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RESEARCH PAPER

Neuropeptide Y modulates effects of bradykinin and prostaglandin E_2 on trigeminal nociceptors via activation of the Y_1 and Y_2 receptors

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Background and Purpose: Although previous studies have demonstrated that neuropeptide Y (NPY) modulates nociceptors, the relative contributions of the Y_1 and Y_2 receptors are unknown. Therefore, we evaluated the effect of Y_1 and Y_2 receptor activation on nociceptors stimulated by bradykinin (BK) and prostaglandin E_2 (PGE_2).
Experimental approach: Combined immunohistochemistry (IHC) with *in situ* hybridization (ISH) demonstrated that Y_1- and Y_2-receptors are collocated with bradykinin $_2$ (B_2)-receptors in rat trigeminal ganglia (TG). The relative functions of the Y_1 and Y_2 receptors in modulating BK/PGE_2-evoked CGRP release and increased intracellular calcium levels in cultured TG neurons were evaluated.
Key results: The Y_1 and Y_2 receptors are co-expressed with B_2 in TG neurons, suggesting the potential for direct NPY modulation of BK responses. Pretreatment with the Y_1 agonist [Leu31,Pro34]-NPY, inhibited BK/PGE_2-evoked CGRP release. Conversely, pretreatment with PYY(3-36), a Y_2 agonist, increased BK/PGE_2 evoked CGRP release. Treatment with NPY evoked an overall inhibitory effect, although of lesser magnitude. Similarly, [Leu31,Pro34]-NPY inhibited BK/PGE_2-evoked increases in intracellular calcium levels whereas PYY(3-36) increased responses. NPY inhibition of BK/PGE_2-evoked release of CGRP was reversed by the Y_1 receptor antagonist, BIBO3304, and higher concentrations of BIBO3304 significantly facilitated CGRP release. The Y_2 receptor antagonist, BIIE0246, enhanced the inhibitory NPY effects.
Conclusions and implications: These results demonstrate that NPY modulation of peptidergic neurons is due to net activation of inhibitory Y_1 and excitatory Y_2 receptor systems. The relative expression or activity of these opposing receptor systems may mediate dynamic responses to injury and pain.
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Keywords: neuropeptide Y; Y_1 receptor; Y_2 receptor; pain; bradykinin; sensory neuron; inflammation; neurogenic inflammation; CGRP; NPY

Abbreviations: B_2, bradykinin$_2$ receptor; BK, bradykinin; CGRP, calcitonin gene-related peptide; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglia; GPCR, G-protein coupled receptors; HBBS, Hank's balanced salt solution; IHC, immunohistochemistry; ISH, *in situ* hybridization ; NPY, neuropeptide Y; PGE_2, prostaglandin E_2; RIA, radioimmunoassay; SES, standard extracellular solution; TG, trigeminal ganglia

Introduction

Neuropeptide Y (NPY) is expressed in the peripheral and central nervous systems and modulates several physiological functions including satiety, anxiety and vascular tone (Pedrazzini *et al*., 2003; Heilig, 2004; Feletou *et al*., 2006). It is also reported that NPY modulates sensory neuron activity. However, the mechanism of modulation appears complex as NPY has been shown to both facilitate and inhibit peripheral neuronal activity (Hua *et al*., 1991; Xu *et al*., 1994; White, 1997; Hokfelt *et al*., 1999; Naveilhan *et al*., 2001b; Ossipov *et al*., 2002; Taiwo and Taylor, 2002). Perhaps contributing to the complexity of the modulatory effects of NPY is the finding that the expression of NPY and NPY receptors is dynamic and dramatically altered after peripheral injury, including nerve injury and inflammatory injury (Wakisaka *et al*., 1991; Mantyh *et al*., 1994; Xu *et al*., 1994; Zhang *et al*., 1994b; Hokfelt *et al*., 1999; Marchand *et al*., 1999; Shi *et al*., 1999; Honore *et al*., 2000; Benoliel *et al*., 2001; Naveilhan *et al*., 2001a; Ossipov *et al*., 2002; Taiwo and Taylor, 2002). Therefore, the effect of NPY on sensory neurons may be dependent upon the receptors expressed,

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as well as the signalling cascade the receptor activates, and it is likely that certain types of injury alter these variables.

