

Regulatory Effect of Neurotropin on Nasal Mucosal Hypersensitivity in Guinea Pigs Caused by SART (Intermittent Exposure to Cold) Stress

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ABSTRACT—We stated that SART-stressed guinea pigs showing nasal mucosal hypersensitivity would serve as an animal model for the *in vivo* evaluation of antiallergic drugs. In the present study, the mode of action of Neurotropin on nasal allergy compared with those of antiallergic drugs was studied by using SART-stressed guinea pigs. Daily administrations of Neurotropin improved the methacholine-induced hypersecretion and histamine-evoked sneeze response, which are parameters of nasal mucosal hypersensitivity, and increased the density of muscarinic ACh receptors located on the nasal mucosa caused by SART stress. In addition, the inhibitory action on nasal secretion in a passively sensitized model was more intense in SART-stressed guinea pigs than in normal ones. Ketotifen and tranilast were also found to have marked effects on nasal secretion in the passively sensitized SART-stressed model, but only had weak effects on nasal mucosal hypersensitivity. Neurotropin significantly potentiated the action of ketotifen or tranilast. Thus, at least part of the inhibitory effect of Neurotropin on nasal symptoms such as watery secretion and sneezing is thought to have been brought forth through the regulatory action on nasal mucosal hypersensitivity, and its combined use with antiallergic drugs would be a very effective therapeutic regimen for nasal allergy.

Keywords: Neurotropin, Antiallergic drug, Stress, Nasal allergy, Nasal hypersensitivity

Neurotropin, a non-proteinous extract containing biological active substances formed by immunoinflammatory reactions, which is isolated from cutaneous tissue of rabbits inoculated with vaccinia virus, has been clinically used in Japan as an analgesic and antiallergic drug (1), and it is known to be particularly effective on nasal allergy.

Since Neurotropin exhibits little antiallergic activity (2, 3) in conventional allergic models such as an antagonistic effect on chemical mediators, an inhibitory effect on the release of chemical mediators from rat peritoneal mast cells and passive cutaneous anaphylaxis in animals, the mechanisms responsible for its antiallergic effect in clinical studies have to date remained unclear.

In our previous paper (4), we reported that nasal mucosal hypersensitivity could be induced in guinea pigs by intermittent exposure to cold, i.e., SART (specific stress caused by alteration of rhythm in temperature) stress (5) and that SART-stressed guinea pigs

would be useful for studying nasal allergy.

The primary purpose of the present work is to examine the effect of Neurotropin on nasal mucosal hypersensitivity and nasal allergic symptoms in our *in vivo* experimental system of SART-stressed guinea pigs, and furthermore, to compare its mode of action with conventional antiallergic drugs.

MATERIALS AND METHODS

Experimental animals

Male Hartley guinea pigs weighing 400–600 g were used. The animals were housed in a temperature-controlled room at 24°C with a 12-hour light cycle (lights on from 08:00 to 20:00), and a standard diet and water were given *ad libitum*.

Stress loading

For SART-stress loading (4, 5), guinea pigs were

kept alternately at 24 and 0°C for one hour periods from 10:00 to 17:00 and then constantly at 0°C from 17:00 to 10:00 on the following morning. This treatment was usually repeated for 5 consecutive days. Stress loading was discontinued on the morning of the 6th day, and the animals were subjected to the subsequent experiments.

Drugs and other chemicals

Drugs were obtained from the following companies: Neurotropin[®] (Nippon Zoki), ketotifen fumarate (Zaditen, Sandoz) and tranilast (Rizaben, Kissei).

The radioligand and chemicals were purchased from the following companies: fluorescein sodium (uranin, Kishida), methacholine chloride, histamine dihydrochloride, atropine sulfate (Wako), ovalbumin (Egg albumin, Seikagaku Kogyo) and *l*-quinuclidinyl[phenyl-4-³H]benzylate ([³H]QNB, 46 Ci/mmol, Amersham).

