Suppression of Substance P Biosynthesis in Sensory Neurons of Dorsal Root Ganglion by Prodrug Esters of Potent Peptidylglycine α-Amidating Monooxygenase Inhibitors*

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Substance P as well as many other neuropeptides are synthesized as glycine-extended precursors and converted to the biologically active C-terminal amides by posttranslational modification. The final step of posttranslational processing is catalyzed by peptidylglycine α-amidating monooxygenase (PAM). In a previous study, N-substituted homocysteine analogs were found to be potent inhibitors of PAM partially purified from conditioned medium of cultured rat medullary thyroid carcinoma CA-77 cells. These compounds, however, were only modest inhibitors of substance P production in cultured dorsal root ganglion cells, possibly because of poor cell penetration. Several ester derivatives of hydrocinnamoyl-phenylalanine-homocysteine, one of the most potent PAM inhibitors, were prepared to increase the intracellular accessibility of these compounds. Hydrocinnamoyl-phenylalanine-(S-benzoyl-homocysteine) benzyl ester was identified as the most potent compound, inhibiting substance P biosynthesis in dorsal root ganglion cells with an IC<sub>50</sub> of 2 μM. Inhibition of PAM resulted in a concomitant increase in the glycine-extended substance P (substance P-Gly) precursor peptide. In the presence of 3 μM benzyl ester derivative, the intracellular substance P-Gly level was 2.4-fold higher while the substance P level was 2.1-fold lower than the corresponding peptides in control cells. These results suggest that PAM inhibition represents an effective method for suppression of substance P biosynthesis and, therefore, may have therapeutic utility in conditions associated with elevated substance P levels. Furthermore, PAM inhibition may also prove useful in decreasing other amidated peptides.

The C-terminal amide is a prerequisite for full biological activity of many neuropeptides (1). These neuropeptides are typically synthesized as glycine-extended precursors and converted to the mature peptides by a family of enzymes involved in posttranslational modifications, e.g. O-glycosylation, phosphorylation, sulfation, and hydroxylation, as well as in proteolytic processing, e.g. endopeptidase and exopeptidase (2). The final step of posttranslational processing is catalyzed by an enzyme originally identified as peptidylglycine α-amidating monooxygenase (PAM)¹ (3–5). PAM is localized in secretory granules and requires copper, ascorbate, and molecular oxygen for activity (2, 6, 7). Recent studies have shown that PAM is actually a bifunctional enzyme that contains two distinct enzymatic activities and catalyzes the C-terminal amidation in a sequential manner. The first enzyme, peptidylglycine α-hydroxylating monooxygenase (PHM) (EC 1.14.17.3), requires the cofactors for activity and catalyzes the peptidylglycine α-hydroxylation reaction while the second enzyme, peptidyl α-hydroxyglycine α-amidating lyase (PAL) (EC 4.3.2.5), converts the intermediate into an α-amidated peptide and glyoxylate (8–11).

Among the glycine-extended neuropeptides examined, glycine-extended substance P (substance P-Gly) has been demonstrated to possess the highest affinity for PAM partially purified from conditioned medium of cultured rat medullary thyroid carcinoma CA-77 cells (12). Substance P has been implicated in the pathogenesis of neurogenic inflammation (13, 14). For example, elevated levels of substance P have been observed during inflammation, and depletion of substance P by chronic treatment of animals with capsaicin has been shown to lessen the severity of the inflammatory response (15–17). Furthermore, substance P may also play a role in rheumatoid arthritis (18). It has been shown that substance P stimulates the release of collagenase and prostaglandin E<sub>2</sub> from synoviocytes, resulting in a loss of cartilage, development of lesions in the adjacent bone, and perpetuation of the inflammatory process in the artritic joint (19). The involvement of substance P in the pathophysiology of rheumatoid arthritis has been further supported by the observations that the release of substance P from the dorsal horn of polyarthritic rats is significantly accelerated and that the severity of arthritis in rats is increased upon infusion of substance P into the knee joints (20, 21). Thus, suppression of substance P biosynthesis through PAM inhibition may be beneficial to diseases such as neurogenic inflammation and rheumatoid arthritis.