Of the five cloned NPY receptors, only the Y_1 and Y_2 receptor subtypes have been identified in sensory neurons (Ji *et al*., 1994; Mantyh *et al*., 1994; Zhang *et al*., 1994a, b, 1997; Marchand *et al*., 1999; Brumovsky *et al*., 2002). Both receptors are pertussis toxin-sensitive G-protein-coupled receptors (GPCRs) that, presumably, signal through Gi/o proteins to inhibit cyclic adenosine monophosphate accumulation by inhibiting adenylyl cyclase. Depending upon the cell type, activation of Y_1 or Y_2 receptors can lead to increased or decreased intracellular Ca^{2+} levels, and both display similar affinity for NPY in the low nM range (Walker *et al*., 1988; Herzog *et al*., 1992; Larhammar *et al*., 1992; Balasubramaniam, 1997; Abdulla and Smith, 1999; Silva *et al*., 2002; Pedrazzini *et al*., 2003). The receptors are localized to distinct populations of sensory neurons with the Y_1 receptor found in small to medium sized neurons and Y_2 located in medium to large sized neurons (Mantyh *et al*., 1994; Zhang *et al*., 1994a; Zhang *et al*., 1997). Both receptors are frequently found co-expressed with calcitonin gene-related peptide (CGRP), an important neurotransmitter in mediating nociception and neurogenic inflammation (Brain *et al*., 1985; Zhang *et al*., 2001; Sun *et al*., 2004). Strong evidence exists for an inhibitory function of the Y_1 receptor on nociceptive behaviour, the mechanism of which appears to be due to, at least in part, Y_1 receptor-mediated inhibition of neurosecretion (Duggan *et al*., 1991; Naveilhan *et al*., 2001b; Taiwo and Taylor, 2002; Gibbs *et al*., 2004, 2006). The function of theY_2 receptor has not been fully evaluated, although evidence exists for an excitatory effect of Y_2 receptor activation on sensory neurons (Abdulla and Smith, 1999; Xu *et al*., 1999). Therefore, NPY could have an excitatory or inhibitory effect on sensory neurons depending on which receptor type they express.

In this study, we tested the hypothesis that exogenous NPY modulates sensory neuron activity by activating both the Y_1 and Y_2 receptors. We evaluated the effects of Y_1 and Y_2 receptor stimulation in modulating the activation of nociceptors induced by a combination of the pro-inflammatory algogenic substances bradykinin (BK) and prostaglandin E_2 (PGE_2), using both CGRP release and intracellular calcium levels as outcome measures.

Methods

Animals

Twenty-eight adult male Sprague–Dawley rats (Charles River, Wilmington, MA, USA) weighing 250–300 g were used in this study. The animals were housed for 1–2 weeks before the experiment and maintained under 12-h light/dark cycle at room temperature (20–25°C) with food and water available *ad libitum*. All animal study protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and conformed to International association for the study of pain (IASP) and federal guidelines.

Production of antiserum

In order to conduct immunohistochemical (IHC) analysis of the Y_2 receptor in sensory neurons, a C-terminal peptide (amino acids 367–381) of the Y_2 receptor was synthesized, conjugated to keyhole limpet haemocyanin, and injected into two rabbits (Sigma-Genosys, The Woodlands, TX, USA). Sera were screened for immunoreactivity, and a sample was affinity purified (Sigma-Genosys). Specificity of the immunoreactivity for the Y_2 receptor was established using pre-adsorption with the cognate peptide sequence (Figure 1e and f), as well as collocation with Y_2 receptor mRNA using *in situ* hybridization (ISH) (data not shown).

In situ *hybridization and immunohistochemistry*

In studies with combined ISH/IHC analysis, coverslips containing cultured trigeminal ganglion (TG) neurons were fixed with 4% formaldehyde, made permeable with 0.5% Triton X-100, acylated in acetic anhydride, dehydrated in alcohol and the lipids removed with chloroform. ISHs (55°C) were carried out using a DIG-cRNA probe against the bradykinin$_2$ receptor (B_2; position 102–500, accession no. NM_173100) that was designed and synthesized in our lab (Patwardhan *et al*., 2005). The coverslips were treated with RNAse, washed with decreasing concentrations of standard sodium citrate (SSC) buffer (final wash $0.1 \times$ SSC at 55°C) and the resulting hybridization was detected using a standard alkaline phosphate-based reaction (substrates BCIP-NBT, Roche, Indianapolis, IN, USA). Controls consisted of missense and sense riboprobes. Slides/coverslips were then incubated with rabbit primary antibody against either the Y_1 receptor (Immunostar, Hudson, WI, USA, 1:100, directed towards aa 356–382) or the Y_2 receptor (1:500) overnight at 4°C and detected using an Alexa-488-conjugated fluorescent secondary antibody (1:300, Molecular Probes, Eugene, OR, USA). The IHC protocol included 4% formaldehyde fixation, 0.2% Triton X-100 for permeability, 10% goat serum block and phosphate-buffered saline washes. Double ISH/IHC images were acquired using a Nikon E600 microscope (Melville, NY, USA) equipped with appropriate filters and a Photometrics SenSys charge-coupled device-cooled digital camera (Roper Scientific, Tuscan, AZ, USA) connected to a computer equipped with Metamorph V4.1 image analysis software (Universal Image Corporation, Downingtown, PA, USA).

For IHC on native rat sensory neurons, TG were carefully removed from the dead animal (killed by decapitation) and embedded in Tissue-Tek OCT (Sakura Finetek, Torrence, CA, USA), frozen, cut into 20 μm sections and thaw mounted onto Superfrost plus glass slides. The IHC protocol followed was the same as described above. To test for antibody specificity, tissues were preincubated with $5\times$ blocking peptide before antibody incubation.

