Microinjections of subpicomolar amounts of NPY(13–36) into the nucleus tractus solitarius of the rat counteract the vasodepressor responses of NPY(1–36) and of a NPY Y₁ receptor agonist

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Microinjections of neuropeptide Y (NPY) (1–36) and of the NPY Y₂ agonist [Leu³¹,Pro³⁴]NPY into the caudal dorsomedial part of the nucleus tractus solitarius (Sol) in the anaesthetized rat led to the development of dose-related vasodepressor and bradycardic responses. The threshold dose of the NPY Y₂ agonist NPY(13–36) (50 fmol) significantly counteracted the vasodepressor actions of a close to ED₅₀ dose of NPY(1–36) (2.5 pmol) and of the NPY Y₁ agonist (5 pmol). These results indicate that NPY Y₁ receptor activation in the Sol leads to the development of a vasodepressor response, which can be counteracted by NPY Y₂ receptor activation in the Sol. The results support the existence of a Y₂/Y₁ receptor–receptor interaction in the Sol, via which NPY Y₂ receptors may reduce transduction over NPY Y₁ receptors.

Intraventricular injections of neuropeptide Y (NPY) (1–36) have previously been shown to elicit marked and highly significant vasodepressor and bradycardiac actions⁹,¹⁰,¹⁵, while intraventricular injections of the C-terminal NPY fragment and the Y₂ receptor agonist, NPY(13–36), lead to the development of dose-related vasopressor actions¹²,⁸,²³. Threshold doses of NPY(13–36) were capable of counteracting the vasodepressor actions of NPY(1–36) upon intraventricular injections². It was therefore postulated that Y₂ receptors in cardiovascular centers counteract the activity of Y₁ receptors, possibly through receptor–receptor interactions¹⁴. We have recently postulated that this interaction may take place in the nucleus tractus solitarius (Sol), since microinjections of NPY(1–36) in this area lead to the development of vasodepressor responses⁵,¹³,²⁴, while NPY(13–36) in subpicomolar amounts elicits vasopressor actions within the Sol²². In higher doses however, microinjections of NPY(13–36) into the Sol also induce vasodepressor responses (picomolar amounts)⁴,²². The Sol is also known to contain Y₁ and Y₂ receptors as shown by quantitative receptor autoradiography¹⁰,¹¹,¹⁴,²¹.

In the present paper we have tested this hypothesis by comparing the actions of microinjections of NPY(1–36) and of a Y₁ receptor agonist in the Sol and especially by testing if threshold amounts of NPY(13–36) co-injected into the Sol could counteract the vasodepressor actions of NPY(1–36) and of the Y₁ receptor agonist [Leu³¹,Pro³⁴]NPY⁷.

Eighty-nine adult male specific pathogen-free Sprague–Dawley rats (220–300 g body wt., ALAB, Stockholm, Sweden) were used. The animals were kept under regular lighting conditions (lights on at 06.00 h and off at 20.00 h) and had free access to food pellets and tap water.

Rats were anaesthetized with a mixture of α-chloralose (35 mg/kg i.p.) and urethane (1 g/kg i.p.). The trachea was cannulated so that the airway was unobstructed, when the head was flexed. A heparinized
(Heparin, 50 IU/ml in 0.9% saline) catheter was inserted in the femoral artery and connected to a Statham PC23 DC transducer (Statham Co., Puerto Rico), adapted to a Grass polygraph (model 7, Grass Instruments, MA, USA) to monitor arterial blood pressure and heart rate. The transducer and polygraph recorder were calibrated with a mercury manometer prior to the blood pressure recordings. Another catheter was inserted in the oesophagus and connected to the Grass polygraph via a pressure transducer to record respiratory rate. The animals were then placed in a stereotaxic frame (Kopf, USA) and the head was adjusted to a 45° angle from the horizontal plane. The electrocautery of the neck muscles was conducted to expose the posterior atlanto-occipital membrane and then fine dissection was employed to reveal the caudal medulla in the region of the obex and calamus scriptorius. During the experiment, the animals breathed freely and body temperature was maintained at 37.5 ± 0.5°C by means of a thermostatic blanket.

