EFFECT OF STIMULATION OF THE SUPERIOR CERVICAL GANGLION ON CATECHOLAMINE FLUORESCENCE OF THE CEREBRAL ARTERIES IN CATS

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In order to elucidate the effect of stimulation of the cervical sympathetic nerve on catecholamine (CA) fluorescence of the cerebral arteries, unilateral superior cervical ganglia (SCG) of 18 adult cats were stimulated under pentobarbital anesthesia while 15 cats were used as controls. Short segments of the large arteries of the cerebral base were taken out immediately after various periods of electrical stimulation, processed through Falck-Hillarp's method, and embedded in paraffin, then 8μ thick cross sections were serially cut. Fluorescence photomicrographs were taken on both sides of the respective vessels and the intensities of CA fluorescence were compared with each other. The results obtained were as follows:

1. CA fluorescence was identified in the cerebral vascular walls of the controlled animals and there was no significant difference in CA fluorescence observed between the two sides of the respective vessels.
2. In the experimental animals, CA fluorescence was identified on both sides and it generally increased in the vascular walls of the stimulated side as compared with the unstimulated side. The increase of CA fluorescence on the stimulated side was prominent in the internal carotid arteries, while, it was noted least marked in the posterior cerebral arteries.

The existence of adrenergic nerve terminals in the walls of the cerebral blood vessels is well known (12, 13, 19, 20, 21, 22, 24, 25, 27, 31, 32). However, the results of earlier studies have occasionally been in conflict as to the function of these vascular nerves (1, 4, 5, 9, 14, 17, 24, 26, 28, 29, 33). The nerves that invest cerebral arteries were studied in various animals by light microscopy (25, 31). Penfield (25) demonstrated by silver impregnation staining that nerve fibres lie on or in the adventitia of intracerebral pial arteries and that they lose their myelin sheath as they leave the pia on to intracerebral vessels. Electron microscopy has demonstrated adrenergic and nonadrenergic nerve terminals in the adventitial medial junction of the cerebral blood vessels (13, 19, 27). On the other hand, by using the histochemical fluorescence technique developed by Falck and Hillarp (6, 7), abundant CA-containing nerve plexuses have been demonstrated in the arterial walls of the

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circle of Willis which disappeared after cervical sympathectomy (12, 22, 24).

Since Forbes and Wolff (8) reported constriction of the pial artery by cervical sympathetic stimulation, many investigators have reported various degrees of vascular response and change in cerebral circulation in response to stimulation of cervical sympathetic nerves (7, 14, 17, 24, 28, 29, 33). There have been as many reports which demonstrate the absence of response (1, 4, 9, 26). These studies were mostly carried out by means of physiological techniques. The present study was designed to demonstrate histochemically the changes in CA fluorescence in the walls of cerebral arteries in response to stimulation of the superior cervical ganglion.

MATERIALS AND METHODS

Thirty-three adult cats of both sexes weighing about 2 to 4 kg were used for the study. Fifteen cats were used as control; six unanesthetized and nine anesthetized. The remaining eighteen cats received electric stimulation of SCG on either one of two sides, nine receiving it on the right side and nine on the left. These eighteen experimental animals and anesthetized controls were injected with pentobarbital (30 mg/kg, i.v.), mechanically ventilated with a respirator (Takahashi type) via an endotracheal tube. The cervical sympathetic trunk and the ipsilateral SCG were isolated on one side. After the sympathetic trunk was cut cranial to the stellate ganglion, SCG was stimulated continuously with 5–10 volts, 10–20 Hz, 2 msec square waves for 1 min to 14 hr. Effectiveness of sympathetic stimulation was confirmed by noting pupillary dilation during electrical stimulation. The animals were decapitated immediately after stimulation was terminated and the short segments of the intracranial arteries, i.e. internal carotid, anterior cerebral, middle cerebral, posterior cerebral and basilar arteries, were quickly but carefully taken out with the aid of an operating microscope. The anesthetized control cats were decapitated after being anesthetized for about 1 hr, which corresponded approximately to the total anesthesia time for the stimulated animals except for the cats stimulated for 3 and 14 hr. Unanesthetized control animals were decapitated without any treatment. The specimens obtained were quickly frozen in isopentane cooled to its melting point at -160°C by liquid nitrogen according to the technique of Nagata *et al.* (18). They were then dried *in vacuo* at -50°C for 24 hr (Freeze-Drying Apparatus Model 3-SE), followed by several hours of gradual raise in temperature to 30°C , treated with formaldehyde gas at 80°C for 1 hr in a closed gas vessel containing paraformaldehyde according to Falck-Hillarp's method (6, 7) and infiltrated with paraffin at 60°C for 20 min. Cross sections were cut serially at $8\ \mu$ thickness and mounted in liquid paraffin after removal of paraffin with xylene. 150 serial cross sections were made from each arterial specimen and the first 5 sections of every 30 sections were observed with the fluorescence microscope and photographed under the same conditions. The microscope used was an Olympus FLM type equipped with an HBA-200 W/4 high pressure mercury lamp, BV excitation filter (transmission range; 320–500 nm, peak at 410 nm), dark field condenser for immersion oil, and Y-50 barrier filter (50% transmission at 500 nm).

