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Implication of nigral tachykinin NK_3 receptors in the maintenance of hypertension in spontaneously hypertensive rats: a pharmacologic and autoradiographic study

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1 The role of nigral tachykinin NK_1, NK_2 and NK_3 receptors in central cardiovascular regulation was studied by measuring the effects of selective agonists and antagonists on mean arterial pressure (MAP) and heart rate (HR) after bilateral microinjection into the substantia nigra of spontaneously hypertensive rats (SHR). Quantitative *in vitro* autoradiography was also performed in the midbrain of SHR and Wistar-Kyoto (WKY) with the NK_3 receptor ligand [^{125}I]-HPP-Senktide.

2 Tachycardia was elicited by the NK_1 ([Sar^9,Met(O$_2$)^{11}]SP) and NK_2 ([βAla^8]NKA(4-10)) agonists at 25 and 100 pmol while the NK_3 agonist (senktide, 50 and 100 pmol) had no significant effect. The three agonists had no effect on behaviour, and increases in MAP were elicited by the NK_1 agonist only.

3 Whereas antagonists at NK_1 (RP 67580, 500 pmol) and NK_2 (SR 48968, 500 pmol) receptors had no significant effect on MAP and HR, the NK_3 antagonist (R-820, 500 pmol) reduced MAP for over 3 h in SHR. That anti-hypertensive effect did not occur after intracerebroventricular or intravenous injection of R-820. Also, R-820 had no cardiovascular effect in WKY.

4 The affinity (K_D: 0.7 nM) and densities of specific NK_3 receptor binding sites measured in the substantia nigra, ventral tegmental area, hippocampus and amygdala were not significantly different in SHR and WKY.

5 It is concluded that endogenous tachykinins exert a tonic activity on NK_3 receptors in the substantia nigra of SHR to maintain high blood pressure. Hence, nigral tachykinin NK_3 receptors may represent a promising therapeutic target in the treatment of arterial hypertension.
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Abbreviations: aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; HR, heart rate; i.c.v., intracerebroventricular; i.v., intravenous; MAP, mean arterial blood pressure; NKA, neurokinin A; SHR, spontaneously hypertensive rats; SN, substantia nigra; SP, substance P; WKY, Wistar-Kyoto.

Introduction

Compelling evidence suggests a role for tachykinin receptors in central autonomic cardiovascular regulation (Unger *et al*., 1988; Culman & Unger, 1995; Culman *et al*., 1997; Cellier *et al*., 1997; 1999) and in the modulation of the nigro-striatal dopaminergic system (Reid *et al*., 1990; Humpel *et al*., 1991; Humpel & Saria, 1993; Bannon & Whitty, 1995; Nalivaiko *et al*., 1997; Marco *et al*., 1998). Tachykinins are known to modulate nigro-striatal dopaminergic neurons by stimulating the release, turnover and metabolism of striatal dopamine *via* the activation of neurokinin-1 (NK_1) and neurokinin-3 (NK_3) receptors in the substantia nigra (SN) (Reid *et al*., 1990; Humpel *et al*., 1991; Humpel & Saria, 1993; Bannon & Whitty, 1995; Marco *et al*., 1998). Autoradiographic, immunohistochemical, *in situ* and/or solution hybridization and single cell reverse transcription-polymerase chain reaction techniques have shown the presence of substance P (SP) and neurokinin A (NKA) containing nerve terminals and tachykinin NK_1 and NK_3 receptors and/or their mRNA in the SN (Helke *et al*., 1990; Stoessl & Hill, 1990; Stoessl, 1994; Bannon & Whitty, 1995; Whitty *et al*., 1995; Shughrue *et al*., 1996; Futami *et al*., 1998; Chen *et al*., 1998; Ribeiro-Da-Silva *et al*., 2000). Also, SP and NKA are released *in vivo* (Lindefors *et al*., 1989) and *in vitro* (Jessel, 1978; Humpel & Saria, 1989) in the SN from striato-nigral projecting neurons.

Lesion of the nigro-striatal dopaminergic pathway attenuates the development of hypertension in young spontaneously hypertensive rats (SHR) that points to a putative role for this neuronal pathway in the development of hypertension (Van den Buuse *et al*., 1991; Linthorst *et al*., 1994).

Previous studies have shown that central activation of tachykinin NK_3 receptors by i.c.v. injection of selective agonists (senktide or [MePhe^7]NKB) leads to increases of mean arterial pressure, heart rate and behavioural activity in conscious rats (Itoi *et al*., 1992; Picard *et al*., 1994; Cellier *et al*., 1997). Intravenous pre-treatment with the dopamine D_2 receptor antagonist haloperidol blocked those cardiovascular and behavioural changes and unmasked a vasopressin-

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dependent bradycardia (Cellier & Couture, 1997). Although anatomical and physiological studies suggest an interaction between tachykinins and the nigro-striatal dopamine pathway in normotensive rats, there is no information regarding the implication of tachykinins in hypertension at the level of the SN.

With the purpose to follow up the recent pharmacological evidence suggesting that the SN is a potential site of modulation of cardiac autonomic activity by tachykinins in the normotensive rat (Lessard & Couture, 2001), the present study was undertaken to assess the cardiovascular effects of selective tachykinin agonists and antagonists bilaterally injected into the SN of SHR. The respective agonists and antagonists were: [Sar^9,$Met(O_2)^{11}$]SP (Regoli *et al*., 1988) and RP 67580 (Garret *et al*., 1991) for NK_1 receptors; [β-Ala^8]NKA(4-10) (Rovero *et al*., 1989) and SR 48968 (Advenier *et al*., 1992; Emonds-Alt *et al*., 1992) for NK_2 receptors; senktide (Wörmser *et al*., 1986) and R-820 (Regoli *et al*., 1994) or SR 142801 (Emonds-Alt *et al*., 1995) for NK_3 receptors. To avoid the spurious effects of anaesthesia and the stress induced by immobilization, these studies were carried out in awake, unrestrained rats. Moreover, *in vitro* autoradiography was performed to evaluate the density and dissociation constant (K_D) of NK_3 receptor binding sites in the SN and other midbrain areas of SHR and normotensive control Wistar-Kyoto rats (WKY).

Methods

Animal source and care

Male SHR (15 weeks, $n=88$) and age-matched WKY ($n=17$) were purchased 3–5 days prior to experiments from Charles River, St-Constant, Québec, Canada and housed four to five per cage under a 12 h light–dark cycle in a room with controlled temperature (20°C), humidity (53%) with food (Charles River Rodent) and tap water available *ad libitum*. The care of animals and research protocols conformed to the guiding principles for animal experimentation as enunciated by the Canadian Council on Animal Care and approved by the Animal Care Committee of our University.

