STUDIES ON THE SITE OF ACTION OF NEUROTROPIN IN EXPERIMENTS ON DENERVATION-SUPERSENSITIVITY OF THE DUODENUM AND VAS DEFERENS OF RATS AND MICE

Tomitaro KITA, Taeko HATA, Akio NAMIMATSU and Eiji ITOH
Department of Pharmacology, Faculty of Pharmacy, Kinki University, Kowakae 3 Chome 4-1, Higashi-Osaka 577, Japan

Accepted October 25, 1982

Abstract—In order to clarify to a greater extent its action on peripheral nerves, various experiments were conducted using denervated animals to determine the effects of the nervous sedative, Neurotropin (NSP, containing many types of polysaccharides), on the site considered to be the periphery of autonomic nerves and the central nervous system. The increase in response to ACh due to bilateral cardiac vagotomy was significantly inhibited by a daily administration of NSP, but the increase in the response to noradrenaline (NA) due to celiac sympathectomy was hardly affected by this administration. The supersensitivity to the muscarinic action of ACh or methacholine of a denervated rat vas deferens, owing to ablation of the serous membrane, was significantly inhibited by the daily administrations of NSP. However, supersensitivity to NA was hardly affected. NSP never had any effect on the increase in the NA response of the duodenum and vas deferens isolated from mice given 6-hydroxydopamine, an adrenergic degenerator. Thus, an inhibitory action of NSP on denervation-supersensitivity is conceivably exerted on the parasympathetic nerves, rather than on the sympathetic nerves, and on a muscarinic receptor site, instead of a nicotinic site.

Neurotropin (NSP) is a drug containing active substances, many types of polysaccharides and is extracted from the inflammatory skin tissues of rabbits inoculated with cowpox virus. According to Takino (1), NSP is strongly effective in patients suffering from abnormalities in the autonomic nervous system in clinical use.

In animal experiments, NSP prevented (2) various abnormal conditions in a SART stressed animal (3) and showed inhibitory action on the central nervous system (4). A SART stressed animal is one suffering from stress resulting from periodic change in temperature and is considered to be a model animal for autonomic imbalance. Though the acetylcholine (ACh) response decreased in a duodenum excised from a SART stressed animal (5), it increased in the duodenum from a restraint and water immersion stressed animal (6). NSP showed marked inhibitory action on both of these changes (6, 7). On comparing these actions (6, 7) with those resulting from autonomic drugs and also psychotropic actions, it is thought that NSP acts on the peripheral autonomic nervous system as well as the central nervous system.

Experiments were carried out in order to obtain a clearer understanding of the action of NSP on the periphery of autonomic nerves, especially its action on the receptors of the cholinergic and adrenergic nerves. After the duodenum or vas deferens of an animal was selectively denervated, the action of NSP was examined for supersensitivity of these organs toward a chemical transmitter, ACh or noradrenaline (NA); these results are presented in this report.
Materials and Methods

Male Wistar rats weighing 200–250 g were used for surgical denervation, and male ddY mice weighing 20–22 g were used for chemical denervation.

Denervated animals were prepared according to the following 4 methods: a) Subdiaphragmatic vagotomy: Rats were immobilized supinely under pentobarbital (Abbott, Nembutal sodium solution) anesthesia (35 mg/kg, i.p.), and the vagus nerve was cut bilaterally at the lower part of the esophagus and about 1 cm above the cardia of the stomach under a stereoscopic microscope (Konan Camera, type M-1, 8 magnifications) according to the method of Toriumi et al. (8). b) Celiac sympathectomy: The celiac ganglions and postganglionic fibers of rats were resected according to the method of Kamata et al. (9). c) Denervation of the rat vas deferens: Denervation was carried out by ablation of the serous membrane surrounding the dextral vas deferens according to Birmingham's method (10). The sinistral vas deferens was left untouched, to serve as a control. d) Chemical denervation (11): A dose of 5 mg/kg of 6-hydroxydopamine hydrobromide (6-OHDA, Aldrich), an adrenergic neuron degenerator, was administered once i.p. to mice.

The success of the operation for decentralization or denervation was confirmed as follows: Success of the vagotomy was confirmed by abnormal distention of the stomach and disappearance of spike bursts on the EMG of the rat duodenum. When the EMG of the rat duodenum was derived according to Kamata et al. (9), spike bursts were scarcely observed in the vagotomized rat duodenum, though continuous spike bursts of 400–1000 nV in amplitude were observed in the normal rat duodenum. From this fact, it is suggested that parasympathetic action was considerably weakened and excitatory impulse from the higher center was decreased; and so, the vagotomy was undoubtedly successful.