The data used for analysis was obtained only from the experimental animals that showed distinct pupillary dilatation on SCG stimulation and that had no

apparent circulatory distress or other complications. The data used for analysis of the paired arteries, i.e. internal carotid, anterior, middle and posterior cerebral arteries, was obtained from fourteen cats, consisting of six controls (two unanesthetized and four anesthetized) and eight cats stimulated on either side of their SCG. Data from ten cats was used for analysis of the basilar arteries (seven stimulated and three controlled). The time of SCG stimulation in these seven cases ranged from 3 min to 14 hr.

FIG. 1. Grading of CA fluorescence. 1A represents extremely dense fluorescence (grade ###), 1B; large number of fluorescent varicosities some of which are confluent (grade ##), 1C; small number of fluorescent varicosities (grade ++), 1D; fluorescence identifiable but minimal (grade +) and 1E; no fluorescence visible (grade -). $\times 110$

RESULTS

CA fluorescence was observed in the adventitial layers of all the arterial vessels examined in the form of various sized granular varicosities of one to several layers. The intensity of CA fluorescence was estimated in 25 enlarged ($\times 200$) photographs from each vessel. For purposes of comparison, a histogram of CA fluorescence was made for each artery. In making a histogram, CA fluorescence was graded into five grades: grade (###) indicates extremely dense fluorescence, grade (##) a large number of fluorescent varicosities, some of which are confluent, grade (++) a small number of fluorescent varicosities, grade (+) fluorescence identifiable but minimal and grade (—) no fluorescence visible (Fig. 1).

1. Control animals without stimulation

In a case of anesthetized control (cat No. 24), there was no difference in CA fluorescence between the two sides of the respective vessels and fluorescence was most intense in the internal carotid arteries (Figs. 2A and 2B), with the anterior (Figs. 2C and 2D) and middle cerebral arteries (Figs. 2E and 2F) showing somewhat less intensity and the posterior cerebral arteries (Figs. 2G and 2H) showing the least. This was generally true in all the remaining cases. The histogram of this case (cat No. 24) is shown in Fig. 3. Of 23 paired vessels from six controlled animals studied, no side-to-side asymmetry of CA fluorescence was observed except in the case of one pair, which were the middle cerebral arteries of an unanesthetized cat. There was essentially no difference in CA fluorescence between anesthetized and unanesthetized controls when the respective vessels were compared with each other.

2. SCG stimulated animals

A. One minute stimulation

In a cat whose right SCG was stimulated for 1 min (cat No. 29), the internal carotid and anterior cerebral arteries showed a difference in CA fluorescence between the two sides. Fluorescence was more intense on the stimulated sides (Figs. 4A and 4C) than on the unstimulated sides (Figs. 4B and 4D). The histogram of this case (cat No. 29) is shown in Fig. 5. Analysis of 8 paired vessels from two cats in this group, both of which were stimulated on the right side, showed an increase in CA fluorescence on the stimulated side of four paired vessels (50%); two in the anterior cerebral, and one in the internal carotid and middle cerebral arteries (Table 2). No difference between the two sides was found in any of the posterior cerebral arteries.

