INTERACTIONS BETWEEN THE SYMPATHETIC AND SENSORY INNERVATION OF THE IRIS¹

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Abstract

The interaction between peptidergic, sensory nerves and sympathetic fibers was examined in the rat iris. The putative peptide neurotransmitter, substance P, was used as an index of the sensory innervation, because the peptide is exclusively localized in the iris to trigeminal sensory fibers. Extirpation of the sympathetic, superior cervical ganglion resulted in an increase in iris content of substance P-like immunoreactivity (henceforth SP), suggesting that sympathetic terminals influence the peptidergic sensory innervation of the iris. The increase in iris peptide after sympathetic ganglionectomy was reversed by implantation of sympathetic ganglia into the anterior chamber of the eye. Pharmacological stimulation or blockade of sympathetic nerve impulse activity and pharmacological blockade of sympathetic axonal transport did not alter iris peptide, suggesting that these procedures did not mediate the sympathetic-sensory interaction. However, injection of nerve growth factor (NGF) systemically or into the anterior chamber increased iris peptide, reproducing the effects of ganglionectomy. Conversely, injection of antiserum to NGF (anti-NGF) into the anterior chamber decreased iris SP suggesting that endogenous trophic protein normally regulates sensory peptide. The effects of anti-NGF were transitory; iris peptide returned to normal after cessation of treatment. Consequently, anti-NGF administration apparently did not lead to sensory neuron destruction, but rather altered either the number of sensory fibers in the iris or the amount of peptide per fiber. Finally, injection of anti-NGF into the anterior chamber reversed the effects of sympathetic ganglionectomy, suggesting that NGF may mediate the sympathetic-sensory interaction. Our observations suggest that competition for target NGF may result in reciprocal regulation of the iris sympathetic and sensory innervation.

Survival and maturation of a variety of neuronal types depend upon interactions with target tissues (Hamburger, 1934; Prestige, 1967; Landmesser and Pilar, 1974). For example, developing neurons deprived of target influences fail to express neurotransmitter phenotypic traits normally (Hendry and Iversen, 1973; Dibner and Black, 1976; Kessler and Black, 1980), and overall neuronal survival is reduced (Hamburger, 1934). Moreover, the maintenance of *adult* phenotypic characters and of organotypic patterns of innervation requires continuing neuron-target interactions during maturity (Olson and Malmfors, 1970; Hendry and Thoenen, 1974).

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Target regulatory processes may be extremely complex, involving interactions among multiple co-innervating neuronal populations and the target itself. For example, we recently found that peptidergic sensory nerves in the iris are modulated by co-innervating sympathetic fibers (Kessler et al., 1983). In the iris, the putative peptide neurotransmitter, substance P (SP), is localized to sensory fibers arising from the trigeminal ganglion (Cuello et al., 1978; Butler et al., 1980; Tervo et al., 1980; Miller et al., 1981; Kessler et al., 1983). Extirpation of the innervating sympathetic, superior cervical ganglion (SCG) increased iris SP, and this rise was abolished by trigeminal ablation. Consequently, sympathetic terminals apparently regulate sensory peptidergic innervation of the iris (Kessler et al., 1983). The present communication examines mechanisms underlying these regulatory interactions.

To facilitate analyses, we define the sympathetic-sensory interaction in the iris in greater detail, using SP to monitor the trigeminal innervation. Recent studies suggest that some innervating neurons and their targets

exert reciprocal effects through the mediation of nerve growth factor (NGF): targets regulate sympathetic (Hendry and Thoenen, 1974) and peptidergic sensory (Kessler and Black, 1980) neurons via this trophic protein. Conversely, sympathetic and sensory innervation appears to influence target elaboration of NGF (Ebendal et al., 1980). Consequently, in the present experiments we focused on the role of NGF in sympathetic-sensory interactions in the iris. We find that NGF increases the levels of SP in iris trigeminal fibers and that the effects of sympathetic ganglionectomy on sensory peptide seem to be mediated by increased availability of NGF. Our observations suggest that the balance between sympathetic and sensory innervation of the iris may be regulated by competition for target NGF.

Materials and Methods

Experimental animals. Sprague-Dawley rats weighing 150 to 175 gm (Charles River Breeding Laboratories) were housed in clear plastic and wire cages and were exposed to 540 to 810 lux of cool white fluorescent illumination from 5 A.M. to 7 P.M. daily. Ralston Purina Lab Chow and water were offered *ad libitum*.

