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Penile erection induced by EP 80661 and other hexarelin peptide analogues: involvement of paraventricular nitric oxide

Maria Rosaria Melis [a], Salvatora Succu [a], Maria Sabrina Spano [a], Antonio Torsello [b], Vittorio Locatelli [b], Eugenio E. Muller [c], Romano Deghenghi [d], Antonio Argiolas [a,*]

[a] *Bernard B. Brodie Department of Neuroscience, University of Cagliari, Via Porcell 4, 09124 Cagliari, Italy*
[b] *Department of Experimental and Environmental Medicine and Biotechnologies, University of Milan-Bicocca, Milan, Italy*
[c] *Department of Pharmacology, University of Milan, Via Vanvitelli 32, 20129 Milan, Italy*
[d] *Europeptides, Bat. Aristote, 9 Avenue du Marais, 95018 Argenteuil Cedex, France*

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Abstract

The effect of GAB-D-Trp(2-Me)-D-Trp(2-Me)-LysNH$_2$ (EP 80661), GAB-D-Trp(2-Me)-D-Trp(2-Me)-D-Trp(2-Me)-LysNH$_2$ (EP 60761), GAB-D-Trp(2-Me)-LysNH$_2$ (EP 91071) and GAB-D-Trp(2-Me)-D-βNal-Phe-LysNH$_2$ (EP 50885), four hexarelin peptide analogues that induce penile erection when injected into the paraventricular nucleus of the hypothalamus of male rats, on the concentration of NO_2^- and NO_3^- in the paraventricular dialysate was studied in male rats. EP peptides (1 μg) induced penile erection and increased the concentration of NO_2^- and NO_3^- in the paraventricular dialysate. In contrast, hexarelin (1 μg) was ineffective on either penile erection or paraventricular NO_2^- and NO_3^-. EP peptide-induced penile erection was prevented by the nitric oxide synthase inhibitor N^G-nitro-l-arginine methylester given into the paraventricular nucleus (20 μg), which also reduced the concomitant increase of NO_2^- and NO_3^- concentration in the paraventricular dialysate. In contrast, the oxytocin receptor antagonist [d(CH$_2$)$_5$Tyr(Me)^2-Orn^8]vasotocin (1 μg) given into the paraventricular nucleus, was ineffective on penile erection and on the NO_2^- and NO_3^- increase induced by EP peptides, despite its ability to prevent the sexual response induced by the above peptides when given into the lateral ventricles. The present results show that EP peptides induce penile erection by activating nitric oxide synthase in the paraventricular nucleus of the hypothalamus, possibly in the cell bodies of oxytocinergic neurons that control penile erection.

Keywords: Penile erection; EP 80661; EP 60761; EP 91071; EP 50885; Hexarelin; Oxytocin; Nitric oxide (NO); Paraventricular Nucleus; (Rat)

1. Introduction

Recently we found that a few peptide analogues of hexarelin, a peptide characterised for its ability to release growth hormone (GH) in animals and man (Deghenghi et al., 1994; Deghenghi, 1996; Muller et al., 1999 and references therein), induce penile erection when injected into the paraventricular nucleus of the hypothalamus or systemically in male rats (Melis et al., 2000b). Structure-activity comparisons revealed that the proerectile effect of these peptides was not related to their effect on GH release or eating behaviour (Melis et al., 2000a). This led us to suggest that these peptides induce penile erection by acting on specific receptors, which differ from those that mediate GH release or eating behaviour (Melis et al., 2000a). Among the analogues tested, GAB-D-Trp(2-Me)-D-Trp(2-Me)-LysNH$_2$ (EP 80661) was active at doses as low as 20 ng in inducing the sexual response. We also found that the proerectile effect of these peptides was not antagonised by the injection into the paraventricular nucleus of oxytocin, dopamine or NMDA receptor antagonists (Melis et al., 2000a,b), but it was reduced by ω-conotoxin-GVIA, a potent *N*-type Ca^{2+} channel blocker (McCleskey et al., 1987) given into the paraventricular nucleus, by N^G-nitro-L-arginine methylester (L-NAME), a potent competitive inhibitor of the Ca^{2+}/calmodulin dependent enzyme nitric oxide (NO) synthase (Rees et al., 1990), given in the lateral ventricles (i.c.v.) or into the paraventricular nucleus, and by [d(CH$_2$)$_5$Tyr(Me)^{2}Orn^8]vasotocin, a potent oxytocin receptor antagonist (Bankowski et al., 1980), given

* Corresponding author. Tel.: +39-070-675-8415; fax: +39-070-657-237.
E-mail address: argiolas@unica.it (A. Argiolas).