B. Three minutes stimulation

In the case of 3 min stimulation of right SCG (cat No. 29, Fig. 6), all paired vessels except the posterior cerebral arteries showed a difference in CA fluorescence; more intense on the stimulated sides (Figs. 6A, 6C and 6E) than the

FIG. 2. Representative photographs of the individual vessels of an anesthetized control (cat No. 24). A & B; internal carotid, C & D; anterior, E & F; middle and G & H; posterior cerebral arteries. Photographs of the right sides of the vessels are in the left column and those on the left sides in the right column. Note that there is no difference in CA fluorescence between the two sides of the respective vessels. Fluorescence is most intense in the internal carotid and least intense in the posterior cerebral arteries. $\times 110$

FIG. 3. Histogram of CA fluorescence of an anesthetized control (cat No. 24). The abscissa indicates the number of photographs in per cent and the ordinate the grade of CA fluorescence. R: right, L: left, IC: internal carotid, AC: anterior cerebral, MC: middle cerebral, PC: posterior cerebral arteries. There is no significant difference between the two sides of the respective vessels.

TABLE 1. Comparison of CA fluorescence between the two sides of the arteries in the control group

Groups	No. of animals	No. of paired vessels	Comparison of CA fluorescence between two sides	
			Different	Not different
Unanesthetized	2	7	1*	6
Anesthetized	4	16	0	16
Total	6	23	1(4%)	22(96%)

* middle cerebral artery

unstimulated (Figs. 6B, 6D and 6F). The histogram of this case is shown in Fig. 7. Of 15 paired vessels from four cats belonging to this group, increase of CA fluorescence on the stimulated side was encountered in 67% and no increase in 34% of the vessels (Table 2). One pair of middle cerebral arteries exhibited less intense fluorescence on the stimulated side. Of the 10 paired vessels which showed increased CA fluorescence on the stimulated sides, four were internal carotid, three middle cerebral, two anterior cerebral and one posterior cerebral arteries. Seven of 11 paired vessels (64%) from three cats whose right SCG were stimulated and

FIG. 4. Representative photographs of the individual vessels of cat No. 29, whose right SCG was stimulated for 1 min. Note increased fluorescence on the stimulated side seen in the internal (4A) and anterior cerebral (4C) arteries. No significant difference between the two sides noted in the middle (4E & 4F) and posterior (4G & 4H) cerebral arteries.

FIG. 5. Histogram of CA fluorescence of cat No. 29, whose right SCG was stimulated for 1 min. Increase in CA fluorescence on the stimulated side is shown in the right internal carotid (IC) and anterior cerebral (AC) arteries. No significant increase is shown in the middle (MC) and posterior (PC) cerebral arteries. Abbreviations are the same as in Fig. 3.

TABLE 2. Comparison of CA fluorescence between the two sides of the arteries in the SCG-stimulated group as classified according to duration of stimulation

Time of stimulation	No. of animals	No. of paired vessels	CA fluorescence	
			Increase on the stimulated sides	No increase
1 min	2	8	4(50%)	4(50%)
3 min	4	15	10(67%)	4(27%)
				1*(7%)
3 & 14 hr	2	8	4(50%)	4(50%)
Total	8	31	18(58%)	13(42%)

* decrease on the stimulated side

3 of 4 paired vessels (75%) from one cat whose left SCG was stimulated showed an increase in CA fluorescence on the stimulated side.

