ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Apr; 22 (4): 349-354
http://www.chinaphar.com

Central norepinephrine pathways are involved in cardiovascular response to intracerebroventricular substance P[1]

QIAN Hong[2], YANG Li-Ping, WEI Zhen-Yu, LIU Ai-Dong, TIAN Hong, YANG Su-Fen[3]
(*Department of Physiology, Zunyi Medical College, Zunyi 563003, China*)

KEY WORDS substance P; dopamine; radioligand assay; blood pressure; heart rate

ABSTRACT

AIM: To study the role of norepinephrine system in the cardiovascular response to intracerebroventricular substance P (SP) in rabbit. **METHODS**: SP was given intracerebroventricularly in anesthetized rabbits pretreated with the catecholaminergic neurotoxin, 6-hydroxydopamine (6-OHDA). The density and affinity of SP receptors on synaptosomal membranes of the hypothalamus and the ventral medulla of rabbits were determined by [^{125}I]SP receptor assay. Arterial blood pressure and heart rate were recorded. **RESULTS**: Intracerebroventricular (icv) pretreatment of rabbits with 6-OHDA, reduced norepinephrine in the hypothalamus (by 86.7 %) and in the ventral medulla (by 77.0 %) respectively. The pressor response and tachycardia of these rabbits to icv SP (3.55 nmol·kg^{-1}) were attenuated. The density and the affinity of SP receptors in the hypothalamus and the ventral medulla of 6-OHDA-lesioned rabbits were decreased. The B_{max} (pmol·g^{-1} protein) of SP receptors in hypothalamus and the ventral medulla are 108 ± 5, 35.9 ± 2.2 in control group, and 42 ± 18, 20 ± 5 in 6-OHDA-lesioned rabbits, respectively. K_d (nmol·L^{-1}) of SP receptors in the two regions are 0.015 ± 0.004, 0.014 ± 0.006 in control group and 0.029 ± 0.001, 0.015 ± 0.003 in 6-OHDA group. There is a significant difference of B_{max} ($P < 0.01$) and K_d ($P < 0.01$, $P < 0.05$) in both regions between 6-OHDA groups and control groups. **CONCLUSION**: The results suggested that central norepinephrine pathways are involved in the cardiovascular response to icv SP.

INTRODUCTION

Substance P (SP), an undecapeptide purified from bovine hypothalamus, was the first neuropeptide to be discovered. It is unevenly distributed in the central nervous system. Numerous studies have shown that SP given intracerebroventricularly (icv) or intrathecally can elicit dose-dependent pressor response and tachycardia, which are mediated by the stimulation of spinal sympathetic preganglionic neurons[1-3]. The central mechanism involved in the cardiovascular response of brain SP has not been clear to date yet. Many researches have suggested an interaction between SP and central catecholamines, in particular, SP-like immunoreactive nerve terminals have been shown to surround most catecholamine-containing cell groups in brain and appear to synapse with catecholamine cells in locus coeruleus and nucleus tractus solitarii, areas known to influence blood pressure[4]. Recent studies reported that the cardiovascular actions of SP were related to α_1-adrenoceptor in the ventral medulla and periventricular structures[5,6]. However, whether the cardiovascular effects of SP involve catecholamine pathways has yet not been confirmed. In the present study, 6-hydroxydopamine (6-OHDA), a neurotoxin, was used to investigate whether destruction of central catecholamine pathways modifies the effects of icv SP on blood pressure and heart rate (HR) in anesthetized rabbits. Furthermore, the density of SP receptor in hypothalamus and ventral medulla which are important for circulation control were determined with [^{125}I]SP receptor binding assay to examine whether SP receptors take changes in the two regions of 6-OHDA-lesioned rabbits.