Several inhibitors of PAM have been identified previously, including acetopyruvate (4), [(4-methoxybenzoyloxy)acetic acid (22), trans-styrylthioacetic acid (23), benzylhydrazine (24), N-formyl amides (25), sulfite (26), and derivatives of organic acids (27). Most of these compounds inhibit PAM with IC<sub>50</sub> values in the low micromolar to sub-millimolar range. Through mechanism-based inhibitor design, Zabriskie et al. (28) have found that N-phenylalanyl-L-phenylalanine-α-vinylglycine, a substrate analog, inhibited PAM with an apparent K<sub>i</sub> of 20 μM. Since substance P-Gly and other glycine-extended neuropeptides have displayed affinities in the low micromolar concentration range.

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¹ The abbreviations used are: PAM, peptidylglycine α-amidating monooxygenase; Boc, N-butoxycarbonyl; DRG, dorsal root ganglion; EDCI, N-[3-(dimethylamino)propyl]-N'-ethylcarbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole; substance P-Gly, glycine-extended substance P; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TFA, trifluoroacetic acid.
Cysteine thiolactone (Compound 2) as a common intermediate. A general procedure for the synthesis of prodrug esters of Compound 1 is described previously. A suspension of 10 g of t-butoxy carbonyl (Boc)-t-phenylalanine (37.7 mmol), 5.75 g of d,L-homocysteine thiolactone hydrochloride (37.6 mmol), and 5.75 g of HOBT hydrate in 300 ml of methylene chloride was cooled in an ice bath. Triethylamine (5.3 ml, 38.1 mmol) was added in 1-ml aliquots followed by 7.25 g (37.9 mmol) of EDCI. The mixture was allowed to warm to room temperature and stirred for 17 h. The solvent was then evaporated in vacuo, and 300 ml each of ether and water were added to the residue. The phases were separated, and the organic layer was washed twice with 200 ml of 0.1 N HCl and once with saturated NaHCO3. The combined organic layers were dried over sodium sulfate/magnesium sulfate and filtered. The solvent was evaporated in vacuo to give 13.68 g of Boc-t-phenylalanyl-D,L-homocysteine thiolactone (99%) as a white foam. A solution of 2 g (5.5 mmol) of the above material and 5 ml (65 mmol) of trifluoroacetic acid was stirred for 45 min. The reaction was diluted with toluene before the solvents were evaporated in vacuo to give the product as white foam. This material was then dissolved in 40 ml of methylene chloride, and triethylamine (1.3 ml, 9.3 mmol) was added in portions. The pH was monitored to make sure that the reaction was basic. To this mixture was added 824 mg of hydrocinnamic acid (5.5 mmol), 840 mg of HOBT hydrate (5.5 mmol), and 1.04 g (5.5 mmol) of EDCI. The mixture was stirred for 90 min. The solvents were evaporated in vacuo, and the residue was taken up in ethyl acetate and water. The resultant elution was filtered through celite, and the phases were separated with water. The organic layer was washed once with water, twice with 0.1 N HCl, and once with saturated NaHCO3. It was then dried over sodium sulfate/magnesium sulfate and filtered, and the solvent was evaporated in vacuo. The resultant solid was dissolved in warm methylene chloride and purified by flash chromatography on silica gel (25% ethyl acetate/hexane) to give 1 g (51% overall, 3 steps) of hydrocinnamoyl-t-phenylalanyl-D,L-homocysteine thiolactone as a white solid.

Chemical Syntheses—Hydrocinnamoyl-phenylalanyl-D,L-homocysteine Benzyl Ester (Compound 3)—To a solution of 200 mg (0.5 mmol) of Compound 2 in 15 ml of degassed methanol was added 27 mg (0.68 mmol) of sodium hydride (60% dispersion in mineral oil). After stirring for 30 min at room temperature, the mixture was quenched with water and extracted with ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, and evaporated in vacuo. The residue was recrystallized in ethyl acetate/hexane to give 124 mg (58%) of the product as a white solid (m.p. 111–113 °C).

