Limited neuropeptide Y precursor processing in unfavourable metastatic neuroblastoma tumours

P Bjellerup1, E Theodorsson3, H Jörnvall4 and P Kogner2

1Department of Clinical Chemistry and 2Childhood Cancer Research Unit, Department of Woman and Child Health, Karolinska Institutet and Karolinska Hospital, S-171 76 Stockholm; 3Department of Clinical Chemistry, University Hospital, S-581 85 Linköping; 4Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden

Summary  Neuropeptide Y (NPY) is found at high concentrations in neural crest-derived tumours and has been implicated as a regulatory peptide in tumour growth and differentiation. Neuroblastomas, ganglioneuromas and pheochromocytomas with significant concentrations of NPY-like immunoreactivity were investigated for different molecular forms of NPY and for significance of proNPY processing. Gel-permeation chromatography identified intact NPY (1–36) in all tumours, whereas proNPY (69 amino acids) was detected only in control adrenal tissue and malignant neuroblastomas. Purification of NPY-like immunoreactivity in tumour extracts and structural characterization revealed that both NPY (1–36) and the truncated form NPY (3–36) was present. The degree of processing of proNPY to NPY in tumour tissue was lower in advanced neuroblastomas with regional or metastatic spread (stage 3 and 4) (P = 0.004). MYCN oncogene amplification was also correlated to a low degree of proNPY processing (P = 0.025). In summary, a low degree of proNPY processing was correlated to clinical advanced stage and poor outcome in neuroblastomas. ProNPY/NPY processing generated molecular forms of NPY with known differences in NPY-receptor selectivity, implicating a potential for in vivo modulation of NPY-like effects in tumour tissue. © 2000 Cancer Research Campaign

Key words: neuropeptide Y; neuroblastoma; pheochromocytoma; NPY processing; NPY (3–36); proNPY; prohormone processing

Neuroblastoma is the most common extra-cranial tumour in children. Its clinical behaviour ranges from spontaneous regression or complete remission after minimal drug therapy to unfavourable outcome from aggressive tumour growth despite intensive multimodal therapy (Katzenstein and Cohn, 1998). The benign differentiated counterpart, ganglioneuroma, usually has an excellent prognosis. Both tumours originate from the sympathetic nervous system and like all tumours of neuroendocrine origin they are able to produce different neuropeptides (Langley, 1994). Measurement or identification of these neuropeptides has become increasingly important in screening, diagnosis and follow-up of several tumours or tumour syndromes (Pollak and Schally, 1998; Lee and Evans, 1997). Intervention by peptide analogues is a well established treatment in several modalities of neuroendocrine tumours and can relieve symptoms (Öberg, 1998).

Neuropeptide Y (NPY), a 36-residue peptide present throughout the peripheral and central nervous systems, is synthesized as a preprohormone, preproNPY (97 residues), first cleaved to proNPY (69 residues), and eventually to the biologically active, C-terminally amidated peptide, NPY, (1–36) (Lundberg et al, 1982; Adrian et al, 1983a; Allen, 1990). Both proNPY and NPY as well as other, not structurally identified, molecular forms of NPY-like immunoreactivity have been identified in neuroblastoma and pheochromocytoma tumours (Adrian et al, 1983b; O’Hare and Schwartz, 1989b; Kogner et al, 1993; deS Senanayake et al, 1995). Plasma concentrations, but not tumour concentrations, of NPY have been shown to be of value to monitor follow-up of treatment in neuroblastoma, and high concentrations have been associated with poor outcome (Kogner, 1995; Dötsch et al, 1998). Five different receptor subtypes for NPY have been cloned with different affinity profiles for NPY, truncated forms of NPY, and NPY-related peptides and several different physiological effects has been attributed to NPY through these receptors (Michel et al, 1998). NPY has also been implicated in cellular events, e.g. to be mitogenic in vascular smooth muscle cells and in neuroblastoma cells in vitro (Zukowska-Grojec et al, 1993; Shorter and Pence, 1997) and to be angiogenic both in vitro and in vivo, an effect mediated through the Y2-receptor (Zukowska-Grojec et al, 1998).