Quantitative measurement of nasal secretion

According to the method of Namimatsu et al. (6), based on capillary action, nasal secretion was measured by using defatted cotton threads (No. 40/2; Yokota, Japan). The threads were dyed with 10% fluorescein sodium in saline at one end and were cut to yield 100-mm-long pieces, of which 10 mm from one end was colored. Ten minutes after nasal provocation, the guinea pig was immobilized under handling, and nasal secretion was measured with the dyed thread, one end of which was inserted into the unilateral anterior naris and kept there for 60 sec. The stretch of color on thread due to nasal secretion was regarded to indicate the quantity of nasal secretion.

Measurement of reactivity and threshold of sensitivity in nasal mucosa

Nasal mucosal reactivity and sensitivity were tested by dropping 10 μ l of methacholine into the unilateral (left) anterior naris or 10 μ l of histamine into the anterior nares of both sides. A series of diluted methacholine or histamine (dissolved in phosphate-buffered saline) solutions were consecutively applied at intervals of 10 min to determine the reactivity and sensitivity of individual animals. When the length of the stretch of color due to nasal secretion induced by methacholine reached beyond 5 mm or a sneeze response induced by histamine appeared, we regarded the methacholine or histamine concentration used then as being the threshold of sensitivity (6).

Anti-ovalbumin sera

According to the method of Terada et al. (7), the guinea pigs were immunized intraperitoneally with 20

μ g of ovalbumin and 10 mg of aluminum hydroxide in 1 ml of physiological saline, seven times every 2 weeks. Then airway sensitization was performed for 5 consecutive days by ultranebulization of 2 ml of 0.25% ovalbumin in saline. One week thereafter, nasal provocation was performed by applying 50 μ l of 1% ovalbumin on both anterior nares, and sera were collected from guinea pigs in which typical nasal symptoms were observed. The titers of the pooled antisera were 1:2¹³ and 1:2¹⁰ as estimated by 4 hr and 7 days homologous passive cutaneous anaphylaxis, respectively.

Nasal provocation in passively sensitized guinea pigs

Nasal symptoms were provoked by application of 50 μ l of 1% ovalbumin on the unilateral (left) anterior naris 2 days after intraperitoneal sensitization with 2 ml of anti-ovalbumin serum diluted eightfold with saline. Sensitization of SART-stressed guinea pigs was carried out on the 4th day, and nasal symptoms were provoked on the 6th day from the start of stress loading.

Preparation of membranes

Guinea pigs were decapitated and their nasal mucosae were removed. The nasal mucosa was weighed and immediately frozen at -80°C and stored until preparation of membrane protein samples. The nasal mucosa was homogenized in 100 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) by a Polytron. The homogenate was filtered through nylon mesh to remove connective tissue and then centrifuged at 50,000 \times *g* for 20 min. The pellet was resuspended in 10 ml of incubation buffer (50 mM Tris-HCl buffer, 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, pH 7.4) and then chilled in an ice bath until use.

Receptor binding assay

Radioligand binding assay for muscarinic ACh receptors (m-ACh-R) was carried out by the method of Ishibe et al. (8), with slight modifications. The standard binding assay was carried out in 1 ml incubation buffer containing approximately 1 mg membrane protein and [³H]QNB in the presence or absence of an excess of 2 \times 10⁻⁶ M atropine. Specific receptor binding is defined as the difference between the total binding of [³H]QNB and the nonspecific binding observed in the presence of atropine. Specific binding was usually about 70% of the total binding. The binding reaction was initiated upon addition of the membrane protein and incubation was allowed to proceed for 30 min at 25°C for [³H]QNB binding in a shaking water bath. After incubation, the mixture of membrane protein and radioligand was rapidly filtered in vacuo through a Whatman GF/F glass filter, which was then washed 3 times with 3 ml of

ice-cold 50 mM Tris-HCl buffer. The filter was transferred to a scintillation vial and after drying at 60°C for 5–12 hr, 5 ml of toluene-based scintillator (AL-1, Dojin) was added, and radioactivity was counted in a liquid scintillation counter. The protein content of the membrane samples was determined by the method of Lowry et al. (9), using bovine serum albumin as a standard.