Animal preparation

Rats were anaesthetized with an intraperitoneal (i.p.) injection of 65 mg kg^{-1} sodium pentobarbitone (Somnotol; M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada) and then positioned in a stereotaxic frame (David Kopf Instrumentation, Tujunga, CA, U.S.A.) with the incisor bar set at 3.3 mm below the interaural line. The skull was exposed, cleaned and a hole was drilled above each SN (coordinates: 5.3 mm posterior to the bregma, 2.0 mm lateral to the midline, 6.3 mm ventral from the skull surface for SHR and WKY; Paxinos & Watson, 1998). Two 23-gauge stainless steel guide cannulae targeted 2 mm dorsal to each SN were implanted and fixed with two screws and dental cement to the skull. An additional group of SHR was implanted with one 23-gauge stainless steel guide cannulae into the right lateral ventricle (coordinates: 1 mm posterior to the bregma, 1.4 mm lateral to the midline, 3.0 mm ventral from the skull surface, Paxinos & Watson, 1998). Stylets (31-gauge stainless steel) were inserted into the guide cannulae to avoid their obstruction and the loss of cerebrospinal fluid. Then, the skin was replaced and sutured. Animals were housed in individual plastic cages ($40\times23\times20$ cm) in the same controlled conditions and allowed to recover for 7 days. Then, the rats were re-anaesthetized with sodium pentobarbitone (65 mg kg^{-1}, i.p.) and an intravascular siliconized (Sigmacote, Sigma-Aldrich Canada) polyethylene tubing PE-60 catheter (Intramedics, Clay Adams, NJ, U.S.A.), filled with physiological saline containing 100 i.u. ml^{-1} heparin sodium salt (Sigma-Aldrich Canada), was inserted into the abdominal aorta *via* the right femoral artery for direct blood pressure recording. The catheter was tunnelled subcutaneously to emerge at the back of the neck. Another group of SHR was implanted with an intravenous (i.v.) siliconized polyethylene tubing PE-10 catheter, filled with physiological saline containing 100 i.u. ml^{-1} heparin sodium salt, into the right jugular vein for i.v. injection. The catheter was tunnelled subcutaneously to emerge with the femoral catheter at the back of the neck. Before surgery, the animals received Ethacilin (5 mg kg^{-1}, i.m., rogar/S.T.B. Inc., London, Ontario, Canada) and Ketoprophen (anafen, 10 mg kg^{-1}, i.m., Merial Canada Inc., Baie d'Urfé, Québec, Canada). Recovery from anaesthesia was monitored closely under a warming lamp to maintain the body temperature of animals. Thereafter, rats were housed individually in polyethylene cages with a top grid and returned to their resident room. Rats with apparent abnormal behaviour (loss of $>25\%$ of body weight, anorexia, weaknesses) were immediately humanely killed with an overdose of pentobarbitone. Experimental protocols were initiated 48 h after the final intervention, in conscious and unrestrained rats.

Measurement of cardiovascular parameters

During all experiments, continuous direct recordings of blood pressure and heart rate were made respectively with a Statham pressure Transducer (P23ID) and a cardiac tachometer (model 7P4) (triggered by the arterial blood pressure pulse) coupled to a Grass polygraph (model 79; Grass Instruments Co., Quincy, MA, U.S.A.). Cardiovascular responses were measured 1 h after the rats were transported to an isolated and quiet testing-room, where only the experimenter had access. Rats remained in their resident cage but the top grid was removed and they had no more access to the food and water for the duration of the experiment, which lasted for a period of 3–6 h. When resting blood pressure and heart rate were stable, microinjections were made simultaneously into each SN of undisturbed, freely moving rats using two hand-held Hamilton microsyringes (5 μl, Fisher Scientific Ltd, Montréal, Québec, Canada) connected to 60 cm length polyethylene tubing PE-10. Five to 10 min prior to injection, two 31-gauge stainless steel injectors, pre-connected to the PE-10 tubing, were inserted into the guide cannulae without handling the rats. All solutions for microinjections were freshly prepared and injected (volume of 0.1 μl in each SN, 1 μl for i.c.v. and 1 ml kg^{-1} for i.v.) over a period of 1 min.

Measurement of behavioural parameters

Behavioural activity was measured as previously reported (Picard *et al*., 1994). Briefly, during every consecutive period

of 15 s, a score of 1 or 0 was given systematically depending on whether the animal showed the specific type of behaviour or not, whatever its frequency, intensity or duration during that period. Summation of scores for the first 30 min period following SN injection gave the behavioural scores for face washing, grooming, sniffing and rearing. The maximal theoretical score was 120 (15 s intervals $\times 30$ min). Wet dog shakes and locomotion (number of complete exploratory circles within the cage) behaviours were measured according to the number of episodes or frequency during the first 30 min period.

Histology

At the end of the experiments, the rats received 0.1 μl of Evans Blue dye (Sigma-Aldrich Canada) in each SN and they were immediately sacrificed with an overdose of sodium pentobarbitone. The brains were removed and fixed with 10% (v v^{-1}) formol and 20% (w v^{-1}) sucrose. Coronal sections (40 μm, cut on a freezing microtome) were mounted on glass slides and stained with cresyl violet for histological examination of the microinjection sites. Twenty-three out of 69 SHR (33%) implanted in the SN were rejected, either for evidence of cerebral haemorrhage (17 rats) or because the microinjection site was outside the accepted area (6 rats) (Figure 1). The latter six rats displayed no cardiovascular effect to agonists ($n=2$) or R-820 ($n=4$). Thus, numbers indicated in results represent only rats which were included in the study.

Experimental protocols

Experiment 1: cardiovascular effects of SN microinjection of selective tachykinin agonists SHR ($n=8$) and WKY ($n=3$) received initially a microinjection of artificial cerebrospinal fluid (aCSF) into the SN followed 60 min later by the injection of 25 or 50 pmol of the selective NK_1 agonist [Sar^9,$Met(O_2)^{11}$]SP, the selective NK_2 agonist [β-Ala^8]NKA(4-10) and the selective NK_3 agonist senktide at random. Additional SHR received only the NK_2 and NK_3 agonists ($n=2$) or only the NK_3 agonist ($n=3$). The dose of 100 pmol of each agonist was given to four SHR at random and two additional rats received only NK_2 and NK_3 agonists. Only one dose of the same agonist was given to any animal. Agonists were injected at a minimum of 60 min intervals to enable blood pressure and heart rate to return to baseline values. Cardiovascular effects induced by agonists were not significantly different whatever their order of injection or if given alone or together to the same animal, suggesting the absence of cross desensitization between the three agonists. Each dose refers to the summation of doses given on each side of the SN. Doses of agonists were chosen on the basis of our previous study in Wistar rats (Lessard & Couture, 2001).