Compounds

NPY, the Y_1 agonist [Leu31,Pro34]-NPY, and the Y_2 agonist PYY(3–36) (Bachem Labs, Torrence, CA, USA) were all made up in stock solutions of deoxygenated ddH_2O with 1% ascorbic acid and BK (Sigma-Aldrich, St Louis, MO, USA)

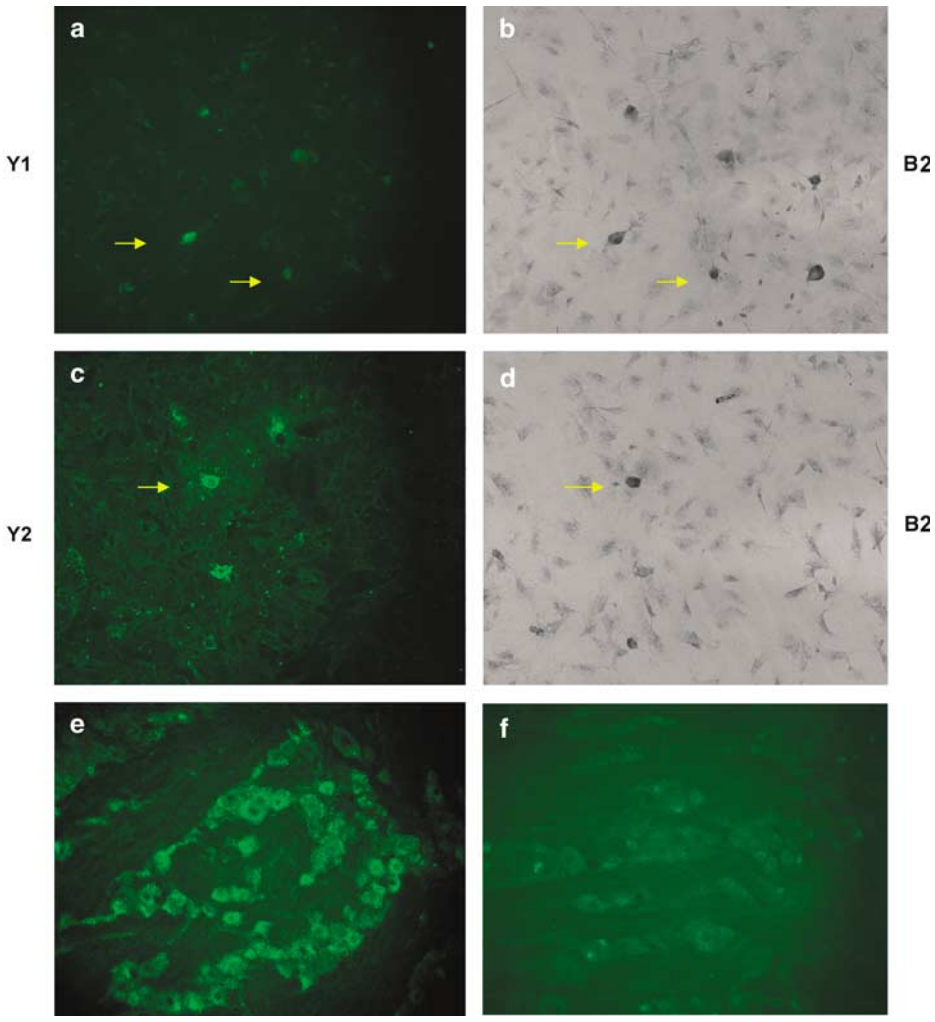

Figure 1 Representative photomicrographs showing collocation of NPY receptors with the BK B_2 receptor in cultured trigeminal neurons. Y_1 immunoreactive cells (**a**, green) colocalize with cells containing B_2 mRNA (**b**). Examples of cells containing both Y_1 immunoreactivity and B_2 mRNA hybridization are highlighted with yellow arrows. Y_2 immunoreactive cells (**c**, green) colocalize with cells containing B_2 mRNA (**d**). Examples of cells containing both Y_2 immunoreactivity and B2 mRNA hybridization are highlighted with yellow arrows. Representative photomicrograph of Y_2 immunoreactivity in native TG (**e**) and near elimination of the signal by pretreatment with Y_2 blocking peptide (**f**).

was diluted in ddH_2O immediately before the experiment. PGE_2 (Cayman Chem, Ann Arbor, MI, USA) was made in stock solutions with EtOH and diluted in buffer immediately before the experiment (final 0.01% EtOH). BIBO3304 ((*R*)-*N*-([4-{aminocarbonylaminomethyl}-phenyl]methyl)-N^2-(diphenylacetyl)-argininamide trifluoroacetate), a non-peptide Y_1 antagonist, and BIIE0246 ((*S*)-N^2-([1-{2-(4-[(*r,s*)-5, 11-dihydro-6(6H)-oxodibenz[*b,e*]azepin-11-yl]-1-piperazinyl)-2-oxoethyl}cyclopentyl]acetyl)-*N*-(2-[1,2-dihydro-3,5(4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl)-argininamide), a non-peptideY_2 antagonist (both kindly donated by Boehringer Ingelheim Pharma KG), were dissolved in H_2O with the aid of a sonicator, stored at −20°C, and aliquots used as needed.