Administration of drugs

To evaluate the acute effects on nasal provocation, drugs to be used perorally were suspended in 0.5% carboxymethylcellulose-Na and were administered to guinea pigs in a volume of 5 ml/kg body weight 60 min prior to nasal provocation. Atropine to be injected subcutaneously was dissolved in saline and was injected in a volume of 1 ml/kg body weight 30 min prior to nasal provocation. When chronic effects of the drugs were examined, in addition to the above-mentioned administration, drugs were given once daily for 5 consecutive days of the SART stress period.

Analysis of data

Scatchard analyses (10) were used to obtain values for the maximum number of binding sites (B_{\max}) and dissociation constants (K_D). The data in the figures are expressed as the mean \pm S.E. Statistical significance of difference was determined by Student's *t*-test or a one-way analysis of variance with Dunnett's multiple range test.

RESULTS

Effects on nasal mucosal hypersensitivity in SART-stressed guinea pigs

As parameters for nasal mucosal hypersensitivity in guinea pigs, we used the threshold of sensitivity to methacholine in causing nasal secretion or to histamine in evoking sneeze response.

As shown in Fig. 1, the threshold to methacholine in the SART-stressed control group was significantly lowered. Daily administrations of Neurotropin dose-dependently prevented the decrease in the threshold. On the other hand, ketotifen and tranilast had only slight effects, whereas atropine showed a marked effect.

As shown in Fig. 2, the threshold to histamine in the SART-stressed control group was also significantly lowered. Neurotropin dose-dependently prevented the decrease in the threshold. Ketotifen was also found to prevent the decrease.

Effects on increase in density of m-ACh-R in nasal mucosa of SART-stressed guinea pigs

The maximum binding (B_{\max}) of [^3H]QNB to the m-ACh-R in SART-stressed control group increased significantly compared with that in the normal group. Daily administrations of Neurotropin dose-dependently prevented the increase in density of m-ACh-R as seen in Fig. 3. Ketotifen and tranilast at the dose used in this study had no effect.

Group & Drug	Dose (mg/kg/day)	Methacholine (g/ml)					Threshold (g/ml)
		3×10^{-3}	10^{-2}	3×10^{-2}	10^{-1}	3×10^{-1}	
Normal control							7.31×10^{-2}
SART-stressed Control							1.12×10^{-2}
Neurotropin	50						2.31×10^{-2}
	100						7.31×10^{-2} *
Ketotifen	1						2.69×10^{-2}
Tranilast	300						2.01×10^{-2}
Atropine	0.5×1						$> 30 \times 10^{-2}$ **

Fig. 1. Effects of Neurotropin and antiallergic drugs on hypersensitivity to methacholine in causing nasal secretion in nasal mucosa of SART-stressed guinea pigs. Atropine was administered s.c. 30 min prior to nasal provocation. Other drugs were administered p.o. once daily for 5 consecutive days of the SART stress period, and the last administrations were carried out 60 min prior to nasal provocations on the 6th day. Asterisks denote significant differences from the SART-stressed control at * $P < 0.05$ and ** $P < 0.01$, respectively. Data represent the mean values \pm S.E. of 8 guinea pigs.

Effects on nasal secretion in passively sensitized guinea pigs

In normal and SART-stressed guinea pigs, passively sensitized with anti-ovalbumin serum, we examined the effects of Neurotropin and ketotifen on nasal secretion induced by allergen.

Daily administrations of Neurotropin at a dose of 200 mg/kg/day in normal guinea pigs showed a weak inhibitory action of only about 16% (Fig. 4). In SART-stressed guinea pigs in which an increase in quantity of nasal secretion was observed in comparison with that in the normal group, a single medication of Neurotropin

at a dose of 200 mg/kg had an inhibitory effect of about 34% (Fig. 5), and its daily administrations at doses of 25 to 100 mg/kg/day showed a further pronounced inhibitory effect in a dose-dependent manner (Fig. 6). The inhibitory action of daily administrations of ketotifen was also found to be stronger in SART-stressed guinea pigs than in normal ones, but no difference was found with its action by a single administration.