Experiment 2: cardiovascular effects of SN microinjection of selective tachykinin antagonists In order to assess the contribution of endogenous tachykinins and their receptors, SHR were microinjected into the SN with one of the three antagonists (anti-NK_1, RP 67580, 500 pmol, $n=5$; anti-NK_2, SR 48968, 500 pmol, $n=6$; anti-NK_3, R-820, 500 pmol, $n=12$ or SR 142801, 500 pmol, $n=4$). Each rat received only one antagonist, and doses were chosen on the basis of their ability to block in a selective and reversible manner the cardiovascular effects induced by the over-mentioned tachykinin agonists following their SN injection in Wistar rats (Lessard & Couture, 2001).

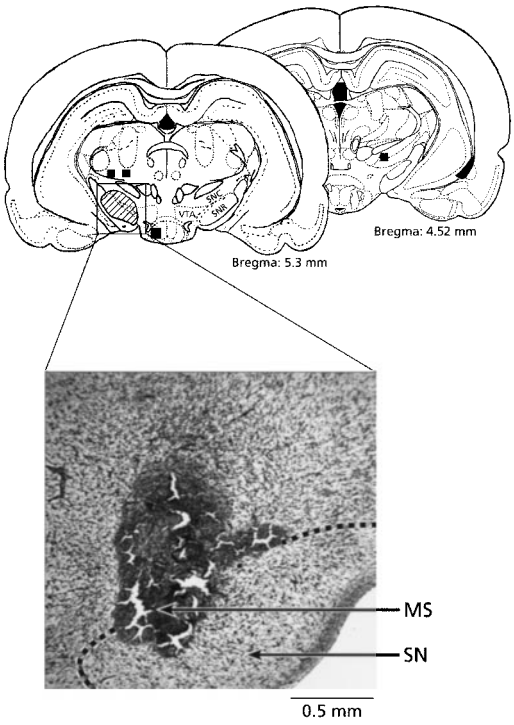

Figure 1 Identification of the microinjection sites in the substantia nigra (SN) following post-mortem histological examination of microinjected Evans Blue dye (0.1 μl bilaterally). The hatched zone represents accepted microinjection sites and the squares represent microinjection sites of R-820 ($n=4$) excluded from the results and kept as negative control for spread. A rat was considered successfully injected when both cannula tips were shown to be slightly above SN or within a distance of 0.5 mm of the SN (AP = 5.3 mm posterior to the bregma) (Paxinos & Watson, 1998). Abbreviations: MS, microinjection site; SN, substantia nigra; SNC, SN pars compacta; SNR, SN pars reticulata; VTA, ventral tegmental area. Scale: 0.5 mm.

Since intranigral microinjection of RP 67580 and SR 48968 had no cardiovascular effect in SHR (present study) and Wistar rats (Lessard & Couture, 2001), while R-820 reduced blood pressure in SHR, WKY received an intranigral microinjection of R-820 (500 pmol, $n=10$) only. To control for the site of the anti-hypertensive action of R-820, two groups of SHR received R-820 into the right lateral ventricle (500 pmol, $n=8$) and the right jugular vein (500 pmol, $n=6$).

Tissue preparation for autoradiography

SHR ($n=5$) and WKY ($n=4$) used for autoradiography had previously received i.c.v. injection (500 pmol) of the NK_1 (RP 67580) and the NK_2 (SR 48968) antagonists which caused no cardiovascular effect in a parallel study. At least 2 days after the last i.c.v. injection, rats were sacrificed by an intra-arterial injection of an overdose of sodium pentobarbitone. Brains were immediately removed and frozen in 2-methyl butane cooled at $-45-55^{\circ}$C with liquid nitrogen, and then stored at -80°C until use. Coronal sections (20 μm) were cut on a cryostat and fixed at a temperature between -11 and -13°C. Slices were thaw-mounted on 0.2% gelatine/0.033%

chromium potassium sulphate coated glass slides and stored at −80°C.

Peptide iodination

Iodination of HPP-Senktide was performed according to the chloramine T method (Hunter & Greenwood, 1962). Briefly, 5 μg of peptide were incubated in 0.05 M phosphate buffer for 30 s in the presence of 0.5 mCi (18.5 MBq) of $Na^{125}I$ and 220 nmol of chloramine T in a total volume of 85 μl. The mono-iodinated peptide was then immediately purified by high pressure liquid chromatography on a C4 Vydac column (0.4 × 250 mm) (The Separations Group, Hesperia, CA, U.S.A.) with 0.1% trifluoroacetic acid and acetonitrile as mobile phases. The specific activity of the iodinated peptide was calculated as 2000 c.p.m. $fmol^{-1}$ or 1212 Ci $mmol^{-1}$.

In vitro *receptor autoradiography*

Sections were thawed at room temperature, pre-incubated for 30 s in 25 mM PIPES buffer (pH 7.4; 4°C) and incubated at room temperature for 90 min in 25 mM PIPES buffer (pH 7.4; 4°C) containing: 1,10-phenanthroline 1 mM, dithiothreitol 1 mM, bacitracin 0.014%, captopril 0.1 mM, BSA 0.2% (protease free) and magnesium chloride 7.5 mM in the presence of 0.7–1.7 nM of [^{125}I]-HPP-Senktide in order to construct a saturation curve. The non-specific binding was determined in the presence of 1 μM of unlabelled HPP-Senktide or senktide. At the end of the incubation period, slides were transferred sequentially through four rinses of 4 min each in 25 mM PIPES (pH 7.4, 4°C) and dipped for 15 s in distilled water (4°C) to remove the excess of salts and air-dried. [^{3}H]-Hyperfilm was juxtaposed onto the slides in the presence of [^{125}I]-microscales and exposed at room temperature for 24 h. The films were developed in D-19 (Kodak developer) and fixed in Kodak Ektaflo. Autoradiograms were quantified by densitometry using an image analysis system (MCID™, Imaging Research Inc., Ontario, Canada). Standard curves from [^{125}I]-microscales were used to convert density levels into fentomoles per milligram of tissue (fmol mg^{-1} tissue). Specific binding was determined by subtracting non-specific labelling from total binding taken from adjacent sections. Quantification of total and non-specific binding was made on 200 and 160 tissue sections in 5 SHR and 4 WKY, respectively that correspond to eight tissue sections per rat for each of the five concentrations of the radioligand. Anatomical parameters and nomenclature were determined according to Paxinos & Watson (1998).