Success of the sympathectomy was confirmed by the near failure of the excised duodenum to relax after tyramine (tyramine hydrochloride, Wako) (12), which shows a sympathomimetic action through the release of endogenous noradrenaline from the sympathetic nerve ending. Though \(10^{-3}\) M tyramine caused a similar relaxation of 2.5–4 mm to the excised normal rat duodenum as that by \(1-2\times10^{-6}\) M noradrenaline (NA), relaxation by tyramine was scarcely observed in the isolated duodenum from sympathectomized rats. From this, sympathectomy was verified.

And complete decentralization and denervation were confirmed moreover with the naked eye by laparotomy following sacrifice on the final day of the experiment.

After a given period following the operation, the duodenum and vas deferens was excised from the treated rats and mice. The duodenum about 10 cm in length following the stomach was isolated from the stomach, rinsed out several times with Tyrode solution and soaked in fresh Tyrode solution at room temperature. About 10 min after, a piece of duodenum about 20 mm in length was excised from about 5 mm distal to the gastric pylorus and suspended in an organ bath filled with 20 ml of Tyrode solution and bubbled with air for about 20 min. Relaxation of 0.5–1 mm was observed in the suspended duodenum specimen after 20 min by calculation on the chart, and no significant difference was observed in the degree of relaxation among normal, vagotomized and sympathectomized rat duodenau. The specimen was restored in length after removal of drugs, and no significant difference (below 0.3–0.7 mm in length) was observed among specimens of the above 3 groups. Now, there was no significant difference in the whole length of
the small intestines among normal, vagotomized and sympathectomized rats.

On the other hand, the entire length of the vas deferens, namely, from the end of the seminal vesicle to the testis, was isolated. Next procedures were the same as those used for the duodenum.

After soaking in Tyrode solution in the organ bath for 20 min, the isotonic contraction and relaxation were recorded on a recorder (Yokogawa Electric Works, YEW type 3056) through an isotonic transducer (Nihon Kohden Kogyo, TD 112S). The load was 1 g and 500 mg with the rat and mouse duodenum, respectively, and 400 mg and 300 mg with the rat and mouse vas deferens, respectively.

The duodenum was first examined with respect to responsiveness to ACh (acetylcholine chloride, Daiichi Seiyaku, Ovisot®) using the single dose method; and then, after an interval of 30 min, the cumulative response to NA (Sankyo, Nor-Adrenalin) until the maximal relaxation could be found.

The vas deferens, however, showed a biphasic contractile response to ACh (13): a fast phase contraction due to the nicotinic action of ACh and a slow phase contraction due to the muscarinic action. ACh was applied at about 15-min intervals by the single dose method; the resulting biphasic contractions were separated into the two contractions, and dose-response curves were prepared for both the nicotinic and muscarinic action. After the vas deferens had been allowed to rest, methacholine (MCh, Wako, methacholine chloride) was applied as a selective muscarinic receptor stimulant, and then NA was applied cumulatively as an adrenergic agonist.

As a test drug, a dried preparation of NSP (Nippon Zoki) was dissolved in saline; and doses of 50 and 100 mg/kg/day, unless otherwise specified, were each administered, i.p., daily, to the rats and mice from the day of denervation until the time the experiments were carried out.

Results

1. Daily changes in drug-responsiveness of the excised duodenum from denervated rats: Figures 1 and 2 show the relationship between the day following denervation and the responsiveness to ACh and NA of the excised rat duodenum. In Figs. 1 and 2, the abscissas show molar concentrations of ACh and NA, and the ordinates show reaction percentages with the average value of the maximal heights of untreated rats set at 100.

Figure 1 shows that vagotomy increased the responsiveness of the duodenum to ACh, reaching a maximum value after 10 days.
later. This value was considerably more than that for either 5 or 15 days. Responsiveness to NA, however, underwent no significant change throughout the period.

The results of the sympathectomy, however, as can be seen from Fig. 2, differ from those of the vagotomy in that the responsiveness to NA increased particularly. This increase was pronounced at 5 and 10 days later, but there was hardly any difference for either of these times. Recovery to nearly the same degree as in the case of untreated animals could be observed after 15 days. A slight increase in responsiveness to ACh was observed after 5 days.