Combined inhibitory effects of ketotifen or tranilast with Neurotropin on nasal secretion in passively sensitized SART-stressed guinea pigs

Group & Drug	Dose (mg/kg/day)	Histamine (g/ml)						Threshold (g/ml)
		3×10^{-4}	10^{-3}	3×10^{-3}	10^{-2}	3×10^{-2}	10^{-1}	
Normal control				○ ○	○ ○	○ ○	○	1.49×10^{-2}
SART-stressed Control		● ●	● ●	● ●	●			0.13×10^{-2}
Neurotropin	50		■	■ ■	■ ■	■		0.73×10^{-2} *
	100		■	■	■ ■	■ ■	■	1.73×10^{-2} **
Ketotifen	1			▲	▲ ▲	▲ ▲	▲ ▲	2.72×10^{-2} **

Fig. 2. Effects of Neurotropin and ketotifen on hypersensitivity to histamine in evoking the sneeze response in nasal mucosa of SART-stressed guinea pigs. Other explanations are as in Fig. 1.

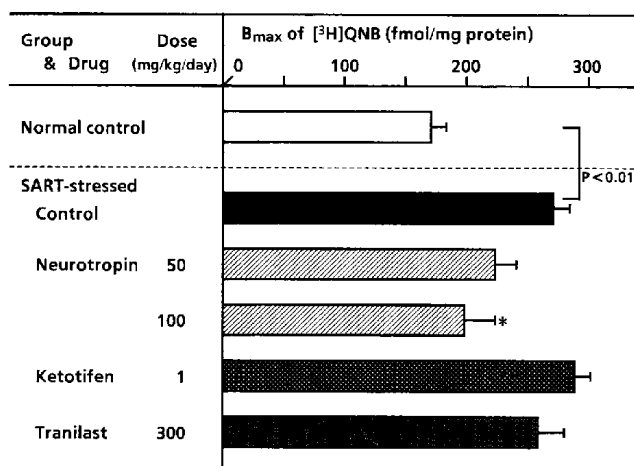


Fig. 3. Effects of Neurotropin and antiallergic drugs on changes in muscarinic ACh receptors in nasal mucosa of SART-stressed guinea pigs. Drugs were administered p.o. once daily for 5 consecutive days of the SART stress period. Asterisks denote significant differences from the SART-stressed control at * $P < 0.05$. Other explanations are as in Fig. 1.

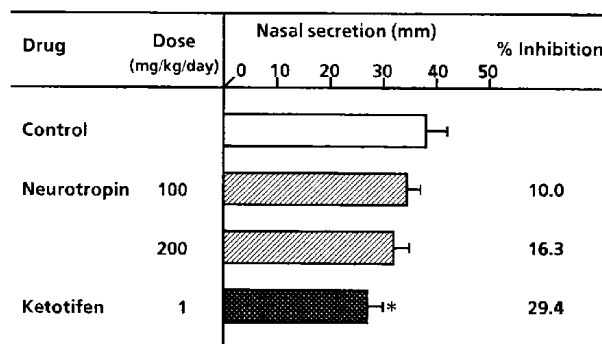


Fig. 4. Effects of Neurotropin and ketotifen on nasal secretion induced by allergen in passively sensitized normal guinea pigs. Drugs were administered p.o. once daily for 5 consecutive days, and the last administrations were carried out 60 min prior to nasal provocations on the 6th day. Asterisks denote significant differences from the control at * $P < 0.05$. Other explanations are as in Fig. 1.

