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Preferential expression of the neuropeptide Y Y_1 over the Y_2 receptor subtype in cultured hippocampal neurones and cloning of the rat Y_2 receptor

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1 Neuropeptide Y (NPY) and NPY receptors are most abundant in the hippocampal formation where they modulate cognitive functions. Expression of NPY receptors in rat cultured primary hippocampal cells was investigated in the present study by use of combined molecular, pharmacological and immunohistochemical approaches, including the cloning of the rat Y_2 receptor described here for the first time.

2 More than 70% of the hippocampal neurones were endowed with [^{125}I]-[Leu^{31},Pro^{34}]PYY Y_1-like receptor silver grain accumulations and Y_1 receptor immunostaining. These radio- and immuno-labelling signals were distributed over cell bodies and processes of bipolar, stellate and pyramidal-like neuronal cells, as confirmed by neurone-specific enolase and MAP-2 staining.

3 Competition binding profiles revealed that specific [^{125}I]-[Leu^{31},Pro^{34}]PYY binding was competitively displaced according to a ligand selectivity pattern prototypical of the Y_1 receptor sub-type with [Leu^{31},Pro^{34}]substituted NPY/PYY analogues $>>$ C-terminal fragments = pancreatic polypeptides, with the non-peptide antagonist BIBP3226 being most potent. This profile excludes the possible labelling by [^{125}I]-[Leu^{31},Pro^{34}]PYY of the newly cloned Y_4, Y_5 and Y_6 receptors.

4 The expression of the genuine Y_1 receptor was confirmed by RT–PCR in hippocampal cultures. In contrast, negligible levels of Y_2-like/[^{125}I]-PYY_{3-36} binding were detected in these cultures in spite of the presence of its mRNA, as characterized by RT–PCR. The expression of both the Y_1 and the Y_2 receptor mRNAs was also noted in normal embryonic hippocampal tissues showing that signals expressed in cultured neurones were also present *in utero*.

5 Taken together, these results suggest that the Y_1 receptor subtype may be of critical importance in the normal functioning of the rat hippocampus, especially during brain development and maturation.

Keywords: Primary culture; immunofluorescence; emulsion receptor autoradiography; RT–PCR; [Leu^{31},Pro^{34}]PYY; PYY_{3-36}; rat Y_2 receptor cloning; Y_1 receptor expression

Introduction

Neuropeptide Y (NPY) belongs to a large family of peripheral and central peptides including peptide YY (PYY) and pancreatic polypeptides (PP) (Tatemoto *et al*., 1982). The widespread distribution of NPY in the brain suggests putative roles in a variety of physiological responses, notably in cognition (for reviews, see Wahlestedt *et al*., 1990; Dumont *et al*., 1992; Wahlestedt & Reis, 1993; Colmers & Bleakman, 1994). The hippocampal formation, a region critical to learning and memory processes (Milner, 1966; Gustafsson & Wigström, 1988), contains significant amounts of NPY. Immunohistochemical studies have detected the presence of NPY immunoreactivity within or adjacent to the pyramidal cell layer of all subfields of Ammon's horn of various species (Woodhams *et al*., 1985; Chan-Palay *et al*., 1986; DeQuidt & Emson, 1986; Köhler *et al*., 1986). Some groups have shown that intra-hippocampal injection of NPY increases memory retention, retrieval and consolidation, while NPY antibodies have the opposite effect (Morley & Flood, 1990; Nakajima *et al*., 1994). Moreover, NPY appears to be an important modulator of excitability in the CNS by inhibiting the frequency of neuronal epileptic discharges, particularly in the hippocampal formation (Sperk *et al*., 1992; Rizzi *et al*., 1993; Colmers & Bleakman, 1994; Vezzani *et al*., 1994; Erickson *et al*., 1996). Electrophysiological studies have demonstrated that NPY exerts a presynaptic inhibitory effect on excitatory synaptic transmission of pyramidal cells of the CA1 and CA3 subfields (Colmers *et al*., 1987; 1988; Bleakman *et al*., 1992). Additionally, NPY potentiates N-methyl-D-aspartate (NMDA)-induced neuronal activation in CA3 pyramidal neurones (Monnet *et al*., 1992; but see McQuiston & Colmers, 1992). However, the cellular mechanisms and receptor subtypes underlying the physiological actions of NPY in the hippocampus are still not fully understood.

Recent studies revealed that NPY induces its effects by acting via a plethora of NPY receptor subtypes called Y_1, Y_2, Y_3 (not cloned yet), Y_4, Y_5 and Y_6 (Eva *et al*., 1990; Herzog *et al*., 1992; Krause *et al*., 1992; Larhammar *et al*., 1992; Wahlestedt & Reis, 1993; Bard *et al*., 1995; Gerald *et al*., 1995; 1996; Jacques *et al*., 1995; Lundell *et al*., 1995; Rose *et al*., 1995; Gehlert *et al*., 1996a; Weinberg *et al*., 1996). Early on, autoradiographic studies have shown that the adult rat and human hippocampal formation are enriched with the Y_2 over the Y_1 receptor subtypes (Dumont *et al*., 1990; 1993; 1996;

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Aicher *et al*., 1991; Gehlert *et al*., 1992; Larsen *et al*., 1993; Widdowson, 1993). Indeed, Y_2 binding sites are particularly concentrated in the strata radiatum and oriens with very low levels seen in the pyramidal cell layer of the CA1 and CA3 subfields. In contrast, Y_1 binding sites are mostly localized in the granular layer of the dentate gyrus with moderate levels in the pyramidal cell layer of the CA1 and CA3 subfields (Dumont *et al*., 1990; 1993; 1996; Gehlert *et al*., 1992; Larsen *et al*., 1993; Widdowson, 1993; Jacques *et al*., 1996). However, it is not clear at this time which cell type expresses a given NPY receptor subtype in the hippocampal formation. Primary cultures of embryonic hippocampal cells have been used extensively to investigate various functional aspects of hippocampal neurotransmission (Abele *et al*., 1990; Ferreira *et al*., 1994; Arancio *et al*., 1995) and are known to develop similar morphological and biochemical features as those seen *in vivo* (Dotti *et al*., 1988; Mattson & Kater, 1989; Fletcher *et al*., 1994). Indeed, these cultures are known to be enriched mostly with pyramidal-like neuronal cells, and to a lesser extent with bipolar and stellate neurones (Banker & Cowan, 1977; Mattson & Kater, 1989; Fletcher *et al*., 1994). We thus hypothesized that an embryonic hippocampal neuronal culture might provide an excellent approach to study the cellular localization of NPY receptors and reveal mechanisms underlying the physiological effects of NPY in hippocampal neurones.

In the present study, we performed binding assays and emulsion receptor autoradiography to determine and localize specific NPY receptor subtypes on cultured primary hippocampal cells. We used two different double-labelling approaches to identify the cellular type bearing the receptors. Moreover, the identity of the NPY receptors expressed in primary hippocampal cells was ascertained by reverse transcription-polymerase chain reaction (RT–PCR), following the cloning of the rat Y_2 receptor gene to investigate further the presence of the Y_2 receptor mRNA in our preparations. We show here that the Y_1 receptor subtype is preferentially expressed in a primary culture of hippocampal cells, being especially localized on pyramidal-like, bipolar and stellate neurones.

Methods

Primary hippocampal cultures

Cell cultures were prepared from hippocampi of 18–19 day-old rat embryos as described by Banker and Cowan (1977) with some modifications (Alonso *et al*., 1994). Hippocampi were dissociated enzymatically by treatment with trypsin (0.25% for 10 min at 37°C) and mechanically by repeated passages through Pasteur pipettes in Ca^{2+}- Mg^{2+}- free Hank's balanced salt solution (HBSS) containing 15 mM HEPES. The cells were resuspended in the culture medium minimum essential medium (MEM) supplemented with 10% (v/v) foetal bovine or calf sera, 20 mM KCl, 28 mM glucose, 15 mM HEPES, 2 mM L-glutamine, 110 mg l^{-1} sodium pyruvate, 10 u ml^{-1} penicillin, 10 μg ml^{-1} streptomycin and 0.250 μg ml^{-1} fungizone. Cellular suspensions were plated (day *in vitro*; DIV = 0) at low (50 000–60 000 cells cm^{-2}) or high density (275 000–350 000 cells cm^{-2}) on poly-D-lysine (25 μg ml^{-1}) coated 24-well tissue culture plates. Cultures were maintained at 37°C in a humidified 10% CO_2/90% atmosphere and the media were replaced at DIV 3 with a fresh one. Experiments were performed on mature, 8 to 10 days, hippocampal cultures (Basarsky *et al*., 1994; Fletcher *et al*., 1994). Although the dentate gyrus is part of the embryonic hippocampal formation, hippocampal cultures are enriched with pyramidal cells and interneurones since granular cells of the dentate gyrus mainly develop postnatally (Banker & Cowan, 1977; Kuhn *et al*., 1996).