From the above results, the effects of NSP on denervated animals were examined 10 days following denervation on the basis of responsiveness to ACh and NA for vagotomy and sympathectomy, respectively.

2. Effects of NSP on responsiveness of denervated rat duodenum to drugs: Figure 3 shows that there is an inhibiting effect of NSP on an increase in the response of the duodenum excised from vagotomized rats to ACh. The maximal contraction rate due to ACh was 142.9% in the vagotomized control group, a significant increase (P<0.001) compared with that in the untreated control group; and the maximal contraction rate was 128.7% and 118.3%, respectively, in the groups given 50 and 100 mg/kg/day of NSP. Thus an increase in responsiveness to ACh due to vagotomy was dose-dependently and significantly (P<0.05–0.001) inhibited by NSP.

Figure 4 shows the effects of NSP observed in the sympathectomy. In Fig. 4, the maximal relaxation rate due to NA shows an increase in responsiveness to the same extent as that found for both the sympathectomized control group and the groups given 50 and 100 mg/kg/day of NSP.
kg/day of NSP. Thus, no significant effect of NSP was observed in any of these cases.

When 100 mg/kg/day of NSP was administered daily to normal rats for 10 days, the extent of responsiveness to either ACh or NA of the excised duodenum was the same as that in the untreated group.

3. Effects of NSP on denervation supersensitivity in the rat vas deferens: In accordance with Birmingham's method (10), the rat vas deferens was used 7 days following denervation.

Since the denervated vas deferens showed no fast phase contraction due to the nicotinic action of ACh from depletion of catecholamine, only the slow phase contraction, that is, the response to muscarinic action, was used as an index, as shown in Fig. 5. The lower figure in Fig. 5 shows the % contraction, as in the foregoing figure, while the upper figure in Fig. 5 shows the conversion of this into the co-ordinate $[\log X, \log \{Y/(Y_{\text{max}}-Y)\}]$. X shows the molar concentration of ACh and Y the % contraction height.

Figure 5 shows that the dose-response curve of the muscarinic action of ACh shifts to the left owing to denervation, but such a shift to the left was remarkably inhibited in the group to which NSP was administered daily.

When NSP was administered in the same manner for 7 days to innervated rats, re-
sponsiveness to ACh of the excised vas deferens did not differ from that of the untreated control for either the fast phase (not shown in the figure) or the slow phase.

Though not shown in the figure, supersensitivity of the vas deferens to MCh due to denervation was significantly ($P<0.001$) inhibited by administering 50 mg/kg/day of NSP for 7 days. This is the same as the case of the muscarinic action of ACh.

Figure 6 shows the effects of NSP on denervation-supersensitivity to NA. The dose-response curve of NA shifts to the left owing to denervation, and the group given NSP shows nearly the same curve, indicating that NSP hardly has any influence on supersensitivity to NA.

4. Effects of NSP on chemically denervated mice: While the above experiments were conducted on surgically denervated rats, the following examinations were made on chemically denervated mice with 6-OHDA.

Responsiveness to ACh and NA of the excised duodenum and vas deferens from 6-OHDA-treated mice was examined daily, and the results are shown in Figs. 7 and 8.

Figure 7 shows that the administration of 6-OHDA increased the response of the duodenum to NA, the degree of this increase attaining a maximum in 2 days; and after 4 days later the response became the same as that of the untreated group.

Figure 8 shows that as in the case of the vas deferens, a maximal increase in response...
to NA took place 2 days following its administration.

On the other hand, the response to ACh was not affected in either organ.

With the NA response of the duodenum and vas deferens 2 days after the 6-OHDA administration as an index, effects of NSP on the changes in response were examined, and the results are shown in Fig. 9. The group given NSP consisted of 2 groups: a group to which 100 mg/kg/day of NSP was administered once a day, for a total of two times, over a period ranging from the day of the 6-OHDA treatment to the day of the experiment (1st-1st) and a group to which the same dose of NSP was administered daily over a 6 day period ranging from 4 days before the 6-OHDA treatment to the day of the experiment (1st-1st).

Figure 9 shows that an increase in NA responsiveness of the duodenum and vas deferens due to the administration of 6-OHDA tended to be slightly inhibited only in the group given NSP for 6 days; however, this was insignificant.