Surgical procedures. All rats were anesthetized with halothane (3% in 100% oxygen at 2.5 liters/min), and the two major postganglionic nerves of the SCG were exposed using a dissecting microscope. Axotomy was performed as described previously (Kessler and Black, 1979). Injections of drugs were given subcutaneously to the nape of the neck except when otherwise noted.

Implantation of colchicine-impregnated Silastic cuffs on the postganglionic nerves of the SCG was performed as described previously (Kessler and Black, 1979). Postoperatively, each animal was carefully examined for pupillary equality, pupillary reactivity to light, and elevation of the eyelid. Occasional animals (approximately 5%) showed some signs of ptosis or miosis and were discarded.

Transplantation of ganglia to the anterior chamber of the eye was performed as described by Olson et al. (1979).

Radioimmunoassay. SP-like immunoreactivity (henceforth SP) was measured by immunoassay as described previously (Kessler and Black, 1980).

Preparation of NGF. β -NGF was prepared from adult male mouse salivary glands by the method of Mobley et al. (1976). NGF was inactivated by heating it to 70°C for 30 min.

Statistics. Data were analyzed by the Student's *t* test or by analysis of variance (Anova).

Results

Effects of ganglionectomy. Previously, we reported that superior cervical ganglionectomy increased SP in trigeminal fibers innervating the iris (Kessler et al., 1983). To define the time course of this process, unilateral ganglionectomy was performed on adult rats, and the ipsilateral and contralateral (control) irides were examined after varying periods for SP content.

The effects of ganglionectomy on iris peptide developed slowly, leading to a chronic elevation of SP. There was no significant difference between the operated and control sides at days 1 or 4 postganglionectomy (Fig. 1).

Figure 1. Time course of the effects of sympathectomy on iris SP. Unilateral extirpation of the superior cervical ganglion was performed (*open circles*), and the contralateral, unoperated side acted as control (*solid circles*). After varying periods of time, the irides were removed and examined for content of SP. Each value represents eight animals and is expressed as mean picograms of SP per iris $\pm$ SEM. *, Differs from respective control at $p < 0.02$; **, differs from respective control at $p < 0.01$.

At 8 days and thereafter, however, ipsilateral iris SP was significantly elevated, exceeding control by about 55%. Thus ganglionectomy elevated iris SP to a new plateau level, suggesting that the SCG normally exerts an inhibitory influence on iris peptide.

Effects of SCG transplantation into the anterior chamber. Sympathetic ganglia transplanted into the anterior chamber of the eye elaborate neurites and innervate the host iris (Olson and Malmfors, 1970). To determine whether the increase in iris SP after ganglionectomy could be reversed by sympathetic reinnervation, ganglia were transplanted into the eyes of previously ganglionectomized animals. Surgery was performed in two stages. Unilateral superior cervical ganglionectomy or sham ganglionectomy was followed 7 days later by transplantation of two neonatal superior cervical ganglia into the ipsilateral anterior chamber. Three weeks after transplantation, the ipsilateral and contralateral irides were examined for SP content. Sham transplantation did not alter the expected increase in SP after ganglionectomy (Fig. 2). However, transplantation of sympathetic ganglia reversed the effects of ganglionectomy; SP levels after combined ganglionectomy and transplantation were reduced to control values. Finally, transplantation of sympathetic ganglia into animals with intact sympathetic innervation had no effect on iris peptide. These observations suggest that the increase in iris peptide subsequent to sympathetic ganglionectomy is reversible.

Role of nerve impulse activity. The inhibitory influence of the SCG on iris SP could be mediated by a variety of factors, including: (1) nerve impulse activity, (2) orthograde axonal transport and release of a substance inhibitory to trigeminal SP, or (3) competition by sympathetics with trigeminal terminals for a stimulatory factor. To help distinguish among the alternatives, we initially sought to determine the role of nerve impulse activity in regulating iris peptide. Animals were treated

with a variety of drugs which modulate sympathetic activity and/or adrenergic receptor function. To facilitate interpretation of the results, the drugs administered are listed according to the major action on the SCG, although it is recognized that many of them have multiple effects. For example, reserpine not only increases sympathetic nerve impulse activity, but also has other prominent actions, such as depletion of catecholamine. The drugs examined included:

1. α receptor blockade: Phenoxybenzamine (10 mg/kg, i.p., b.i.d. for 8 days); phentolamine (15 mg/kg, s.c., b.i.d. for 8 days).

2. β receptor blockade: Propranolol (25 mg/kg, i.p., q.i.d. for 8 days).