Unilateral microinjections were made stereotaxically into the dorsomedial part of the Sol with a glass micropipette (tip diameter 40–50 µm) connected to a Hamilton micro-syringe22. The coordinates for microinjections were 0.5 mm rostral and 0.5 mm lateral to the calamus scriptorius, and 0.5 mm below the dorsal surface of the brainstem. NPY(1–36) (Peninsula Lab., Belmont, CA, USA), NPY(13–36) (Peninsula Lab., Belmont, CA, USA) and [Leu31,Pro34]NPY (Peninsula Lab., Belmont, CA, USA) were freshly prepared in artificial cerebrospinal fluid (aCSF) (0.12 M NaCl, 0.02 M NaHCO3, 2 mM KCl, 0.5 mM KH2PO4, 1.2 mM CaCl2, 1.8 mM MgCl2, 0.5 mM Na2SO4, 5.8 mM d-glucose) (pH 7.2–7.4). aCSF was used for control injections. Drug and control solutions were injected in 50 nl during a period of 10 seconds. Only rats with a stable baseline (S.E.M. were <5% of mean basal values) were used and each animal received only one microinjection. Seven different doses of NPY(1–36) and six different doses of [Leu31,Pro34]NPY were injected into the dorsomedial Sol (Fig. 1) in order to evaluate a possible dose-dependent effect and the effects were compared with the aCSF group. In the other two experiments, a close to ED50 dose of NPY(1-36) or of [Leu31,Pro34]NPY for the vasodepressor response was administered together with a threshold dose (50 fmol) of NPY(13–36), determined in a previous paper22, to study possible interactions between NPY Y1 and Y2 receptors in cardiovascular regulation. Registrations were performed as described earlier15. Basal values were registered every 5 min during a period of 15 min before the microinjection. Measurements of mean arterial blood pressure (MAP), heart rate (HR), respiratory rate (RR) were made during the following 1 h time interval, and the area created by the curve was calculated for each parameter and for each animal using an IBM-XT computer and a program developed by Guna Consult, Stockholm, Sweden. The area values (overall effects) were expressed as absolute values in arbitrary units, mainly reflecting the duration of the effect under 60 min, and the peak effects (maximal responses) were shown as percent changes from the respective mean basal values. The ED50 values were calculated using iterative, non-linear curve fitting procedures6. The possible dose–response relationship was evaluated with the Jonckheere-Terpstra test and the one-way ANOVA with the Fisher’s LSD test was used to compare peak and overall effects between experimental groups and the control group. For comparisons between groups in the interaction experiments the unpaired Student’s t-test was used.

After the completion of each microinjection experiment, the injection sites and the diffusion area were evaluated by injecting Evans blue dye (50 nl) or NPY(1–36). Briefly, transcardiac perfusion was performed 10 min after the microinjection of NPY(1–36) (5 pmol in 50 nl) under the same conditions as above by using a fixative consisting of 4% paraformaldehyde dissolved in 0.1 M sodium phosphate buffer (pH 7.3). The brain was then removed and postfixed by immersion in the fixative solution for 90 min. Vibratome sections (30 µm thick) were cut and subsequently immunostained by using the avidin-biotin complex technique with 3,3′-diaminobenzidine as chromogen, resulting in brownish staining of the NPY(1–36) injected in the ipsilateral Sol16,17.

By comparing the two sides of the Sol, it was possible to differentiate exogenous NPY from endogenous NPY. As seen in Fig. 1, the microinjection site of NPY peptides was located in the caudal dorsomedial part of the Sol, an area where the baroreceptor afferents terminate18,19. The diffusion area of Evans blue (50 nl) and of the injected NPY(1–36) (50 nl, 5 pmol, 10 min) as evaluated by NPY immunoreactivity (IR) was clearly demonstrated and was shown to involve the entire medial part of the ipsilateral Sol. Only rats with this type of diffusion area in the medial Sol were included in the cardiovascular analysis. Interestingly some nerve cell bodies and their dendrites in the Sol appeared to have internalized and accumulated high concentrations of exogenous NPY-like immunoreactivity with a cytoplasmic and nuclear location (Fig. 1).

As seen in Table I, microinjections of NPY(1–36) into the medial Sol in the dose range of 1–100 pmol led to the development of a dose-related reduction of MAP, of HR and of RR as evaluated from the peak
changes and from the area values under the curves (ED$_{50}$ for vasodepressor action: 2.8 pmol; ED$_{50}$ for bradycardia: 1.5 pmol; ED$_{50}$ for bradypnea: 2.5 pmol based on peak changes). The vasodepressor action was fully developed within 5 min and a partial recovery had taken place 15 min following the injection, after which