Rat TG primary culture

Cultures of TG neurons used for CGRP release were generated using a protocol modified from Vasko *et al.* (Burkey *et al.*, 2004) in which rat TGs were quickly removed after the animal had been killed (by decapitation) and placed in ice-cold balanced Hank's balanced salt solution-calcium and magnesium free (HBBS, Gibco Carlsbad, CA, USA) and washed twice with HBBS. Ganglia were then treated with 5 mg ml^{-1} collagenase (Sigma-Aldrich) for 30 min and 0.1% trypsin (Sigma-Aldrich) for 15 min before homogenization. The TG were then treated with 10 U of DNAse I (Roche, Indianapolis, IN, USA), centrifuged at 2000 r.p.m. for 2 min and resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco), that also contained 1 × pen-strep (Gibco), 1 × glutamine (Gibco) 3 μg ml^{-1} 5-fluoro-2-deoxyuridine (5-FDU) and 7 μg ml^{-1} uridine (Sigma-Aldrich), 10% foetal calf serum (Gibco) and 100 ng ml^{-1} nerve growth factor (NGF, Harlan, Indianapolis, IN, USA). The tissue was gently triturated and cells from six ganglia were plated on one 48 well poly-D-lysine-coated plate or onto poly-D-lysine-coated coverslips (BD biosciences, Bedford, MA, USA) yielding ~4000 cells well^{-1}. The cell culture media was changed 1

and 3 days after culturing and experiments were performed on day 5.

CGRP release assay
All release experiments were performed at 37°C, using modified Hank's buffer (Gibco; 10.9 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 4.2 mM sodium bicarbonate, 10 mM dextrose and 0.1% bovine serum albumin were added to 1 × Hanks). After two initial washes with the buffer, a 15 min baseline sample was collected for radioimmunoassay (RIA) analysis of CGRP content. For Y receptor agonist experiments, the cells were treated with the selected agonist (NPY, [Leu31,Pro34]-NPY, or PYY(3–36)) or vehicle for 15 min. Cells were then stimulated with the combination of BK (10 μM) and PGE_2 (1 μM) for 15 min. In experiments using a Y receptor antagonist, the cells were pre-exposed to BIIE0246 or BIBO3304 for 15 min before NPY agonist application (15 min) and stimulation with BK/PGE_2 (15 min). After stimulation all samples were analysed using RIA for CGRP content.

Immunoreactive calcitonin gene-related peptide (iCGRP) RIA
Aliquots of the superfusate were incubated for 24 h at 4°C with 100 μl of CGRP antisera (kindly donated by Dr M Iadarola, NIDCR, NIH; Bethesda, MD, USA; final dilution 1:1 000 000). After 48 h, 100 μl of ^{125}I-labelled $CGRP_{28-37}$ (approximately 20 000–25 000 c.p.m.) and 50 μl of goat anti-rabbit antisera coupled to magnetic beads (PerSeptive Diagnostics; Cambridge, MA, USA) were added to the tubes, which were then vortexed and allowed to incubate for another 24 h. Bound and free ^{125}I-labelled $CGRP_{28-37}$ were then separated by immunomagnetics. All drugs were tested for interference with the RIA, and no crossreactivity was observed in this study.

Calcium imaging
Trigeminal ganglia cells cultured on coverslips were loaded with the cell-permeable calcium sensitive dye FURA 2-AM (1 μg ml^{-1}) (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C in standard external solution (SES) of the following composition in mM: 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES, pH 7.4. Coverslips containing cells were placed in a chamber with constant infusion of SES. Images were detected by a Nikon Eclipse TE-2000 microscope fitted with a × 20/NA 0.75 Fluor objective. Fluorescence images were collected at 5 s intervals throughout the experiment, analysed, and the F_{340}/F_{380} ratio calculated by the Methafluor Software (MethaMorph, Web Universal Imaging Corporation, Downingtown, PA, USA). [Leu31, Pro34]-NPY or PYY(3–36) was delivered into the bath solution, whereas BK and PGE_2 were delivered locally to the cells. The magnitude of calcium influx was determined by subtracting the averaged baseline 30 s before the capsaicin stimulus from the peak achieved by the capsaicin stimulation for each cell (ratiometric method, $\Delta F_{340}/F_{380}$).