Chemical Syntheses—Hydrocinnamoyl-phenylalanyl-D,L-homocysteine n-Butyl Ester (Compound 4)—To a solution of 200 mg (0.5 mmol) of Compound 2 in 5 ml of tetrahydrofuran was added 320 mg of mercury (II) bistrifluoroacetic acid. The mixture was stirred for 45 min. The reaction was diluted with toluene before the solvents were evaporated in vacuo to give the product as white foam. This material was then dissolved in 40 ml of methylene chloride, and triethylamine (1.3 ml, 9.3 mmol) was added in portions. The pH was monitored to make sure that the reaction was basic. To this mixture was added 824 mg of hydrocinnamic acid (5.5 mmol), 840 mg of HOBT hydrate (5.5 mmol), and 1.04 g (5.5 mmol) of EDCI. The mixture was stirred for 90 min. The solvents were evaporated in vacuo, and the residue was taken up in ethyl acetate and water. The resultant elution was filtered through celite, and the phases were separated with water. The organic layer was washed once with water, twice with 0.1 N HCl, and once with saturated NaHCO3. It was then dried over sodium sulfate/magnesium sulfate and filtered, and the solvent was evaporated in vacuo. The resultant solid was dissolved in warm methylene chloride and purified by flash chromatography on silica gel (25% ethyl acetate/hexane) to give 1 g (51% overall, 3 steps) of hydrocinnamoyl-t-phenylalanyl-D,L-homocysteine thiolactone as a white solid.

Materials—N-[3-(dimethylamino)propyl]-N’-ethycarbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole (HOBT) were obtained from Aldrich. Substance P and substance P antiserum were products of Cambridge Research Biochemicals (Wilmington, DE). N-Tris(hydroxyethyl)methyl)methyl-2-aminoethanesulfonic acid (Tes), catalase, and rabbit liver esterase were purchased from Sigma. Cytosine arabinoside and methyl methanethiosulfonate (MTS) were products of Unigene Laboratories (Fairfield, NJ), respectively. Substance P and substance P antiserum were products of Cambridge Research Biochemicals (Wilmington, DE). N-Tris(hydroxyethyl)methyl)methyl-2-aminoethanesulfonic acid (Tes), catalase, and rabbit liver esterase were purchased from Sigma. Cytosine arabinoside and methyl methanethiosulfonate (MTS) were products of Unigene Laboratories (Fairfield, NJ), respectively.

Chemical Syntheses—Hydrocinnamoyl-phenylalanyl-D,L-homocysteine benzyl ester (Compound 3) was synthesized as described previously. A general procedure for the synthesis of prodrug esters of Compound 1 is shown in Scheme 1, using hydrocinnamoyl-t-phenylalanyl-D,L-homocysteine thiolactone (Compound 2) as a common intermediate.

EXPERIMENTAL PROCEDURES

Materials—N-[3-(dimethylamino)propyl]-N’-ethycarbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole (HOBT) were obtained from Aldrich. Substance P and substance P antiserum were products of Cambridge Research Biochemicals (Wilmington, DE). N-Tris(hydroxyethyl)methyl)methyl-2-aminoethanesulfonic acid (Tes), catalase, and rabbit liver esterase were purchased from Sigma. Cytosine arabinoside and partially purified PAM were obtained from Uijohn (Kalamazoo, MI) and Unigene Laboratories (Fairfield, NJ), respectively.

Inhibition of SP Biosynthesis by Esters of PAM Inhibitors

In a previous study, N-substituted homocysteine analogs were found to be potent inhibitors of PAM partially purified from conditioned medium of cultured rat medullary thyroid carcinoma CA-77 cells (30). Studies in cultured dorsal root ganglion (DRG) cells, however, showed only modest inhibition of substance P bioconversion with these compounds (30). Since PAM is localized in secretory granules, these results suggest that the inhibitors were not accessible to the intracellular compartment of the cells. In the present study, several ester derivatives of hydrocinnamoyl-phenylalanylhomocysteine, one of the most potent PAM inhibitors, were synthesized to improve the intracellular accessibility of these compounds.

Hydrocinnamoyl-phenylalanyl-d,l-homocysteine Methyl Ester (Compound 3)—To a solution of 200 mg (0.5 mmol) of Compound 2 in 15 ml of degassed methanol was added 27 mg (0.68 mmol) of sodium hydride (60% dispersion in mineral oil). After stirring for 30 min at room temperature, the mixture was quenched with water and extracted with ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, and evaporated in vacuo. The residue was recrystallized in ethyl acetate/hexane to give 124 mg (58%) of the product as a white solid (m.p. 111–113 °C).