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i.c.v. but not into the paraventricular nucleus (Melis et al., 2000a,b). Similar results are found when penile erection is induced by dopamine receptor agonists, oxytocin and NMDA (Argiolas and Melis, 1995; Melis et al., 1996, 1997a,b) and penile erection occurs in physiological contexts (see Melis et al., 1998, 1999a and references therein). This suggests that these peptides activate oxytocinergic neurons mediating penile erection by activating NO synthase in the paraventricular nucleus, as found for dopamine receptor agonists, oxytocin, NMDA and NO donors (Argiolas and Melis, 1995; Melis et al., 1996, 1997a,b). To provide further support to this hypothesis, we studied the effect of EP peptides that induce penile erection on NO production in the paraventricular nucleus in vivo. This was achieved by measuring the concentration of NO_2^- and NO_3^- which represent the reaction products of newly formed NO with O_2, in the dialysate collected from a vertical microdialysis probe implanted in the paraventricular nucleus. This provides an indirect but reliable indicator of NO production in vivo (see Melis et al., 1996 and references therein). For comparison, the effect of hexarelin, which does not induce penile erection (Melis et al., 2000b), was also studied.

2. Material and methods

2.1. Animals

Male Sprague Dawley rats (250–300 g)(Charles River, Como, Italy) were used in all the experiments. The animals were caged in groups of 4–6 at 24°C, humidity 60%, lights on from 07:00 to 19:00 h with water and standard laboratory food ad libitum. The experiments were performed between 09:00–13:00 h.

2.2. Drugs and peptides

Hexarelin and analogues were prepared by conventional solid phase synthesis and purified by high pressure liquid chromatography (HPLC) by one of us (RD)(see Table 1 for amino acid sequence). N^G-nitro-L-arginine methylester (L-NAME), sulfanilamide and *N*-(1-naphtyl)-ethylenediamine were purchased from Sigma (St. Louis, MO, USA), and [d$(CH_2)_5$Tyr(Me)^2-Orn^8]vasotocin from Peninsula Eur., St Helens/Merseyside, UK. All other reagents were of the highest available purity.

Table 1
Structure of hexarelin and peptide analogues used in this study

Hexarelin	His-D-Trp(2-Me)-Ala-Trp-D-Phe-LysNH_2
EP 60761	GAB-D-Trp(2-Me)-D-Trp(2-Me)-D-Trp(2-Me)-LysNH_2
EP 80661	GAB-D-Trp(2-Me)-D-Trp(2-Me)-LysNH_2
EP 91071	GAB-D-Trp(2-Me)-LysNH_2
EP 50885	GAB-D-Trp(2-Me)-D-βNal-Phe-LysNH_2

Abbreviations: GAB = γ-aminobutyryl; βNal = β-(2-naphthyl)alanine.

2.3. Microinjections and microdialysis in the paraventricular nucleus

Microinjections and microdialysis were performed in the paraventricular nucleus of the same male rat by using microdialysis probes (approximately 1 mm of free surface for dialysis), glued with an epoxy resin to a microinjection cannula made with fused capillary silica tubing, ending adjacent to the U-shaped dialysis membrane, which were prepared as previously described (Melis et al., 1996, 1997a,b). The probes were implanted stereotaxically (Stoelting, Wood Dale, IL, USA) into the paraventricular nucleus under chloral hydrate anaesthesia, 2 days before the experiments (coordinates: 0.2 mm anterior to bregma, 0.4 mm lateral to midline and 2 mm ventral to dura) (Pellegrino and Cushman, 1971). The animals were given 2 days to recover from surgery, and each rat was used only once. For microinjection into the paraventricular nucleus, the microinjection cannula was connected by polyethylene tubing to a 10 μl Hamilton syringe driven by a Stoelting microsyringe pump. The probe was perfused with Ringer's solution, containing 147 mM NaCl, 3 mM KCl and 1.2 mM $CaCl_2$, pH 6.5, at a constant flow rate of 2 μl/min by using a Stoelting 200 microsyringe pump. After a 2-h equilibration period, dialysate was collected every 20 min in fractions of 40 μl, in polyethylene tubes kept at 10–15°C for the determination of NO_2^- and NO_3^-, as described below. After the collection of three dialysate aliquots, one of the EP peptides was dissolved in Ringer's solution and injected into the paraventricular nucleus in a volume of 0.3 μl. Rats were observed for 80 min, during which four additional dialysate fractions of 40 μl each were collected every 20 min and penile erection episodes were counted. In those experiments in which L-NAME or [d$(CH_2)_5$Tyr(Me)^2-Orn^8]vasotocin was used, these compounds were dissolved in saline and microinjected into the paraventricular nucleus in a volume of 0.3 μl over a period of 2 min, 15 min before the EP peptide.