Data analysis
The data were analysed by one-way or two-way analysis of variance (ANOVA) with repeated measures followed by Duncan's multiple range test to determine differences between groups. Data are presented as '% of control iCGRP release' by normalizing values to the group receiving vehicle + BK/PGE_2 evoked release for each experiment. Data are presented as mean ± s.e.m. and were considered statistically significant when $P<0.05$. Nonlinear regression curves were generated using GraphPad Prism Software Ver 4.0 (San Diego, CA, USA). Experiments were repeated at least three times.

Results

Collocation of the BK B_2 receptor with the Y_1 and Y_2 receptors in cultured sensory neurons
In order to test the hypothesis that Y1 and Y2 receptor agonists were capable of modulating BK/PGE2-evoked iCGRP release by a direct mechanism of action, we first evaluated the collocation of NPY receptors with the BK B_2 receptor using combined ISH with IHC. We focused on the B_2 receptor, as BK is a recognized pro-nociceptive inflammatory mediator and pharmacological studies performed in our laboratory have demonstrated that the BK effect of BK is abolished in TG cultures pretreated with the B_2 antagonist HOE140 (data not shown). The anatomical studies demonstrated examples of Y_1 and Y_2 immunoreactive neurons that also expressed B_2 mRNA (Figure 1a–d). Collectively, these data suggest that activation of the neuronal Y_1 or Y_2 receptors might modulate responses to BK

Effect of NPY receptor agonists on iCGRP release from cultured sensory neurons
We next evaluated the effect of pretreatment with selected NPY receptor agonists on BK/PGE_2-evoked iCGRP release from TG cultures. Pretreatment with vehicle followed by application of BK/PGE_2 caused a two- to threefold increase in iCGRP release as compared to basal release levels and this value was normalized to 100% (Figure 2). Pretreatment with the Y_1 agonist [Leu31,Pro34]-NPY induced a significant and concentration-dependent inhibition of evoked iCGRP release with a maximum inhibition of ~80% and an EC_{50} of 10 nM ($F(9,100)=3.85$, $P<0.0001$) (Figure 2a). In contrast, pretreatment with the Y_2 agonist PYY(3–36), elicited a significant enhancement of BK/PGE_2 release with a maximum facilitation approximately 60% greater than vehicle control and an EC_{50} of 25 nM ($F(6,98)=3.69$, $P<0.01$) (Figure 2b). Pretreatment with NPY, a peptide that binds both Y_1 and Y_2 at nM affinities, had no significant overall effect on BK/PGE_2-evoked release, although a trend towards inhibition (Figure 2c) was observed ($F(9,137)=0.49$).

Effect of NPY receptor antagonists on NPY-mediated inhibition of BK/PGE_2-evoked iCGRP release from cultured sensory neurons
We next evaluated the hypothesis that the NPY effect was mediated by dual activation of Y receptors with opposing