C. Prolonged stimulation

Vessels taken from two cats stimulated for 3 and 14 hr (cats Nos. 17 and 16, respectively) generally showed low intensity of CA fluorescence, and an increase

FIG. 6. Representative photographs of the individual vessels of cat No. 25, whose right SCG was stimulated for 3 min. Letters A through H indicate the same named arteries as in Fig. 2. Increase in CA fluorescence on the right side is noted in the internal carotid (6A), anterior (6C) and middle cerebral (6E) arteries. $\times 110$

FIG. 7. Histogram of CA fluorescence of cat No. 25, whose right SCG was stimulated for 3 min. Increase in CA fluorescence is noted on the right side in the internal carotid (IC), anterior (AC) and middle cerebral (MC) arteries. Abbreviations are the same as in Fig. 3.

in fluorescence was observed in 50% of stimulated sides (Table 2). As can be seen in Fig. 8 in which the internal carotid and anterior cerebral arteries of these two cases are shown, CA fluorescence was generally less intense and increased CA fluorescence on the stimulated side was noted in the internal carotid arteries of both animals (Figs. 8A, 8B, 8E and 8F). The histogram of cat No. 17 is shown in Fig. 9.

3. CA fluorescence of the basilar artery

CA fluorescence of the basilar arteries (Figs. 10A and 10B) was generally more intense than that of the posterior cerebral arteries (Figs. 10D, 10F and 10H) but less than that of the internal carotid arteries (Figs. 10C, 10E and 10G). It was noted that unilateral SCG stimulation showed no marked effect with regard to either the side or the duration of stimulation (Figs. 10A and 10B).

FIG. 8. Representative photographs of the cases of prolonged stimulation. The upper 4 photographs are from cat No. 17, whose left SCG was stimulated for 3 hr and the lower 4 photographs are from cat No. 16, whose right SCG was stimulated for 14 hr. A, B, E & F are internal carotid and C, D, G & H are anterior cerebral arteries. The photographs of the right sides of the arteries are in the left column and those of the left sides in the right column. Note that CA fluorescence is generally low in intensity, yet differences in intensity between the sides are observed in the internal carotid arteries of both animals (B, A & E, F).

FIG. 9. Histogram of CA fluorescence of cat No. 17, whose left SCG was stimulated for 3 hr. Increase in CA fluorescence on the left side is noted in the internal carotid (IC) and middle cerebral (MC) arteries. Also note the general decrease in CA fluorescence. Abbreviations are the same as in Fig. 1.

4. Quantitative analysis

The overall increase rate (58%) of CA fluorescence on the stimulated side in Table 2 as compared with the control values in Table 1 was stochastically significant by Chi-square test ($p < 0.01$). Likewise, each one of the groups stimulated for various periods of time shown in Table 2 was significant ($p < 0.01$). As for the various vessels, the internal carotid artery demonstrated the highest frequency (88%) of increased CA fluorescence on the stimulated side, followed by the middle, anterior and posterior cerebral arteries (Table 3). Of these increase rates, all except that of the posterior cerebral artery were significant by Chi-square test ($p < 0.01$). The difference in rate of increased CA fluorescence on the stimulated

FIG. 10. Representative photographs of the basilar arteries. A is the basilar artery of an unanesthetized control (cat No. 33) and B is that of cat No. 12, whose left SCG was stimulated for 1 hr. There is no difference in CA fluorescence between the two basilar arteries. C is the left internal carotid artery of control cat No. 30 and D is the left posterior cerebral artery of control cat No. 28. E & G are right and left internal carotid arteries respectively and F & H are right and left posterior cerebral arteries respectively of cat No. 23, whose right SCG was stimulated for 3 min. Note that CA fluorescences of both basilar arteries (A & B) are more intense than those of the posterior cerebral arteries (D, F & H) and less than those of the internal carotid arteries (C, E & G).

TABLE 3. *Number of arteries showing increased CA fluorescence on the stimulated side as classified with respective vessels in the SCG-stimulated group*

Arteries	No. of paired vessels examined	No. of vessels showing increased CA fluorescence
Internal carotid	8	7(88%)
Anterior cerebral	7	4(57%)
Middle cerebral	8	6(75%)
Posterior cerebral	8	1(13%)
Total	31	18(58%)

side between the internal carotid and posterior cerebral arteries was significant by confidence limit (confidence 90%) as was the difference between the middle and posterior cerebral arteries. Thirteen out of 23 vessels (56%) from a total of six cats whose right SCG were stimulated and 5 out of 8 vessels (62%) from a total of two cats whose left SCG were stimulated showed an increase in CA fluorescence on the stimulated side. The difference between these two rates was not stochastically significant ($p > 0.05$).