2.4. Determination of NO_2^- and NO_3^- in the paraventricular dialysate

The concentration of NO_2^- and NO_3^- was measured in the paraventricular dialysate by a modification of the Griess reaction, as already described in detail (Melis et al., 1996, 1997a,b). Briefly, NO_2^- in the dialysate was used for the diazotization of sulfanilamide and subsequent coupling to *N*-(1-naphtyl)-ethylene-diamine. The azo dye was then quantified by high-pressure liquid chromatography (HPLC) from its absorbance at 546 nm. The sensitivity of the assay was 0.1 μM and the response was found to be linear, with increasing concentrations of NO_2^- up to 25 μM. For the determination of NO_3^- in the dialysate, NO_3^- was previously reduced to NO_2^- with copper-cadmium, as already described (Melis et al., 1996). Total NO_2^- was then measured as described above and the NO_3^- content

was calculated by subtracting that of NO_2^- found in the dialysate without copper-cadmium reduction. The sensitivity of the method was 3 μM, and the response was linear with NO_3^- up to 30 μM.

2.5. Behavioural studies

Rats were placed individually in Plexiglas cages (30 × 30 × 30 cm). After a 30-min habituation period, the microdialysis probe was connected via polyethylene tubing to a 10 μl Hamilton microsyringe driven by a Stoelting microsyringe pump on one end and to the polyethylene collecting loop on the other hand. The cannula for paraventricular injections was also connected to a 10 μl Hamilton microsyringe driven by a microinfusion pump via polyethylene tubing. After a 2-h equilibration period of perfusion of the dialysis probe with Ringer's solution, each one of the EP peptides was given in the paraventricular nucleus over a 2-min period. After treatments, rats were observed for the entire duration of the experiment to replace filled loops with empty ones every 20 min and to count penile erection episodes. Penile erections were scored when the penis emerged from the penile sheath, which was usually accompanied by penile grooming and hip flexions. In those experiments in which L-NAME or [d$(CH_2)_5$,Tyr(Me)^2, Orn^8]vasotocin was given, these were injected in the paraventricular nucleus over a 2-min period, 15 min before the EP peptide.

2.6. Histology

At the end of the experiments, the animals were killed by decapitation, the brains were immediately removed and stored in 2% aqueous formaldehyde for 10–12 days. To localise the position of the probe tip, 50 μm transverse brain sections were prepared by means of a freezing microtome, stained with Neutral Red and inspected on a phase contrast microscope. The site of the probe tip was localised by following the probe tract through a series of brain sections. Only those animals found to have the probe tip positioned correctly in the paraventricular nucleus of the hypothalamus were considered for the statistical evaluation of the results.

2.7. Statistics

For the statistical evaluation of the results, the area under the curves (AUC) obtained by plotting penile erection, NO_2^- and NO_3^- values vs. time in each animal was first calculated with the classical trapezoidal rule. The AUCs were then statistically compared between groups with the Manney–Whitney *U*-test in order to show significant differences between the groups that received a different pharmacological treatment. A $P < 0.025$ was considered significant. (Statistica for Windows, 4.0, Statsoft, Tulsa, OH, USA).