[^{125}I]-HPP-Senktide was chosen instead of [^{3}H]-Senktide used in previous autoradiographic studies (Dam *et al*., 1990; Stoessl & Hill, 1990; Stoessl, 1994; Langlois *et al*., 2001) because the iodinated ligand has the advantage to display greater specific activity (1212 Ci $mmol^{-1}$) over the tritiated ligand (50–63.5 Ci $mmol^{-1}$). Hence, the time of tissue exposure to the [^{3}H]-Hyperfilm can be reduced from 8–10 weeks with [^{3}H]-Senktide to 1 day with [^{125}I]-HPP-Senktide without affecting the selectivity of the agonist for the NK_3 receptor.

Chemicals and materials

The composition of aCSF was, in mM: NaCl 128.6, KCl 2.6, $MgCl_2$ 2.0 and $CaCl_2$ 1.4; pH adjusted to 7.2. [Sar^9,Met$(O_2)^{11}$]SP was obtained from Peninsula Lab. Inc. (San Carlos, CA, U.S.A.), while [β-Ala^8]NKA (4-10) and senktide were purchased from Bachem Bioscience Inc. (King of Prussia, PA, U.S.A.). The non-peptide antagonists RP 67580 (racemic form of 7,7-diphenyl-2[1-imino-2(2-methoxy-phenyl)-ethyl]-perhydroisoindol-4-one (3aR, 7aR), SR 48968 ((S)-N-methyl-N-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)-butyl]benzamide) and SR 142801 ((S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-N-methylacetamide) were provided by Dr C. Garret (Rhone Poulenc, Paris, France) and X. Emonds-Alt (Sanofi Recherche, Montpellier, France), respectively. The antagonist R-820 (3-Indolyl-carbonyl-Hyp-Phg-N(Me)-Bzl) was generously provided by Dr J.L. Fauchère (Servier, Paris, France). [Sar^9,Met$(O_2)^{11}$]SP was solubilized in aCSF while senktide, [β-Ala^8]NKA (4-10) and all antagonists were solubilized in 1–15% (v v^{-1}) DMSO (Fisher Scientific, Montréal, Québec, Canada). The solution was then completed with aCSF (i.c.v. and SN) or saline (i.v. injection) which contained 20% of 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich Canada). Stock solutions (10 mg ml^{-1}) of agonists and antagonists were stored in aliquots of 100 μl at −20°C until use. In all experiments, vehicle (aCSF containing 10% DMSO) was injected as control and had no significant effect on any parameters when compared to baseline values. HPP-Senktide (3-4 hydroxyphenyl-propionyl-Asp-Asp-Phe-N-MePhe-Gly-Leu-Met-NH_2 (MW:1006.5) is derived from the selective NK_3 receptor agonist senktide (Wörmser *et al*., 1986). It was synthesized by W. Neugebauer (Department of Pharmacology, Université de Sherbrooke, Canada). Autoradiographic [^{125}I]-microscales (20 μm) and [^{3}H]-Hyperfilm (single-coated, 24 × 30 cm) were purchased from Amersham Pharmacia Biotech Canada. Piperazine-N,N′-bis[2-ethanesulphonic-acid] (PIPES), 1,10-phenanthroline, dithiothreitol, bacitracin, captopril and bovine serum albumin (BSA) (protease free) were purchased from Sigma-Aldrich Canada.

Statistical analysis of data

Results are expressed as means ± s.e.mean of (n) rats. Results were analysed for statistical significance using a two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni confidence intervals. Statistical analysis for specific binding sites was performed with a Student's t-test for unpaired samples. Only probability values (P) less than 0.05 were considered to be statistically significant.

Results

Cardiovascular response induced by the NK_1 agonist [Sar^9,Met$(O_2)^{11}$]SP

The effects on MAP and HR of two doses of [Sar^9,Met$(O_2)^{11}$]SP in SHR are shown in Figures 2A, 3A and 4B. [Sar^9,Met$(O_2)^{11}$]SP (25 pmol and 100 pmol) evoked increases in HR and MAP which were significant ($P < 0.05$) when compared to vehicle (aCSF) values ($n = 8$). Thus, the tachycardia was significant at 25 pmol (5–45 min, $n = 8$) and 100 pmol (5–15 min, $n = 4$). The maximal rise in HR was 71 ± 24 b.p.m.

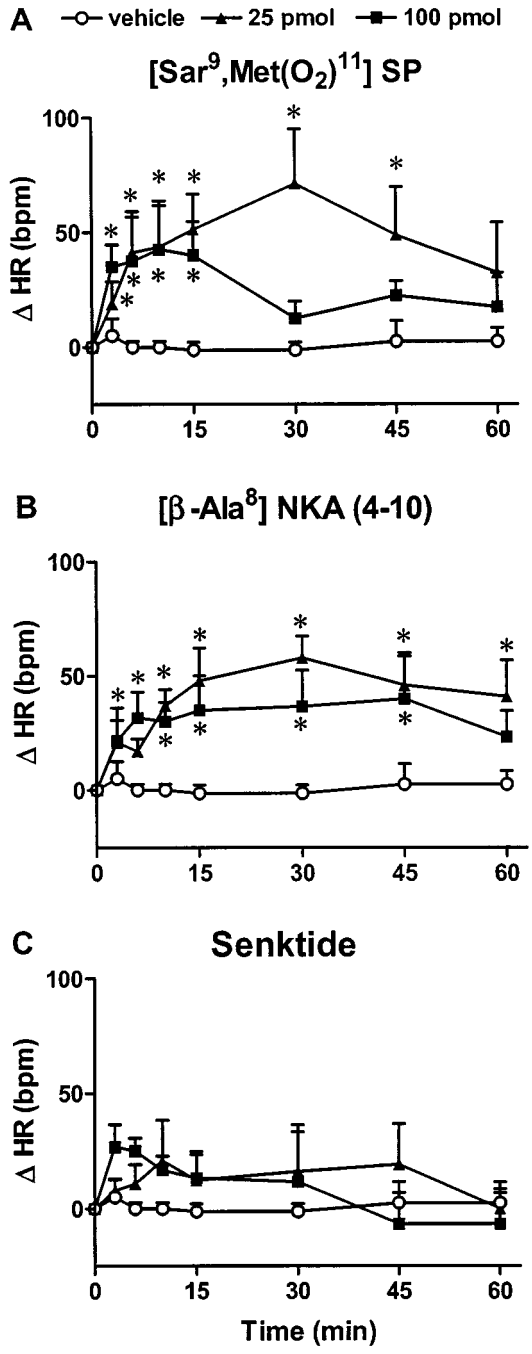

Figure 2 Changes in heart rate (Δ HR) induced by SN microinjection of the vehicle (aCSF, $n=8-13$) or (A) the NK_1 agonist [Sar^9,$Met(O_2)^{11}$]SP (25 pmol ($n=8$), 100 pmol ($n=4$)); (B) the NK_2 agonist [β-Ala^8]NKA (4-10) (25 pmol ($n=10$), 100 pmol ($n=6$)) and (C) the NK_3 agonist senktide (50 pmol ($n=13$), 100 pmol ($n=6$)) in SHR. Each point represents the mean$\pm$s.e.mean of (n) rats. Comparison to vehicle values is indicated by $^*P<0.05$.