Peptide iodination

PYY, PYY_{3-36} and [Leu^{31},Pro^{34}]PYY were iodinated with $Na^{125}I$ by using a modified chloramine T/sodium metabisulphite method as described in detail by our group (Dumont *et al*., 1995). With this procedure it was possible to obtain, following high performance liquid chromatography (h.p.l.c.) purification, monoiodinated radioligands. The specific activity of [^{125}I]-PYY, [^{125}I]-PYY_{3-36} and [^{125}I]-[Leu^{31},Pro^{34}]PYY was assumed to be of the theoretical value (2 000 Ci $mmol^{-1}$).

Receptor binding assays

All binding assays performed in that study were conducted on whole cell of primary hippocampal cultures. [^{125}I]-PYY substituted in position 31 and 34, [Leu^{31},Pro^{34}]PYY, a selective agonist of the Y_1 subtype (Fuhlendorff *et al*., 1990; Dumont *et al*., 1994; 1995), was used for Y_1 binding studies. [^{125}I]-PYY_{3-36}, a rather specific Y_2 agonist (Wahlestedt *et al*., 1986; Sheik *et al*., 1989), was used as Y_2 radioligand. PYY-derived radioligands were chosen to prevent binding of the putative Y_3 receptor subtype (Dumont *et al*., 1994). Radioligands were added to fresh Krebs Ringer HEPES buffer (KRH; composition in mM: NaCl 125, KCl 4.8, HEPES 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.2 and glucose 10, at pH 7.4) supplemented with 0.1% BSA (v/v) and 0.05% bacitracin (v/v) and used optimal binding conditions (150 min, at room temperature; Dumont *et al*., 1995). For saturation experiments, cells were grown at high cellular density in 24-well tissue culture plates for 8–9 DIV. Medium was removed and culture plates washed twice with KRH. Binding was performed with increasing concentrations (24 to 3350 pM) of [^{125}I]-[Leu^{31},Pro^{34}]PYY and [^{125}I]-PYY_{3-36} in the presence (non-specific binding) or absence (total binding) of 1 μM NPY. Competition binding experiments were completed, under similar conditions, in order to establish the ligand selectivity of [Leu^{31},Pro^{34}]PYY binding. Specific [^{125}I]-[Leu^{31},Pro^{34}]PYY binding was displaced by increasing concentrations (10^{-11} to 10^{-6} M) of unlabelled competitors: [Leu^{31},Pro^{34}]PYY (a putative Y_1 agonist with affinity for the Y_4 and Y_5 receptors), [Leu^{31},Pro^{34}]NPY (a purported Y_1 agonist with possible affinity for the Y_5 receptor), NPY (a non-selective agonist), BIBP3226 (a selective Y_1 antagonist), PYY_{3-36} (a preferential Y_2 agonist with affinity for the Y_4 and Y_5 receptors), NPY_{13-36} (a Y_2 agonist with some affinity for the Y_5 receptor), rPP (a Y_4 agonist) and hPP (a Y_4 and Y_5 receptor agonist) (Wahlestedt *et al*., 1986; Sheik *et al*., 1989; Fuhlendorff *et al*., 1990; Dumont *et al*., 1994; 1995; Rudolf *et al*., 1994; Bard *et al*., 1995; Jacques *et al*., 1995; Lundell *et al*., 1995; Gehlert *et al*., 1996b; Gerald *et al*., 1996). Non-specific binding was determined in the presence of 1 μM NPY. At the end of the incubation period, cells were rapidly washed three times with ice-cold KRH. Cells-sequestering radioactivity were lifted off with NaOH 0.1 N and the radioactivity quantified by use of a gamma-counter (Cobra, Canberra-Packard Canada Ltd.) with 80% efficiency.

Emulsion receptor autoradiography and NSE immunocytochemistry

Rat hippocampal cells were grown either on 18-mm German glass or Aclar plastic poly-D-lysine-coated coverslips in 24-well

tissue culture plates at low (50 000–60 000 cells cm^{-2}) and very low (25 000–30 000 cells cm^{-2}) cellular density in order to visualize isolated hippocampal cells. The medium was then removed and cultures washed twice with KRH. Hippocampal cells were incubated (150 min at room temperature) with 170 pM of either [^{125}I]-PYY, [^{125}I]-[Leu^{31},Pro^{34}]PYY or [^{125}I]-PYY_{3-36} in the presence (non-specific binding) or absence (total binding) of 1 μM NPY. At the end of the incubation period, cells were washed three times with ice-cold KRH and then fixed with cold 2.5% glutaraldehyde dissolved in 0.1 M sodium phosphate buffer (PBS: pH 7.4) for 30 min. For combined experiments with emulsion receptor autoradiography and immunocytochemistry, cells were fixed with a cold mixture of 4% paraformaldehyde (PFA) and 2.5% glutaraldehyde after the incubation with [^{125}I]-[Leu^{31},Pro^{34}]PYY. Immunostaining with the NSE antibody (1 : 500) to visualize neuronal cells was performed following pre-incubation in 0.1% Triton X-100. Cells were then incubated with biotinylated secondary antibodies (ABC method) and stained with the addition of 0.25% DAB and 0.003% H_2O_2. Coverslip-mounted cells were dehydrated with increasing concentrations of alcohol and pasted on slides with histology mountant DPX. All slides were dipped in Kodak NTB2 photographic emulsion, dried and exposed in the dark at 4°C. Following appropriate exposure, slides were developed at 15°C in D19 developer (Kodak), washed in water and fixed (Kodak rapid fixer). Finally, slides were washed in water for 20 min and counterstained with cresyl violet. Silver grains were quantified with an MCID (M4) automated image analysis system adapted for grain counting (Imaging Research Inc., St-Catherines, Ontario, Canada). Photographs were taken with a Leitz orthoplan microscope, under dark- and bright-field illumination, equipped with a DAGE 72 camera. Photomicrographic montages were constructed on a FUJI Pictrography 3000 printer assisted by a computer (Adobe Photoshop).

Y_1 receptor and MAP-2 immunocytochemistry

Rat hippocampal cells were grown at low cellular density on poly-D-lysine-coated 18 mm or Aclar plastic coverslips in 24-well tissue culture plates. Medium was removed and culture plates washed twice with 0.1 M PBS and then fixed for 30 min with 4% PFA. Coverslip-mounted cells were washed twice and preincubated with 0.1% Triton X-100 and 3% goat serum for 15 min at room temperature. The cells were incubated for 18 h at 4°C with a mixture of Y_1 receptor antiserum (1 : 5000) (Zhang *et al*., 1994) and MAP-2 antibody (1 : 4000) in PBS supplemented with 1% goat serum. Cells were then washed, incubated with a mixture of Texas red-conjugated donkey anti-rabbit (1 : 100) and FITC-conjugated donkey anti-mouse (1 : 100) immunoglobulins in 0.1 M PBS/1% goat serum, and finally washed and mounted in aquamount. Slides were visualized and photographed with a Leica confocal laser scanning microscope (CLSM), configured with a Leica diaplan inverted microscope equipped with an argon/krypton laser assisted with a VME bus MC 68020/68881 computer system. For double labelling studies, fluorescence images were simultaneously acquired with two different channels, one for FITC and the other for Texas-red. Photomicrographs were taken and treated as above.

