent of the nucleus (bregma -1.8 mm to bregma -4.16 mm, Fig. 2 [Paxinos and Watson, 1998]) were imaged at $10\times$ magnification using laser excitation of visualization of the Y1r fluorescent signal. Y1r-ir signal was chosen for this purpose since the borders of the BLA were clearly visualized when using this antibody. The borders of the nucleus were manually outlined for each section based on the distribution of Y1r-ir within the confines of the nucleus (Fig. 3) using an Olympus Fluoview 300 confocal microscope (Melville, NY) equipped with a motorized x - y - z stage control. The BLA was defined as including the following: the dorsolateral subdivision of the lateral amygdalar nucleus (Ldl), ventro-medial subdivision of the lateral amygdalar nucleus (Lvm), posterior subdivision of the basolateral amygdalar nucleus (BLp), and anterior subdivision of the basolateral amygdalar nucleus (BLa).

StereoInvestigator software (MBF Bioscience, Williston, VT) was used to implement the optical fractionator counting procedure (West et al., 1991; Peterson, 1999) and generate unbiased counting frames as described below. At each systematically, randomly selected site, a serial confocal stack (1 μm step, 14 μm total thickness) of each fluorophore was individually captured on the appropriate emission channel using a $60\times$ oil immersion objective (1.4 numerical aperture). The following excitation wavelengths were used: 488 nm for the secondary fluorophore FITC, 568 nm for Cy3, and 647 nm for Cy5. Stacks were collected for each fluorophore, merged, and saved for counting offline. Colocalization was determined by overlapping signals observed at several focal planes throughout each cell. Any cell whose top came into focus within the inclusion limits of the unbiased counting frame was counted. Section thickness was recorded at three sites per section and the average section thickness per animal was determined for stereological estimate calculations. StereoInvestigator software was used to calculate the total neuron number, the numbers of counted neurons, and the corresponding sampling probability.

Brightness and contrast of the photomicrographs presented here were adjusted using Adobe Photoshop 6.0 (San Jose, CA) to ensure the highest quality images for publication.

Projection neurons: colocalization of Y1r with CaMKII—To assess the expression of Y1r on projection neurons in the BLA, Y1r-, and CaMKII-ir cells were counted at predetermined intervals (sampling grid: $x = 500$ μm ; $y = 500$ μm) with an optical dissector counting frame (100 $\mu\text{m} \times 100$ μm) overlaid on a collected serial confocal stack. The counting frame thickness was 10 μm with a 2 μm guard zone on either side of the counting frame to avoid bias from “lost caps” (Schmitz and Hof, 2005). These parameters resulted in a sampling fraction of 1/500th. For Y1r/CaMKII, staining was examined in seven animals with an average of 57 ± 1 collection sites per animal.

Interneurons: colocalization of Y1r with CCK and CR or PV and SOM—To assess the expression of Y1r on BLA interneurons, sections were immunostained for Y1r with a combination of CCK and CR or PV and SOM. Due to the low number of cells expressing interneuronal markers relative to Y1r-ir cells, these populations were estimated using two independently sized counting frames imposed on the same sampling grid. Utilizing appropriately sized counting frames for each phenotype allowed the collection of the fewest number of sites while maintaining adequate sampling for each cell population. The stereological parameters for estimates of BLA interneurons (single-labeled and colabeled with Y1r) were as follows: sampling grid: $x = 325$ μm ; $y = 350$ μm , counting frame: 267×200 μm , sampling fraction 1/50th. Single-labeled cells for Y1r were counted in

the same grid but in a counting frame of $50 \times 50 \mu\text{m}$, resulting in a sampling fraction of 1/1,000th (for Y1r single-labeled cells). Confocal stacks were collected at $1\text{-}\mu\text{m}$ intervals for $14 \mu\text{m}$ ($10 \mu\text{m} + 2 \mu\text{m}$ guard zone on either side). For each combination of markers staining was examined in six animals with an average of 104 ± 3 (PV/SOM/Y1r) and 95 ± 3 (CCK/CR/Y1r) collection sites per animal.