**Discussion**

Up to the present, NSP has been considered to act primarily on the central nervous system (2, 4, 14) and to some extent on the autonomic nervous periphery (6, 7). Our present experiment on the isolated duodenum indicates that the site of action of NSP is also the autonomic nervous periphery and not at the periphery of sympathetic nerves, but at the periphery of the parasympathetic nerves. NSP had hardly any influence on the nicotinic action of ACh on the vas deferens excised from normal rats (15). The influence on nicotinic action should moreover be studied.

The increase in the response to NA of chemically sympathectomized mouse duodenum and vas deferens with 6-OHDA (11) was slightly inhibited by NSP, but this is statistically insignificant.

**Denervation-supersensitivity** is an increasing change in response to a chemical transmitter situated at the postsynaptic receptor site on the peripheral smooth muscle induced by chronic depletion of the chemical transmitter at the presynaptic site (16, 17). Denervation-supersensitivity of the above-mentioned organs to a muscarinic receptor stimulant was not affected by a single administration of NSP, but remarkably so by the daily administrations of NSP. This finding suggests that NSP has a directly preventive action on the muscarinic ACh receptor site at the site of cholinergic nerve
endings, unless there is another tract for transmission from the central nervous system to the peripheral organs other than the autonomic nerve.

It has been not confirmed electron-microscopically by us whether presynaptic sites fall morphologically into hypofunction when the response of the organs to ACh is made to increase by denervation or decentralization. For the present, we feel inclined to believe that the target sites of NSP are receptor sites.

On the other hand, with the duodenum excised from SART animals, a decrease in the amount of a muscarinic ACh receptor has been reported in the results of a receptor binding assay by Uchida et al. (18). We have already reported that daily administrations of NSP for SART stressing significantly inhibit a decrease in ACh response in the SART animal duodenum (7). This change resulting from SART stress may belong to subsensitivity (16), and NSP was observed to have a preventive action on changes in the postsynaptic receptor sites in the case of subsensitivity (7) as in the case of denervation-supersensitivity.

With ACh contraction of the vagotomized rat duodenum shown in Fig. 3, the intrinsic activity based on the maximal contraction was mainly observed to increase, and an increase of affinity was hardly observed. This means that a quantitative change in receptor was mainly caused more than a qualitative change. On the other hand, with ACh contraction of the denervated rat vas deferens in Fig. 5, the leftward shift of dose-response curve to ACh was remarkably observed without any change in maximal contraction of intrinsic activity. This means an increase in affinity, leading us to believe that a qualitative change in receptor occurred mainly. Thus, the level of change in intrinsic activity and affinity differs with various organs, but an explanation for this curious phenomenon has not yet emerged. On the other hand, with ACh contraction of the SART stressed animal duodenum, the dose-response curve slightly shifted to the right, and a remarkable decrease in intrinsic activity and only a small decrease in affinity was shown contrary to denervation.

All of the changes in the decentralized rat duodenum, denervated rat vas deferens and SART stressed rat and mouse duodena are receptor changes. These facts indicate that NSP acts protectively on changes in both the intrinsic activity and affinity of the receptor. In regard to the protective effects of NSP on receptor sites, it is yet unknown whether NSP acts primarily on changes in affinity or intrinsic activity or on the relation between affinity and intrinsic activity and whether there are any other explanations. It seems reasonable to consider that NSP acts in such a way as to normalize the muscarinic ACh receptor site in the parasympathetic nerve.

We are planning to carry out an electron-microscopical observation of nerve ending sites in SART stressed and denervated animals. Thus, it is hoped that the remaining problems in our present data can be solved. Following this, we intend to carry out experiments on the action of NSP on nicotinic receptors in denervated skeletal muscles (19–22).

References

1) Takino, M.: The role of the autonomic nervous system in pathogenesis of allergy. Japan. J. Allergol. 20, 637–652 (1971)
2) Kita, T., Hata, T., Okage, T., Yoneda, R. and Hoshino, K.: Effect of neurotropin upon SART-stress states in mice and rats. Folia Pharmacol. Japon. 71, 211–220 (1975) (Abs. in English)
3) Kita, T., Hata, T., Yoneda, R. and Okage, T.: Stress state caused by alternation of rhythm in environmental temperature, and the functional disorders in mice and rats. Folia Pharmacol. Japon. 71, 195–210 (1975) (Abs. in English)
4) Hata, T., Kita, T. and Yoneda, R.: Central activity, antihypertensive action and antiulcerogenic
effects of Neurotropin. Folia Pharmacol. Japon. 72, 879-890 (1976) (Abs. in English)