The aim of the present study was to investigate the processing of proNPY and its correlation to different clinical parameters. Analysis of proNPY processing was carried out using gel-permeation chromatography and an antiserum specific for the mid-portion of NPY. Peptides with NPY-like immunoreactivity (NPY-LI) were isolated, chromatographically purified and characterized by amino acid sequence analysis and mass spectrometry.

MATERIAL AND METHODS

Patient material and sample handling

Samples from primary tumours from 18 children with neuroblastoma and one child with ganglioneuroma containing significant
concentrations of NPY-LI (30–4000 pmol g⁻¹), (Table 1) were selected from a series of tumours (Kogner, 1995). The tumours were diagnosed according to international criteria and neuroblastomas of all clinical stages were included (Brodeur et al., 1993). One metastasis, one healthy adrenal gland from a child with neuroblastoma, one ganglioneuroma and five phaeochromocytoma tumours from adult patients were also investigated. Tumour tissue, obtained at surgery, was frozen on solid CO₂ and kept at –70 °C until extraction. Six of the children with neuroblastoma died within 4–38 months from diagnosis, whereas the remaining children have been followed for 48–118 months without any evidence of disease. The study was approved by the ethics committee of Karolinska Institutet, Stockholm, Sweden.

Analytical peptide extraction

Extraction was performed by homogenization and boiling the tumour samples for 10 min in 10 volumes (minimum 2 ml) acetic acid (1 mol l⁻¹). After centrifugation at 2000 g for 15 min, the supernatants were lyophilized and dissolved in RIA-buffert.

Preparative peptide extraction

Preparative extraction was performed on two samples from children with neuroblastoma. After homogenization, boiling, centrifugation and decantation, trifluoroacetic acid (TFA) was added to the supernatant to a final concentration of 0.1% and the material was loaded onto SepPak cartridges (Waters, Milford, MA, USA), primed with 10 ml 80% methanol, 0.1% TFA, and equilibrated with 10 ml water, 0.1% TFA. After loading the sample, the cartridge was flushed with 2 ml water, 0.1% TFA and immunoreactive material was eluted with 4 ml 80% methanol, 0.1% TFA. The eluates were dried under vacuum at 40 °C, and dissolved in formic acid (1 mol l⁻¹), 0.02% sodium azide, before application onto gel-permeation chromatography.

Gel-filtration and reversed-phase high-pressure liquid chromatography (RP-HPLC)

A Sephadex G-50 superfine column (2.6 × 95 cm) (Amersham Pharmacia Biotech, Sweden) was eluted with formic acid (1 mol l⁻¹), 0.02% sodium azide, 0.1% bovine serum albumin (BSA) (BSA was excluded in preparative runs) at a flow rate of 0.5 ml min⁻¹. The column was calibrated using synthetic NPY (1–36) and 3H-NPY (1–36) (Peninsula, Belmont, CA, USA). Void volume (V₀) was determined with Blue dextran and total volume (Vₜ) with 22Na. Fractions (2 ml in analytical and 8 ml in preparative runs) were collected and lyophilized for subsequent analysis for NPY-LI. For structural analysis, fractions were lyophilized and dissolved in 0.1% TFA, pooled and further purified by RP-HPLC.

Table 1 Results from measurements of NPY-LI and clinical data for individual patients.