Reproducibility of stereological estimates—There was no significant difference in the optical fractionator estimate of the total Y1r population (sum of single and double-labeled cells, $P > 0.05$) between experiments that quantified Y1r-ir neuron number (one-way analysis of variance, ANOVA [$F = 3.152$, $r^2 = 0.2826$, $P = 0.0701$]). Additionally, in these studies the coefficient of error (CE, Gundersen $m = 0$), a measure of the precision of stereological estimates (Gundersen and Jensen, 1987), ranged from 0.08–0.18. These low CE values demonstrate the high degree of reproducibility of our stereological procedures. Data are reported as mean $\pm$ SEM.

RESULTS

Characterization of Y1r antibody in WT and KO mice

To further verify the specificity of our Y1r antibody, Y1r-ir was assessed in WT and Y1r KO mice. Y1r-ir was observed in the BLA of WT animals (Fig. 1A). Immunopositive cells had a homogenous rostral-caudal and dorsal-ventral distribution in the BLA and heterogeneous sizes and shapes similar to those seen in rat. Both small nonpyramidal, presumably GABAergic interneurons (horizontal arrow, Fig. 1A), and larger pyramidal-shaped, likely glutamatergic projection, cells were seen (vertical arrows, Fig. 1A). As expected, no specific Y1r signal was seen in the BLA of KO mice (Fig. 1B).

Stereological analysis of pyramidal neurons and interneurons in the BLA

Confocal stereology was employed to assess the degree of NPY Y1 receptor expression on pyramidal neurons and interneurons in the BLA. While there was extensive labeling of CaMKII-ir and GABA-ir throughout the BLA, coexpression of GABA and CaMKII was not observed, demonstrating that CaMKII is a reliable marker for glutamatergic neurons in the BLA (Fig. 4). Numerous CaMKII-ir cells were homogeneously distributed throughout both the rostral-caudal and dorsal-ventral axis of the BLA. All CaMKII-ir cells exhibited a pyramidal shape but heterogeneous sizes with a range of $15.83\text{--}21.67 \mu\text{m}$ (mean: $17.34 \pm 0.27 \mu\text{m}$; $n = 26$, Fig. 4A). The CaMKII-ir pyramidal neuron population was stereologically estimated to be $66,763 \pm 3,326$ cells (Table 2).

GABA-ir neurons were scattered throughout the BLA with $\approx 20\text{--}30$ neurons visible per section. GABA-ir cells exhibited a variety of shapes and sizes including small round cells (Fig. 4B), medium-sized fusiform shaped cells, and large multipolar cells. To more specifically characterize Y1r-ir on the different populations of BLA interneurons, CaBPs (CR, PV) and neuropeptides (CCK, SOM) were used as markers for specific GABAergic neuronal subpopulations in the BLA (McDonald and Mascagni, 2001a, 2002; Mascagni and McDonald, 2003). All of the interneuronal markers studied were evenly, if sparsely, distributed throughout the BLA and did not exhibit labeling with CaMKII (results not shown). The largest population of interneurons contained PV, followed by the CCK/CR colabeled population and SOM-ir cells, with CR and CCK single-labeled cells being the least numerous populations (Table 3, $n = 6$). Although some pyramidal cells contain low levels of CR, these cells were easily distinguished from the GABAergic interneurons based on size and shape as well as signal intensity (McDonald and Mascagni, 2001a). Generally, the PV- and neuropeptide-containing cells exhibited the largest diameters of the nonpyramidal interneurons, while CR-ir cells were the smallest. Specifically, the majority of

PV-ir cells were large and multipolar with diameters ranging from 15.83–28.33 μm and an average diameter of $19.22 \pm 0.61 \mu\text{m}$ ($n = 22$). SOM-ir cells were frequently a fusiform shape with an average length across the longest diameter of $16.52 \pm 0.56 \mu\text{m}$ (range: 13.33–27.92 μm , $n = 20$). Two populations of CCK-ir neurons were seen: CCK single-labeled cells, which were large multipolar cells ($16.47 \pm 0.65 \mu\text{m}$, range: 11.67–24.17 μm ; $n = 19$) and CCK-ir/CR-ir double-labeled cells that were typically small and round ($11.9 \pm 0.49 \mu\text{m}$, range: 10.00–19.17 μm , $n = 11$). The size of these CCK/CR double-labeled cells was the same as CR single-labeled cells ($11.9 \pm 0.31 \mu\text{m}$, range: 10.00–18.33 μm , $n = 22$).