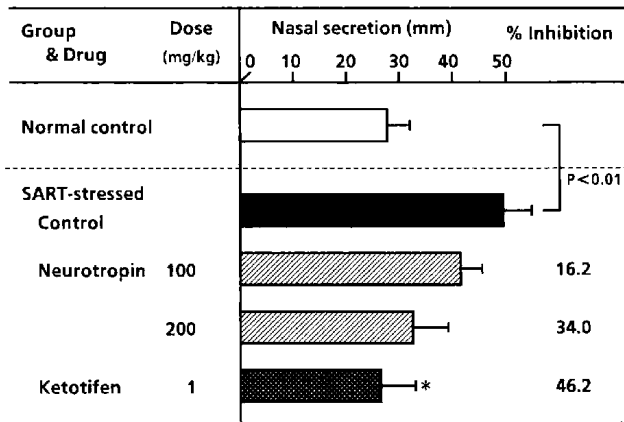


Fig. 5. Effects of a single administration of Neurotropin and ketotifen on nasal secretion induced by allergen in passively sensitized SART-stressed guinea pigs. Drugs were administered p.o. 60 min prior to nasal provocations on the 6th day. Asterisks denote significant differences from the SART-stressed control at **P* < 0.05. Data represent the mean values \pm S.E. of 7 guinea pigs.

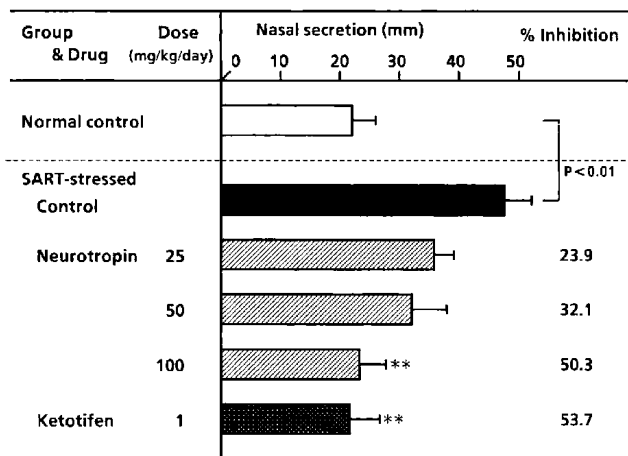


Fig. 6. Effects of daily administrations of Neurotropin and ketotifen on nasal secretion induced by allergen in passively sensitized SART-stressed guinea pigs. Asterisks denote significant differences from the SART-stressed control at ***P* < 0.01. Other explanations are as in Fig. 1.

Firstly, a dose-related inhibitory action of ketotifen or tranilast was examined as seen in Fig. 7.

Ketotifen at doses of 0.3 to 3 mg/kg/day showed a dose-dependent inhibitory action, but at a dose of 10 mg/kg/day, there was a plateau of the maximal inhibitory action. Tranilast also showed a dose-dependent inhibitory action.

Subsequently, combined actions of ketotifen or tranilast with Neurotropin were investigated.

Ketotifen alone at a dose of 0.3 mg/kg/day had a weak inhibitory action of only about 22%. When it was

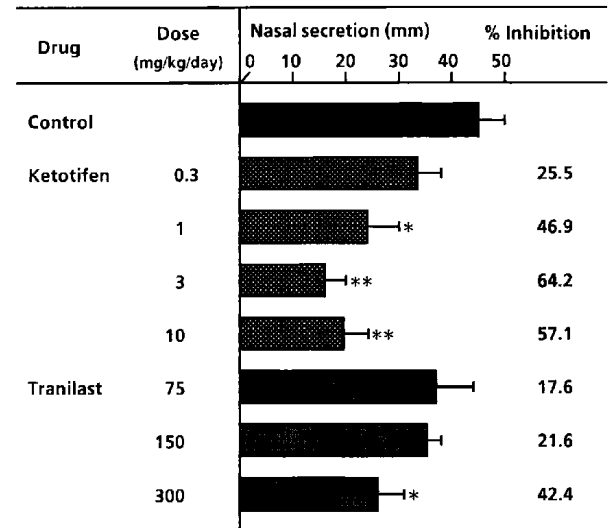


Fig. 7. Effects of ketotifen and tranilast on nasal secretion induced by allergen in passively sensitized SART-stressed guinea pigs. Other explanations are as in Fig. 1.

combined with Neurotropin at 100 mg/kg/day, however, a marked inhibitory effect was observed, which was comparable to that of a single administration of ketotifen at doses of 3 to 10 mg/kg/day. Neurotropin also potentiated the action of 3 mg/kg/day of ketotifen beyond the range of the maximal effect that can be attained by a single medication of ketotifen alone (Fig. 8).