| Pat. | Diagnosis | Age at diagnosis (months) | Gender | Follow-up (months) | Status | NPY-LI (pmol g⁻¹) | ProNPY (fmol) | NPY (fmol) | ProNPY- proc. (%) | MYCN ampl. (⁺⁻) |
|------|-----------|--------------------------|--------|-------------------|--------|-------------------|-------------|------------|-----------------|----------------|
| T61  | AD        | 41                       | F      | 88                | NED    | 9.6               | 1487        | 806        | 35.1            | –              |
| T41  | GN        | 31                       | F      | 94                | NED    | 2133              | 614         | 9570       | 93.8            | –              |
| T63  | MET       | 41                       | F      | 88                | NED    | 545               | 2370        | 1979       | 45.5            | –              |
| T90  | NB 1      | 13                       | M      | 61                | NED    | 128               | 2162        | 4769       | 68.8            | –              |
| T107 | NB 1      | 18                       | F      | 60                | NED    | 682               | 2159        | 9755       | 81.9            | –              |
| T29  | NB 2      | 0                        | F      | 118               | NED    | 262               | 0           | 3392       | 100.0           | –              |
| T92  | NB 2      | 12                       | M      | 62                | NED    | 92                | 79          | 6204       | 98.7            | –              |
| T116 | NB 2      | 33                       | F      | 52                | NED    | 31.2              | 317         | 2692       | 89.5            | –              |
| T65  | NB 2      | 103                      | F      | 73                | NED    | 195               | 0           | 423        | 100.0           | –              |
| T26  | NB 3      | 3                        | F      | 81                | NED    | 451               | 2046        | 7904       | 79.4            | –              |
| T64  | NB 3      | 6                        | M      | 80                | NED    | 119               | 1248        | 676        | 35.1            | –              |
| T93  | NB 3      | 21                       | M      | 6                 | DOD    | 203               | 1798        | 1644       | 47.8            | +              |
| T38  | NB 4      | 8                        | F      | 4                 | DOD    | 282               | 2171        | 300        | 12.1            | +              |
| T88  | NB 4      | 11                       | F      | 4                 | DOD    | 143               | 1437        | 483        | 25.2            | +              |
| T50  | NB 4      | 24                       | M      | 10                | DOD    | 226               | 6135        | 3149       | 33.9            | –              |
| T52  | NB 4      | 32                       | M      | 18                | DOD    | 116               | 634         | 6919       | 91.6            | –              |
| T62  | NB 4      | 41                       | F      | 88                | NED    | 78                | 0           | 328        | 100.0           | –              |
| T51  | NB 4      | 60                       | M      | 48                | NED    | 177               | 0           | 1289       | 100.0           | –              |
| T01  | NB 4      | 60                       | F      | 38                | DOD    | 220               | 1330        | 300        | 18.4            | –              |
| T66  | NB 4S     | 0                        | M      | 80                | NED    | 50                | 580         | 3861       | 86.9            | –              |
| T95  | NB 4S     | 2                        | F      | 58                | NED    | 55                | 32          | 901        | 96.6            | –              |
| 8302 | PHEO      |                           |        |                   |        | 1003              | 0           | 900        | 100.0           | –              |
| 8305 | PHEO      |                           |        |                   |        | 259               | 0           | 1116       | 100.0           | –              |
| P7   | PHEO      |                           |        |                   |        | 305               | 293         | 3356       | 92.0            | –              |
| P8   | PHEO      |                           |        |                   |        | 3955              | 0           | 2122       | 100.0           | –              |
| P9   | PHEO      |                           |        |                   |        | 281               | 172         | 2842       | 94.3            | –              |

Healthy adrenal (AD), phaeochromocytomas (PHEO), ganglioneuromas (GN), neuroblastomas (NB) of different clinical stages (1–4 and 45) and metastasis (MET). No evidence of disease (NED), dead of disease (DOD).
Structural analysis
The primary structure of peptides was investigated by N-terminal amino acid sequence analysis using an Applied Biosystems 476 instrument (Perkin-Elmer, Norwalk, CT, USA) and phenylthiohydantoin detection with a 120 analyser. Molecular mass were determined by MALDI-TOF mass spectrometry (Finnigan, San Jose, CA, USA). Both methods were carried out according to the manufacturers’ instructions.

Fractions containing NPY immunoreactivity with a larger Stokes radius were lyophilized after gel-permeation chromatography and reconstituted in Veronal buffer, pH 8.6. Endoproteasease Lys-C (Sigma, St. Louis, MO, USA), 16 ng in 10 μl, was added prior to incubation at 37°C for 60 min.

Radioimmunoassay (RIA)
NPY-LI was analysed with RIA performed under non-equilibrium conditions using intact porcine NPY (1–36) as standard. The antiserum was raised in rabbit and had specificity for the mid-portion of NPY (Theodorsson-Norheim et al, 1985). The cross-reactivity for human NPY (1–36) and (3–36) was 65.2 and 19.2%, respectively.

MYCN oncogene amplification by Southern blot analysis
MYCN oncogene copy number was determined by Southern blot analysis performed as previously described (Hedborg et al, 1992) using the MYCN clone pNB 19–21. MYCN amplification was scored when the gene copy number was three or more in each haploid genome (Seeger et al, 1985).