Colocalization of Y1r with markers for pyramidal neurons and interneurons in the BLA

Pyramidal neurons—The distribution of Y1r-ir on pyramidal neurons in the BLA is presented in Figure 5. Single- and double-labeled populations of cells were assessed using stereological techniques. Y1r-ir cells ($79,839 \pm 4,795$ cells) exhibited a homogenous rostral-caudal and dorsal-ventral distribution in the BLA consisting of heterogeneous sizes and shapes.

Y1r-ir cells exhibited a high degree of colocalization with CaMKII-ir (Fig. 5A–C). Double-labeled cells were noted throughout the entire BLA and no change in the proportion of colocalization was noted in rostral-caudal or dorsal-ventral directions. In most CaMKII-ir cells Y1r-ir appeared as diffuse cytoplasmic signal. Virtually all CaMKII-ir cells expressed Y1r (99.9%), contributing to 83.6% of the total Y1r-ir cell population in the BLA (Table 2). Many Y1r-ir cells that were not CaMKII-ir were also observed ($13,129 \pm 1,609$, Fig. 5B,C). These single-labeled Y1r-ir cells were of varying sizes and shapes, some being smaller or larger than the neighboring CaMKII-ir cells. All single-labeled Y1r-ir cells were nonpyramidal in shape, likely representing GABAergic interneurons. Based on the size of these single-labeled (Y1r-ir) nonpyramidal neurons, the smaller cells would be expected to be immunoreactive for CR and it is probable that the larger cells are PV- or neuropeptide-ir.

Interneurons—The initial presence of Y1r-ir on interneurons was assessed using a GAD antibody to detect GABA-producing cells in the BLA (Fig. 5D–F). While these cells were not assessed stereologically, there was a high degree of coexpression of Y1r-ir in GAD-immunopositive cells (> 85% of the GAD-ir cells) counted in atlas-matched sections through the BLA (Paxinos and Watson, 1998). In order to determine if Y1r-ir could be identified on specific subpopulations of GABAergic interneurons, we performed a series of immunocytochemical experiments using antibodies against Y1r and the BLA interneuronal markers described above: CR, CCK, PV, SOM. Each of these interneuron subpopulations showed appreciable Y1r-ir in most cells (Table 3). Colabeled cells were evenly distributed throughout the BLA and were not confined to a particular subnucleus. CCK (Fig. 6A), CR (Fig. 6C), SOM (Fig. 6E), and PV (Fig. 6G)-containing interneurons exhibited Y1r-ir that appeared mostly cytoplasmic (Fig. 6D,H). Of all the interneuronal markers examined in this study, the PV-ir population comprised the greatest percentage of total nonpyramidal Y1r-ir cells and also the greatest proportion of single-labeled cells. When combined, these groups of Y1r-expressing interneurons account for $\approx 7.5\%$ of the total Y1r-ir in the BLA. Sections stained for PV displayed a dense immunoreactive neuropil (blue) that formed baskets around pyramidal-shaped, Y1r single-labeled cells (green; Fig. 6I–K, angled arrows).