3. Results

3.1. Effect of EP peptides on the concentration of NO_2^- and NO_3^- in the paraventricular dialysate and on penile erection

As found in previous studies (Melis et al., 2000a,b), EP 80661, but not hexarelin, injected into the paraventricular nucleus at the dose of 1 μg increased spontaneous penile erection episodes from 0.43 ± 0.12 to 3.26 ± 0.42 when compared to saline (Fig. 1). Accordingly, the AUC of EP

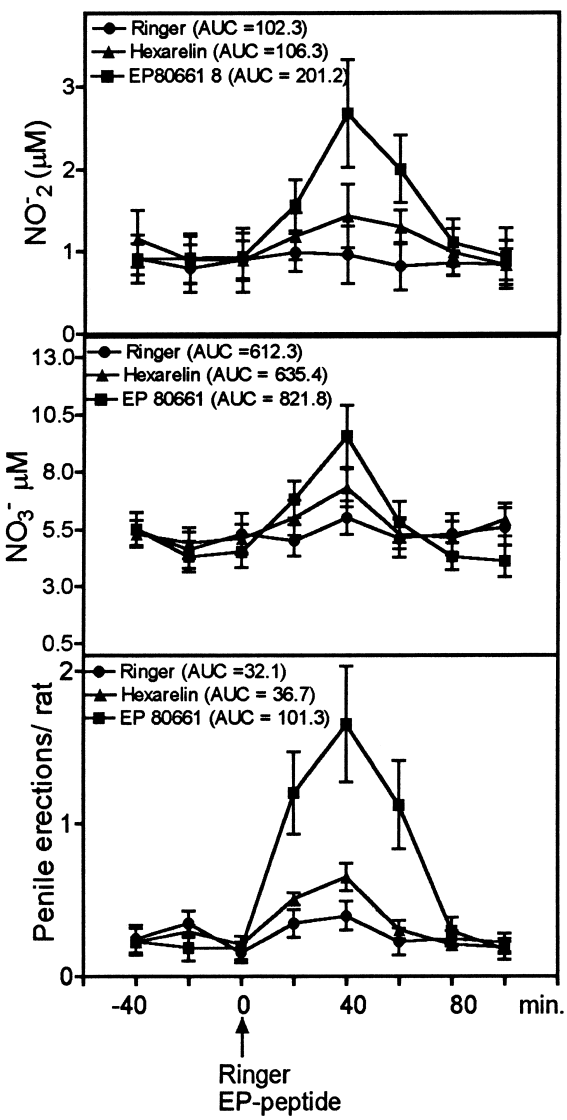

Fig. 1. Effect of hexarelin peptide analogues injected into the paraventricular nucleus on penile erection, and on the NO_2^- and NO_3^- concentration in the paraventricular dialysate of male rats. After 2 h of perfusion, 0.3 μl of Ringer's solution alone or containing 1 μg of EP 80661 or hexarelin was injected into the paraventricular nucleus. After treatment, the male rats were observed for 80 min to count penile erections and to replace filled loops with empty ones every 20 min for the determination of NO_2^- and NO_3^- concentration in the dialysate. Values are means ± SEM of seven rats. The areas under the curve (AUC), obtained by plotting penile erections, NO_2^- and NO_3^- concentrations vs. time for each group shown were calculated with the trapezoidal rule as described in Section 2.

80661-treated rats for penile erection was significantly higher than that of Ringer's solution-treated rats ($P < 0.01$). Penile erections started within 4–6 min after the injection of the peptides and the effect lasted for 60–80 min. Penile erection occurred concomitantly with an increase in the concentration of NO_2^- and NO_3^- in the paraventricular dialysate. The NO_2^- concentration increased from 0.81 ± 0.12 to 2.56 ± 0.12 μM and that of NO_3^- from 4.23 ± 0.42 to 8.43 ± 1.12 μM. (Fig. 1). Accordingly, the AUC of EP 80661-treated rats for NO_2^- and NO_3^- was significantly different from those of Ringer's solution- and hexarelin-treated rats ($P < 0.01$). Similar results were obtained with EP 60761, EP 91071 and EP 50885 (not shown).