Calculation of processing ratio and statistics
The extent of proNPY-processing was calculated by dividing the amount of NPY-LI eluting at the position of NPY (1–36) with the amount of all NPY-LI. Significance was calculated according to Fischer’s exact test, Mann–Whitney U test, Spearman and Pearson correlation coefficients. Survival probability was calculated according to Kaplan–Meier and compared using the logrank test.

RESULTS
Gel-permeation chromatography identified two molecular forms of NPY-LI with different Stokes radii (Figure 1). Some of the neuroblastomas, the ganglieneuroma and the pheochromocytomas contained only little or nothing of the larger form of NPY-LI (first peak), (Table 1). Incubation with Endoproteasease Lys-C of the material with NPY-LI from the first peak (representing a larger Stokes radius) and subsequent gel-permeation chromatography, showed cleavage of the high molecular precursor to a component detected at a position slightly earlier than intact NPY (1–36) (data not shown). Incubation with 6 mol l⁻¹ urea for eliminating the material with NPY-LI from the first peak (representing a larger Stokes radius) and subsequent gel-permeation chromatography was carried out. The second peak material with a smaller Stokes radius was further purified by RP-HPLC in several steps before amino acid sequence analysis. The result showed an amino acid sequence identical with that of human NPY (1–36) which was followed for 21 and 36 cycles, respectively. In the latter tumour, the amino-acid sequence also revealed an N-terminally truncated form of NPY, NPY (3–36). This was confirmed by mass spectrometry, showing the molecular masses of the oxidized, C-terminally amidated forms of NPY (1–36) and NPY (3–36) (Figure 2).

The processing ratio in the healthy adrenal was 35% (Figure 3), (Table 1). In the ganglieneuroma and the adult pheochromocytomas it was more than 90% (98%, 92–100%, median, range), whereas the ratio in the neuroblastomas varied (80%, 12–100%). Metastatic tissue, available from one child, showed a processing ratio of 46%, as compared to 100% in the corresponding primary tumour. A significantly lower degree of proNPY processing was seen in neuroblastomas at advanced stage (stages 3 and 4) (41%, 12–100%) as compared to stages 1, 2 and 4S (93%, 69–100%, P = 0.012) (Figure 3). A processing of less than 50% was significantly correlated with poor prognosis (P = 0.004) and with a significant difference in survival probability (P < 0.001, χ² = 12.63) as analysed according to Kaplan–Meier (Figure 4). MYCN oncogene amplification by Southern blot analysis
amplification was also significantly correlated to prognosis \((P = 0.025)\) and to a low degree of proNPY processing \((P = 0.025)\) but not to tumour stage \((P = 0.147)\) and there was a significant difference in survival probability as analysed according to Kaplan–Meier \((P < 0.001, \chi^2 = 12.63)\) (Figure 3). No other correlation was identified for the data in Table 1, e.g. there was no correlation between the concentration of NPY-LI and the processing degree using either Pearson \((r^2 = 0.05, P > 0.2)\) or Spearman \((P > 0.7)\) coefficients of variation.

### DISCUSSION

Pheochromocytomas and neuroblastomas may produce large amounts of NPY and the concentration of NPY in plasma can be a valuable marker in the diagnosis and monitoring of these diseases (Adrian et al, 1983b; Kogner et al, 1993; deS Senanayake et al, 1995). In the present material, a decreased intracellular processing of proNPY was significantly correlated with both widespread disease and poor outcome. Furthermore, a truncated form of NPY was identified indicating that the processing of proNPY and NPY in neuroblastoma may increase the possibility to modulate the interaction with different NPY receptors.

In this series of 25 tumours and one control adrenal, two major molecular forms of NPY-LI were identified (Figure 1). The larger molecular form of NPY-LI was deduced to be proNPY as was indicated by Endoproteinase Lys-C (Jekel et al, 1983). Endoproteinase Lys-C cleaves the peptide bond C-terminally of the Lys-residue, and can thereby be expected to cleave the 69 amino acid precursor proNPY C-terminally of the only lysine-residue present (position 38) in proNPY, generating the two fragments NPY (1–36)-Gly-Lys and Arg-CPON (C-flanking Peptide Of NPY) (O'Hare and Schwartz, 1989a).