DISCUSSION

NPY, via activation of Y1r receptors, exerts anxiolytic effects (Heilig et al., 1989; Heilig, 1995; Kask et al., 2001; Redrobe et al., 2002; Sorensen et al., 2004). While there are a number of anatomical sites where NPY elicits changes in behavior (Flood et al., 1989; Kask et al., 1998, 2002), the activation of these receptors in the BLA is critical for NPY's

anxiolytic action (Sajdyk et al., 1999b, 2008; Redrobe et al., 2002). Until now, the cell-specific expression of Y1 receptors in the BLA was unknown. To more fully understand the circuitry through which NPY could be producing anxiolysis, we used multiple-label immunocytochemistry for Y1 receptors combined with markers for identification of BLA neuron subtypes. The results of the current study demonstrate the presence of NPY Y1 receptors on both pyramidal neurons and nonpyramidal interneurons in the rat BLA, suggesting that NPY can directly influence the activity of both neuron subpopulations.

The present studies are the first to stereologically address the phenotypes of neurons in the basolateral amygdala complex that includes the Ldl, Lvm, BLp, BLA. As the population of BLA neurons is heterogeneous in both size and shape, the use of stereology as an analytical tool was important to provide an accurate assessment of NPY receptor expression among the different cell types. Traditional nonstereological quantification of cellular populations often results in a sampling bias, with larger cells being sampled with a higher probability than smaller cells present at the same frequency (Peterson, 1999). The stereological techniques employed here allowed efficient and highly reproducible quantification of neuron number. Furthermore, the proportion of pyramidal cells ($\approx 90\%$) and interneurons ($\approx 10\%$) found in these studies matches well with those previously reported ($\approx 85\%/\approx 15\%$ [McDonald, 1982, 1992]). Additionally, the proportion of interneurons expressing each marker was in line with previous studies that examined the distribution within specific subregions of the nucleus (McDonald and Mascagni, 2001a; McDonald and Betette, 2001; Mascagni and McDonald, 2003; McDonald et al., 2005).

Distribution of Y1r-ir on pyramidal cells in the BLA

To further elucidate the circuitry underlying NPY's role in regulating BLA output and anxiety, we first investigated the colocalization of Y1r-ir with CaMKII-ir to identify pyramidal cells. Expression of NPY receptor-ir was observed on virtually all BLA pyramidal neurons. The high levels of Y1r expression on pyramidal neurons in the BLA suggests that NPY may directly influence the activity of this cell type and hence the output of the BLA. In a current collaboration using electrophysiological approaches, we demonstrated that activation of NPY Y1 receptors in the BLA hyperpolarizes pyramidal cells (Giesbrecht, Urban, Colmers, pers. commun.). These actions of NPY are due to a direct effect on the cells and are not mediated via a GABAergic mechanism. Additionally, application of NPY also decreased the excitability of projection neurons in the lateral amygdala through activation of an inwardly rectifying potassium current (Sosulina et al., 2008). These inhibitory effects of NPY on BLA projection neurons are consistent with an anxiolytic mode of action (Pesold and Treit, 1995; Sajdyk et al., 1999b; Redrobe et al., 2002; Sornenson et al., 2004) and may be one mechanism through which NPY decreases anxiety-related behavior.

Distribution of Y1r-ir on interneurons in the BLA

While the determination of NPY receptor expression on pyramidal neurons, due to the ubiquitous expression of CaMKII-ir in this population being fairly straightforward, the investigation of Y1r expression on interneuronal populations required a more detailed analysis. The BLA interneuronal markers used in this study included CaBPs (PV, CR) and peptides (SOM, CCK) based on previous characterizations of these cells (McDonald and Betette, 2001; McDonald and Mascagni, 2001a, 2002; McDonald et al., 2005). Interneurons provide the main inhibitory influence on pyramidal cells in the BLA and each marker signifies a functionally different type of interneuron (Sorvari et al., 1998; Muller et al., 2003, 2007a; McDonald et al., 2005; Rainnie et al., 2006; Chung and Moore, 2007). While each interneuron subtype did express Y1r-ir, the majority of interneurons expressing Y1r were PV-ir. Consistent with previous reports (McDonald et al., 2005), we found that PV-ir cells

comprised $\approx 45\%$ of the interneuron population. These cells are known to have extensive axonal arborizations that form pericellular baskets around pyramidal soma. In return, PV-ir cells receive reciprocal connections from pyramidal neurons and are uniquely positioned to have strong synaptic control over pyramidal neuron activity. In the BLA, PV-ir cells may innervate 100 pyramidal cells (Muller et al., 2006) and each pyramidal cell may receive innervation from at least 10 PV-ir cells. The presence of NPY receptors on this population of interneurons could therefore have profound effects on the synchronous activity of the BLA (Rainnie et al., 2006).