Hydrocinnamoyl-phenylalanyl-d,l-homocysteine n-Butyl Ester (Compound 4)—To a solution of 200 mg (0.5 mmol) of Compound 2 in 5 ml of tetrahydrofuran was added 320 mg of mercury (II) bistrifluoroacetic acid (0.75 mmol) and 37 mg (0.5 mmol) of sodium methoxide (0.75 mmol) in 1 ml of tetrahydrofuran was added 320 mg of mercury (II) bistrifluoroacetic acid (0.75 mmol) and 37 mg (0.5 mmol) of sodium methoxide (0.75 mmol) in 1 ml of tetrahydrofuran.
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When the DRG cells were dissociated and grown one day in culture, both the neuronal cells and non-neuronal cells such as glial cells and fibroblasts were seen (results not shown). The growth of the non-neuronal cells could be suppressed almost entirely after treatment of the cells with cysteine arabinoside during the second and third days of culture, resulting in a nearly homogenous population of neuronal cells that were inter-connected with neurites (results not shown). The effects of PAM inhibitors on substance P biosynthesis were assessed using these cells.

Compound 1 was previously found to be a potent PAM inhibitor in vitro, with an IC50 of 10 nM. However, this compound was not effective in suppressing the biosynthesis of substance P in DRG cells; only 25% inhibition was observed at 10 μM (Table 1). The homocysteine moiety was absolutely required for inhibition of substance P production in these cells; no significant inhibition was obtained when it was replaced by cysteine (results not shown). Since compounds that contain a charged group, such as a carboxylic acid, frequently exhibit poor cell penetration, we attributed the large difference in the potencies between the in vitro isolated enzyme and the cell assays to the inability of Compound 1 to enter DRG cells. One approach to circumvent this problem was to increase the hydrophobicity of the compound by cyclizing the sulfhydryl group with the C-terminal carboxyl group. As expected, the resulting thioactone (Compound 2) was inactive in the in vitro PAM assay since a free sulfhydryl group was necessary to coordinate the active site copper ion. Unfortunately, this compound only showed a slight improvement in the DRG cell assay; substance P biosynthesis was inhibited by 37% at 10 μM (Table 1). Therefore, a number of prodrug esters of Compound 1 were synthesized. The methyl (Compound 3), butyl (Compound 4), and benzyl (Compound 5) esters were 200- to 500-fold weaker in potency in the in vitro PAM assay when compared with the parent compound; their respective IC50 values were 2.3, 1.9, and 5.4 μM. In DRG cells, the prodrug esters inhibited substance P biosynthesis with increased potency. The results also indicate that increased hydrophobicity of the esterified group led to better cellular activity. For example, Compound 5 inhibited the biosynthesis of substance P (Table 1).
Compound 5 was selected for further modification. Its sulfhydryl group was replaced with thionicotinate (Compound 6), thioacetate (Compound 7), or thiobenzoate (Compound 8). These compounds were inactive in the in vitro PAM assay as expected. Surprisingly, Compound 6 showed a decreased activity in the DRG cell assay when compared with Compound 5 (Table I). Also, replacement with a thioacetate group did not result in an improved inhibition of substance P biosynthesis when compared with the results obtained with Compound 5. Compound 8 was the most potent in this series; it inhibited the production of substance P in DRG cells with an estimated IC$_{50}$ of 2 $\mu$M (Fig. 1).

In a previous study, Compound 9 was also identified to be a potent inhibitor of PAM in vitro, and it was more effective than Compound 1 in the DRG cell assay (Tables I and II). Using the same strategy as described in Table I, the benzyl ester derivative of Compound 9 (Compound 10) was synthesized. Unfortunately, this compound was less potent than its parent compound in the DRG cell assay (Table II). Therefore, optimization of this series of compounds was not pursued further.