The molecular form of NPY-LI with a smaller Stokes radius eluted at the same position as intact NPY. By subsequent purification and amino acid sequence analysis, intact NPY was identified in one tumour whereas the other tumour was shown to contain both intact NPY and an N-terminally truncated form of NPY, NPY (3–36), also confirmed with mass spectrometry (Figure 2).

The processing degree of proNPY to NPY in neuroblastoma was significantly higher in the more differentiated tumours as a processing of less than 50% was seen only in the most unfavourable tumours (stage 3 and 4) with regional or metastatic spread \((P = 0.012)\) (Figure 3). A processing of less than 50% was also significantly correlated with a poor survival probability as five of the six children in this group died within 38 months from...
diagnosis ($P = 0.004$) (Figure 4). In the group of children with a processing of 50\% or more, 11 out of 12 children were alive without evidence of disease during a follow-up time of 48 to 118 months.

Variation in the degree of processing is in agreement with an in vitro study using eight different neuroblastoma cell lines where the processing degree varied between 33 and 72\% (O’Hare and Schwartz, 1989a). However, they are in contrast to another study by the same group where three neuroblastoma tumours all had more than 90\% processing (O’Hare and Schwartz, 1989b). The difference in these results may be due to the fact that cell lines are most often raised from more malignant tumours, whereas the clinical tumours in the study may be of more benign stages (not specified in the articles).

Prohormone processing has been investigated in a number of endocrine tumours but has so far not been implicated to be of prognostic value (Rehfeld et al, 1996). The significant differences, although analysed in a limited number of samples, may indicate that biochemical events involved in the formation of NPY may be a part of an aggressive phenotype and that proNPY processing may be an indicator of prognosis in children with neuroblastoma.

The conversion of proNPY to the active peptide, NPY (1–36) occurs intracellularly within secretory vesicles involving several different enzymes. Among these enzymes the prohormone convertases (PC) 1 (also called PC 3) and PC 2 seem to be both the most substrate-specific and tissue-specific for proNPY processing (Wulff et al, 1993; Hook et al, 1996; Paquet et al, 1996). The present results may be derived from variation in PC-expression in the tumours; further studies are needed to elucidate this issue.

A low degree of proNPY processing in the more aggressive neuroblastomas may seem to contradict to the previous reported correlation between elevated concentrations of NPY-LI in plasma and poor outcome and relapse of disease (Kogner, 1995; Dötsch et al, 1998). However, the degree of proNPY processing is not correlated to the total amount of NPY-LI present (Table 1). Furthermore, no correlation between NPY mRNA expression and NPY-LI plasma concentration (tumour release) has been found (Dötsch et al, 1998). Thus, translation, transcription and cellular release are not necessarily linked.

The five pheochromocytomas from adult patients did not show significant amounts of proNPY. This is in agreement with previous studies of pheochromocytoma tumour extracts showing only one immunoreactive form of NPY (Adrian et al, 1983b; Corder et al, 1984; Allen et al, 1987; O’Hare and Schwartz, 1989b). No classification of the pheochromocytomas has been indicated in these previous studies, and also not in the present material. However, the amounts detected in all samples indicate that the processing degree in general, is high in pheochromocytoma tumour tissue.

Amplification of the MYCN oncogene is an established predictor of poor prognosis in neuroblastoma and is strongly correlated with clinically advanced stages (Seeger et al, 1985; Brodeur et al, 1992). In the present study, all three tumours with MYCN amplification showed a low degree of proNPY processing, were of advanced clinical stage and the children died during follow-up (Table 1 and Figure 3). However, a low degree of proNPY-processing had a higher correlation to advanced tumour stage and poor outcome than MYCN amplification.

By amino acid sequence analysis and mass spectrometry, intact NPY was identified in one tumour investigated, whereas another tumour was shown to contain both intact NPY and an N-terminally truncated form of NPY, NPY (3–36) (Figure 2). Evidently, none of the chromatographic methods, or the RIA, was able to separate intact NPY from NPY (3–36). This lack of specificity is probably a feature that is in common with most, if not all, other investigations.