DISCUSSION

From the results obtained in the present study, it was demonstrated that CA fluorescence in the form of varicosities was present in all the vascular walls of the arteries examined regardless of anesthesia or the side of cervical sympathetic stimulation. Adrenergic nerve terminals demonstrated as fluorescent varicosities were only found in the adventitial layers of the arteries most prominent at the adventitial medial junction. Iwayama (12) divided fluorescent fibres into two groups; one located at the outer edge of the adventitia and the other closer to the media-adventitia border. Owman *et al.* (24) histologically demonstrated that both adrenergic and cholinergic nerve plexuses were running in close correspondence in the same whole-mount preparations of pial vessels. Electron micrographs of the nerve terminals have shown an adrenergic varicosity containing electron-dense vesicles located in close apposition to a cholinergic varicosity containing electron-lucent synaptic vesicles, offering a structural possibility for an interaction mechanism between the two types of axon terminals (21, 24). Adrenergic nerve terminals have not been found in the media of the cerebral arteries (12, 13, 19, 21, 24, 27). Outside the cerebral arteries, however, Tsunekawa *et al.* (32) demonstrated CA fluorescence in the media of small to medium size (0.5 mm to 3 mm) systemic arteries of the dog. Falck-Hillarp's method was employed after pretreatment of animals with nialamide (monoamine oxydase inhibitor) and noradrenaline. They could not, however, demonstrate CA fluorescence in the inner half of the media of the systemic arteries just described.

Falck-Hillarp's method has been widely used to demonstrate biogenic monoamines. With the recent introduction of the glyoxylic acid method and Vibratome, it has become possible to demonstrate catecholamines more rapidly, simply and with higher sensitivity (2, 11, 16). The system of vascular adrenergic nerves, however, was equally well visible with the use of Falck-Hillarp's formaldehyde

and the glyoxylic acid methods (28). Because of individual differences in the animals used and the non-uniformity of the fluorescing effects in the different experiments, determination of the side-to-side differences in intensity of CA fluorescence in the walls of the arteries was most useful in the present study.

The status of the autonomic nervous system at the time of sacrificing the animals may not have been "unanesthetized control" in the sense we had expected because the cats may have been under extreme psychological tension. However, it was noted that there was no essential difference in CA fluorescence between unanesthetized and anesthetized controls. It may be that the difference was not marked enough to be detected by the method employed in the present study.

CA fluorescence was most intense in the internal carotid and least intense in the posterior cerebral arteries with the remaining circle of Willis arteries falling somewhere in between (Figs. 2 and 10). Differences in the intensity of CA fluorescence in the different named arteries have been studied histochemically by whole stretch preparations (20, 22, 28) and the results are in accord with those of the present study except that the anterior cerebral arteries have at least as rich a sympathetic nerve supply as the internal carotid arteries. The morphological and functional significance of the differences in intensity of CA fluorescence among different named vessels is not clear. In order to compare the intensities of CA fluorescence in the walls of different vessels, Sercombe *et al.* (28) counted the numbers of fluorescent fibres crossing an imaginary circumferential line of the longitudinally cut-open stretch preparations of the arteries. Edvinsson *et al.* (5) measured concentration of noradrenaline in pial vessels after sympathetic ganglionectomy, obtaining significant reduction of its concentration. These methods were not used, however, for sympathetic stimulation.

General increase in CA fluorescence on the stimulated sides as shown in the present results (Tables 2 and 3) indicates that the adrenergic nerve fibres innervating the large arteries in the base of the brain are under the influence of the superior cervical ganglion. The adrenergic nerve fibres of the pial arteries were proved both histochemically and electron microscopically to originate from the superior cervical ganglion by demonstrating specific and complete degeneration of the adrenergic nerves following bilateral superior cervical ganglionectomy (12, 20). The fact that a side-to-side difference in CA fluorescence was encountered relatively infrequently in the anterior and posterior cerebral arteries (Table 3) could partly be explained by probable bilateral contribution of the adrenergic nerve in the anterior cerebral artery and basilar artery from which the posterior cerebral artery issues (20). On the other hand, in the present study an increase in CA fluorescence on the side of SCG stimulation was encountered in the majority of the internal carotid and middle cerebral arteries (Table 3).