(25 pmol, 30 min) and 43$\pm$21 b.p.m. (100 pmol, 15 min). [Sar^9,$Met(O_2)^{11}$]SP produced an increase in MAP at 25 pmol (7–45 min, $n=8$) and 100 pmol (5–45 min, $n=4$) in SHR. The maximal rise in MAP was 13$\pm$6 mmHg (25 pmol, 30 min) and 11$\pm$7 mmHg (100 pmol, 15 min). There was no significant effect on behaviour. Baseline HR and MAP values were 293$\pm$11 b.p.m. and 160$\pm$7 mmHg, respectively.

Cardiovascular response induced by the NK_2 agonist [β-Ala^8]NKA (4-10)

The effects on MAP and HR of two doses of [β-Ala^8]NKA (4-10) in SHR are shown in Figures 2B, 3B and 4C. [β-Ala^8]NKA (4-10) evoked a significant tachycardia ($P<0.05$) which was similar at 25 pmol (10–60 min, $n=10$) and 100 pmol (7–45 min, $n=6$) when compared to vehicle (aCSF with 7% DMSO) values ($n=10$). The maximal rise in HR was 58$\pm$10 b.p.m. (25 pmol, 30 min) and 40$\pm$19 b.p.m. (100 pmol, 45 min). [β-Ala^8]NKA (4-10) (25 and 100 pmol) had no significant effect on MAP when compared to vehicle values. There was no significant effect on behaviour. Baseline HR and MAP values were 323$\pm$31 b.p.m. and 161$\pm$9 mmHg, respectively.

Cardiovascular response induced by the NK_3 agonist senktide

The effects on MAP and HR of two doses of senktide in SHR are shown in Figures 2C, 3C and 4D. Senktide at the dose of 50 pmol ($n=13$) and 100 pmol ($n=6$) had no significant effect on MAP and HR when compared to vehicle values ($n=13$). There was no significant effect on behaviour. Baseline HR and MAP values were 338$\pm$25 b.p.m. and 160$\pm$10 mmHg, respectively.

Effect of tachykinin agonists in WKY

In WKY ($n=3$), the NK_1 [Sar^9,$Met(O_2)^{11}$]SP, 25 pmol), NK_2 ([β-Ala^8]NKA (4-10), 25 pmol) and NK_3 (senktide, 50 pmol) receptor agonists evoked increases in HR which were similar in amplitude and duration to the tachycardia measured in Wistar rats (Lessard & Couture, 2001). As observed in Wistar rats, 25 pmol of these agonists failed to alter MAP and behaviour in WKY (data not shown).

Effects of tachykinin antagonists in SHR and WKY

The effects on MAP of the NK_1 receptor antagonist RP 67580 (500 pmol, $n=5$) and the NK_2 receptor antagonist SR 48968 (500 pmol, $n=6$) in SHR are shown in Figure 5A. Neither of the two antagonists caused significant changes in MAP compared to vehicle values (aCSF with 10% DMSO, $n=6$). Both antagonists also failed to alter HR (data not shown). Moreover, SN microinjection of the NK_3 antagonist SR 142801 (500 pmol, $n=4$) was devoid of cardiovascular effect in this study (data not shown).

The effects on MAP of the NK_3 receptor antagonist R-820 (500 pmol) microinjected into the SN of SHR ($n=12$) and WKY ($n=10$) are shown in Figure 5B. Whereas R-820 failed to alter MAP when compared to vehicle values (aCSF with 10% DMSO, $n=12$) in WKY, it reduced significantly ($P<0.05$, 10 min–3 h) MAP in SHR for a period that lasted over 3 h post-injection when compared to vehicle values ($n=12$). The maximal fall in MAP was -18 ± 5 mmHg at 55 min. However, R-280 did not modify HR in both WKY and SHR (data not shown). Contrarily to SN microinjection, i.c.v. (500 pmol, $n=8$) or intravenous (500 pmol, $n=6$) injection of R-820 failed to elicit significant changes in MAP from time 0 to 3 h post-injection when compared to vehicle values (data not shown).

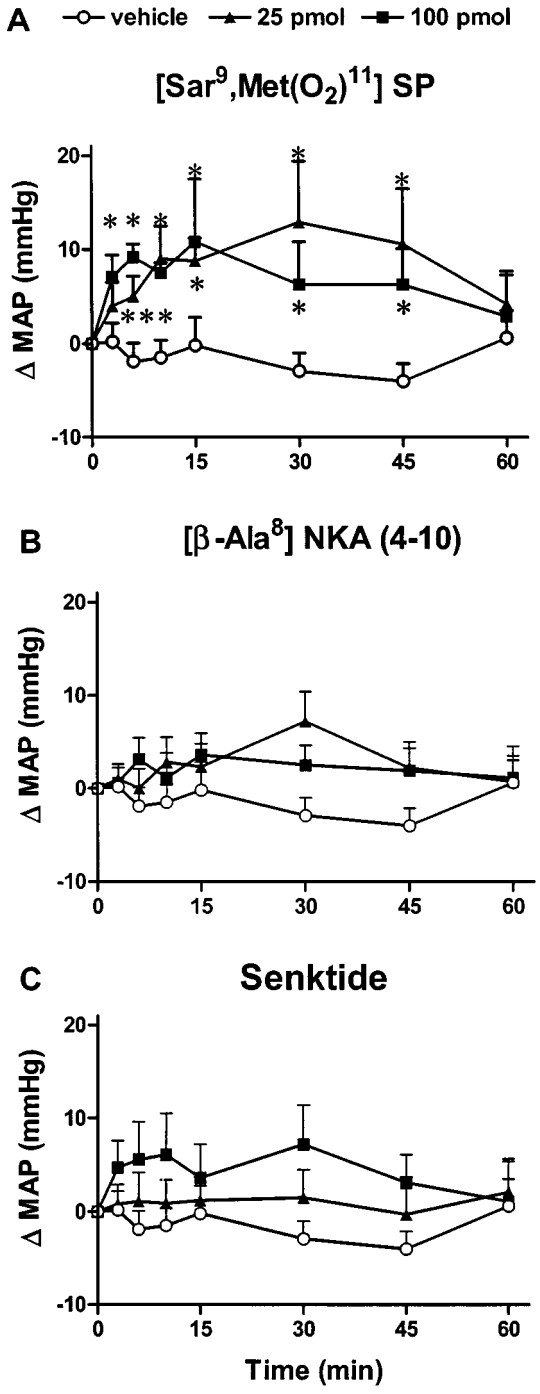

Figure 3 Legend as Figure 2 for changes in mean arterial pressure (Δ MAP) in the same animals.