In the combination of tranilast and Neurotropin, Neurotropin also significantly potentiated the action of tranilast (Fig. 9).

DISCUSSION

Recently, it has been widely recognized that the nasal surface basophilic cells, consisting of basophil leucocytes in the mucous blanket and mast cells in the epithelium of the nasal mucosa, increase in nasal allergy and play a key role in the appearance of nasal symptoms (11, 12). Therefore, Okuda et al. (12) emphasized that effective drugs for nasal allergy must affect at least one of the following three important factors involving in the nasal allergic reaction: the number of nasal surface basophilic cells, the sensitivity of the basophilic cells to allergen, and the nasal sensitivity to released chemical mediators. Of the therapeutic drugs for nasal allergy, topical steroids, antiallergic and antihistaminergic drugs may act on each of the factors, respectively. Neurotropin, on the other hand, exhibits little antihistaminergic activity and a weak inhibitory effect on allergen-induced histamine release from murine peritoneal mast cells, guinea pig pulmonary tissue, and human leukocytes (2, 3),

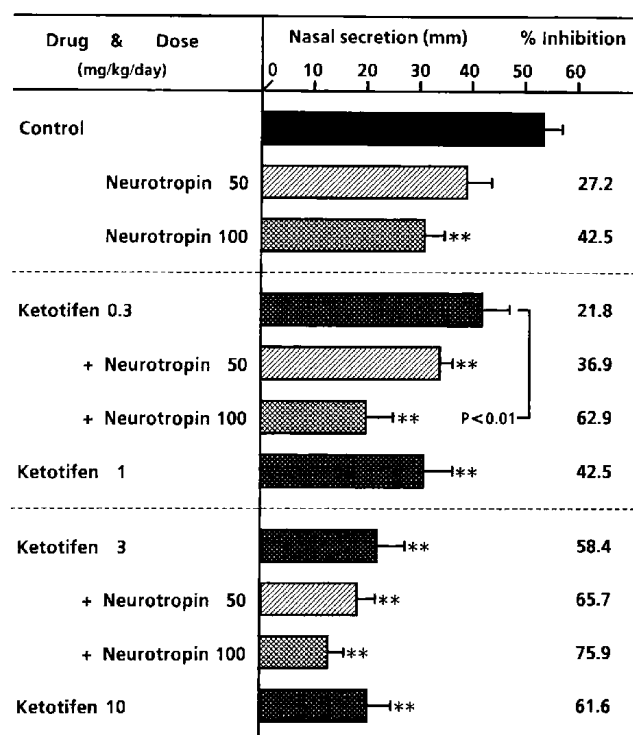


Fig. 8. Combined inhibitory effect of ketotifen with Neurotropin on nasal secretion induced by allergen in passively sensitized SART-stressed guinea pigs. Asterisks denote significant differences from the SART-stressed control at $^{***}P < 0.01$. Other explanations are as in Fig. 1.

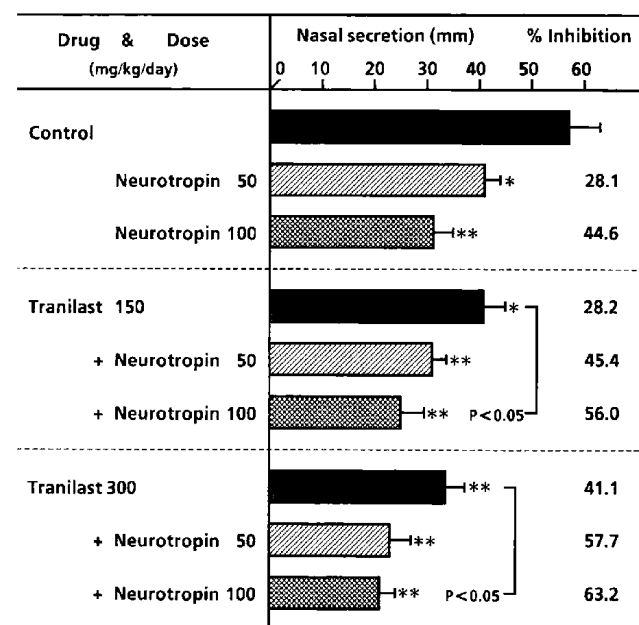


Fig. 9. Combined inhibitory effect of tranilast with Neurotropin on nasal secretion induced by allergen in passively sensitized SART-stressed guinea pigs. Other explanations are as in Fig. 1.