MATERIALS AND METHODS

Animals and drugs SP (Arg-Pro-Lys-Pro-Gln-

[1] Supported by the Natural Science Foundation of Guizhou Province, № 19963084.
[2] Correspondenceto Dr QIAN Hong. Now in *Department of Physiology, Medical College of Southeast University, Nanjing 210009, China*.
[3] Now in *Department of Pharmacology, Zunyi Medical College, Zunyi 563003, China*.
Phn 86-25-331-8787, ext 49924. E-mail njqh@21cn.com
Received 2000-07-03 Accepted 2001-02-06

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Apr; 22 (4)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

Gln-Phe-Phe-Gly-Leu-Met-NH_3) and 6-OHDA ($C_8H_{11}NO_3$) were products of Sigma Company. SP (1 g·L^{-1}) was dissolved in artificial cerebrospinal fluid (aCSF). 6-OHDA hydrobrimide was dissolved in aCSF containing ascorbic acid 2 g·L^{-1}. The concentration of 6-OHDA was 59.2 mmol·L^{-1}. The aCSF consists of NaCl 152, KCl 2.8, $MgCl_2$ 1.2, $CaCl_2$ 1.1 mmol·L^{-1} (pH 7.2)[7]. [^{125}I]SP (34 Gbq·mol^{-1}) was purchased from Peking Union Medical College and Chinese Academy of Medical Sciences. The experiments were performed on 16 male or female rabbits (Grade II) weighing 2.10 kg ± 0.05 kg. The animals were anesthetized with urethane (1 g·kg^{-1}) intravenously. Supplemental doses of the anesthetic were given when required. One group of animals (n = 8) received four doses of 6-OHDA (300 μg·kg^{-1} in 30 μL, icv) at intervals of 12 h and the other group (n = 8) received vehicles 30 μL·kg^{-1}.

Central administration of drugs Each animal was anesthetized and the bregma was exposed. Two small holes were drilled through the skull in the position of AP1, L5, R5 and H5 according to Sawyer's atlas[8]. Two cannulas with an external diameter of 0.6 mm and a internal diameter of 0.4 mm were inserted into the right hole and the left hole, respectively. It was a symbol of successful insertions of cannulas into the lateral ventricles that cerebrospinal fluid came out from the cannulas. 6-OHDA or vehicle was injected into the two lateral ventricles via polypropylene tube through the cannula connecting to 50 μL syringe. The left lateral ventricle alternated with the right ventricle received the drugs every 12 h within 2 d. One cannula was obstructed by a needle which had a compatible diameter to the cannula and kept in a small plastic bags on the rabbits' head and the other cannula was removed. Each animal received three doses of penicillin (400 kU/d) and gentamycin (40 kU/d) during three days after the completion of the operation. All animals were raised in isolated cage in the same room. On the tenth day after the first injection of 6-OHDA or vehicle, SP was given icv (3.55 nmol·kg^{-1} in 4.8 μL) to each animal.

Arterial blood pressure was monitored through an arterial catheter in left femoral artery. Arterial blood pressure and electric cardiogram (ECG) were recorded on the computer with BL-310 biosoftware purchased from Tai Meng Company in Chengdu, China.

Preparation of synaptosomal membranes from the hypothalamus and the ventral medulla[9] The procedure was performed at 0 – 4 ℃. The hypothalamus and ventral medulla were removed and homogenized in ice-cold sucrose solution 0.32 mmol·L^{-1} (pH 7.5, w/v = 1∶10). The homogenate was centrifugated at 1000 × g at 4 ℃ for 10 min. The supernatant was collected and centrifugated at 20 000 × g at 4 ℃ for 20 min. The pellet was suspended in the same amount of phosphate buffer saline (PBS) 50 mmol·L^{-1} (pH 7.4) containing 41 mL Na_2HPO_4 0.5 mol·L^{-1}, 9 mL NaH_2PO_4 0.5 mol·L^{-1}, and 450 mL distilled water after centrifugation again at 1500 × g at 4 ℃ for 10 min. The pellet was resuspended in 2 mL PBS 50 mmol·L^{-1} (pH 7.4). The solution may be stored at 4 ℃ in aliquots (containing 1 mL per tube) for 2 weeks which was designed to quantify the total protein in brain tissue and SP receptors.