All tachykinin receptor antagonists tested in this study were devoid of behavioural activity in SHR and WKY. Four rats that had injection sites beside the SN after histological post-mortem examination failed to evoke any significant changes in MAP with 500 pmol R-820 (Figure 1).

Midbrain NK_3 receptor binding sites in SHR and WKY

Densities of total, non-specific and specific NK_3 receptor binding sites in the SN of SHR according to the concentration of [^{125}I]-HPP-Senktide are shown in Figure 6. Non-specific binding defined in the presence of 1 μM unlabelled senktide accounted for approximately 30% of total binding at ligand concentration of 1.3 nM. In all midbrain areas, only small amounts of specific NK_3 receptor binding sites were detected (Figure 7, Table 1). Specific binding with [^{125}I]-HPP-Senktide in SN displayed a B_{max} which was not significantly different in SHR (11.5 fmol mg^{-1} of tissue) and WKY (10 fmol mg^{-1} tissue). Likewise, no difference was seen between SHR and WKY regarding densities of specific NK_3 receptor binding sites in the ventral tegmental area, the hippocampus and the amygdala whatever the concentration of the radioligand (Table 1). Furthermore, the affinity of the binding as defined by the dissociation constant (K_D) was identical in all these regions (0.7 nM) from both SHR and WKY.

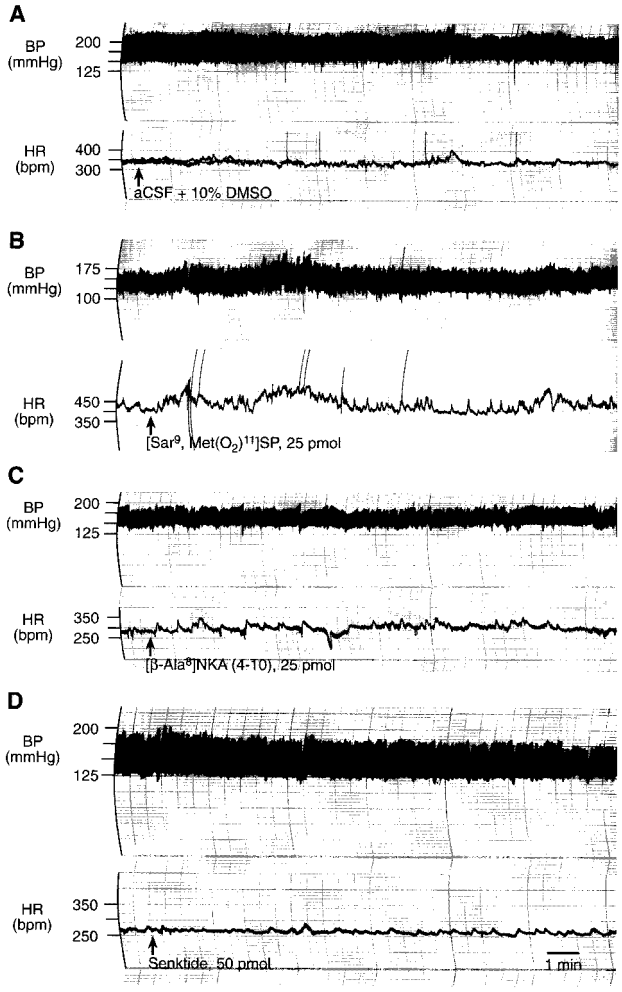

Figure 4 Original traces showing changes in heart rate (HR) and blood pressure (BP) induced by SN microinjection of (A) vehicle (aCSF with 10% DMSO), (B) the NK_1 agonist [Sar^9,Met(O$_2$)^{11}]SP (25 pmol), (C) the NK_2 agonist [β-Ala^8]NKA (4-10) (25 pmol) and (D) the NK_3 agonist senktide (50 pmol) in SHR.

Discussion

The profile of the cardiovascular responses elicited by bilateral microinjection of low doses (pmol range) of selective

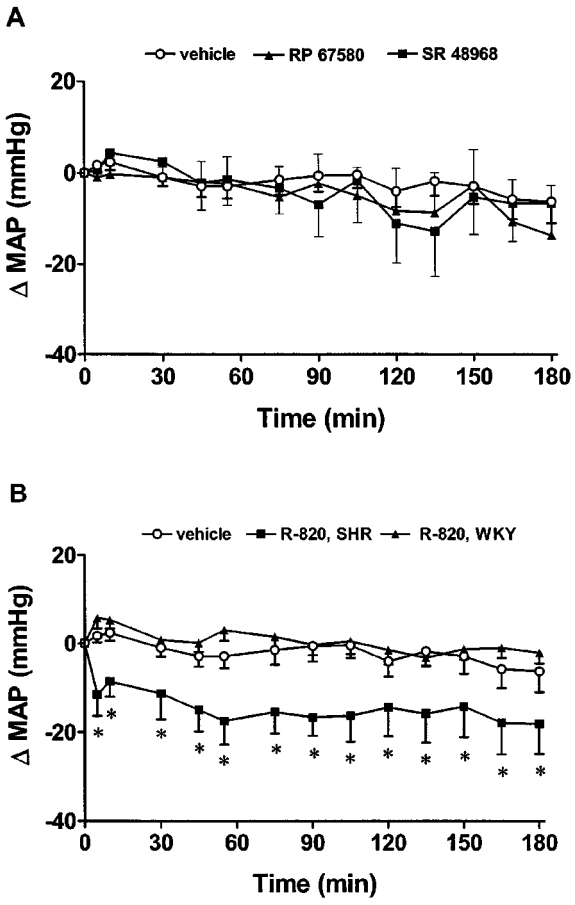

Figure 5 Time-course effects on changes in mean arterial pressure (Δ MAP) induced by SN microinjection of (A) vehicle (aCSF with 10% DMSO, $n=6$), RP 67580 (500 pmol, $n=5$) and SR 48968 (500 pmol, $n=6$) in SHR, and (B) vehicle (aCSF with 10% DMSO, $n=12$) and R-820 (500 pmol) in SHR ($n=12$) and WKY ($n=10$). Each point represents the mean ± s.e.mean of (n) rats. Comparison to vehicle values is indicated by $^*P<0.05$.

tachykinin NK_1 and NK_3 receptor agonists into the SN of SHR is quite different from that obtained in normotensive rats using the same approach (Lessard & Couture, 2001). While a tachycardia occurred without changes in MAP with the NK_1 agonist in normotensive Wistar rats (Lessard & Couture, 2001) and WKY, same doses of agonist increased both heart rate and MAP in SHR. These results are consistent with a previous report showing a 3 fold increase in pressor effect and a marked heart rate increase following i.c.v. injection of substance P in SHR when compared with age-matched WKY (Unger *et al*., 1980). While the NK_3 agonist evoked a marked heart rate increase in the absence of blood pressure changes in Wistar rats (Lessard & Couture, 2001) and WKY, this agonist was found inactive on both cardiovascular parameters in SHR. However, the NK_2 agonist elicited a similar tachycardia in Wistar, WKY and SHR with no effect on blood pressure.