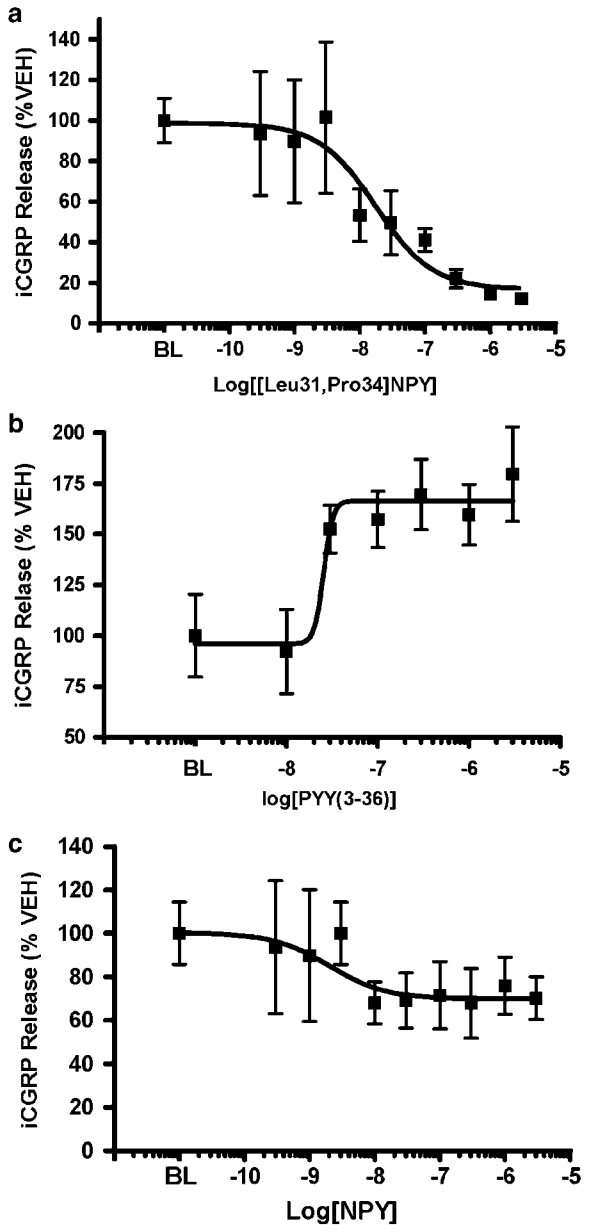

Figure 2 Effect of [Leu31,Pro34]-NPY, PYY(3–36) and NPY on BK/PGE_2 stimulated iCGRP release. After washing and collection of baseline CGRP levels (5–6 fmol), trigeminal neuronal cultures were treated with various concentrations of [Leu31,Pro34]-NPY (**a**), PYY(3–36) (**b**) and NPY (**c**) for 15 min followed by 15 min stimulation by BK(10 μM) and PGE_2 (1 μM). Samples were then measured for iCGRP content using RIA. Data are presented as percent of Veh where 100% represents the two- to threefold increase in CGRP release, which occurs in response to BK/PGE_2 treatment. Each point represents the mean and vertical lines show s.e.m.; $n=12$–20.

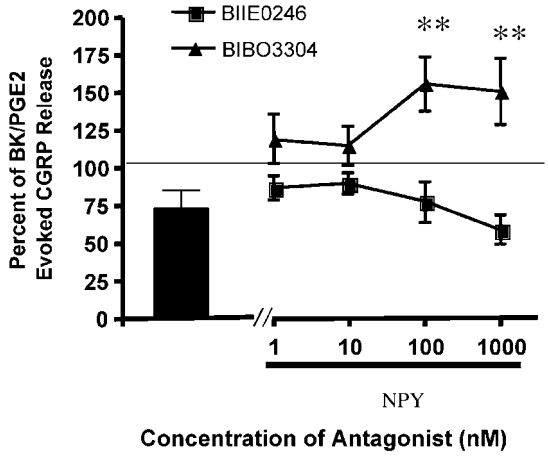

Figure 3 Effect of BIBO3304 and BIIE0246 on NPY-mediated inhibition of BK/PGE_2-evoked iCGRP release. After washing and collection of baseline CGRP levels (5–6 fmol), trigeminal neuronal cultures were pretreated with either BIBO3304 (Y_1 antagonist) (1 nM–1 μM) or BIIE0246 (Y_2 antagonist) (1 nM–1 μM) or vehicle for 15 min, followed by NPY (100 nM) for 15 min before stimulation with BK (10 μM) and PGE_2 (1 μM). Samples were then analysed for iCGRP content using RIA. NPY significantly inhibited BK/PGE_2-evoked iCGRP release ($P<0.05$; see bar). Pretreatment with various concentrations of BIBO3304 reversed the NPY-mediated inhibition of iCGRP release and at higher concentrations, actually facilitated iCGRP release. Pretreatment with BIIE0246-enhanced NPY-mediated inhibition of iCGRP release. Data are presented as mean and vertical lines show s.e.m. ($n=12$). *Post hoc* analysis using Dunnett's test: $P<0.01$ (**100 nM BIBO3304/NPY/BK/PGE_2 vs NPY/BK/PGE_2; 1 μM BIBO3304/NPY/BK/PGE_2 vs NPY/BK/PGE_2).

actions. TG cultures were pretreated with specific Y_1 and Y_2 receptor antagonists followed by NPY the altered rates of BK/PGE_2-evoked iCGRP release evaluated (Figure 3). Pretreatment with NPY (100 nM) significantly inhibited BK/PGE_2-evoked release by about 30% when compared with vehicle control samples (bar, Figure 3). Pretreatment with the Y_1 antagonist BIBO3304 blocked NPY-mediated inhibition, leading to the emergence of a significant and concentration-dependent facilitation of iCGRP release (as revealed by ANOVA: $F(5,65)=4.89$, $P<0.001$) (Figure 3). In contrast, pretreatment with the Y_2 antagonist BIIE0246 caused a significant and concentration-dependent enhancement of NPY-mediated inhibition of iCGRP release (as revealed by ANOVA: $F(5,65)=2.48$, $P<0.05$). These findings are consistent with a simultaneous overall inhibitory effect of Y_1 receptor activation and an excitatory effect of Y_2 receptor activation in sensory neurons.

Effects of Y_1 and Y_2 receptor agonists on calcium levels in sensory neurons

To confirm these findings, we evaluated the effects of selective Y_1 and Y_2 agonists on BK/PGE_2-evoked accumulation of intracellular $[Ca^{2+}]_i$ levels (Figure 4) by measuring single-cell calcium influx. The local application of a combination of 10 μM BK and 1 μM PGE_2 evoked a significant calcium influx in 69.7 ± 6.7% of all vehicle-treated cells. The pretreatment of cells with either [Leu31,Pro34]-NPY (100 nM) or PYY(3–36)(100 nM) for 10 min did not alter basal intracellular calcium levels on their own (data not shown). However, similar to the results from the CGRP release experiments, pretreatment with [Leu31,Pro34]-NPY evoked a significant decrease in the mean BK/PGE_2-evoked peak calcium influx (Figure 4a) as well as a 25% decrease in the number of cells responding to the BK/PGE_2 stimulus (52.3 ± 7% of total cells) (Figure 4b). In contrast, pretreatment of cells with PYY(3–36) caused a significant increase in the mean BK/PGE_2-evoked