Determination of the total membrane protein in brain tissue extracts Protein of brain extracts was determined with $CBBG_{250}$ protein assay supported by Nanjing Biological Technical Institute, China. The contents of membrane protein were (1.24 ± 0.20) g·L^{-1}.

Substance P receptor binding assay[10] [^{125}I]SP at the final concentrations ranging from 3.9 – 23.4 nmol·L^{-1} were used to label SP receptors in saturation binding experiment. The result of saturation binding experiment showed that specific binding values were 71.2 % of total binding values at the concentration of [^{125}I]SP 15.6 nmol·L^{-1} than others. So, [^{125}I]SP 15.6 nmol·L^{-1} was employed to analysed changes of SP-receptor binding sites in hypothalumus and ventral medulla of 6-OHDA lesioned rabbits. Tissue hemogenate 200 μL was added to tubes containing 20 μL [^{125}I]SP 195 nmol·L^{-1}. The nonspecific binding was defined by presence of unlabeled competitor (SP 74.6 μmol·L^{-1}). Incubation was initiated by adding PBS (50 mmol·L^{-1}, pH 7.4) to tubes to yield a final assay volume of 0.4 mL. Nonspecific binding and total binding were defined by [^{125}I]SP at its final concentration 15.6 nmol·L^{-1}. After incubation at 33 ℃ for 20 min, incubations were terminated by ice-cold water bath and the mixture was centrifugated (2000 × g at 4 ℃, 10 min) immediately. The pellet was rinsed with PBS 50 mmol·L^{-1} (pH 7.4) and recentrifugated (2000 × g at 4 ℃, 10 min). The pellet was rinsed once again with PBS 50 mmol·L^{-1} pH 7.4. The tissue bound [^{125}I]SP was seperated from free radioligand by centrifugation and counted by γ scintillation spectroscopy. All assays were run in triplicate. B_{max} and K_d values of SP receptor to [^{125}I]SP could be calculated by Scatchard Equation.

Determination of norepinephrine and dopa-

mine contents in brain[11] Dopamine and norepinephrine were determined by fluoroscent spectroscopy as follows: The tissues (100 mg) were removed, homogenized, and sonicated in $HClO_4$ 0.4 mmol·L^{-1}. The supernatant was collected and recentrifugated in 5 % $HClO_4$ at 1000 × g at 0 ℃ for 10 min after centrifugated with 0.1 mL KOH-HCOOH solution at 15 000 × g at 0 ℃ for 30 min. The catecholamines were extracted using Sephadex G-10 (made in Sweden) and oxidized by KI. G-10 was presoaked in distilled water for 24 h and washed three times with NaOH 0.5 mol·L^{-1}, HCl 0.5 mol·L^{-1}, and distilled water, respectively. The contents of norepinephrine and dopamine were determined under 385/485 nm and 310/390 nm wavelength by spectrophotofluorometer. Then, the contents of norepinephrine and dopamine in the brain were calculated by standard curves of norepinephrine and dopamine.

Data statistics The data were expressed as $\bar{x} \pm s$, and were analyzed paired or unpaired t-test. Statistical significance was accepted when $P < 0.05$. Binding data was analysed using Prism 3.0.

RESULTS

Effects of 6-OHDA pretreatment on the cardiovascular response to intracerebroventricular SP

6-OHDA depleted the norepinephrine in both regions examined. Norepinephrine in hypothalamus was reduced to only 13.3 % and that in ventral medulla to 23.0 % of the control ($P < 0.01$). The significant change of dopamine level in both regions was not observed (Fig 1).