NPY (3–36) has earlier been identified in a human somatostatinoma of the pancreas but has, to our knowledge, not been found in normal human tissue or blood (Shaw et al, 1993). NPY has an N-terminally tyrosine followed by a prolyl residue. This structure renders the peptide resistant to generalized aminopeptidase degradation but susceptible to more specialized enzymes (Mentlein, 1988). Several enzymes have recently been investigated and human dipeptidyl amino peptidase (DPP) IV was the only enzyme with high activity for NPY generating Tyr-Pro dipeptides (Mentlein et al, 1993). DPP IV has been identified on the surface of endothelial cells, T-lymphocytes (CD 26), hepatocytes and in the intestinal and kidney brush border membranes, but adrenal or tumour tissue has not been investigated (Mentlein et al, 1993).

The multitude of receptors and ligands indicate that the processing of proNPY and intact NPY offers a potential to modulate the physiological and biological actions of NPY (Michel et al, 1998). Processing of NPY to NPY (3–36) generates a molecule, which does not bind to the Y1 receptor (Grandt et al, 1996). The presence of both intact NPY and NPY (3–36) in the same tumour tissue has not been reported earlier. These findings indicate the presence of DPP IV on the surface of cells in neuroblastoma tumours, converting non-receptor selective intact NPY into Y2-, Y3- and Y5 receptor-selective NPY (3–36), thereby creating a potential for modulation, or even a shift, in biological actions. The Y1 and Y2 receptor may be expressed in neuroblastoma cell lines (Wahlestedt et al, 1992), and formation of NPY (3–36) in neuroblastoma tumours may be of particular significance since the Y2 receptor mediates the angiogenic activity of the ligand NPY (3–36) in vitro and in vivo (Zukowska-Grojec et al, 1998).

In conclusion, a low degree of proNPY processing in tumour tissue was correlated to advanced disease with regional or metastatic spread and poor outcome in neuroblastoma. ProNPY/NPY processing within the same tumour tissue generated NPY (3–36), a molecule known to have different receptor selectivity in comparison with intact NPY. Simple methods are needed to specifically measure the different forms of NPY-LI that are present in neuroblastoma tumour tissue.

**ACKNOWLEDGEMENTS**

This work was supported by The Children’s Cancer Foundation of Sweden, The Cancer Research Fund (1806, 2313, and 3165) and The Swedish Society for Medical Research.

**REFERENCES**

Adrian TE, Allen JM, Bloom SR, Ghatei MA, Rosor MN, Roberts GW, Crow TJ, Tatemoto K and Polak JM (1983a) Neuropeptide Y distribution in human brain. *Nature* 306: 584–586

Adrian TE, Allen JM, Terenghi G, Bacarese-Hamilton AJ, Brown MJ, Polak JM and Bloom SR (1983b) Neuropeptide Y in pheochromocytomas and ganglioneuroblastomas. *Lancet* 2: 540–542

Allen JM (1990) Molecular structure of neuropeptide Y. *Ann N Y Acad Sci* 611: 86–98

Allen JM, Yeats JC, Causon R, Brown MJ and Bloom SR (1987) Neuropeptide Y and its flanking peptide in human endocrine tumours and plasma. *J Clin Endocrinol Metab* 64: 1199–1204
Brodeur GM, Azar C, Brother M, Hiemstra J, Kaufman B, Marshall H, Moley J, Nakagawara A, Saylors R, Scavarda N, Schneider ES, Wasson J, White P, Seeger R, Look T and Castlebury R (1992) Neuroblastoma. Effect of genetic factors on prognosis and treatment. Cancer 70: 1685–1694

Brodeur GM, Pritchard J, Berthold F, Carlsten NL, Castel V, Castellanry RP, De Bernardi B, Evans AE, Favrot M, Heidberg F, Kaneko M, Kemshead J, Lampert F, Lee REJ, Look AT, Pearson ADJ, Philip T, Roald B, Sawada T, Seeger RC, Toucheida Y and Voite PA (1993) Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment [see comments]. J Clin Oncol 11: 1466–1477