5) Hata, T., Kita, T., Iida, J., Yoshida, H., Uchida, S. and Ishida, S.: Decrease of ACh response in isolated duodenum from repeated cold stressed (SART stressed) mice. J. Pharmacobiodyn. 1, 338-340 (1978)

6) Yoneda, R., Kita, T., Hata, T. and Namimatsu, A.: Experimental partial sympathicotonia, and effects of some drugs on it in restraint and water immersion stressed animals. J. Pharmacobiodyn. 3, 692-701 (1980)

7) Kita, T., Hata, T., Iida, J. and Ishida, S.: Decrease of ACh response in isolated duodenum from SART stressed (repeated cold stressed) mice. Folia Pharmacol. Japon. 75, 33-44 (1979) (Abs. in English)

8) Toriumi, T., Nakamura, N. and Nagao, F.: Curative effect of a vagotomy on acetic acid ulcers. In Experimental Ulcers. Edited by Umehara, S., Takagi, K., Nagao, F. and Matsuo, Y., p. 355-362, Japan Medical Center, Tokyo (1976) (in Japanese)

9) Kamata, K., Watanabe, M. and Kasuya, Y.: Changes in the response to drugs of the rat stomach after a chronic vagotomy and a sympathectomy. Folia Pharmacol. Japon. 74, 225-238 (1978) (Abs. in English)

10) Birmingham, A.T.: Sympathetic denervation of the smooth muscle of the vas deferens. J. Physiol. 206, 646-661 (1970)

11) Porter, C.C., Totaro, J.A. and Stone, C.A.: Effect of 6-hydroxydopamine and some other compounds on the concentration of norepinephrine in the hearts of mice. J. Pharmacol. Exp. Ther. 140, 308-316 (1963)

12) Grobecker, H., Holtz, P. and Jonsson, J.: Effects of tyramine on the isolated small intestine. Naunyn Schmiedebergs Arch. Pharmacol. 255, 481-500 (1966)

13) Ozawa, H., Aihara, K., Abe, F. and Sugawara, K.: Pharmacological analysis of acetylcholine-induced contraction in mouse vas deferens. Japan. J. Pharmacol. 24, 814-816 (1974)

14) Shirafuji, Y. and Go, K.: A study on the change of GSR on patients with Neurotropin Tablets (NT). Gendai-no-Shinryo 23, 633-644 (1981) (in Japanese)

15) Kasuya, Y. and Suzuki, N.: Regional differences in the distribution of cholinergic receptors in the rat vas deferens. Japan. J. Pharmacol. 29, 313-315 (1979)

16) Fleming, W.W., McPhillips, J.J. and Westfall, D.P.: Postjunctional supersensitivity and subsensitivity of excitable tissues to drugs. Rev. Physiol. Biochem. Pharmacol. 68, 55-119 (1973)

17) Westfall, D.P., McPhillips, J.J. and Foley, D.J.: Inhibition of cholinesterase activity after postganglionic denervation of the rat vas deferens: Evidence for prejunctional supersensitivity to acetylcholine. J. Pharmacol. Exp. Ther. 189, 493-498 (1974)

18) Uchida, S., Takeyasu, K., Noguchi, Y., Yoshida, H., Hata, T. and Kita, T.: Decrease in muscarinic acetylcholine receptors in the small intestine of mice subjected to repeated cold stress. Life Sci. 22, 2197-2203 (1978)

19) Lomo, T. and Westgaard, R.H.: Control of acetylcholine sensitivity in rat muscle fibers. Cold Spring Harbor Symp. Quant. Biol. 40, 263-274 (1975)

20) Ko, P.K., Anderson, M.J. and Cohen, M.W.: Denervated skeletal muscle fibers develop discrete patches of high acetylcholine receptor density. Science 196, 540-542 (1977)

21) Cangiano, A. and Lutzemberger, L.: Partial denervation affects both denervated and innervated fibers in the mammalian skeletal muscle. Science 196, 542-544 (1977)

22) Froehner, S.C., Reiness, C.G. and Hall, Z.W.: Subunit structure of the acetylcholine receptor from denervated rat skeletal muscle. J. Biol. Chem. 252, 8589-8596 (1977)