Rat Y_2 cDNA isolation and nucleotide sequence determination

Total RNA (3 mg) from the Y_2 expressing cell line MSN-SMS was used as a template to synthesize random primed single-stranded cDNA. The cDNA was used in a PCR assay together with oligonucleotide primers corresponding to position 294–316 and 886–910 in the Y_2 human cDNA (Rose *et al*., 1995). PCR conditions: 30 cycles at 95°C for 1 min, 63°C for 1 min and 72°C for 1 min. The reaction product was gel purified and subcloned for sequencing into the Bluescript vector (Stratagene) for confirmation of the sequence. A Lambda-ZAP cDNA libraries derived from rat hypothalamus (Stratagene) was screened with the ^{32}P labelled human cDNA PCR fragment as a probe under stringent hybridization conditions. Two strongly hybridizing clones were isolated and the Bluescript vectors were excised. The larger of the two cDNA clones (Hz19) containing a 1.3 kb fragment was completely sequenced and shown to encode the entire open reading frame (381 amino acids) of the rat Y_2 receptor (see Figure 6).

Reverse transcription-polymerase chain reaction

Total RNA was extracted from adult rat cerebral cortex (50–70 mg), adult rat hippocampus, non-dissociated embryonic hippocampal tissues (three to five 18–19 day-old embryos) and primary hippocampal cell cultures ($\sim$10 mg protein) with the TRIzol Reagent (GIBCO) following the manufacturer's protocol according to Chomczynski and Sacchi (1987). Cerebral cortex and hippocampus were homogenized with a polytron (Brinkman, Westbury, NY) while embryonic tissues and cultured cells were lyzed by successive passages through a Pasteur pipette and a 1 ml syringe (25G) filled with the TRIzol Reagent. RNA samples (3–20 μg) were treated with RQ1 DNase (30 min at 37°C) to remove residual genomic DNA, and then purified by extraction in phenol/chloroform and precipitation in ethanol before resuspension in water. These purified RNAs were used as templates for the synthesis of complementary DNA (cDNA) by reverse transcriptase.

Reverse transcription RNA template was primed with random primers (200 ng μg^{-1} RNA) (70°C for 10 min) and reverse transcribed (15 min at 20–25°C, 60 min at 42°C) in reverse transcription buffer (final volume of 50 μl) containing 0.5 mM of each dNTP, 80u RNase inhibitor, 10 mM DTT in the presence (RT sample) or absence (non-RT sample) of 2u avian myeloblastosis virus-reverse transcriptase enzyme (AMV–RT). The non-RT reaction was prepared for each RNA sample in order to control for any genomic DNA contamination.

Oligonucleotide primers Primers for the Y_1 and the Y_2 receptors were designed according to the published sequence of the rat Y_1 receptor (Eva *et al*., 1990) and the cloned rat Y_2 receptor described here (see Figure 6). They respectively amplified a 637 base pairs (bp) fragment spanning from the extracellular loop between the fourth and fifth transmembrane domains to the 3′-untranslated region of the Y_1 receptor, and a 501 bp fragment spanning from the fifth and sixth transmembrane domains to the 3′-untranslated region of the Y_2 receptor. The primers were synthesized (Intersciences Inc., Markham, Ontario, Canada) as follows. Primers for the Y_1 receptor were: Y_1-forward; a 21-mer complementary to bases 661–682 flanked by the BamHI restriction site TCAGGATCC and Y_1-reverse; a 19-mer complementary to bases 1263–1282 flanked by the EcoRI restriction site GGAATTC. Primers for the Y_2 receptor were: Y_2-forward; a 20-mer complementary to bases 784–804 flanked by the ECoRI restriction site TGAATTC and Y_2- reverse: a 21-mer complementary to bases 1250–1270 flanked by the BamHI restriction site CTGGATCC. The specificity of the Y_1 primers was confirmed

with pBluescript II-KS and the PCR-II plasmids bearing the coding sequence of the Y_1 and Y_4 receptors, respectively.

PCR amplification The cDNA (5–10 μl) of RT and non-RT reactions or plasmids (2 μl) containing the Y_1 or Y_2 sequences were amplified with *Taq* polymerase with the Y_1 or the Y_2 receptor primers. The PCR reaction mixture (final volume of 50 μl) contained $MgCl_2$ (1.5 mM for the Y_1 and 3.0 mM for the Y_2), 0.5 mM of each dNTP, 4% DMSO, 500 nM of each primer and 2 u of *Taq* polymerase. Forty cycles of amplification were performed with a MJ Research Thermal Minicycler under the following conditions: 94°C for 40 s (Y_1 and Y_2), 58°C (Y_1) or 55°C (Y_2) for 40 s and 72°C for 1 min (Y_1) or 40 s (Y_2) with a pre- and a post-incubation of 94°C (Y_1) or 72°C (Y_2) for 5 min and 72°C for 5 min (Y_1 and Y_2). An aliquot (Y_1: 7–10 μl; Y_2: 15–20 μl) of the PCR-derived products was size-fractionated by electrophoresis on a 1.8% agarose gel stained with ethidium bromide, visualized under u.v. light and photographed. Cortex and hippocampal tissues both from adult rat, known to express Y_1 and Y_2 receptor mRNA, respectively, were run in parallel with the embryonic hippocampal tissues and cultured cells to control for the specificity and efficiency of the PCR method.

Restriction mapping The PCR products were identified by restriction enzyme analysis. The products were extracted from the gel by use of a Glassmax DNA extraction kit (GIBCO) and were then incubated (37°C for 180 min) with enzymes which yielded 2 fragments of predicted size on gel electrophoresis. The generated fragments were electrophoretically separated on a 1.8% agarose gel to confirm their size. Y_1- and Y_2-PCR products were digested by HincII (2 fragments of 357 and 280 bp each) and XhoI (2 fragments of 437 and 200 bp each), and HindIII (2 fragments of 224 and 277 bp each) and PvuII (2 fragments of 62 and 439 bp each), respectively. Photomicrographs were taken and treated as above.

Materials

Female Sprague Dawley pregnant rats obtained from Charles River (St-Constant, Québec, Canada) were kept on a 12 h light-dark cycle (light on at 7 h 00 min) in temperature and humidity controlled rooms. Animals were fed with standard laboratory chow and had access to tap water *ad libitum*. Animal care was according to protocols and guidelines approved by McGill University and the Canadian Council of Animal Care.

Analogues and fragments of PYY and NPY were generously provided by Drs S. St-Pierre and A. Fournier (INRS-Santé, Pointe-Claire, Canada) and BIBP3226 (N^2-(diphenylacetyl)-N-[4-hydroxyphenyl)methyl]-D-arginine amide) by Dr H. Doods (Karl Thomae GmbH, Biberach, Germany). Human and rat pancreatic polypeptides (hPP and rPP) were obtained from Bachem California (Torrance, CA). $Na^{125}I$ (400 mCi ml^{-1}) was purchased from ICN Biochemical (Mississauga, Ontario, Canada). Bacitracin, bovine serum albumin (BSA), poly-D-lysine, uridine, 5-fluorodeoxyuridine (FDU) and 3,3′-diaminobenzidine (DAB) were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture media and reagents, trypsin, penicillin, streptomycin and fungizone were purchased from GIBCO-BRL (Burlington, Ontario, Canada). Tissue culture plates were obtained from Corning (Corning Costar Corporation, Cambridge, Massachusetts) while 18-mm German glass and Aclar plastic coverslips were provided from Fisher Scientific Co. (Montréal, Québec, Canada) and ProPlastics Inc. (Linden, NJ), respectively. Triton X-100 was obtained from Amersham (Airlington Heights, IL), paraformaldehyde and H_2O_2 from Fisher Scientific Co. (Montréal, Québec, Canada). Neurone specific enolase (NSE) antibody was purchased from Dakopatts (Dimension Laboratories, Mississauga, Ontario, Canada), ABC kits from Vector Laboratories (Burlingham, CA) and the histology mountant DPX was obtained from Fluka Chemical Corp. (New York, U.S.A.). The Y_1 receptor antiserum was kindly supplied by Drs X. Zhang and T. Hökfelt (Karolinska Institute, Stockholm, Sweden). Texas red- and fluorescein (FITC)-conjugated donkey anti-rabbit and anti-mouse immunoglobulins (multiple labelling grade) were purchased from Jackson Immuno Research and the MAP-2 antibody from Boehringer Mannheim. Glutaraldehyde was supplied from J.B.E.M. Services Inc. (Pointe-Claire, Québec, Canada) and Kodak NTB2 photographic emulsion from Intersciences (Markham, Ontario, Canada). TRIzol Reagent was obtained from GIBCO-BRL (Burlington, Ontario, Canada) while RQ1 DNase, dNTP (mixture of dATP, dGTP, dCTP and dTTP), RNase inhibitor, DTT, avian myeloblastosis virus-reverse transcriptase enzyme (AMV–RT), *Taq* DNA polymerase were provided from Promega (Madison, WI, U.S.A.). pBluescript II-KS and the PCR-II plasmids were generously supplied by Dr Y. Tong (Biotechnology Research Institute, Montréal, Canada). All other chemicals were of analytical grade and were purchased from Fisher Scientific Co. (Montréal, Canada) or Sigma Chemical Co. (St. Louis, MO).