Corder R, Emerson PC and Lowry PJ (1984) Purification and characterization of human neuropeptide Y from adrenal-medullary phaeochromocytoma tissue. Biochem J 219: 699–706

deSennayake P, Denker J, Bravo EL and Graham RM (1995) Production, characterization, and expression of neuropeptide Y by human phaeochromocytoma. J Clin Invest 96: 2503–2509

Dotsch J, Christianen H, Hanze J, Lampert F and Rascher W (1998) Plasma neuropeptide Y of children with neuroblastoma in relation to stage, age and prognosis, and tissue neuropeptide Y. Regul Pept 75–76: 185–190

Grand D, Schimickez M, Rascher W, Feth F, Shively J, Lee TD, Davis MT, Reeve JR, Jr and Michel MC (1996) Neuropeptide Y 3–36 is an endogenous ligand selective for Y2 receptors. Regul Pept 67: 33–37

Heidberg F, Lindgren PG, Johansson I, Kogner P, Samuelsson BO, Bekassy AN, Olsen L, Kreuger A and Pålman S (1992) N-myc gene amplification in neuroblastoma: a clinical approach using ultrasound guided cutting needle biopsies collected at diagnosis. Med Pediatr Oncol 20: 292–300

Hook YV, Schiller MR and Azaryan AV (1996) The processing proteases prohormone thiore proteinase, PC1/3 and PC2, and 70-kDa aspartic proteinase characterization, and expression of neuropeptide Y by human neuropeptide Y in human and murine neuroblastoma and pheochromocytoma cell lines. Cancer Res 49: 7015–7019

O’Hare MM and Schwartz TW (1989a) Expression and precursor processing of neuropeptide Y in human and murine neuroblastoma and pheochromocytoma tumors. Cancer Res 49: 7010–7014

Oberg K (1998) Advances in chemotherapy and biotherapy of endocrine tumors. Curr Opin Oncol 10: 58–65

Paquet L, Massie B and Mains RE (1996) Proneuropeptide Y processing in large dense-core vesicles: manipulation of prohormone convertase expression in sympathetic neurons using adenoviruses. J Neurosci 16: 964–973

Pollak MN and Schally AV (1998) Mechanisms of antineoplastic action of somatostatin analogs. Pinn Soc Exp Biol Med 217: 143–152

Rheinfeld JF, Bardram L and Hilsted L (1996) Gastroenteropancreatic tumours and prohormones. Scand J Gastroenterol Suppl 216: 39–45

Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY and Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY and Hamilton D (1985) Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 313: 1111–1116

Shaw C, Cormican K, Thim L, Maage AG, Sloan JM and Buchanan KD (1993) Neuropeptide Y and neuropeptide Y 3–36: isolation from human pancreatic endocrine tumours. Regul Pept 45: 387–394

Shorter NA and Pence JC (1997) Retinoic acid-induced regulation of neuropeptide Y receptor expression and function in the neuroepithelioma line SK-N-MC. J Pediatr S 32: 721–723

Theodorsson-Norheim E, Hemsen A and Lundberg JM (1985) Radioimmunoassay for neuropeptide Y (NPy): chromatographic characterization of immunoactivity in plasma and tissue extracts. Scand J Clin Lab Invest 45: 355–365

Wahlstedt C, Regnathan S and Reis DJ (1992) Identification of cultured cells selectively expressing Y1-, Y2-, or Y3-type receptors for neuropeptide Y/reptide YY. Life Sci 50: PL7–12

Wulf BS, Johansen TE, Dalboge H, O’Hare MM and Schwartz TW (1993) Processing of two homologous precursors, pro-neuropeptide Y and pro-polypeptide Y, in transfected cell lines expressing different precursor convertases. J Biol Chem 268: 13327–13335

Zakowska-Grojec Z, Karwatowska-Prokoczek E, Rose W, Rome J, Movafagh S, Ji H, Yeh YY, Chen WT, Kleinman HK, Grounemann E and Grant DS (1998) Neuropeptide Y – A novel angiogenic factor from the sympathetic nerves and endothelium. Circ Res 83: 187–195

Zakowska-Grojec Z, Pruszczczyk P, Colton C, Yao J, Shen GH, Myers AK and Wahlstedt C (1993) Mitogenic effect of neuropeptide Y in rat vascular smooth muscle cells. Peptides 14: 263–268