3. Ganglion (synaptic) blockade: Chlorisondamine (10 mg/kg, s.c., b.i.d. for 8 days).

4. Ganglion stimulation: Nicotine (2.5 mg/kg, s.c., q.d. for 8 days); reserpine (2.5 mg/kg, s.c. q.o.d. for 8 days); tyramine (15 mg/kg, i.p., b.i.d. for 8 days).

None of these drugs significantly altered iris SP. Moreover, decentralization (denervation) of the SCG also had no effect on iris peptide. These observations *in toto* suggest that sympathetic impulse activity or receptor stimulation does not mediate the effects of sympathetics on iris SP.

Effects of colchicine cuff implantation and axotomy. Axonal transport in postganglionic nerves of the SCG was blocked by local application of colchicine to examine the role of this process in regulating iris SP. Colchicine was applied by implanting Silastic cuffs impregnated with the drug (Kessler and Black, 1979). Four groups of animals were studied: (1) unilateral colchicine cuffs; (2) unilateral blank (no drug) cuffs; (3) unilateral postganglionic axotomy; (4) sham operation (control). Animals with colchicine cuffs and with blank cuffs were main-

Figure 3. Effects of colchicine cuffs or axotomy on iris SP. Unilateral implantation of colchicine-impregnated cuffs or blank (drug-free) cuffs or axotomy was performed on the postganglionic nerves of the SCG, and the contralateral side acted as control. Two weeks after surgery the irides were examined for SP content, which is expressed as mean picograms per iris $\pm$ SEM. $N = 10$. *, Differs from all other groups at $p < 0.05$.

tained for 14 days without ptosis or miosis, indicating that electrophysiological integrity was maintained. In contrast, rats subjected to unilateral axotomy exhibited the expected ipsilateral ptosis and miosis, which persisted for the entire 14-day period. Iris peptide was not significantly altered by either colchicine cuffs or blank cuffs (Fig. 3). By contrast, axotomy increased SP 58%, reproducing the effects of ganglionectomy.

Effects of nerve growth factor. The foregoing experiments suggested that neither impulse activity nor axonal transport mediated the effects of the SCG on trigeminal SP. We sought, therefore, to determine whether the SCG-trigeminal interaction might result from competition by sympathetic terminals for a substance stimulatory to trigeminal SP. NGF seemed a likely candidate, because sympathetic nerve terminals actively take up the trophic protein (Hendry et al., 1974) and because the iris may produce NGF (Ebendal et al., 1980).

To determine whether trigeminal SP is influenced by NGF, neonates were injected with the protein or buffer, and the irides were examined for SP. Inasmuch as systemic injection of adults requires large quantities of NGF, neonates were used to conserve the protein. Injection of NGF significantly increased SP in the trigeminal ganglion (218% of control) and also in the iris (171%) and cornea (158%), trigeminal target organs (Fig. 4).

Effects of NGF injection into the anterior chamber. To determine whether local concentrations of NGF can influence iris SP, the protein was injected directly into the anterior chambers of adults. Unilateral injections were performed, and the contralateral eyes served to control for systemic effects. Animals were anesthetized and injected with either NGF or heat-inactivated NGF, and a third group was anesthetized but not injected. After 7 days of treatment, the ipsilateral and contralateral irides were examined for SP content. Injection of

Figure 2. Effects of combined sympathectomy and transplantation of sympathetic ganglia to the anterior chamber. Unilateral superior cervical ganglionectomy was performed, and the contralateral, unoperated side acted as control. One week later, the animals received either ipsilateral transplantation of two neonatal superior cervical ganglia into the anterior chamber or sham transplantation. Three weeks after the transplantations, the irides were examined for content of SP, which is expressed as mean picograms per iris $\pm$ SEM. $N = 16$. *, Differs from all other groups at $p < 0.025$.

heat-inactivated NGF did not significantly affect iris SP (Fig. 5). However, injection of NGF significantly increased SP by 43% in the ipsilateral iris without a change in the contralateral iris, suggesting that local concentrations of NGF stimulate iris SP.

Figure 4. Effects of nerve growth factor administration. Neonates were injected with either NGF (100 μ g/gm of body weight, s.c.) or saline daily for 7 days. The trigeminal ganglia, irides, and corneas were then examined for content of SP, which is expressed as mean picograms $\pm$ SEM. $N = 10$. *, Differs from respective control at $p < 0.001$; **, differs from respective control at $p < 0.01$.