Fig. 1. Representative microinjection site (→), (A) in the dorsomedial part of the nucleus tractus solitarius (Sol) shown by darkfield technique. B and C represent coronal vibratome sections (30 μm) injected with 50 nl of NPY(1–36) (dose 5 pmol; time 10 min after injection). NPY immunocytochemistry was performed according to the avidin-biotin complex technique (B) (see text). Interestingly, in addition to extracellular diffusion in the dorsomedial Sol exogenous NPY(1–36) accumulates in distinct nerve cell bodies (→) and their dendrites (→) in the Sol (C). 10, dorsal motor nucleus vagus; AP, area postrema; Gr, gracile nucleus; Sol, nucleus solitary tract; sol, solitary tract. Bars = 200 μm (A and B), 100 μm (C).
TABLE I

Cardiovascular and respiratory effects of microinjections of different doses of NPY(1–36) and [Leu^{34},Pro^{34}]NPY into the Sol in the anaesthetized male rat

Means ± S.E.M. are shown, n = 4–9 rats in each group. The area values formed under the curves are expressed in arbitrary units. The maximal peak effects are expressed as % change from the respective mean basal value. The one-way ANOVA with the Fisher's LSD test was used to compare the experimental group with the control group (aCSF). * P < 0.05, ** P < 0.01. The Jonkheere-Terpstra test (JT) for ordered alternatives was used to evaluate the dose–response effect of the peptides.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MAP Basal value (mmHg)</th>
<th>Decreases</th>
<th>HR Basal value (beats/min)</th>
<th>Decreases</th>
<th>RR Basal value (breaths/min)</th>
<th>Decreases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak</td>
<td>Area</td>
<td>Peak</td>
<td>Area</td>
<td>Peak</td>
<td>Area</td>
</tr>
<tr>
<td></td>
<td>(%)</td>
<td>(arbitrary units)</td>
<td>(%)</td>
<td>(arbitrary units)</td>
<td>(%)</td>
<td>(arbitrary units)</td>
</tr>
<tr>
<td>aCSF (50 nl)</td>
<td>94 ± 1</td>
<td>-1.6 ± 0.8</td>
<td>-27 ± 14</td>
<td>366 ± 9</td>
<td>0.5 ± 0.3</td>
<td>-28 ± 23</td>
</tr>
<tr>
<td>NPY(1–36) 0.05 pmol</td>
<td>83 ± 2</td>
<td>-4.1 ± 1.3</td>
<td>-16 ± 7</td>
<td>378 ± 19</td>
<td>-3.8 ± 1.8</td>
<td>-230 ± 129</td>
</tr>
<tr>
<td>NPY(1–36) 0.1 pmol</td>
<td>92 ± 2</td>
<td>-2.8 ± 0.5</td>
<td>-40 ± 14</td>
<td>373 ± 10</td>
<td>-3 ± 0.9</td>
<td>-186 ± 70</td>
</tr>
<tr>
<td>NPY(1–36) 1 pmol</td>
<td>85 ± 2</td>
<td>-8.8 ± 1.3</td>
<td>-107 ± 18</td>
<td>414 ± 21</td>
<td>-5.9 ± 2.1</td>
<td>-568 ± 223</td>
</tr>
<tr>
<td>NPY(1–36) 2.5 pmol</td>
<td>90 ± 4</td>
<td>-14 ± 1.5 **</td>
<td>-186 ± 34 **</td>
<td>394 ± 16</td>
<td>-3.6 ± 0.6</td>
<td>-183 ± 48</td>
</tr>
<tr>
<td>NPY(1–36) 12.5 pmol</td>
<td>90 ± 1</td>
<td>-24 ± 0.7 **</td>
<td>-300 ± 68 **</td>
<td>406 ± 16</td>
<td>-14 ± 3.8 **</td>
<td>-1319 ± 341**</td>
</tr>
<tr>
<td>NPY(1–36) 30 pmol</td>
<td>90 ± 2</td>
<td>-39 ± 5.2 **</td>
<td>-358 ± 23 **</td>
<td>418 ± 18</td>
<td>-15 ± 1.8 **</td>
<td>-1418 ± 292**</td>
</tr>
<tr>
<td>NPY(1–36) 100 pmol</td>
<td>90 ± 6</td>
<td>-34 ± 1.1 **</td>
<td>-442 ± 94 **</td>
<td>406 ± 20</td>
<td>-13 ± 2.7 **</td>
<td>-666 ± 126**</td>
</tr>
<tr>
<td>[Leu^{34},Pro^{34}]NPY 2.5 pmol</td>
<td>90 ± 4</td>
<td>-8.3 ± 3.1 *</td>
<td>-70 ± 31</td>
<td>449 ± 10</td>
<td>-3.6 ± 1.3</td>
<td>-166 ± 74</td>
</tr>
<tr>
<td>[Leu^{34},Pro^{34}]NPY 5 pmol</td>
<td>88 ± 2</td>
<td>-11 ± 0.6 **</td>
<td>-179 ± 33 *</td>
<td>404 ± 15</td>
<td>-5.8 ± 1.4</td>
<td>-424 ± 164</td>
</tr>
<tr>
<td>[Leu^{34},Pro^{34}]NPY 12.5 pmol</td>
<td>88 ± 1</td>
<td>-19 ± 0.3 **</td>
<td>-209 ± 5.8 *</td>
<td>379 ± 13</td>
<td>-6.1 ± 0.5</td>
<td>-347 ± 104</td>
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<tr>
<td>[Leu^{34},Pro^{34}]NPY 30 pmol</td>
<td>91 ± 5</td>
<td>-22 ± 1.6 **</td>
<td>-267 ± 50 **</td>
<td>384 ± 13</td>
<td>-8.3 ± 1.1 **</td>
<td>-520 ± 149</td>
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<tr>
<td>[Leu^{34},Pro^{34}]NPY 100 pmol</td>
<td>91 ± 5</td>
<td>-28 ± 2.6 **</td>
<td>-484 ± 85 **</td>
<td>374 ± 18</td>
<td>-8.8 ± 1.5 **</td>
<td>-726 ± 202**</td>
</tr>
<tr>
<td>[Leu^{34},Pro^{34}]NPY 300 pmol</td>
<td>91 ± 3</td>
<td>-26 ± 5.2 **</td>
<td>-399 ± 134 **</td>
<td>412 ± 20</td>
<td>-12 ± 5.9 **</td>
<td>-841 ± 578**</td>
</tr>
</tbody>
</table>