Data analysis

All binding experiments were performed in triplicate for each experimental condition; each experiment was repeated two to four times in separate culture preparations. Data obtained from the saturation curves were analysed by nonlinear regression with GraphPad Prism InPlot (GraphPad Prism software v2.2, San Diego, CA). While binding parameters of the saturation curves were estimated by a linear regression, IC_{50} values of the various competitors were calculated from the competition curves with the Lundon Software Receptor Fit Competition (Lundon Software Inc. Chagrin Falls, OH). Differences in silver grain accumulations were evaluated by analysis of variance (ANOVA) followed by Scheffe's *a posteriori* test with a level $P<0.05$ being considered significant.

Results

In the present study, all experiments were performed between days eight and ten, periods at which the embryonic rat hippocampal cell cultures were found to exhibit morphological and functional maturation (Dotti *et al*., 1988; Basarsky *et al*., 1994; Fletcher *et al*., 1994) with the great majority (80–90%) of grown cells being of neuronal phenotype, as revealed by NSE immunostaining.

Determination of NPY receptor subtype in primary cultures

As shown in Figure 1, the purported Y_1-like radioligand, [^{125}I]-[Leu^{31},Pro^{34}]PYY binds to an apparent single class of high affinity ($K_d = 0.58 \pm 0.05$ nM), low capacity ($B_{max} = 14.8 \pm 0.7$ fmol mg^{-1} protein) sites in cultured primary hippocampal cells. In contrast, the putative Y_2-like radiolabelled probe [^{125}I]-PYY_{3-36} did not reveal any significant level of binding, despite its ability to label the Y_2 binding sites in adult rat brain homogenates, demonstrating the functionality of the ligand used and assay conditions

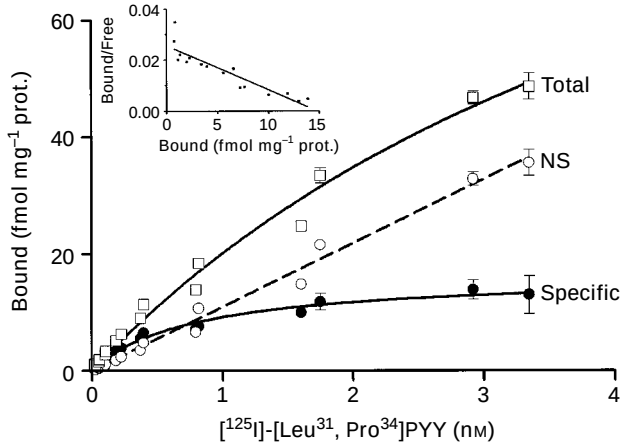

Figure 1 Saturation binding curves of [^{125}I]-[Leu^{31},Pro^{34}]PYY in primary hippocampal cultures in the presence or absence of 1 μM NPY. Specific binding was determined as the difference between total and nonspecific binding. Inset: Scatchard analysis of these data. Each point represents the mean of data obtained from four separate experiments, each performed in triplicate (vertical lines show s.e.mean). Maximal density of binding sites (B_{max}) = 14.8 ± 0.7 fmol mg^{-1} protein and dissociation constant (K_d) = 0.58 ± 0.05 nM).

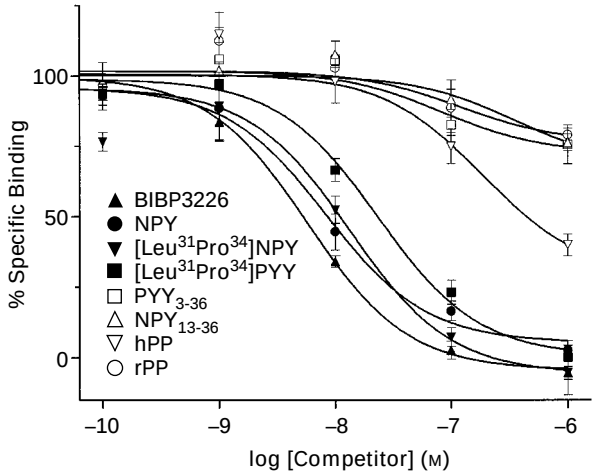

Figure 2 Comparative competition binding profile of NPY, derivatives and homologues, and the non-peptide antagonist BIBP3226 against specific Y_1-like/[^{125}I]-[Leu^{31},Pro^{34}]PYY binding in primary hippocampal cultures. Each point represents the mean of data obtained from two to four separate experiments (vertical lines show s.e.mean), each performed in triplicate and expressed as the percentage of specific binding determined using 1 μM NPY. IC_{50} values are shown in Table 1.

(data not shown). Reduction of cellular density by plating cells from a high (~350 000 cells cm^{-2} = 160 ± 13 μg protein/well) to a low (~60 000 cells cm^{-2} = 76 ± 3.4 μg protein/well) density still generated significant specific binding with the Y_1-like radioligand, while specific binding with the Y_2-like radioligand failed to be detected. The ligand selectivity profile of specific [^{125}I]-[Leu^{31},Pro^{34}]PYY binding was established next. As shown in Figure 2, BIBP3226 ≥ NPY ≥ [Leu^{31},Pro^{34}]NPY ≥ [Leu^{31},Pro^{34}]PYY > > PYY$_{3-36}$ ≥ NPY$_{13-36}$ ≥ hPP ≥ rPP. This profile is prototypical of the Y_1 receptor subtype excluding labelling of [^{125}I]-[Leu^{31},Pro^{34}]PYY to Y_2, Y_3, Y_4, Y_5 and Y_6 receptors on the basis of the high affinity of the antagonist BIBP3226 and the low affinities of both hPP and rPP (Table 1).

Table 1 Competition binding parameters of NPY and related molecules against [^{125}I]-[Leu^{31},Pro^{34}]PYY binding in primary hippocampal neurones

Competitors	*IC_{50}* (nM)	*Relative potency* (%)
NPY	5.6 ± 3.8	100
[Leu^{31},Pro^{34}]NPY	8.8 ± 4.3	64
[Leu^{31},Pro^{34}]PYY	25.2 ± 8.9	22
NPY$_{13-36}$	2710 ± 1552	0.20
PYY$_{3-36}$	2020 ± 689	0.27
hPP	507 ± 214	1.1
rPP	2130 ± 268	0.26
BIBP3226	5.1 ± 0.4	110

Data represent the mean ± s.e.mean of two to four separate experiments, each performed in triplicate. IC_{50} values reflect the concentration of competitors needed to inhibit 50% of the specific binding of the radioligand.

Localization of NPY receptor sites on primary hippocampal cells

Emulsion receptor autoradiography was used to determine further the localization of specific [^{125}I]-PYY, [^{125}I]-[Leu^{31},-Pro^{34}]PYY and [^{125}I]-PYY$_{3-36}$ binding sites on primary hippocampal cells. As shown in Figure 3, after incubation with [^{125}I]-PYY (a) or [^{125}I]-[Leu^{31},Pro^{34}]PYY (b) accumulations of silver grains were mainly seen over neuronal processes, and to a lesser extent over perikarya of cultured hippocampal cells. These signals were specific, being reduced to background levels in the presence of a saturating concentration of 1.0 μM NPY (Figure 3a,b; NS panels). In contrast, no specific [^{125}I]-PYY$_{3-36}$ binding was detected under similar conditions (Figure 3d). Quantification of silver grain densities confirmed the enrichment in Y_1-like, but not Y_2-like, binding sites in our preparations and indicated that [^{125}I]-PYY labelled the same quantity of sites as [^{125}I]-[Leu^{31},Pro^{34}]PYY (Figure 4).