Figure 5. Effects of nerve growth factor injection into the anterior chamber of the eye. Unilateral injections of NGF (3 μ g in 10 μ l) or heat-inactivated NGF were performed daily into the anterior chamber of the eye. A third group of animals was anesthetized without being injected. After 7 days of treatment, the ipsilateral and contralateral irides were examined for content of SP, which is expressed as mean picograms per iris $\pm$ SEM. $N = 10$. SP in the contralateral irides (not depicted) in each group did not differ significantly from the uninjected control. *, Differs from other groups at $p < 0.05$.

Figure 6. Effects of anti-NGF injection into the anterior chamber of the eye. Unilateral injections of anti-NGF (75 units in 10 μ l) or control serum were performed daily into the anterior chamber of the eye. A third group of animals was anesthetized without being injected. After 7 days of treatment, the ipsilateral and contralateral irides were examined for content of SP, which is expressed as mean picograms per iris $\pm$ SEM. $N = 10$. SP in the contralateral irides (not depicted) in each group did not differ significantly from the uninjected control. *, Differs from other groups at $p < 0.02$.

Figure 7. Recovery from effects of anti-NGF injection into the anterior chamber of the eye. Unilateral injections of anti-NGF (75 units in 10 μ l) (open circles) or control serum (solid circles) were performed daily for 7 days into the anterior chamber of the eye. The ipsilateral irides were examined at varying periods of time after cessation of treatment for content of SP, which is expressed as mean picograms per iris $\pm$ SEM. *, Differs from control at $p < 0.01$.

Effects of anti-NGF injection. To determine whether endogenous NGF normally regulates iris peptide, unilateral injections of antiserum to NGF (anti-NGF) were performed. The anterior chambers of adult rats were injected with anti-NGF or control serum daily for 7 days. A third group of animals was anesthetized but not injected. After 7 days, the ipsilateral and contralateral irides were examined for SP content. Injection of control serum did not significantly change iris peptide (Fig. 6).

Figure 8. Effects of combined sympathectomy and injection of anti-NGF into the anterior chamber of the eye. Unilateral superior cervical ganglionectomy or sham operation was performed, and the contralateral, unoperated side served as control. Ipsilateral injections of anti-NGF (75 units in 10 μ l) or control serum were performed daily into the anterior chamber of the eye. After 7 days of treatment, the ipsilateral and contralateral irides were examined for content of SP, which is expressed as mean picograms per iris $\pm$ SEM. $N = 12$. *, Differs from control serum groups at $p < 0.05$; **, differs from all groups at $p < 0.025$; ***, differs from control serum groups at $p < 0.03$. Does not differ from sham and anti-NGF group.

However, anti-NGF administration significantly decreased SP by 31% ipsilaterally, without contralateral alteration, suggesting that local, endogenous NGF normally regulates iris peptide.

To determine whether the effects of anti-NGF are reversible, the anterior chambers of adult rats were injected with either anti-NGF or control serum daily for 7 days. Treatment was then stopped, and the irides were examined after varying periods of time (Fig. 7). Iris peptide returned to normal after cessation of anti-NGF treatment. Although iris SP was reduced by 34% on the last day of treatment, peptide levels were normal 2 weeks later.

To determine whether NGF mediates the increase in iris SP after sympathetic ganglionectomy, rats were treated with anti-NGF after surgery. Immediately subsequent to unilateral ganglionectomy or sham operation, the ipsilateral anterior chambers were injected with either control serum or anti-NGF. Seven days postoperatively the ipsilateral and contralateral irides were assayed. Iris peptide in the contralateral (control) irides was the same in all groups, excluding systemic effects of anti-NGF. Injection of control serum did not alter the expected increase in iris SP after ganglionectomy (Fig. 8). However, anti-NGF injection completely blocked the effects of ganglionectomy and, in fact, decreased iris peptide to the same level in ganglionectomized and sham-operated animals (Fig. 8).

Discussion

Cellular interactions critically regulate neuronal ontogeny, as well as maintenance of neuronal function in adults. Target regulation of neuronal survival and of the quantitative expression of transmitter phenotypic characters is a generalized phenomenon found throughout

the peripheral (Hamburger, 1934; Hendry and Iversen, 1973; Landmesser and Pilar, 1974) and central (Cowan, 1970; Bjorkland et al., 1975) nervous systems. Less clearly understood are interactions which occur among different neuronal populations innervating the same target. In the present study we have examined the relationship between sympathetic and peptidergic sensory nerves in the iris. Our observations suggest that sympathetic and sensory fibers compete for NGF within the target iris.