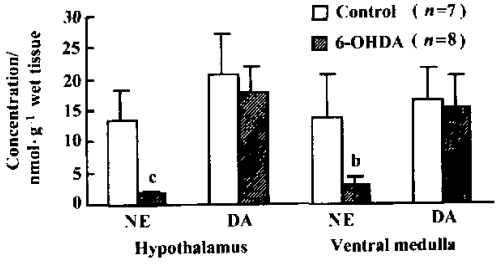

Fig 1. Effects of 6-OHDA or vehicle pretreatment on catecholamine levels in the hypothalamus and ventral medulla. NE, norepinephrine; DA, dopamine. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

The resting mean arterial pressure (MAP) and HR were observed for 10 min prior to SP. The average of resting MAP and HR in 6-OHDA group were (13.8 ± 0.5) kPa and (271 ± 40) beats·min^{-1}, and (13.5 ± 2.0) kPa and (297 ± 49) beats·min^{-1} in the control group, respectively. There was no statistic significance in resting MAP and HR between two groups. Icv SP elicited an increase of MAP and tachycardia in rabbits with pretreatment of vehicle. However, the pressor response was attenuated in rabbits pretreated with the neurotoxin 6-OHDA ($P < 0.05$). Tachycardia was not be induced by icv SP in 6-OHDA-lesioned rabbits (Fig 2).

Changes of SP-receptor binding sites of hypothalamus and the ventral medulla in rabbits pretreated with 6-OHDA Saturation binding experiments were conducted over a range of 3.9 – 23.4 nmol·L^{-1}. Saturation binding curves (Fig 3A) showed, specific binding sites of [^{125}I]SP were saturated by [^{125}I]SP 15.6 nmol·L^{-1}, but the total binding and nonspecific binding sites were not saturated at the highest concentration (23.4 nmol·L^{-1}) used. Therefore, the saturation binding curve could not fit to low affinity sites and Scatchard plot analysis of the data using Prism 3.0 yielded a best fit to a one site model of high binding affinity (Fig 3B, $K_d = 0.20$ nmol·L^{-1}, $B_{max} = 46.5$ pmol·g^{-1} protein). All data of saturation binding experiment were obtained from six normal rabbits studied in independent experiments.

In the receptor radioligand experiment using [^{125}I] SP 15.6 nmol·L^{-1} revealed that four doses of 6-OHDA administered icv can decrease the maximum binding (B_{max}) of SP receptors and increased the K_d of SP receptor in hypothalamus and ventral medulla compared to the control group ($P < 0.01$, Tab 1). The B_{max} (108 ± 5) pmol·g^{-1} protein of SP receptor in hypothalamus was higher than that (35.9 ± 2.2) pmol·g^{-1} protein in the ventral medulla in control group.

DISCUSSION

More and more evidence have suggested that there is an interaction between central catechola mines and SP. In the present study, icv 6-OHDA, a catecholaminergic neurotoxin, depleted norepinephrine by over 70 % in the ventral medulla while norepinephrine almost disappeared from the hypothalamus. In contrast, dopamine was not affected in these regions in 6-OHDA lesioned rabbits. Another result in present study was that there was a great reduction of the pressor response to icv SP after pretreatment with 6-OHDA. This differed from the previous

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Apr; 22 (4)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