Visualization of Y_1 receptors on hippocampal neurones

Two double-labelling experiments were performed to determine the cellular type expressing the Y_1 receptor subtype. Firstly, combined emulsion receptor autoradiography and NSE immunocytochemistry revealed the accumulation of [^{125}I]-[Leu^{31},Pro^{34}]PYY binding sites over immunostained neuronal cells (Figure 3c). Secondly, double immunostaining with Y_1 receptor (Zhang *et al*., 1994) and MAP-2 antibodies confirmed the presence of the Y_1 receptors on neuronal cell bodies and processes (Figure 5). Three neuronal cell types were positively immunostained or radiolabelled with the two Y_1 receptor probes used, especially the pyramidal-like (Figure 5), stellate (Figure 3c) and bipolar (data not shown) neurones. Similar proportions (70–80%) of neurones bearing Y_1 silver grain accumulations and Y_1-immunostaining were noted, indicating further an enrichment with the Y_1 receptor in rat hippocampal neuronal cultures.

Cloning of the rat Y_2 receptor subtype

The rat Y_2 receptor gene was cloned in order to perform mRNA detection of this receptor in primary hippocampal cultures and embryonic hippocampal tissues. Several human Y_2 cDNAs have recently been isolated by expression cloning techniques (Rose *et al*., 1995; Gerald *et al*., 1995; Gehlert *et al*., 1996a). We used a RT–PCR technique to amplify sequences of this cDNA from total RNA of the Y_2 expressing human cell line MSN-SMS in order to use it as

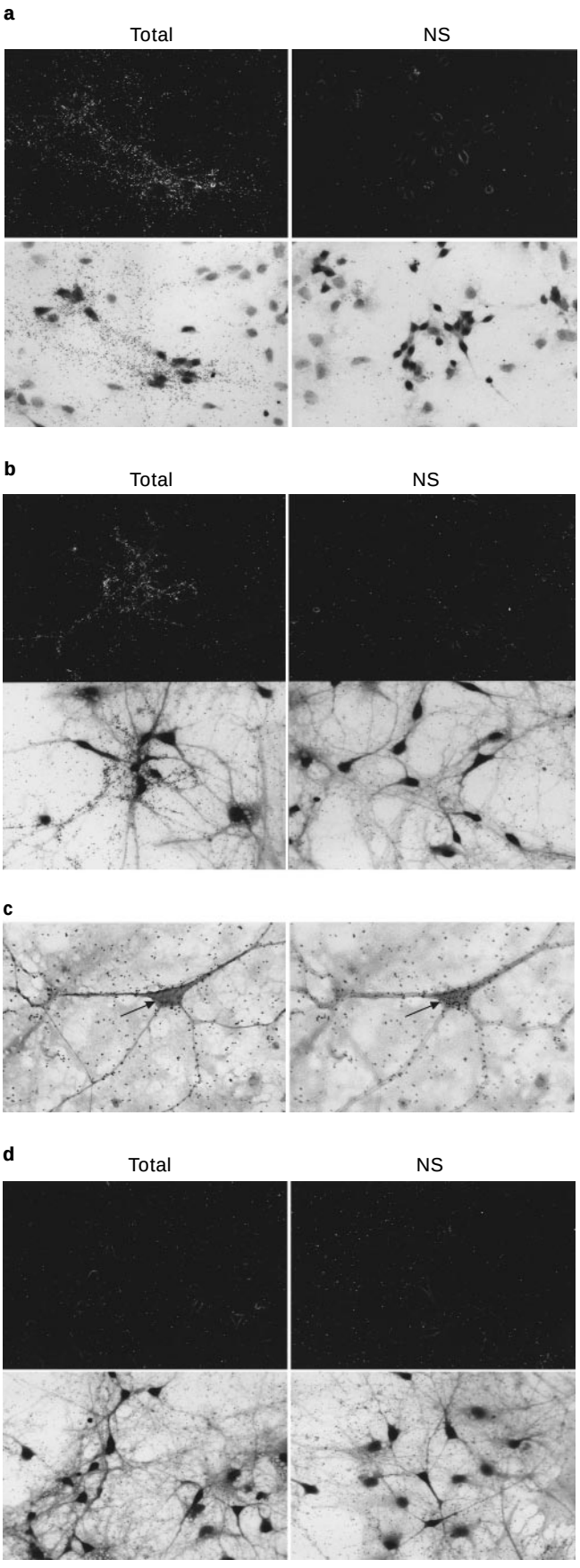

Figure 3 Representative photomicrographs of emulsion receptor autoradiography experiments on cultured primary hippocampal cells plated at low cellular density (50 000–60 000 cells cm^{-2}). Total binding was obtained with 170 pM of either the non-selective ligand [^{125}I]-PYY (a), the Y_1-like ligand [^{125}I]-[Leu^{31},Pro^{34}]PYY (b) and the Y_2-like ligand [^{125}I]-$PYY_{3\text{-}36}$ (d). In (a) and (b), dark-field (top panels) photomicrographs show silver grain accumulations of [^{125}I]-PYY and Y_1-like/[^{125}I]-[Leu^{31},Pro^{34}]PYY binding sites over processes of hippocampal cells disappearing in the presence of 1 μM NPY (non-specific binding, NS). Nissl-counterstained cells, of the same picture, are revealed on the bright-field (low panels) photomicrographs showing silver grain accumulations on neuronal processes. Double-labelling of [^{125}I]-[Leu^{31},Pro^{34}]PYY binding and NSE immunopositive staining show the presence of Y_1 binding sites over neurites (left panel) and cell body (right panel) of the same stellate neuron (arrow in c). In (d), both dark- and bright-field photomicrographs display no silver grain accumulation with the Y_2-like/[^{125}I]-$PYY_{3\text{-}36}$ radioligand. Magnification 30× (a, b, d) and 50× (c).

a probe to screen rat cDNA libraries. Oligonucleotide primers corresponding to positions 294–316 and 886–910 on the human Y_2 cDNA were used to amplify a 616 bp fragment which was subcloned and sequenced. The ^{32}P labelled human fragment was used to screen under stringent conditions several rat cDNA libraries. Two strongly hybridizing clones were isolated and sequenced from a rat hypothalamic library, with the longest of these (Hz19–1.3 kb) encoding a complete open reading frame of 381 amino acids (Figure 6). Analysis of the amino acid sequence of Hz19 confirmed that it represents the rat homologue of the human Y_2 sequence with an overall identity of 92%.

Expression of the Y_1 and Y_2 receptor genes

PCR products of the expected size (637 bp) for the Y_1 receptor were observed by gel electrophoresis in six different primary hippocampal culture preparations (Figure 7; lanes 1–6). Since hippocampal cells were grown under artificial cell culture conditions (hence possibly inducing Y_1 receptor expression) Y_1 receptor RT–PCR experiments were also performed with *in situ*, non-dissociated embryonic hippocampal tissues (from E18–19 day-old embryos). Signals for the Y_1 receptor were observed in six normal, non-dissociated embryonic hippocampal tissues (Figure 7; lanes 8–13) as well as in the adult rat frontal cortex used here as a positive control (Figure 7; lane 7).

The presence of mRNAs encoding for the cloned rat Y_2 receptor was also determined by RT–PCR in the same preparations as used for the Y_1 even if only negligible amounts of Y_2 binding sites were detected (see above). As shown in Figure 8, PCR products of the expected size (501 bp) for the Y_2 receptor were detected in seven primary hippocampal cultures (lanes 1–7), six embryonic hippocampal tissues (lanes 8–13) as well as in the adult rat hippocampus used here as positive control (lane 14). Contamination by genomic DNA was excluded since no band was detected in agarose-gel from the non-RT samples (Figures 7 and 8; lanes –).

Validation of the PCR products by restriction mapping

Identification of the various PCR products from primary hippocampal cultures and embryonic hippocampal tissues was achieved by restriction mapping. HincII (lane 2) and XhoI (lane 3), respectively digested Y_1-cDNA PCR products of 637 bp (lane 1) in two fragments of 357/280 bp and 437/200 bp (Figure 9a) whereas HindIII (lanes 5 and 8) and PvuII (lanes 6 and 9) digested Y_2-cDNA PCR products of 501 bp (lanes 4 and 7) in two fragments of 277/224 bp and 439/62 bp, respectively (Figure 9b). The generated fragments exactly matched those predicted from knowledge of restriction maps of the Y_1 and Y_2 receptor genes and the locations of the primer templates.