3.2. Effect of L-NAME and [d(CH$_2$)$_5$Tyr(Me)^{2}Orn^8]-vasotocin on the paraventricular NO_2^- and NO_3^- increase and on penile erection induced by EP 80661, EP 60761, EP 91071 and EP 50885

L-NAME (20 μg) given into the paraventricular nucleus 10 min before EP 80661 (1 μg), reduced both penile erection and the increase of NO_2^- and NO_3^- in the paraventricular dialysate induced by the peptide. In contrast, [d(CH$_2$)$_5$Tyr(Me)^{2}Orn^8]vasotocin (1 μg), given into the paraventricular nucleus 10 min before EP 80661, was ineffective on the increase of penile erection and of NO_2^- and NO_3^- concentration induced by EP 80661. Accordingly, the AUC of L-NAME + EP 80661-treated rats for NO_2^- and penile erection was significantly lower from those of EP 80661-treated rats ($P < 0.01$), while those of [d(CH$_2$)$_5$Tyr(Me)^{2}Orn^8] vasotocin + EP 80661-treated rats were not ($P > 0.1$). Similar results were found with EP 60761, EP 91071 and EP 50885 (Table 2).

Table 2
Effect of L-NAME and [d(CH$_2$)$_5$Tyr(Me)^{2}Orn^8]vasotocin (Oxy Ant) given into the paraventricular nucleus on penile erection and on the NO_2^- increase that occurs concomitantly in the paraventricular dialysate induced by EP 80661 and other peptide analogues

Treatment	NO_2^- (AUC)	Penile erections (AUC)
Ringer + Ringer	91.71 ± 9.32	30.21 ± 4.41
Ringer + EP 80661	203.71 ± 14.32[a]	98.21 ± 8.91[a]
Oxy Ant + EP 80661	191.12 ± 17.43[a]	94.56 ± 7.76[a]
L-NAME + EP 80661	121.34 ± 6.98[b]	39.34 ± 4.87[b]
Ringer + EP 60761	189.45 ± 12.34[a]	90.54 ± 10.43[a]
Oxy Ant + EP 60761	212.06 ± 13.76[a]	102.43 ± 7.98[a]
L-NAME + EP 60761	101.47 ± 11.21[b]	31.41 ± 3.87[b]
Ringer + EP 91071	179.89 ± 8.99[a]	89.23 ± 8.55[a]
Oxy Ant + EP 91071	189.45 ± 10.54[a]	98.43 ± 9.41[a]
L-NAME + EP 91071	78.99 ± 4.65[b]	29.59 ± 3.87[b]
Ringer + EP 50885	179.82 ± 8.27[a]	101.12 ± 10.32[a]
Oxy Ant + EP 50885	191.34 ± 12.34[a]	98.65 ± 5.67[a]
L-NAME + EP 50885	89.26 ± 4.98[b]	30.27 ± 4.29[b]

L-NAME (20 μg) or [d(CH$_2$)$_5$Tyr(Me)^{2}Orn^8]vasotocin (1 μg) was dissolved in Ringer's solution and injected into the paraventricular nucleus 15 min before each EP peptide (1 μg). The other experimental conditions were identical to those described in the legend of Fig. 1. After treatment, rats were observed for 80 min to count penile erections and to replace filled loops with empty ones every 20 min for the determination of NO_2^- concentration in the dialysate. Each value is the means ± SEM of the areas under the curve (AUC) obtained from groups of seven rats by plotting penile erections and NO_2^- concentration vs. time and calculated as those shown in Fig. 1.

[a] $P < 0.01$ with respect to Ringer's solution-treated rats.

[b] $P < 0.01$ with respect to the corresponding group treated with EP peptide alone (Manney–Whitney *U*-test).

4. Discussion

The present study confirms and extends previous findings showing that EP80661, EP60761, EP 91071 and EP 50885, but not hexarelin, induce penile erection when injected into the paraventricular nucleus (Melis et al., 2000a,b). In particular, the results of this study show that EP peptide-induced penile erection occurs concomitantly with a NO_2^- and NO_3^- increase in the paraventricular dialysate of these male rats. This is in line with our previous hypothesis that these EP peptides induce penile erection by activating NO synthase in this hypothalamic nucleus (Melis et al., 2000a,b). A similar increase in paraventricular NO production was found in the paraventricular nucleus of male rats after the injection of compounds that induce penile erection, e.g. dopamine receptor agonists, oxytocin or NMDA (Melis et al., 1996, 1997a,b) and when penile erection occurs in physiological contexts (see Melis et al., 1998, 1999a,b and references therein). That the increase in the concentration of NO_2^- and NO_3^- in the paraventricular dialysate really reflects an activation of paraventricular NO synthase, is further supported by the ability of L-NAME, a potent inhibitor of NO synthase (Rees et al., 1990), given into the paraventricular nucleus to prevent both the NO_2^- and NO_3^- increase and penile erection induced by EP peptides. The findings also confirm that EP peptide-induced penile erection was prevented by L-NAME, given either i.c.v. or into the paraventricular nucleus (Melis et al., 2000a,b).