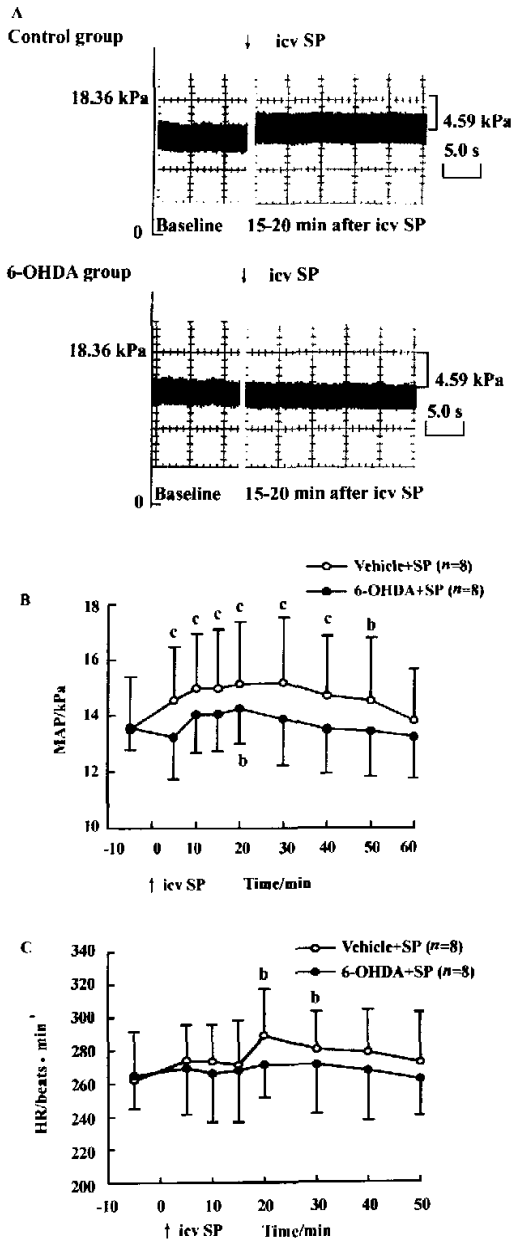

Fig 2. Original recording of the pressor response (A) to icv SP (7.46 nmol) in an anesthetized rabbit pretreated with 6-OHDA or vehicle and effects of 6-OHDA or vehicle pretreatment on changes in mean arterial pressure (MAP, B) and heart rate (HR, C) elicited by icv SP in anesthethized rabbits. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* before icv SP of same group.

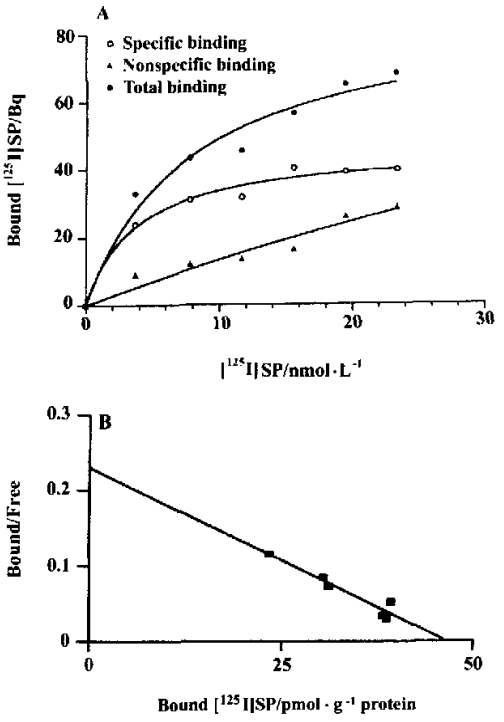

Fig 3. A: Saturation binding curves of [^{125}I]SP to SP receptor in the ventral medulla of rabbits. B: Scatchard plot analysis of binding sites of [^{125}I]SP in the ventral medulla of rabbits. $n=6$.

Tab 1. Changes of B_{max} and K_d of SP receptor in the hypothalamus and the ventral medulla elicited by icv 6-OHDA in rabbits. $n=8$. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

Brain regions	Group	B_{max}/ pmol·g^{-1} protein	K_d/nmol·L^{-1}
Hypothalamus	6-OHDA	42 ± 23^c	0.029 ± 0.001^c
	Control	108 ± 5	0.015 ± 0.004
Ventral medulla	6-OHDA	20 ± 5^c	0.015 ± 0.003^b
	Control	35.9 ± 2.2	0.014 ± 0.006

results that the pressor response to icv injection SP was unchanged[12]. The discrepancy could have resulted from differences in the subject animal, dose of 6-OHDA, method of injection or interval time between two separate doses of 6-OHDA and SP. It is noteworthy that our experiments were performed on anesthetized rabbits instead of rats, and that the blood pressure response of rabbit to SP was more sensitive than that of rat[1]. More importantly, an interesting result in this study was that the density of SP receptor binding sites and the affinity of SP receptor were reduced in hypothalamus and ventral medulla

of 6-OHDA lesioned rabbits, which maybe interpret that the pressor response to icv SP were attenuated by 6-OHDA pretreatment.