Discussion

The major finding of the present study relates to the demonstration of the high expression of Y_1 receptors in cultured primary hippocampal neurones. Although Y_1 receptor mRNA and binding sites have been previously detected in the pyramidal cell layer of the CA1 and CA3 subfields of rat embryos and adult hippocampus (Larsen *et al*., 1993; Tong *et al*., 1997), this result was unexpected at first on the basis of homogenate and receptor autoradiographic binding data revealing that the adult rat hippocampal formation is particularly enriched with the Y_2 receptor subtype (Dumont *et al*., 1990; 1993; 1996; Aicher *et al*., 1991; Gehlert *et al*., 1992; Larsen *et al*., 1993). The expression of the genuine Y_1 receptor subtype in cultured hippocampal neurones was demonstrated by (1) RT–PCR and restriction analysis data showing the presence of mRNAs for the Y_1 subtype; (2) the ligand selectivity profile of specific [^{125}I]-[Leu^{31},Pro^{34}]PYY binding sites revealing that the non-peptide selective Y_1 antagonist BIBP3226 was a most potent competitor while C-terminal NPY or PYY fragments and PPs were not, excluding binding to the Y_2 as well as to the newly cloned Y_4, Y_5 and Y_6 receptors; (3) the lack of significant level of specific [^{125}I]-PYY$_{3\text{-}36}$ binding in hippocampal cultures, as revealed with emulsion receptor autoradiography, while this approach demonstrated the abundance of specific [^{125}I]-[Leu^{31},Pro^{34}]PYY binding over cell bodies and processes and (4) the immunodetection of the Y_1 receptor in hippocampal neurones with a highly specific Y_1

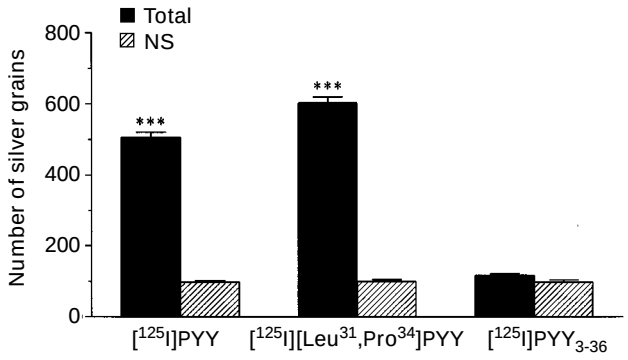

Figure 4 Quantitative analysis of emulsion receptor autoradiography experiments performed on cultured primary hippocampal cells. Total binding was obtained with 170 pM of either [^{125}I]-PYY, [^{125}I]-[Leu^{31},Pro^{34}]PYY or [^{125}I]-PYY$_{3\text{-}36}$ as radioligands. Values are expressed as the mean ± s.e.mean. Each experimental condition was performed four times, each in triplicate. ***$P<0.001$ compared to non-specific (NS) binding determined in the presence of 1 μM NPY.

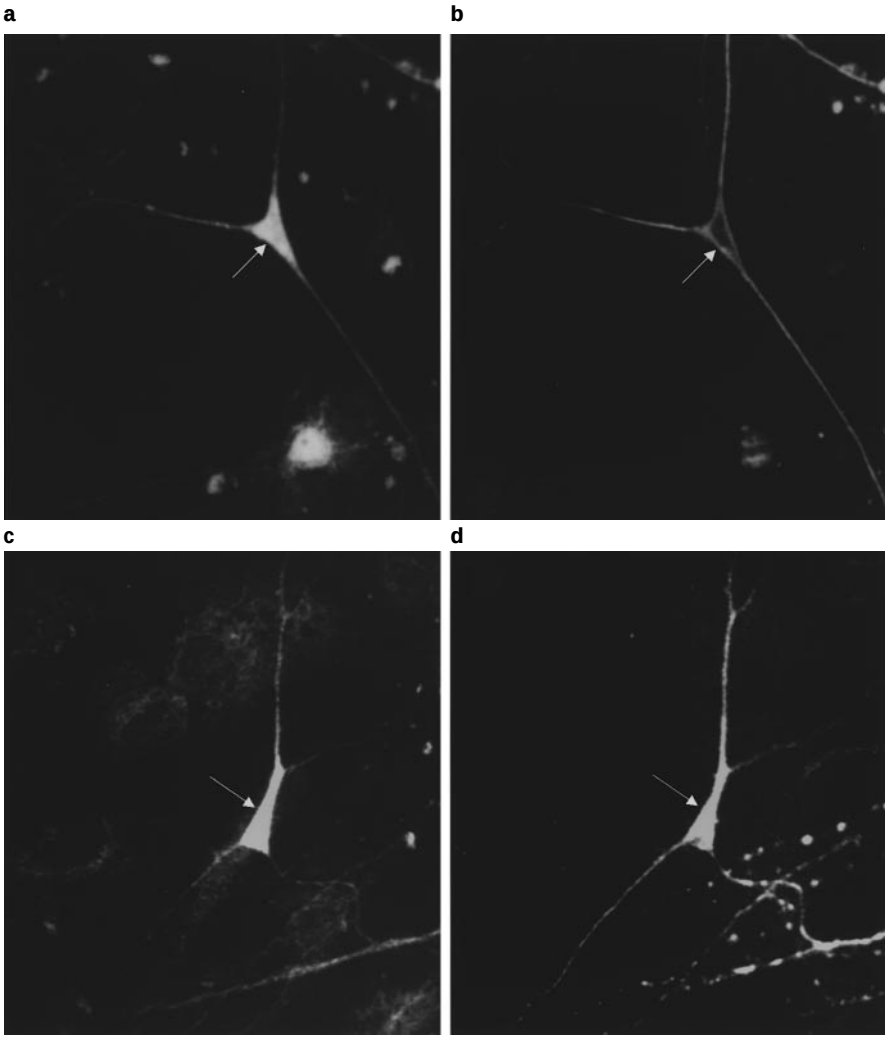

Figure 5 Confocal microscopic images of cultured hippocampal neurones dually labelled with a mixture of a Y_1 receptor antiserum (1 : 5000) (a, c) and a MAP-2 antibody (1 : 4000) (b, d,). Both labelling revealed with a Texas red- and a FITC-conjugated immunoglobulin were acquired through different channels. Note the double immunopositive staining of the cell bodies (arrows) and processes of two pyramidal-like neurones (a, b and c, d).

receptor antibody (Zhang *et al*., 1994). In fact, the majority of cultured hippocampal neurones (70–80%) were enriched with Y_1 receptors.

The expression of the Y_1 receptor subtype is apparently mostly restricted to neurones since [^{125}I]-[Leu^{31},Pro^{34}]PYY binding was present on NSE-positive cells. Moreover, neurones immunopositive to the Y_1 receptor and the MAP-2 antibodies demonstrated the localization of this receptor on perikarya and neurites. Most importantly, the expression of Y_1 receptor mRNA in non-dissociated fresh hippocampal tissues, from 18–19 day-old embryos, demonstrates that the presence of the Y_1 receptor in cultured neurones is not due to the de-repression of the Y_1 receptor gene induced by culture conditions. Bleakman and collaborators (1992) demonstrated