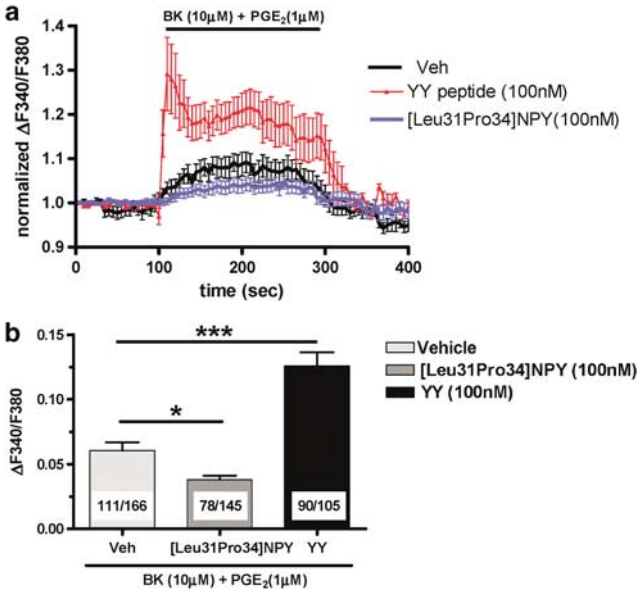

Figure 4 Effect of [Leu31,Pro34]-NPY and PYY(3–36) on BK/PGE_2-stimulated calcium influx in cultured TG neurons. Representative traces of Ca^{2+} imaging experiments performed in cultured TG neurons treated with [Leu31,Pro34]-NPY, PYY(3–36) or vehicle for 15 min followed by a 3 min BK/PGE_2 (30 nM) application are shown in (**a**). [Leu31,Pro34]-NPY and YY (3–36) did not evoke Ca^{2+} influx on their own but significantly inhibited and potentiated BK/PGE_2-evoked flux, respectively (**a**). The cumulative results of Ca^{2+} imaging experiments performed in acutely dissociated TG neurons are shown in (**b**). Data are presented as mean ΔF340/F380 and vertical lines show s.e.m. ($n=75$–111/group, $^*P<0.05$ and $^{***}P<0.001$; one-way ANOVA).

peak calcium influx (Figure 4a) and an 18% increase in the number of cells responding to the BK/PGE_2 stimulus ($85\pm4.5\%$ of total cells) (Figure 4b).

Discussion

This study demonstrates that application of NPY inhibits BK/PGE_2-evoked neuropeptide exocytosis by activation of the Y_1 receptor, but that these effects are diminished by concurrent activation of the Y_2 receptor, which facilitates exocytosis. In support of this finding, we demonstrated that treatment with the Y_1 receptor agonist [Leu31,Pro34]-NPY inhibits BK/PGE_2-evoked iCGRP release, whereas application of the Y_2 receptor agonist PYY(3–36) significantly enhances evoked iCGRP release. In addition, the NPY-mediated inhibition of iCGRP release was enhanced by pretreatment with the Y_2 receptor antagonist BIIE0246, whereas pretreatment with the Y_1 receptor antagonist BIBO3304, significantly increased iCGRP release compared to NPY treatment alone. Collectively, these data indicate that the inhibitory effect of NPY on sensory neurons is probably mediated by Y_1 receptors, whereas the excitatory effect of NPY is likely to be mediated by Y_2 receptors. In the absence of Y subtype selective receptor antagonists, NPY simultaneously activates both receptors and its overall effects appear to be a summation of an inhibitory Y_1 effect and an excitatory Y_2 effect.

This finding was confirmed using calcium imaging. Pretreatment with the Y_1 agonist [Leu31,Pro34]-NPY decreased both the mean peak calcium influx and the percentage of cells responding to the BK/PGE_2 stimulus. This is probably due to complete or partial inhibition of BK/PGE_2-evoked calcium influx below detectable levels in some cells. Conversely, the Y_2 agonist increases both the mean calcium influx and the percentage of cells responding to the BK/PGE_2 stimulus. This is probably due to the potentiation of the overall BK/PGE_2 responses, including some cells that were below detection levels in the vehicle pretreatment group.