In previous experiments, the tachycardiac responses to NK_2 and NK_3 agonists were abolished in the presence of atenolol whereas a treatment with atenolol and atropine was required to block the tachycardiac response of the NK_1 agonist (Lessard & Couture, 2001). It was concluded that the three tachykinin receptors increase the sympatho/adrenal drive to the heart and that the NK_1 agonist can additionally inhibit cardiovagal activity. Therefore, it is likely that the pressor effect of the NK_1 agonist in SHR derived from the well documented enhanced activity and reactivity of the sympathetic nervous system in SHR (De Champlain, 1998). Additionally, the inhibition of vagal activity by the NK_1 agonist might be altered in SHR. Indeed, the greater sympathetic drive in SHR is associated with a reduced vagal activity and baroreceptor reflex (Julius, 1988; Korner, 1989; De Champlain, 1998; Grassi *et al*., 1998).

A quantitative *in vitro* autoradiography was performed to address possible changes in affinity or number of NK_3 receptors into the midbrain of SHR. This was tested as a putative mechanism to account for the loss of senktide-induced tachycardia in SHR. Data reveal that modest densities of NK_3 receptor binding sites are similarly distributed in SN, amygdala, hippocampus and ventral tegmental area in SHR and WKY. This is consistent with autoradiographic and *in situ* hybridization studies regarding the distribution of NK_3 receptor binding sites and mRNA in the midbrain of normotensive rats (Shughrue *et al*., 1996; Langlois *et al*., 2001). These studies revealed moderate density of NK_3 receptor binding sites in the rat midbrain. B_{max} values measured in the SN with [^{125}I]-HPP-Senktide (11.5 and 10 fmol mg^{-1} tissue in SHR and WKY, respectively) are in the same range than the value previously reported with [^{3}H]-Senktide (16 ± 3 fmol mg^{-1} tissue) (Langlois *et al*., 2001). This suggests that both radioligands yield similar results, yet [^{125}I]-HPP-Senktide has the advantage to require only 24 h of exposure to the film contrary to 8–10 weeks needed for [^{3}H]-Senktide which has a much lower specific activity. Moreover, [^{125}I]-HPP-Senktide bound with higher affinity ($K_D=0.7$ nM) to midbrain areas than [^{3}H]-Senktide ($K_D=2.3-2.8$ nM) to rat brain cortex and striatum (Dam *et al*., 1990; Stoessl & Hill, 1990). Because both the dissociation constant (K_D values) and densities of receptors in the four analysed midbrain areas were not significantly different between SHR and WKY, it is concluded that the reduced cardiovascular effect following microinjection of the NK_3 agonist in SHR is unlikely to be attributable to the loss of NK_3 receptors or to changes in affinity.

In SHR, the NK_3 antagonist R-820 reduced MAP for over 3 h when microinjected into the SN while, at the same dose, NK_1 (RP 67580) and NK_2 (SR 48968) antagonists were devoid of any cardiovascular effect. The latter receptor antagonists blocked in a selective and reversible manner the cardiovascular responses induced by i.c.v. or SN injection of the selective tachykinin agonists mentioned above in Wistar rats (Picard *et al*., 1994; Cellier *et al*., 1999; Lessard & Couture, 2001). Likewise, R-820 inhibited the cardiovascular, antidiuretic and antinatriuretic effects induced by i.c.v. and/or nigral injection of senktide or [MePhe^7]NKB (Cellier *et al*., 1997; Yuan & Couture, 1997; Lessard & Couture, 2001), and the thermo-hypoalgesia induced by the intrathecal injection of the two NK_3 agonists in the rat tail-flick test (Couture *et al*., 2000).

In previous studies, we showed that the high affinity human NK_3 receptor antagonist SR 142801 behaves as a tachykinin NK_3 receptor agonist (blocked by R-820) when injected intracerebroventricularly or intrathecally in conscious rats (Cellier *et al*., 1997; Couture *et al*., 2000). When

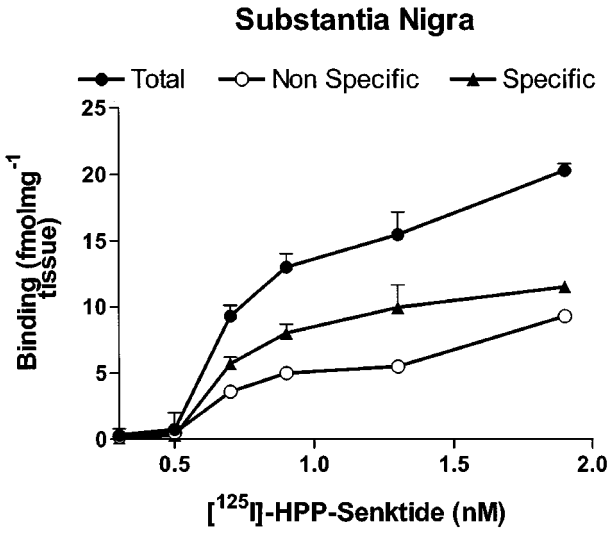

Figure 6 Total, non-specific and specific binding (fmol mg^{-1} tissue) of [^{125}I]-HPP-Senktide as a function of its concentration in the substantia nigra of SHR. Non-specific binding was measured with the addition of 1 μM HPP-Senktide. Quantification was performed on 40 sections (4 sections per animal × 5 SHR) for each concentration (× 5 concentrations) corresponding to 200 sections for total and non-specific binding.