Several reports have suggested that central catecholamines may participate in the pressor response elicited by icv SP. There was other evidence that the synthesis and turnover of central catecholamines was increased by high doses of SP[4]. The histological studies showed that virtualy all catecholamine-containing neurons in the brain surrounded by SP-positive fibres, particularly the dopa minergic cells and dendrites in the substantia nigra and A2 cells in the nucleus tractus solitarii and dorsal motor nucleus of vagus[13-15]. Iontophoretic application of SP enhanced the basal firing rate of cells in the locus coeruleus (LC), which contains predominantly norepinephrine neurons surrounded by medium to dense SP-positive fibre networks[12]. Electric stimulation and chemical stimulation of LC increased blood pressure and renal sympathetic nerve discharge activity in rats, rabbits and cats[16].

The previous studies on the functional role of a central interaction between SP and catecholamines have concentrated on behavioral effects, particularly on the effects of the peptide on the dopaminergic nigrostriatal system. Since SP and catecholaminergic fibres were found together in the areas which are very important to cardiovascular regulation, such as the hypothalamus, nucleus tractus solitarii and dorsal motor nucleus of vagus, the big possibility of a functional interaction in circulatory control has to be considered. Recent reports demonstrated that α_1-adrenoceptor may in part mediate cardiovascular response to SP in the rostral ventral lateral medulla or in the vicinity of the fourth ventricles. However, no attempt has been made to investigate whether SP receptors distribute in the norepinephrine neurons and whether central norepinephrine pathways are involved in cardiovascular actions of SP in rabbit brain. Present study showed that an attenuation of pressor response to icv SP and a reduction of SP receptor density in 6-OHDA lesioned rabbits. Therefore, we speculated that the central catecholaminergic pathways play a major role in SP-induced pressor response.

In conclusion, the present study revealed that SP receptors are most likely to be present in the norepinephrine terminals and SP neurons appear to synapse with catecholaminergic nerve ter minals. The results provide first direct biochemical evidence for functional interaction between SP and NA in blood pressure regulation. The central norepinephrine pathways are involved in the pressor response to intracerebroventricular SP.

ACKNOWLEDGEMENTS The author wish to thank Prof LI Xi-Cheng in the Third Military Medical University and Prof XU Cheng-Tao in Peking Union Medical College, Chinese Academy Medical Sciences for their advice and encouragement.