(JT: P < 0.01) (JT: P < 0.01) (JT: P < 0.01)
vasodepressor action: 6.7 pmol; EDs0 for bradycardia: the figure refer to the significant counteraction of the area reduction pmol) + NPY(13-36) (50 fmol) group (* * P < 0.01). The two stars in the [Leu31,Pro34]NpY (5 pmol) group with the [Leu31,Pro34]NpY (5 0.7 ** (peak reduction), - 42+ 13 ** (area reduction). Means_+ S.E.M., n = 6-8. The unpaired Student's t-test was used to compare the NPY(1-36) (2.5 pmol) group with the NPY(13-36) (50 fmol): 86+2, - 7.4_+ 1.4** (peak reduction), - 61 + 18* (area reduction). Means ± S.E.M., n = 6–8. The two stars in the figure refer to the significant counteraction of the area reduction by NPY(1-36). no further recovery was seen during the 1 h recording period (Fig. 2). As shown in Table I and Fig. 3, similar results were obtained with the Y1 receptor agonist [Leu31,Pro34]NPY, with regard to cardiovascular actions. Thus, in the dose range 2.5–300 pmol, the Y1 receptor agonist induced dose-related vasopressor and bradycardic actions after microinjection into the medial Sol as seen from the peak values (ED50 for vasodepressor action: 6.7 pmol; ED50 for bradycardia: 11.6 pmol) and the area values. However, in contrast to NPY(1-36), the Y1 receptor agonist in this dose range did not induce any significant changes in RR. The vasopressor action of the Y1 receptor agonist was fully developed within 5 min and gradually recovered till the 20 min time interval, after which no further recovery was made (see Fig. 3). As seen in Table I and from the ED50 values, the Y1 receptor agonist had a somewhat lower potency with a tendency also towards a lower intrinsic activity compared to NPY(1-36) with regard to vasopressor and bradycardic actions as seen from the peak changes.

In a previous paper from this laboratory, the threshold dose of NPY(13-36) for the vasopressor actions after microinjections into the Sol was found to be in the order of 50 fmol22. As seen in Figs. 2 and 3, NPY(13–36) in this threshold dose significantly counteracted the vasopressor actions of a close to ED50 dose of both NPY(1-36) and of the NPY Y1 receptor agonist as seen from both the peak values and the area values. However, in the 2.5 and 5 pmol doses used of the NPY peptides for the vasodepressor effect the bradycardic actions were too weak as seen from the peak changes (Table I) to safely evaluate a possible counteractive action of the threshold dose of NPY(13–36).