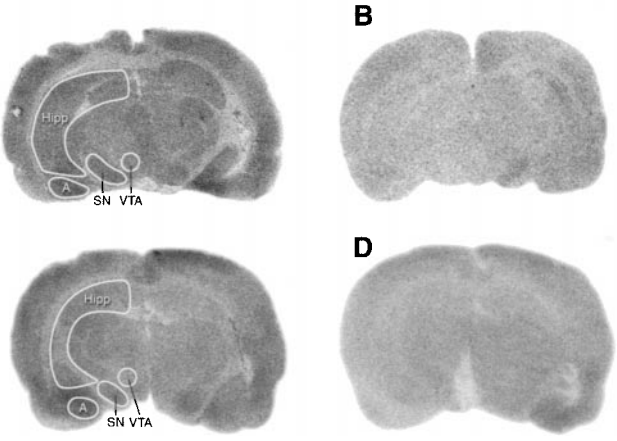

Figure 7 Autoradiographic distribution of [^{125}I]-HPP-Senktide binding sites in the midbrain of SHR (A,B) and WKY (C,D). Total binding measured with 0.7 nM [^{125}I]-HPP-Senktide is shown in panels A and C while the non-specific binding in the presence of 1 μM HPP-Senktide is shown in panels B and D. The areas under study are delimited by a white line. Abbreviations: SN, substantia nigra; VTA, ventral tegmental area; Hipp, hippocampus; A, amygdala.

injected into the SN, SR 142801 did not reproduce the fall in MAP observed with R-820 but was inactive as the agonist senktide. This was expected since this SR compound displays a low affinity at rat NK_3 receptors (Emonds-Alt *et al*., 1995).

In the present study, rats which showed injection sites beside the SN failed to evoke any significant changes in MAP to agonists or R-820. Therefore, the described cardiovascular effects are unlikely to be due to the diffusion of the injection outside the SN. This possibility is also unlikely in this study because the volume (0.1 μl) of injection is 5–10 fold smaller than the volume (0.5 and 1 μl) generally employed in this nucleus (Humpel *et al*., 1991; Humpel & Saria, 1993; Stoessl *et al*., 1995). Furthermore, the cardiovascular responses to agonists are unrelated to changes of behavioural activity which occurred at doses higher than 1 nmol (Stoessl *et al*., 1995; Lessard & Couture, 2001).

A role for nigral tachykinin NK_3 receptors in the tonic control of blood pressure in SHR

Since the three antagonists did not affect resting blood pressure and heart rate upon their central administration (i.c.v. and SN) in Wistar or WKY, it appears that endogenous tachykinins do not play a primary role in the tonic control of blood pressure and heart rate in the brain of normotensive animals. However, data highlight a potential role for tachykinins in the maintenance of hypertension in SHR by activating tonically NK_3 receptors in the SN. Thus, the occupancy of NK_3 receptors by an over-production of endogenous tachykinins may account for the lack of effect with senktide.

The distinctive effects of NK_1 and NK_3 receptor agonists could be related to the activation of different neuronal pathways from the SN. Indeed, *in situ* and/or solution hybridization, autoradiographic and immunocytochemical studies have shown that NK_3 receptors are located mainly on dopaminergic neurons in the substantia nigra pars compacta (Stoessl & Hill, 1990; Stoessl, 1994; Bannon & Whitty, 1995; Whitty *et al*., 1995; Shughrue *et al*., 1996; Chen *et al*., 1998) while NK_1 receptors are mostly located on GABAergic neurons of the substantia nigra pars reticulata (Sivam & Krause, 1992; Stoessl, 1994; Bannon & Whitty, 1995). Electrophysiological and microdialysis studies reveal that senktide activates mostly dopaminergic neurons of the SN pars compacta that leads to an increase of extracellular dopamine concentration in the striatum (Nalivaiko *et al*., 1997; Marco *et al*., 1998). The activation of that dopaminergic pathway by senktide was blocked by selective NK_3 antagonists. Interestingly, an autoradiographic study suggests an up-regulation of dopamine D_1 and D_2 receptors in the caudate-putamen of SHR (Kirouac & Ganguly, 1993). It is tempting to suggest that the tonic activation of nigral NK_3 receptors by endogenous tachykinins is facilitated by a hyperactive dopaminergic nigro-striatal system in SHR. This neuronal pathway could be involved in the maintenance of hypertension in SHR.

Although the SN has been traditionally associated with the central control of motor activity, evidence suggests that this midbrain nucleus exerts a role in central cardiovascular regulation (Barbeau *et al*., 1969; Micieli *et al*., 1987; Van den Buuse *et al*., 1991; Lin & Yang, 1994; Linthorst *et al*., 1994). Electrical or chemical stimulation of the SN leads to increases in blood pressure, heart rate and striatal dopamine levels in anaesthetized rats (Lin & Yang, 1994). On the other hand, orthostatic and postprandial hypotension have been reported in patients with Parkinson's disease who are known to suffer from a degeneration of the nigro-striatal dopaminergic pathway (Barbeau *et al*., 1969; Micieli *et al*., 1987). Also, lesion of the nigro-striatal dopaminergic pathway attenuates the development of hypertension in young SHR (Van den Buuse *et al*., 1991; Linthorst *et al*., 1994). In the present study, an attenuation of the arterial hypertension has been

Table 1 Densities of specific NK_3 receptor binding sites (fmol mg^{-1} tissue) in four midbrain nuclei of SHR and WKY according to the concentration of [^{125}I]-HPP-Senktide

Ligand (nM)	*Substantia nigra* (fmol mg^{-1})		*Ventral tegmental area* (fmol mg^{-1})		*Hippocampus* (fmol mg^{-1})		*Amygdala* (fmol mg^{-1})	
	SHR	*WKY*	*SHR*	*WKY*	*SHR*	*WKY*	*SHR*	*WKY*
0.7	5.7±0.5	5.8±1.0	4.4±0.3	4.1±1.0	5.6±0.7	4.5±1.8	6.8±0.8	6.1±1.2
0.9	8.0±0.7	5.0±0.6	6.0±1.7	4.0±0.6	7.0±0.5	6.0±0.4	10.0±1.2	9.0±0.6
1.3	9.9±1.7	9.0±1.5	6.6±1.2	6.0±1.6	9.2±1.6	7.0±1.6	11.0±1.4	9.0±2.3
1.5	9.1±1.1	10.0±0.7	6.8±1.3	8.0±1.5	8.8±1.0	10.0±1.6	9.3±1.3	12.0±0.8
1.7	11.5±0.5	10.0±0.7	5.4±0.8	5.0±3.5	9.8±1.1	10.0±3.9	10.6±1.3	11.0±4.0

Values represent the mean±s.e.mean of 5 SHR and 4 WKY. No statistical difference was found between strains.

observed in SHR under blockade of nigral tachykinin NK_3 receptors.

Conclusion

With the use of highly selective tachykinin receptor agonists, it was evidenced that the modulation of cardiac activity by NK_3 receptor activation in the SN is markedly altered in SHR which is not the case for NK_2 receptors. This is unlikely to be the consequence of changes in receptor density and affinity. Although significant pressor responses were evoked by activation of NK_1 receptors, the latter do not appear to be tonically active in SHR contrary to NK_3 receptors. Hence, this study using selective antagonists provides the first pharmacological evidence suggesting a pathophysiological role for tachykinin NK_3 receptors in the maintenance of hypertension at the level of the substantia nigra.

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