The effect of PAM inhibition on the relative abundance of substance P and its precursor substance P-Gly in DRG cells was investigated using Compound 8. As described previously (31), cultured DRG cells produce more substance P-Gly than substance P in the absence of exogenous ascorbate. Under this condition, PAM is not fully activated since ascorbate is required for reoxidation of the active site copper during substrate turnover. Addition of 500 $\mu$M ascorbate and incubation of the DRG cells overnight led to a change in the substance P-Gly to substance P ratio from 3.2 to 0.3 (Table III). Compound 8 at 3 $\mu$M significantly inhibited the conversion of substance P-Gly to substance P even in the presence of the reducing agent. The ratio of substance P-Gly to substance P in the DRG cells was 1.5, but the total amount of the two peptides was not significantly different from that obtained in control cells or cells treated with ascorbate overnight (Table III).

**Table I**

| Compound | R$_1$ | R$_2$ | PAM assay IC$_{50}$ | DRG Cell Assay Concentration | Percent inhibition |
|----------|-------|-------|---------------------|-----------------------------|-------------------|
| 1        | H     | H     | 10 $\pm$ 1$^c$     | 10                          | 25 $\pm$ 4$^c$    |
| 2        |       |       | >10,000             | 10                          | 37 $\pm$ 9        |
| 3        | CH$_3$| H     | 2,300               | 10                          | 50 $\pm$ 4        |
| 4        | (CH$_2$)$_2$CH$_3$ | H    | 1,900               | 10                          | 56 $\pm$ 7        |
| 5        | CH$_2$CH$_2$H      | H     | 5,400               | 10                          | 74 $\pm$ 10       |
| 6        | CH$_2$CH$_2$H      | -3-COC$_2$H$_2$N | >10,000          | 10                          | 24 $\pm$ 10       |
| 7        | CH$_2$CH$_2$H      | COCH$_3$ | >10,000            | 10                          | 39 $\pm$ 13       |
| 8        | CH$_2$CH$_2$H      | COCH$_3$ | >10,000            | 3                           | 39 $\pm$ 9        |

$^a$ The IC$_{50}$ values of the prodrug esters in the PAM assay are means of two experiments (<10% error).
$^b$ Percent inhibitions of substance P production in DRG cells are expressed as mean $\pm$ S.E. (n = 3–9).
$^c$ From Ref. 30.

**Table II**

| Compound | R | PAM assay IC$_{50}$ | DRG Cell Assay Concentration | Percent inhibition |
|----------|---|---------------------|-----------------------------|-------------------|
| 9        | H | 10 $\pm$ 3$^c$     | 10                          | 67 $\pm$ 5        |
| 10       | CH$_2$CH$_2$H | 8,000  | 10                          | 54 $\pm$ 3        |

$^a$ The IC$_{50}$ value of the prodrug ester in the PAM assay represents the mean of two experiments (<10% error).
$^b$ Percent inhibitions of substance P production in DRG cells are expressed as mean $\pm$ S.E. (n = 3–5).
$^c$ From Ref. 30.

**Fig. 1.** Dose-dependent inhibition of substance P production in DRG cells by Compound 8. Inhibition of substance P production was performed in the presence of 500 $\mu$M ascorbate as described under “Experimental Procedures.” Each data point represents mean $\pm$ S.E. (n = 3–6).

**Inhibition of SP Biosynthesis by Esters of PAM Inhibitors**

Inhibition of substance P production was performed in the presence of 500 $\mu$M ascorbate as described under “Experimental Procedures.”
TABLE III

Effects of Compound 8 on the amidation of substance P-Gly in DRG cells

| Treatment               | SP   | SP-Gly | Total | SP-Gly/SP ratio |
|-------------------------|------|--------|-------|-----------------|
| Control                 | 74 ± 12 | 240 ± 36 | 320 ± 46 | 3.2 |
| Ascorbate               | 230 ± 66 | 68 ± 35 | 300 ± 74 | 0.3 |
| Compound 8 + Ascorbate  | 110 ± 12 | 160 ± 34 | 260 ± 45 | 1.5 |