Expression of NPY receptors on CCK-ir and CR-ir cells also indicates that NPY may influence the synchronization of GABAergic interneurons as these neurons are known to synapse on other interneuronal populations (Sorvari et al., 1998; Chung and Moore, 2007). In the BLA, SOM-ir terminals arising from interneurons mainly contact distal dendritic spines of glutamate cells and are often seen in close apposition to asymmetrical (excitatory) synapses (Muller et al., 2007a). This location suggests that SOM-ir terminals may serve to shunt incoming excitatory input (Washburn and Moises, 1992). Expression of NPY receptors within the SOM-ir population is, therefore, suggestive of NPY's ability to modulate pyramidal neuron excitability via alteration of interneuron activity.

Functional significance of NPY receptor expression in the BLA

With NPY receptors present on both local inhibitory and excitatory projection cells, the circuitry via which NPY exerts its anxiolytic action is likely to involve more than one mechanism. The presence of NPY receptors on both populations of cell types may appear counterintuitive to the precise regulation of BLA output. However, this expression pattern is certainly not unique, as the cannabinoid receptor (CB1; McDonald and Muscagni, 2001b) and serotonin 2A receptor (5HT_{2A}; McDonald and Muscagni, 2007) are present on both projection cells and interneuronal populations. There are a number of potential explanations as to how NPY functions as an anxiolytic despite the diversity of cell types expressing NPY receptors. It is possible that NPY produces differential effects on pyramidal and interneuron activity. Possible ways this modulation might occur include: 1) differential coupling of Y_{1r} and Y_{5r} to second messenger systems in each cell type (Herzog et al., 1992), 2) differential NPY innervation of interneurons and pyramidal neurons from different brain regions, and 3) functional consequences of heterodimerization by NPY receptor (Y₁ and Y₅) subtypes (Gehlert et al., 2007).

Evidence for differences in receptor function comes from studies with NPY receptor transfected cell lines. Herzog et al. (1992) have shown that Y_{1r} couples to different second messenger system in a manner that is dependent on the cell line used. After stimulation with NPY, CHO cells exhibited an increase in intracellular calcium with no change in adenylate cyclase activity, while HEK293 cells showed no increase in intracellular calcium levels and clear inhibition of adenylate cyclase activity. Additionally, it may be that NPY can exert a direct effect on one cell type yet have only a neuromodulatory effect on the other (Selbie and Hill, 1998). These reports suggest that NPY could act on BLA interneurons and pyramidal neurons via the same receptor but exert differential effects that are dependent on the second messengers to which the receptor is coupled.

Although most BLA neurons express a complement of NPY receptors, they may receive NPY innervation from different sources. These differential NPY inputs would allow for fine tuning control of NPY receptor activation where only a subset of cell types would be activated at any particular time. For example, NPYergic innervation of the BLA arises, at least in part, from local interneurons that are also SOM-ir (McDonald, 1989). Since most SOM-ir fibers synapse on spines and small-caliber dendrites of presumably spiny pyramidal neurons (Muller et al., 2007a), it follows that NPY arising from intrinsic sources also targets

pyramidal cells, and, as mentioned above, is in prime position to shunt incoming excitatory input. A recent study by Cui et al. (2008) demonstrated NPYergic synapses with pyramidal neuron soma and principle dendrites. Further studies employing anterograde tracing techniques and electron microscopy will be necessary to determine if these NPY synapses arise from projection sources. The existence of one or more extra-BLA projection sources of NPY differentially targeting interneurons or pyramidal neurons expressing NPY receptors is likely. That NPY can exert both excitatory and inhibitory effects on a neural system has been posited by Füzezi et al. (2007). The multiplicity of NPY inputs to, and innervation of, subpopulations of hypothalamic CRF neurons in the paraventricular nucleus is postulated to relay a site-specific signal to these neurons which then contributes to the overall activity (inhibition or excitation) of CRF neurons (Füzezi et al., 2007).