This study also shows that EP peptide-induced penile erection and the concomitant increase in the concentration of NO_2^- and NO_3^- in the paraventricular dialysate were not prevented by [d(CH$_2$)$_5$Tyr(Me)^{2}Orn^8]vasotocin, a potent oxytocin receptor antagonist (Bankowski et al., 1980), given into the paraventricular nucleus. This confirms our previous findings that this oxytocin antagonist was unable to prevent EP peptide-induced penile erection when injected into the paraventricular nucleus, despite its ability to prevent this sexual response when given i.c.v. (Melis et al., 2000a,b). Together, these results are in line with the hypothesis that EP peptides induce penile erection by activating NO synthase to increase NO production in the paraventricular nucleus. NO, in turn, activates oxytocinergic neurons projecting to extra-hypothalamic areas, where they release oxytocin, which induces penile erection. A similar explanation was already given for the ability of

dopamine receptor agonists, oxytocin and NMDA to induce penile erection when injected into the paraventricular nucleus (Melis et al., 1996, 1997a,b) and the ability of [d(CH_2)$_5$Tyr(Me)^{2}Orn^8]vasotocin to prevent dopamine receptor agonist-, oxytocin-, NMDA- and NO donor-induced penile erection when injected i.c.v. but not into the paraventricular nucleus (Argiolas and Melis, 1995; Melis et al., 1996, 1997a,b).

The molecular mechanism by means of which the EP peptides used in this study activate oxytocinergic neurons mediating penile erection in the paraventricular nucleus is unknown at present. We have previously suggested that EP peptides induce penile erection by acting on specific receptors, different from those mediating GH release or eating behaviour (both activated by hexarelin), and possibly located in the cell bodies of oxytocinergic neurons mediating penile erection and coupled to Ca^{2+} influx (Melis et al., 2000a,b). This would explain the peptide activation of paraventricular NO synthase found in this study. Support to this hypothesis comes from findings showing that ω-conotoxin-GVIA, a potent blocker of *N*-type Ca^{2+} channels (McCleskey et al., 1987) injected into the paraventricular nucleus prevents EP-peptide induced penile erection (Melis et al., 2000a,b) and the concomitant increase in paraventricular NO production (Melis et al., unpublished data), as shown for dopamine receptor agonist- and oxytocin-induced penile erection (see Succu et al., 1998 and references therein). The existence of specific receptors mediating penile erection and coupled to Ca^{2+} influx for these hexarelin peptide analogues would not be so surprising, since specific receptors for GH-releasing peptides have been identified in the pituitary gland, the hypothalamus and other brain regions (see Pong et al., 1996; Muccioli et al., 1999; Smith et al., 1999 and references therein). Perhaps more relevant to this work, activation of these receptors triggers GH release from the pituitary by increasing Ca^{2+} influx (Sartor et al., 1985; Akman et al., 1993; Deghenghi, 1996; Muller et al., 1999 and references therein) and experimental evidence supports the existence of different sub-populations of GH-releasing peptide receptors. First, the effects of hexarelin and its analogues on GH release are divorced from the eating effects (Torsello et al., 1998). Second, receptors for hexarelin and other GH-releasing peptides, whose activation induces effects independent from the GH-releasing properties of these peptides, have been detected in other tissues, e.g. in the heart (Bodart et al., 1999; Locatelli et al., 1999; Bisi et al., 1999). Third, cloning studies have revealed the existence of several forms of receptors for GH-releasing peptides (see Smith et al., 1999 and references therein). Finally, ghrelin, an endogenous acylated peptide agonist of these receptors, mainly of those that induce GH release, has been recently isolated from the stomach (Kojima et al., 1999). This endogenous GH-releasing peptide is also found to be expressed in different tissues, including the central nervous system and in the hypothalamus (Hosoda et al., 2000). In conclusion, although the molecular mechanism activated by EP peptides at the paraventricular level still has to be clarified, EP 80661 and analogues induce penile erection when injected into the paraventricular nucleus by increasing NO production. This, in turn, activates oxytocinergic neurons projecting to extra-hypothalamic brain areas and mediating penile erection.

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