DISCUSSION

Since PAM requires both ascorbate and copper for activity (33, 34), several investigators have demonstrated changes in the production of amidated peptides by altering the levels of these cofactors in vivo. For example, a diet deficient in vitamin C has been shown to cause a 30-fold increase in the levels of glycine-extended gastrin with a concomitant 2-fold decrease in gastrin in extracts of guinea pig antra (35). Likewise, chronic treatment with a copper chelator N,N-diethyldithiocarbamate or its disulfide dimer disulfiram in rats produced a dose-dependent increase in glycine-extended α-melanotropin in the pituitary (36). Unexpectedly, treatment with disulfiram, but not with N,N-diethyldithiocarbamate, was effective in the reduction of α-melanotropin in rat pituitary neurointermediate lobe and cholecystokinin octapeptide in cerebral cortex (37). Similar treatment with disulfiram in rats also resulted in increased levels of substance P-Gly in various areas of the brain, including preoptic area, medial basal hypothalamus, pons, medulla, and spinal cord (38). However, these treatments may cause other nonspecific effects, since other enzymes such as dopamine β-hydroxylase, superoxide dismutase, and l-lysyl oxidase also require ascorbate and/or copper for activity (39–42). Therefore, specific, potent inhibitors of PAM would be desirable to confirm these observations.

The most potent PAM inhibitors discovered to date are N-substituted homocysteine analogs (30). These compounds were shown to inhibit PAM partially purified from conditioned medium of cultured rat medullary thyroid carcinoma CA-77 cells with IC₅₀ values in the low nanomolar range. Despite their potent activity in the in vitro enzyme assay, the homocysteine analogs were only moderately effective in regulating the biosynthesis of substance P in cultured DRG cells. Since the poor inhibitory activity would be due to poor cell penetration, a number of prodrug esters of hydrocinnamoyl-phenylalanylhomocysteine were prepared to increase the intracellular accessibility of these compounds. Hydrocinnamoyl-phenylalanyl-L-(S-benzyloxyhomocysteine) benzyl ester (Compound 8) was identified as the most potent compound, inhibiting substance P biosynthesis in DRG cells with an estimated IC₅₀ about 2 µM or 1.2 µg/ml (Fig. 1). Although this potency is sufficient to modulate the production of amidated peptides, it is surprising to note that the IC₅₀ value obtained in the DRG cell assay is somewhat higher than expected, considering the parent Compound 1 inhibited PAM with an IC₅₀ of 10 nM in vitro. Several reasons may explain this observed discrepancy in the potencies obtained from the in vitro enzyme and cellular assays. It is possible that Compound 8 is still not optimal for penetration through the DRG cell membrane. Alternatively, the intracellular esterases in the DRG cells might not be effective in converting the prodrug ester to the active PAM inhibitor, or the compound may not be stable throughout the incubation period since dimerization of the compound did occur upon storage (results not shown). Third, since PAM is localized in secretory granules inside the cells, inhibitors would need to cross two membranes to access PAM. If the prodrug esters are converted to the active Compound 1 by intracellular esterases immediately after crossing the plasma membrane, the resulting compound would not be as effective in penetrating through the membrane of the secretory granules to inhibit PAM.

Inhibition of an enzyme frequently results in an excessive accumulation of the precursor and/or up-regulation of the enzyme (43). Under these conditions, the precursor itself may exert biological effects similar to that of the mature product if a sufficient concentration of the precursor is reached to overcome its weak potency. Likewise, up-regulation of enzyme may normalize the biosynthesis of the mature product. Therefore, neither an excessive accumulation of the precursor nor up-regulation of the enzyme would provide the desired pharmacological effects expected for drug intervention. In the present study, a limited quantity of PAM in the scarce DRG cells did not permit an easy assessment of whether PAM was up-regulated in these cells after inhibition. Nevertheless, a significant decrease in the level of substance P in DRG cells after treatment with Compound 8 suggests indirectly that PAM up-regulation was not likely to have occurred. Furthermore, the fact that the total amount of substance P and its precursor, substance P-Gly, remained the same in the presence or absence of ascorbate or PAM inhibitor (Table III) supports the notion that substance P-Gly was not excessively accumulated during PAM inhibition. Thus, inhibition of the α-amidation process appears to be an effective strategy for suppression of substance P biosynthesis. The prodrug esters of PAM inhibitors described here may also be useful to assess the pathological role of other amidated peptides such as neuropeptide Y, a powerful stimulant of food intake (44), whose precursor, glycine-extended neuropeptide Y, has also been shown to be recognized by PAM with high affinity (12).

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