To obtain morphological effects through sympathetic stimulation, Yamaguchi and Waltz (33) stimulated cervical sympathetic trunk for two to four minutes before employing the specimens in radioautographic study. They reported a decrease in regional cerebral blood flow on the stimulated side in at least five of seven cats. The present author obtained the effect in 50% of specimens by 1 min and 75% by 3 min stimulation. Okubo *et al.* (23) studied the effect of prolonged cervical sympathetic stimulation (20 hr or more) by means of an implanted electric stimulator and reported angiographic constriction of the extracranial

carotid arteries and occasionally intracranial large arteries. The two cases of prolonged SCG stimulation in the present study are different, of course, from the animal preparations just mentioned in that in the present study cats were kept anesthetized throughout the period of stimulation so that various systemic parameters, metabolic or autonomic status could be made to markedly deviate from those of awake animals or anesthetized animals for a short period, although systemic blood pressure was monitored and intravenous fluid was maintained. Therefore, in this experimental set-up we may not be seeing the actual effect of SCG stimulation *per se*. It can be said, however, that in these particular animals subjected to prolonged stimulation, CA depletion took place due to a disturbed balance of synthesis-release-uptake mechanism of noradrenaline at the nerve terminals for some reason.

During sustained stimulation of postganglionic nerves, the rate of synthesis or mobilization of noradrenaline increases and a steady state of noradrenaline release per minute is eventually reached (3, 15). In the experiment using cat's spleen, noradrenaline release with continued sympathetic stimulation showed initial outpouring increase followed by gradual decline to a steady state in about 6 min (15). The steady release of the transmitter during maintained nerve impulse activity is maintained mainly by both the synthesis of the transmitter and neuronal uptake of a fraction of the released transmitter (3). Noradrenaline synthesis from tyrosine is enhanced by nerve stimulation (15, 34) while neuronal uptake is not likely to be the dominant factor regulating noradrenaline disposition in blood vessels (30). Therefore, it can be seen that the increase in CA fluorescence on the stimulated sides in the present study was mainly due to a maintained increase of CA synthesis from tyrosine and release of the transmitter during stimulation. On the other hand, in the vessels which did not show increase in CA fluorescence on the stimulated sides, it can be supposed that increased CA synthesis was not properly maintained or that for various reasons CA depletion occurred during continued stimulation.

With regard to the functional aspects of the vascular adrenergic nerves, Edvinsson *et al.* (5) recently reported reduction of cerebral blood volume of mice at the termination of sustained stimulation of preganglionic cervical sympathetic nerves for 40 sec. Sercombe *et al.* (28) reported that preganglionic electrical stimulation of the cervical sympathetic trunk invariably produced a reduction of cerebral blood flow by thermoprobes, which was more pronounced in the caudate nucleus than in the lateral geniculate body. At the same time they demonstrated that the middle cerebral artery supplying the caudate nucleus was more richly innervated by fluorescent nerves than the posterior cerebral artery supplying the lateral geniculate body. These studies strongly suggest that adrenergic nerves originating from SCG take part in the regulation of cerebral circulation.

While adrenergic nerves in the walls of pial vessels have been proved to originate from the superior cervical ganglion, it has recently been proposed on the basis of immunohistochemical localization of dopamine-beta-hydroxylase that intracerebral arteries are also innervated by adrenergic nerves which are nonsympathetic, originating from an intracerebral source which is probably the locus caeruleus (10) and unaffected by bilateral superior cervical ganglionectomy.

The present study indicates the ipsilateral increase of noradrenaline content at the nerve terminals of the large arteries in the cerebral base with SCG stimulation,

suggesting that the function of the large cerebral arteries can be affected by cervical sympathetic activity. At the present time, however, it cannot be concluded that cerebrovascular reactivity or cerebral circulation is solely the function of the autonomic nervous system in physiological state. Further investigation is required to elucidate this point.

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