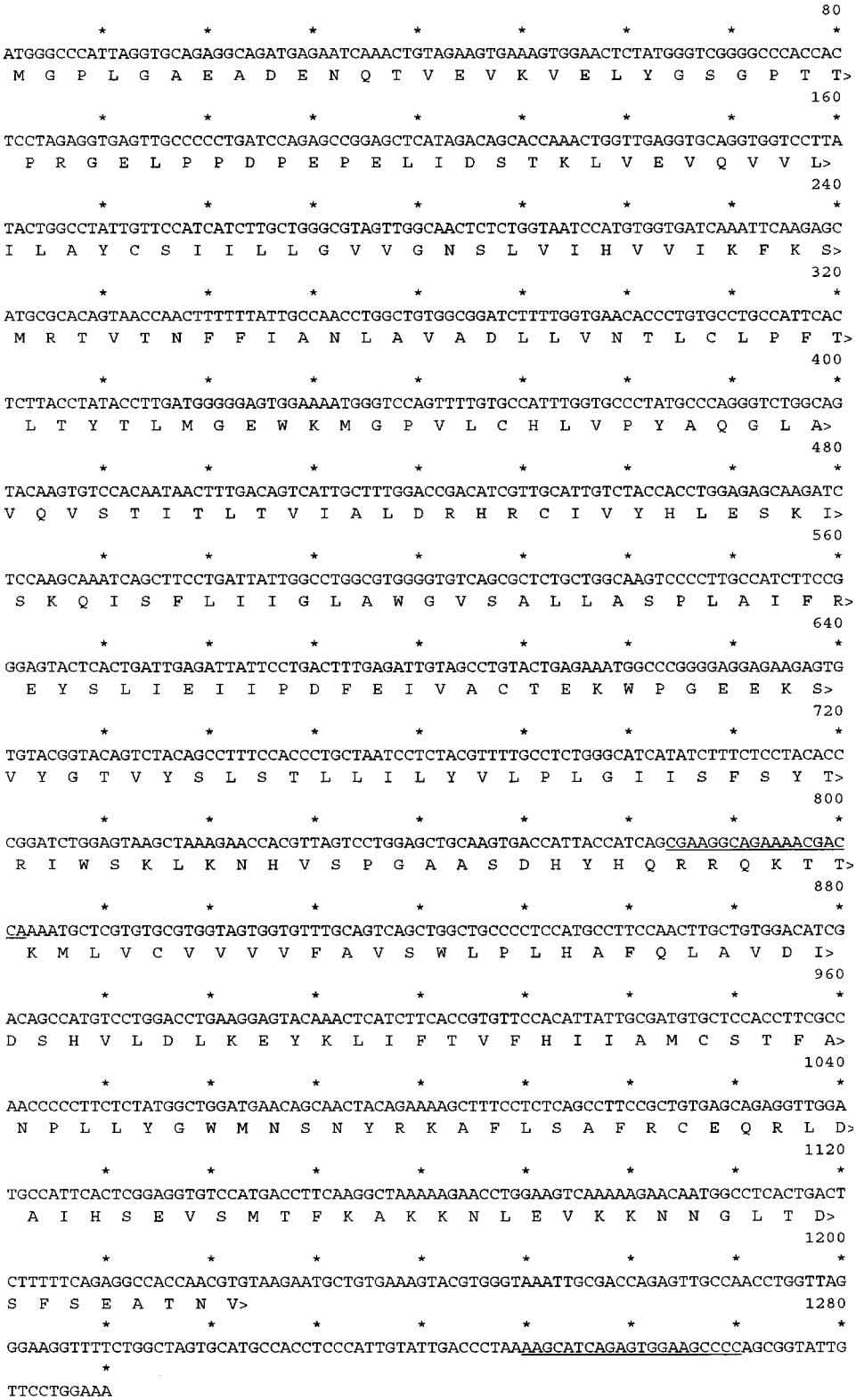

Figure 6 Nucleotide sequence and deduced amino acid sequence of the rat Y_2 receptor cDNA. Oligonucleotide primers used for the RT–PCR experiments on primary hippocampal cultures and normal embryonic tissues are underlined.

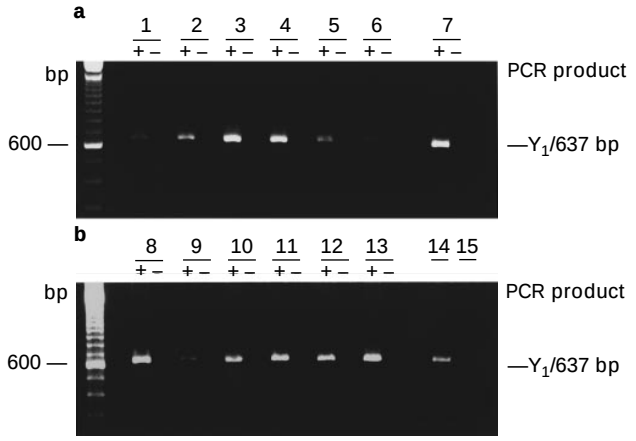

Figure 7 Gel electrophoresis showing the expression of the rat Y_1 receptor mRNA in (a) primary hippocampal cultures and (b) embyronic hippocampi. Total RNA was subjected to reverse transcription followed by PCR amplification with primers specific for the Y_1 receptor subtype; 7–10 μl of reaction product was run on a 1.8% agarose gel stained with ethidium bromide. Fragments of 637 bp indicate the presence of cDNAs for the Y_1 receptor in primary hippocampal cultures (lanes 1–6) and in non-dissociated hippocampal tissues from 18–19 day-old embryos (lanes 8–13). Lanes (+) represent the RT–PCR products of the Y_1 receptor cDNA fragments while lanes (−) represent amplification from the non-RT samples indicating the absence of genomic DNA contamination. Reactions in lanes 14 and 15 contained pBluescript II-KS and the PCR-II plasmids bearing the rat Y_1 (signal) and the rat Y_4 (no signal) receptor gene, respectively. Lane 7 corresponds to a positive control with the adult rat cortex.

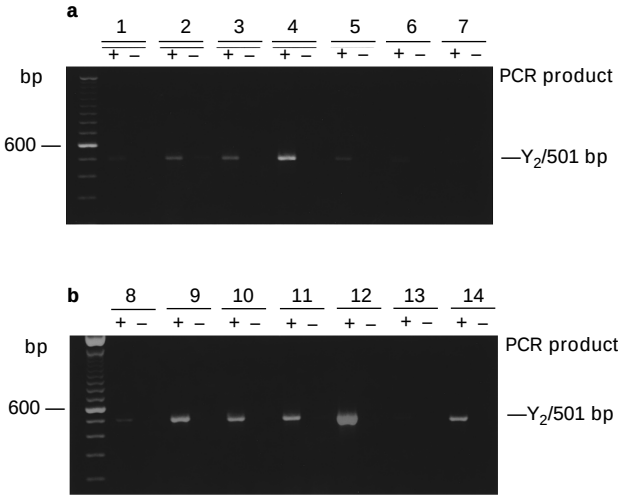

Figure 8 Gel electrophoresis showing the expression of the rat Y_2 receptor mRNA in (a) primary hippocampal cultures and (b) embyronic hippocampi. Total RNA was subjected to reverse transcription followed by PCR amplification with primers specific for the Y_2 receptor subtype; 15–20 μl of each reaction product was run on a 1.8% agarose gel stained with ethidium bromide. Fragments of 501 bp indicate the presence of cDNAs for the Y_2 receptor in primary hippocampal cultures (lanes 1–7) and in non-dissociated hippocampal tissues from 18–19 day-old embryos (lanes 8–13). Lane (+) represent the RT–PCR products of the Y_2 receptor cDNA fragments while lanes (−) represent amplification from the non-RT samples indicating the absence of genomic DNA contamination. Lane 14 corresponds to a positive control with the adult rat hippocampus.

an inhibitory effect of the Y_2 receptor on synaptic transmission in cultured hippocampal neurones. However, interestingly, NPY induced an excitatory response via the Y_1 receptor in a proportion of cultured neurones (Bleakman *et al*., 1992; Colmers & Bleakman, 1994). These authors plated dissociated hippocampal cells onto a monolayer of cortical astrocytes and maintained them in medium containing horse serum for up to six weeks (Bleakman *et al*., 1992). These conditions are markedly different from those used in the present study. Moreover, using binding assays and emulsion receptor autoradiography, we still observed enrichment in Y_1 binding sites in hippocampal neurones plated either on german glass or Aclar plastic coverslips, and maintained for ten days either in foetal bovine, calf or horse sera in the presence or absence of a glial cell proliferation inhibitor (data not shown). It thus appears most likely that long term (6 weeks vs 10 days) culture conditions in the presence of serum and astrocytes are required to express significant levels of the Y_2 receptor proteins in cultured primary hippocampal neurones.