REFERENCES

1 Pernow B. Substance P. Pharmarcol Rev 1983; 35: 86-128.
2 Unger T, Silvia C, Gudrun D, Deltlev G, Rudolfe L, Christiane M, *et al*. Substance P induces a cardio-vascular defense reaction in the rat. Pharmacological characterization. Circ Res 1988; 63: 812-20.
3 Itoi K, Jost N, Culman J, Tschöpe C, Unger T. Further localization of cardiovascular and behavioral actions of substance P in the rat brain. Brain Res 1994; 668: 100-6.
4 Tsou MY, Len WB, Lee TY, Chan SH, Pan WH, Chan JY, *et al*. Participation of noradrenergic neurotransmission in the suppression by substance P of α_2-adrenoceptors at the nucleus reticularis gigantocellularis involved in cardiovascular regulation in the rat. Brain Res 1994; 653: 183-90.
5 Zhang XH, Ni H. Role of rostral ventrolateral medulla in the pressor response to intraventricular (4th) injection of substance P. Acta Physiol Sin 1998; 50: 176-82.
6 Fu SG, Deng ZF, Huang CJ. Presssor effect of substance P applied to the pressor area of ventral surface of medulla oblongata and the underlying mechanism in rabbits. Acta Physiol Sin 1997; 49: 273-80.
7 Zou G. Cerebroventricular perfusion. In: Xu SY, Bian RL, Chen X, editors. Methology of pharmacological experiments. Beijing: People's Medical Publishing House; 1991. p 451.
8 Sawyer CH, Everett JW, Green JD. The rabbit diencephalon in stereotaxic coordinate. J Comp Neurol 1965; 101: 801-24.
9 Xu RB, Wu DL, Song LN. Preparation of synaptosomal membranes. In: Han JS, editor. Principles in neuroscience. Beijing: Beijing Medical University Publisher; 1999. p 70.
10 Buffington CA, Wolfe SA. High affinity binding sites for [^{3}H]substance P in urinary bladders of cats with interstitial cystitis. J Urol 1998; 160: 605-11.
11 Hano T, Jeng Y, Rho J. Norepinephine release and reuptake by hypothalamic synaptosomes of spontaneously hypertensive rats. Hypertension 1989; 13: 250-5.
12 Badoer E, Würth H, Qadri F, Itoi K, Unger T. Central noradrenergic pathways are not involved in the pressor response to intracerebroventricular substance P. Eur J Pharmacol 1988; 154: 105-8.
13 Kawai Y, Takagi H. Pericellular adrenergic neurons receive substance P-ergic inputs in the nucleus of the tractus solitarius of the rat. Brain Res 1989; 479: 344-8.
14 Yeomans DC, Proudfit HK. Projections of substance P-im-

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Apr; 22 (4)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

munoreactive neurons located in the ventromedial medulla to the A7 noradrenergic nucleus of the rat demonstrated using retrograde tracing combined with immunocytochemistry. Brain Res 1990; 532: 329 - 32.

15 Bittencourt JC, Benoit R, Sawchenko PE. Distribution and origins of substance P-immunoreactive projections to the paraventricular and supraoptic nuclei: Partial overlap with ascending catechola minergic rejections. J Chem Neuroanat 1991; 4: 63 - 78.

16 Chen YY, Huang ZS. Stimulation of locus coeruleus increases arterial pressure in rabbits. Acta Pharmacol Sin 1997; 18: 437 - 40.

中枢去甲肾上腺素系统参与脑室注射 P 物质的心血管反应[1]

钱 红[2], 杨俐萍, 魏振宇, 刘爱东, 田 虹, 杨素芬[3]
(遵义医学院生理教研室, 遵义 563003, 中国)

目的: 研究脑内 P 物质(substance P, SP)的心血管反应与去甲肾上腺素能系统的关系. **方法**: 家兔脑室注射 6-羟基多巴胺(6-hydroxydopamine, 6-OHDA) 10 天后注射 SP, 记录平均动脉血压和心率的变化; 放射受体测定法测定下丘脑及腹侧延髓中 SP 受体的密度和亲和力. **结果**: 6-OHDA 预处理后, 下丘脑及腹侧延髓内 NA 的含量分别降低 86.7 % 和 77.0 %, 侧脑室注射 SP 的升压反应显著减弱. 6-OHDA 组下丘脑和腹侧延髓突触小体膜上 SP 受体总数 B_{max}分别为(42 ± 18)和(20 ± 5) pmol·g^{-1} protein, 显著低于对照组(108 ± 5)和(35.9 ± 2.2) pmol·g^{-1} protein, 而 6-OHDA 组[^{125}I]SP 对 SP 受体的平衡解离常数 K_d[(0.029 ± 0.001), (0.015 ± 0.003) nmol·L^{-1}]显著高于对照组[(0.015 ± 0.004), (0.014 ± 0.006) nmol·L^{-1}]. **结论**: 中枢去甲肾上腺素神经通路参与侧脑室注射 SP 的心血管反应.

关键词 P 物质; 多巴胺; 放射配位体测定; 血压; 心率

(责任编辑 朱倩蓉)