The present study confirms previous results showing that microinjections of NPY(1-36) into the Sol elicit monophasic vasodepressor actions as well as monophasic bradycardic effects. The present findings indicate that the major receptor involved in mediating the vasopressor and bradycardic activity is a Y1 receptor, since the actions of NPY(1-36) were mimicked by the actions of the Y1 receptor agonist [Leu31,Pro34]NPY (see ref. 8). Thus, it seems likely that at least in part the vasopressor actions earlier reported upon intraventricular injection of NPY(1-36) are mediated via the activation of Y1 receptors in the Sol9. In line with this view we have recently demonstrated that NPY (13–36), a Y2 receptor agonist, microinjected into the Sol induces vasopressor actions in the dose range 50–500 fmol/rat22, which may inter alia involve inhibition of noradrenaline and adrenaline release20,26. It is only in the higher doses (10–25 pmol) that NPY(13–36) microinjected into the Sol can elicit vasodepressor actions4,12,22. We postulate that the vasodepressor activity of high picomolar amounts of NPY(13–36) involve activation of Y1 receptors possibly through an ability of the NPY(13–36) to act as a partial agonist at Y1 receptors in higher concentrations26. The respiratory effects of NPY(1–36) may instead be induced via the activation of an atypical NPY receptor since they were not seen after the microinjection of the Y1 receptor...
agonist and of the Y_2 receptor agonist. The analysis of diffusion suggests that these effects may involve the medially located respiratory subnucleus of the Sol (intermediate subnucleus). It may be mentioned that in fact one group has postulated that the cardiovascular depression produced by NPY in the Sol is also mediated by an atypical NPY receptor. However, the existence of multiple NPY receptors in the CNS, although likely still remains to be determined.

The demonstration that microinjected NPY(1-36) can be internalized into distinct nerve cell bodies and dendrites of the Sol is of substantial interest, since it was found only in a limited number of nerve cell bodies and their dendrites. In view also of the nuclear localization of this NPY IR the results open up the possibility that extracellular NPY may affect the biochemical machinery of specific nerve cells via internalization and retrograde transport and nuclear translocation to control gene transcription.

The major finding in this study was the ability of a threshold dose of NPY(13-36) cojected with the NPY(1-36) or the Y_1 receptor agonist to significantly counteract the vasodepressor actions of NPY(1-36) and of the Y_1 receptor agonist. These results open up the possibility that Y_2 receptors within the cardiovascular region of the Sol can reduce the transduction of the NPY Y_1 receptors in this region. In line with this hypothesis a previous study has demonstrated that NPY(13-36) can increase the binding of iodinated NPY(1-36) in certain brain areas, which may reflect a reduced coupling to the G-protein, since pertussis toxin treatment has previously been demonstrated to increase the binding of [125I]NPY(1-36) within the Sol.

In previous work NPY(13-36) in threshold doses injected into the Sol has also been demonstrated to counteract the vasodepressor and bradycardic actions of L-glutamate, suggesting that the Y_2 receptors in the Sol also are able to reduce glutamate receptor transduction.

The present findings indicate that the vasopressor actions of NPY(13-36) elicited in the Sol may involve not only effects on glutamate receptor transduction but also on an inhibition of transduction over the NPY Y_1 receptors. Thus, C-terminal fragments formed from NPY(1-36) may effectively counteract the vasodepressor responses in the Sol, since the selective activation of Y_2 receptors may counteract the Y_1 receptor transduction as shown in the present paper as well as the glutamate receptor transduction. Thus, at least in the anesthetized rat, receptor–receptor interactions may allow the elicitation of effective negative feedbacks in the neuronal membranes. In further work using higher doses of NPY(1-36) and the Y_1 receptor agonist leading to elicitation also of clear cut bradycardic actions it may also be possible to demonstrate if such effects, also involving NPY Y_1 receptors, can become modulated by the NPY fragment in the same way as the vasodepressor responses.

In conclusion, NPY Y_1 receptors in the medial Sol area appear to mediate vasodepressor and bradycardic actions. Furthermore, the vasodepressor actions mediated by the Y_1 receptors can become counteracted by Y_2 receptor activation probably due in part to the existence of an antagonistic Y_2/Y_1 receptor interaction in the cardiovascular part of the Sol, leading to a reduction of NPY Y_1 receptor transduction.

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12. Grundemar, L., Wahlestedt, C. and Reis D.J., Neuropeptide Y acts at an atypical receptor to evoke cardiovascular depression...