Another way NPY may differentially influence the activity of interneurons and pyramidal neurons is via formation of heterodimeric receptors. Recent studies show that Y1r/Y5r heterodimers have different signaling properties compared to the individual monomers (Gehlert et al., 2007). For example, in response to certain agonists, cells expressing Y1r and Y5r heterodimers showed greater inhibition of forskolin-induced adenylate cyclase activity compared to cells expressing either receptor alone. These heterodimeric receptors also have different rates of internalization following stimulation compared to monomeric forms of the receptor in the same cell line. Although these studies examined receptor function in HEK293 cells, previous reports from our laboratory show higher than expected molecular weight bands for Y1r on Western blot analysis of hypothalamic tissue, suggesting that these dimers might also exist in vivo (Wolak et al., 2003). While there is a high coexpression of Y1r-ir on pyramidal cells, we also have preliminary evidence to suggest that these cells also express Y5r-ir. Therefore, it can be inferred that the pyramidal neurons expressing Y5r also express Y1r, indicating that these cells have the capacity to form NPY receptor heterodimers. If pyramidal neurons have a higher degree of coexpression of Y1r and Y5r than interneurons, it could indicate that NPY has a stronger or qualitatively different effect on pyramidal neuron activity as compared to interneuronal populations that may only express a single receptor subtype.

These studies provide important information about NPY stress circuitry by presenting detailed information about the cell types expressing NPY Y1 receptors in the BLA. The localization of NPY receptors on BLA pyramidal neurons suggests a prime effect of NPY on this cell type. As both NPY and decreased pyramidal neuron activity are known to cause anxiolysis, these results favor the hypothesis that NPY inhibits BLA output via actions on the pyramidal cell population. The presence of NPY receptors on GABAergic interneurons suggests that NPY may modulate the activity of these neurons as well. These data provide evidence for a complex role, through both direct and indirect (via interneurons) mechanisms, of NPY on pyramidal neuron activity that indicates that not only would NPY modulate the firing of projection neurons but could also modulate the synchronous activity of the BLA through interneurons. As dysregulation of the NPY system has been implicated in the development of stress-related illnesses such as depression and anxiety (Heilig, 2004; Heilig et al., 2004), the present data provide further insight into NPY systems and their regulation under normal conditions is crucial to understanding the etiology and pathophysiology of these disorders as well as the generation of stress resilience (Sajdyk et al., 2008).

Acknowledgments

The authors thank Ms. Gina DeJoseph for expert technical assistance. Antibody #9303 raised against Gastrin/CCK was provided by Dr. Ohning at the CURE Digestive Diseases Research Center, Antibody/RIA Core, UCLA.

Grant sponsor: National Institutes of Health (NIH); Grant number: MH62621.

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Figure 1. Photomicrographs of Y1r-immunoreactivity in the BLA of (A) WT and (B) Y1r KO mice. Robust Y1r-ir was observed in the BLA of WT mice; both pyramidal (vertical arrows) and nonpyramidal (horizontal arrows) shaped cells are evident (A). No specific signal was detected in the BLA of KO animals (B). Scale bar = 20 μ m.

Figure 2.

Photomicrographs of CaMKII-ir sections representative (A) anterior (bregma -1.8 mm), (B) middle (bregma -2.8 mm), and (C) posterior (bregma -4.16 mm) coronal sections of the BLA. These represent typical sections in a 1:6 series used for stereological analysis. BA, basolateral division of the BLA; LA, lateral division of the BLA; CeA, central nucleus of the amygdala; lv, lateral ventricle; HIP, hippocampus. Scale bar = $200\ \mu\text{m}$.

Figure 3. Photomicrograph of Y1r-ir in the BLA. Low-power photomicrograph shows the distribution of Y1r-ir cells throughout the regions of the BLA (bregma -2.30 mm). The borders of the BLA used to define the area for stereological analysis are indicated by arrows. BA, basolateral nucleus; La, lateral amygdala; ec, external capsule. Scale bar = $200\ \mu\text{m}$.