although it improves the increased density of m-AChR observed in the nasal mucosa of patients with nasal allergy (13). Thus, the mechanism by which Neurotropin affects the allergic state seems to be different from those of conventional antiallergic drugs, and its actions have not yet been completely clarified.

It has been empirically known that the nasal mucosa of patients with nasal allergy shows not only specific hyperreactivity to allergens but also nonspecific hyper-sensitivity and hyperreactivity to neuromediators (14, 15), irritants (16) and physical stimuli (17). However, there are very few reports on a nasal allergic animal model with nasal mucosal hypersensitivity. In our previous study on SART-stressed guinea pigs (4), we reported that SART-stressed guinea pigs had nasal mucosal hypersensitivity with an increase in density of m-AChR located at the nasal mucosa and the aggravation of nasal allergic symptoms in a passively sensitized experimental system. Thus, SART-stressed guinea pigs showing nasal mucosal hypersensitivity would serve as an animal model in the *in vivo* evaluation of antiallergic drugs.

To elucidate the mode of action of Neurotropin on nasal allergy, first we studied the effect of Neurotropin on experimental nasal mucosal hypersensitivity and nasal allergy by using SART-stressed guinea pigs. As parameters of nasal mucosal hypersensitivity, the hypersecretion induced by methacholine which directly acts on nasal glands (18, 19) and the sneeze response evoked by histamine which stimulates histamine H_1 receptors located in the sensory nerve ending as trigger (19, 20) were used in our experiments. Daily administrations of Neurotropin improved in a dose-dependent manner the changes in two parameters of nasal mucosal hypersensitivity and increased the density of m-AChR located at the nasal mucosa caused by SART stress. In addition, the inhibitory action of Neurotropin on nasal secretion in the passively sensitized model was more intense in SART-stressed guinea pigs than that in normal ones. Ketotifen and tranilast were also found to exhibit a marked effect on nasal secretion in the passively sensitized SART-stressed model. These drugs, however, had little effect on methacholine-induced hypersecretion and m-AChR density, although ketotifen with antihistaminergic action had a preventive effect on the histamine-evoked sneeze response. From these results, the mode of action of Neurotropin seems to be different from that of ketotifen or tranilast which is an inhibitor of chemical mediator release with or without antihistaminergic action. Thus, at least part of the regulatory effect of Neurotropin on nasal mucosal hypersensitivity is thought to have been brought forth through the normalizing effect on m-AChR density;

and therefore, its regulatory effect in addition to weak inhibitory action on the release of chemical mediators (2) may play a role in improvement of nasal allergic symptoms such as watery secretion and sneezing.

In the second part of the present experiments, inhibitory actions of ketotifen or tranilast combined with Neurotropin was investigated. Neurotropin significantly potentiated the action of ketotifen or tranilast, and their combined effects were of an additive or synergistic nature. Thus, the combined use of antiallergic drugs with Neurotropin, each of which has different mechanisms of action, will be a very effective therapeutic regimen for nasal allergy.

As therapeutic drugs for nasal allergy, antiallergic drugs such as disodium cromoglycate, ketotifen and tranilast, antihistaminergic drugs and topical steroids have been mainly used based on the understanding of the onset of nasal allergy. Since the mechanism of Neurotropin has to date remained unclear, it has been used in a relatively small number of occasions. However, therapeutic effect of Neurotropin through normalization of nasal mucosal hypersensitivity must be considered to be unique compared with other antiallergic drugs. Neurotropin should be used more widely in the future as a new type of therapeutic drug for nasal allergy.

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