The results from the release experiments are consistent with the anatomical collocation experiments, as both the Y_1 and the Y_2 receptors are located with the BK B_2 receptors in our primary TG culture system. The B_2 receptor is the most likely receptor subtype to be involved in these experiments, because our previous studies have shown that BK effects are blocked by pretreatment with HOE140, a B_2 antagonist (Patwardhan *et al*., 2005). Moreover, our unpublished studies have demonstrated minimal expression of the B_1 receptor in TG cultures, as evaluated by reverse transcription–polymerase chain reaction. The B_2 receptor is physiologically relevant because of its involvement in several types of pain (Dray, 1997; Hall, 1997; Calixto *et al*., 2000; Banik *et al*., 2001; Ozturk, 2001). Therefore, collocation of the Y_1 and Y_2 receptor with the B_2 receptor in cultured TG neurons is consistent with the hypothesis that the net result of activation of Y_1 and Y_2 receptors could directly modulate BK signalling.

Under normal conditions, sensory neurons express little-to-no NPY. However, after peripheral nerve injury or certain types of inflammatory injury, NPY expression is significantly increased (Wakisaka *et al*., 1991; Okada *et al*., 1993; Marchand *et al*., 1999; Honore *et al*., 2000; Ma and Bisby, 2000). It has been proposed by several investigators that primary neuronal cultures share certain phenotypic characteristics found in axotomized neurons (Buschmann *et al*., 1998; Kerekes *et al*., 2000; Dussor *et al*., 2003). NPY upregulation has been reported in culture systems before. However, the findings indicating whether the effect occurs in response to NGF are contradictory (Jiang *et al*., 1995; Buschmann *et al*., 1998; Kerekes *et al*., 2000). Although we did not determine whether our cultures express NPY, we found that 15 min pretreatment with a Y_1 or Y_2 antagonist alone did not alter either basal or BK/PGE_2-stimulated CGRP release (data not shown), suggesting that endogenous NPY is not released in sufficient quantities to alter the outcomes measured in these experimental conditions.

The demonstration that NPY has opposing effects on neurosecretion, which are dependent on the relative activation of Y_1 or Y_2 receptors, has implications for the potential role that NPY has in modulating nociceptors after injury. In native dorsal root ganglia (DRG), the Y_1 and Y_2 receptors are localized in distinct neuronal populations; the Y_1 receptors are located in neurons with small to medium diameter cell bodies and the Y_2 receptors found in neurons with medium to large diameter cell bodies. After axotomy, there is a decrease in total Y_1 mRNA expression (Zhang *et al*., 1994b). Conversely, there is an approximate doubling of Y_2 mRNA

both at the level of the DRG and spinal cord after nerve injury in medium to large-sized DRG neurons (Zhang *et al*., 1997). There is probably significant overlap between the populations of neurons expressing the Y_2 receptor and NPY after nerve injury, as NPY upregulation occurs in large diameter-myelinated fibres (Wakisaka *et al*., 1992; Noguchi *et al*., 1993). On the assumption that similar effects occur in the TG, these observations along with other findings (Abdulla and Smith, 1999), indicate a potential contribution of the Y_2 receptor in initiating and/or maintaining neuronal excitability after nerve injury. Conversely, activation of the Y_1 receptor has significant inhibitory effects on neurosecretion. These findings suggest that a Y_2 receptor antagonist may have therapeutic value for treating pain resulting from certain types of neuropathic injury and a Y_1 agonist may have therapeutic use in alleviating conditions of acute pain. Thus, relative levels of expression and activation of Y_1 and Y_2 receptors have the potential for significant and dynamic alterations in nociceptor activity.

Although the present data provide evidence for an inhibitory effect of Y_1 activation and excitatory effect of Y_2 activation on neurosecretion from peptidergic neurons, they do not define the signalling pathways that mediate these effects. The signalling pathways mediating inhibitory effects of Y_1 receptor activation are well characterized. However, it is less clear how Y_2 receptor activation stimulates neurons, as both receptors supposedly couple to Gi/o. There are several possible explanations for Y_2 receptor-mediated activation of neurons. First, Y_2 can couple to other G-proteins, namely Gq, which can lead to excitation under certain conditions (Dautzenberg, 2005; Misra *et al*., 2005). Second, as Y_2 receptors can directly increase the activity of calcium channels, a subsequent increase in neurosecretion could occur (Grouzmann *et al*., 2001; Silva *et al*., 2003). Finally, it is possible that Y_2 increases neuronal exocytosis by heterologous sensitization whereby consecutive stimulation of Gi, Gs and/or Gq leads to increased intracellular message production, which can effect the overall excitability of the cell (Drakulich *et al*., 2003). This is an important area for future research.

In summary, the present results demonstrate that both the Y_1 and Y_2 receptor are co-expressed with the B_2 receptor on cultured sensory neurons and that NPY can signal through both the Y_1 and Y_2 receptor, with activation of the Y_1 receptor resulting in inhibition of BK/PGE_2-evoked exocytosis and calcium influx, and Y_2 receptor activation causing facilitation of these responses. These studies indicate that phenotypic changes in the relative expression of Y_1:Y_2 could dynamically regulate neuronal responses to NPY leading to excitatory or inhibitory signalling and possibly contributing to alterations in nociception after injury.

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Conflict of interest

The authors state no conflict of interest.

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