Figure 4. Photomicrographs of (A) CaMKII and (B) GABA immunoreactivity in the rat BLA. CaMKII, a marker for BLA pyramidal neurons, did not colocalize with GABA, a marker for BLA interneurons (arrowhead). Scale bar = 10 μ m.

Figure 5. Colocalization of Y1r with CaMKII-ir or GAD65-ir in the rat BLA. Both (A) CaMKII-ir and (D) GAD65-ir cell colocalize with (B,E) Y1r-ir as seen in the merged images (C,F). Single-labeled (arrows) and double-labeled (arrowheads) cells are visible. Scale bar = 20 μm .

Figure 6.

Colocalization of Y1r-ir with interneuronal markers in the rat BLA. First row: (A) cholecystokinin, CCK; (B) Y1r; (C) calretinin, CR; (D) merge. A CCK/Y1r double-labeled cell is shown (horizontal arrow), as is a CR-ir cell expressing Y1r (double-headed arrow). Single-labeled Y1r-ir cells are designated with vertical arrows. Y1r-ir was also detected on small round cells containing both CR and CCK (inset top panel). Second row: (E) somatostatin, SOM; (F) Y1r, (G) parvalbumin, PV; (H) merge. A SOM/Y1r double-labeled cell is shown (horizontal arrow), as are PV-ir cells expressing Y1r (double-headed arrow). I–K: Higher-magnification images (third row) illustrate PV-ir fibers observed forming baskets around Y1r-ir single-labeled cells (angled arrows). A PV-/Y1r-ir cell can also be seen (double arrowhead). Scale bar = 20 μm .

TABLE 1

Primary Antibodies Used

Antigen	Immunogen	Description	Dilutions Used
Y1r	Synthetic C-terminal peptide amino acids 363-382	Polyclonal Rabbit (Wolak et al., 2003)	1:1,000 1:1,500
CaMKII	α -subunit of purified human CaMKII	Monoclonal mouse clone 6C9, # MAB8699, Millipore, Billerica, MA	1:6,000
GAD 65	Purified rat brain GAD	Monoclonal mouse MAB351R Millipore	1:1,000
GABA	GABA	Monoclonal mouse clone 5A9, # 693281, MP Biomedicals, Solon, OH	1:2,500
CR	Recombinant human calretinin	Polyclonal goat, # 7699/3H Swant, Bellinzona, Switzerland	1:10,000
PV	Rat muscle parvalbumin	Polyclonal goat, # PVG-214, Swant	1:10,000
SOM	Synthetic peptide amino acids 1-14 of cyclic somatostatin	Monoclonal rat, clone YC7 AB345, Millipore	1:250
CCK	Synthetic Gastrin (peptide 2-17)	Monoclonal mouse AB#9303, generously provided by Dr. G. V. Ohming, CURE Center, UCLA	1:250

TABLE 2

Distribution of Y1r Immunoreactivity on Glutamatergic Pyramidal Neurons in the Rat BLA

	Total population	Double-labeled population	% Double-labeled
CaMKII	66,763 $\pm$ 3,326	66,710 $\pm$ 3,348	99.9
Y1r	79,839 $\pm$ 4,795		83.6

TABLE 3
Distribution of Y1r Immunoreactivity among Distinct Populations of GABAergic Interneurons in the Rat BLA

	Total population	% of Total interneurons	Population expressing Y1r	% of Population expressing Y1r	% of Total Y1r expressing cells
CCK	499 ± 56	7.3	437 ± 50	87.6	0.6
CR	1,458 ± 113	21.6	1,295 ± 128	88.7	1.6
CCK/CR	359 ± 84	5.2	298 ± 72	83.0	0.4
SOM	1,417 ± 124	20.8	1,325 ± 122	93.5	1.7
PV	3,084 ± 98	45.2	2,510 ± 59	81.4	3.2