Emulsion receptor autoradiography and receptor immunocytochemistry revealed that approximately 70–80% of rat cultured hippocampal neurones express specific Y_1 receptors. It is well established that primary cultures of rat embyronic hippocampal neurones predominantly correspond to glutamatergic pyramidal neurones and GABAergic interneurones (Banker & Cowan, 1977; Mattson & Kater, 1989; Fletcher *et al*., 1994). It would now be important to demonstrate if Y_1-expressing neurones are associated with a given neuronal phenotype, since we have determined the presence of Y_1 receptors on pyramidal-like, bipolar and stellate neurones. Studies aiming to investigate the possible modulation by NPY, via the Y_1 receptor, of the action and/or release of the enriched neurotransmitter/neuromodulator would certainly be of interest. Already, some evidences have suggested that NPY can indeed regulate the release of excitatory amino acids

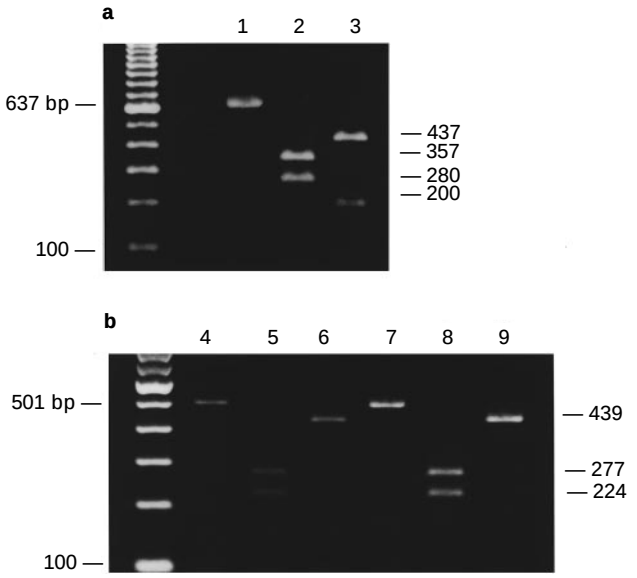

Figure 9 Validation of the PCR products by restriction mapping digestion. Representative PCR samples of the rat Y_1 (a) and rat Y_2 (b) of primary hippocampal cultures and embryonic hippocampal tissues were digested for three hours at 37°C. PCR products and digests were run in parallel to the molecular-weight DNA marker on a 1.8% agarose-gel electrophoresis. HincII (lane 2) and XhoI (lane 3), respectively, digested Y_1-cDNA PCR products of 637 bp (lane 1) in two fragments of 357/280 bp and 437/200 bp whereas HindIII (lanes 5 and 8) and PvuII (lanes 6 and 9) digested Y_2-cDNA PCR products of 501 bp (lanes 4 and 7) in two fragments of 277/224 bp and 439/62 bp (62 bp band was too light), respectively.

(Abele *et al*., 1990; Greber *et al*., 1994) and monoamines (Roman *et al*., 1991) from hippocampal tissues. Interestingly,

we recently observed that a sub-population of Y_1 receptor-bearing neurones also express NPY-like immunoreactivity suggesting the possible existence of a Y_1-autoregulatory mechanism regulating NPY neurotransmission in the rat hippocampus (St-Pierre *et al*., 1995). This is in accordance with data obtained in the peripheral nervous system which have shown that NPY, by acting on presynaptic receptors (usually via Y_2 receptors), can regulate catecholamine and/or acetylcholine release and possibly its own release since NPY has been shown to be contained in adrenergic and cholinergic neurones (Lundberg & Stjarne, 1984; Wahlestedt *et al*., 1986; Potter *et al*., 1989).

In the adult hippocampus, the inhibitory effect of NPY on glutamate-mediated excitatory transmission appears to be mediated by presynaptic Y_2 receptors, possibly by reducing Ca^{2+} influx through N-type Ca^{2+} channels (Colmers *et al*., 1991; Bleakman *et al*., 1992; Klapstein & Colmers, 1992; Toth *et al*., 1993). This selective suppression of excitatory transmission suggests that this neuropeptide might be involved in the control of neuronal excitability. Indeed, NPY knock-out mice were recently shown to be highly prone to epileptic seizures (Erickson *et al*., 1996), supporting the critical role of peptidergic (NPY) inhibitory transmission in that regard. However, the role of the Y_1 receptor subtype in the normal functioning of the hippocampal formation is still unclear. Interestingly, recent data by McQuiston *et al*. (1996) have demonstrated that NPY- and PYY-related molecules, acting on putative Y_1 receptors, have a direct influence on Ca^{2+} conductance by reducing intracellular Ca^{2+} concentrations in the soma of granular cells of the dentate gyrus. This may be taken as an indication that the Y_1 receptor expressed in cultured hippocampal neurones may subserve a similar function. Patch-clamp experiments are currently underway in our laboratories to verify this hypothesis. Moreover, the putative role of the Y_1 receptor subtype in epilepsy could be investigated in our model, since it has been demonstrated that hippocampal pyramidal neurones, establishing networks of excitatory synaptic connections in culture, generate synaptic potentials resembling those of epileptic discharges (Sperk *et al*., 1992; Rizzi *et al*., 1993; Colmers & Bleakman, 1994; Vezzani *et al*., 1994; Erickson *et al*., 1996).

Recently, two isoforms of the gene encoding for the Y_1 receptor have been isolated from mouse bone marrow (Nakamura *et al*., 1995). One form, termed the $Y_{1\alpha}$ receptor, is highly expressed in many organs including the brain whereas the $Y_{1\beta}$ receptor is preferentially expressed during embryonic development, suggesting its potential implication in cellular differentiation (Nakamura *et al*., 1995). Even if the two isoforms upon their transfection and expression in cell lines revealed identical ligand selectivity profiles, differences were noted in signalling pathways. The $Y_{1\alpha}$ receptor induces a pertussis toxin-sensitive increase in intracellular Ca^{2+}, the inhibition of cyclic AMP accumulation and the activation of mitogen-activated protein kinase (MAPK) whereas the $Y_{1\beta}$ receptor evokes no such response (Nakamura *et al*., 1995). Activation of the Y_1 receptor has also been shown to stimulate intracellular Ca^{2+} in peripheral tissues (Mihara *et al*., 1989) and human cell lines (Aakerlund *et al*., 1990; Daniels *et al*., 1992), while inducing opposite effects on cyclic AMP, protein kinase C and MAPK (Herzog *et al*., 1992; Larhammar *et al*., 1992; Nakamura *et al*., 1995). These variant Y_1 receptor transcripts occur at a splice junction in the 5′-end of the gene, a region enriched with multiple promoters (Herzog *et al*., 1993; Ball *et al*., 1995). Possible response elements for the glucocorticoid hormone as well as potential binding sites for the AP-1, AP-2, octamer-binding protein (Oct-1), CREB and NF-κB transcription factors were noted in that region (Ball *et al*., 1995; Nakamura *et al*., 1995). These specific promoter elements are likely to be the molecular basis for the regulation of the expression of the Y_1 receptor isoforms in response to external signals, such as glucocorticoids (Larsen *et al*., 1994), or internal ones, such as NF-κB/Rel proteins (Musso *et al*., 1997). It would thus be of interest to determine whether the respective activation of these various promoters could differentially alter the expression of the Y_1 receptor in cultured hippocampal neurones. Studies of the comparative regulation of the Y_1 and Y_2 receptors in these cultures should also be considered, since their respective mRNAs were detected by RT–PCR while appreciable amounts of the translated proteins were noted only for the Y_1 subtype. For example, it was recently shown that treatment with oestradiol and progesterone had opposite effects on the expression of the Y_2 receptor in the hypothalamus (Parker *et al*., 1996). A similar approach could be used to study the expression of the Y_2 receptor in cultured hippocampal neurones. Finally, the cloning of the rat Y_2 receptor gene was also demonstrated here following its cloning from tissues of human origin (Gerald *et al*., 1995; Rose *et al*., 1995; Gehlert *et al*., 1996a). The rat clone has high homology (92%) to the human counterpart.

In summary, rat primary cultures of embryonic hippocampal cells represent a useful approach to investigate the functional relevance and transcriptional regulatory mechanisms of the expression of the Y_1 and possibly Y_2 receptor subtypes in the hippocampal formation, especially during development and early maturation stages.

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