Previously we reported that surgical or chemical sympathectomy increased SP in the iris and that ablation of the trigeminal ganglion abolished this increase. These observations suggested that sympathetic terminals modulate the sensory peptidergic innervation of the iris by influencing either the number of iris trigeminal fibers or the amount of peptide in each fiber. In the present study we first sought to define the sympathetic mechanisms that depressed SP in iris sensory fibers. This interaction could be mediated by a number of mechanisms, including (1) sympathetic impulse activity, (2) orthograde axonal transport of an inhibitory factor, or (3) sympathetic terminal competition for a stimulatory factor. A number of observations suggested that impulse activity and orthograde axonal transport did not mediate this interaction. To assess the role of impulse activity, various manipulations were performed to either stimulate or block neurotransmission at different anatomical levels in the sympathetic neuraxis. Impulse activity was inhibited by denervating ganglia, blocking ganglionic transmission (chlorisondamine), and blocking noradrenergic receptors (phenoxybenzamine, phentolamine, propranolol). Impulse activity was stimulated by increasing presynaptic activity (reserpine), directly stimulating ganglion cholinergic receptors (nicotine), and stimulating norepinephrine release (tyramine). None of these manipulations

influenced iris peptide, suggesting that sympathetic impulse activity itself did not depress iris SP. Moreover, implantation of sympathetic ganglia into the ocular anterior chamber reversed the effects of ganglionectomy. Inasmuch as implanted ganglia lacked neural input, it seemed unlikely that impulse activity mediated their regulatory influences or iris peptide.

It also seemed unlikely that orthograde axonal transport was involved in the sympathetic-trigeminal interaction, because blockade of transport by colchicine did not affect iris peptide. Although colchicine also blocks *retrograde* axonal transport to the SCG, this treatment presumably does not prevent uptake of material by peripheral terminals. Consequently, we sought to determine whether the remaining possibility, sympathetic competition for a stimulatory factor, mediated the SCG-trigeminal interaction.

NGF was a likely candidate for a number of reasons. First, it is well established that sympathetic terminals in the iris actively take up NGF (Hendry et al., 1974). Moreover, NGF increases SP in dorsal root ganglion sensory neurons and their processes (Kessler and Black, 1980, 1981; Otten et al., 1980), raising the possibility of a similar effect on trigeminal sensory neurons. Finally, the iris itself may be a source of NGF (Ebendal et al., 1980). Consequently, we sought to determine whether NGF might be involved in the sympathetic-trigeminal interaction. In fact, our observations strongly suggest that NGF regulates the sensory innervation of the iris and mediates the effects of sympathectomy on iris peptide.

Treatment of neonates systemically with NGF elevated SP in the trigeminal ganglion and in its targets, the iris and cornea. Consequently, trigeminal sensory neurons apparently possess NGF receptors, the activation of which increases SP. Furthermore, injection of NGF into the ocular anterior chamber of adults increased SP in the ipsilateral iris, suggesting that iris sensory fibers can take up the trophic material.

To determine whether endogenous NGF *normally* regulates the iris peptide, anti-NGF was injected into the anterior chamber. Treatment significantly decreased iris SP, suggesting that endogenous NGF normally maintains elevated levels of the sensory peptide. Because the effects of anti-NGF were transitory, the decrease in SP after treatment presumably did not represent cell death but rather a more selective alteration of neuronal metabolism.

The effects of anti-NGF on iris peptide implied that there was a source of NGF in the eye. Although it was possible that the innervating nerves themselves elaborated the trophic protein, it was more likely that the target itself elaborated NGF. Ebendal et al. (1980) were unable to detect NGF in the normally innervated iris but readily detected the protein after sympathetic or sensory denervation. The authors suggested, therefore, that the iris made NGF only when denervated. As they noted, however, it is also possible that the innervated iris produced NGF, which was promptly removed by sympathetic and sensory terminals, precluding detection. This interpretation is consistent with our observations and provides insight into the mechanisms mediating the sympathetic-sensory interaction.

Injection of anti-NGF into the anterior chamber prevented the increase in trigeminal SP after sympathectomy, suggesting that NGF mediated the rise. Consequently, sympathectomy may increase the availability of NGF to trigeminal fibers by removing a source of competition for the trophic protein. It is thus possible that sympathetic and trigeminal sensory fibers compete for NGF elaborated by the target iris and thereby exert reciprocal regulatory influences. More generally, targets may regulate innervating fibers derived from different neuronal populations by elaborating a single molecular species. Conversely, nerve terminals derived from functionally dissimilar populations may interact indirectly by competing for the same trophic substance elaborated by a mutual target.

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