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Effects of Stimulating the Nucleus Basalis of Meynert on Blood Flow and Delayed Neuronal Death Following Transient Ischemia in the Rat Cerebral Cortex

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Abstract: An increase in cortical cerebral blood flow (CBF), independent of metabolic vasodilation, via the activation of cholinergic neurons originating in the nucleus basalis of Meynert (NBM) in the basal forebrain and projecting to the widespread cortices was recently demonstrated. In the present study, we aimed to clarify whether the increase in CBF following a stimulation of the NBM can improve delayed death of the cortical neurons following transient ischemia in rats. CBF was measured with a laser Doppler flowmeter, and the delayed neuronal death of the cerebral cortex produced by intermittent (every 5 s) occlusions of the unilateral common carotid artery for 60 min was measured histologically in the cortical hemisphere at 3 different coronal levels (6 μm thickness). In control rats without occlusion there were 6,000–8,000 intact neurons and 9–19 damaged neurons in the cortical hemisphere at each coronal level. During the occlusions, CBF ipsilateral to the occluded artery decreased by 13–32% of the preocclusion level. Five days after the occlusions, the numbers of damaged neurons were increased to 75–181. Repetitive electrical stimulation was delivered to the NBM, ipsilateral to the occluded artery, starting 5 min before the occlusions and finishing around the end of them. The increase in CBF induced by NBM stimulation prevented the occlusion-induced decrease in CBF in all 3 of the cortices. The delayed death of the cortical neurons previously observed after the occlusions was scarcely observable in all the cortices when NBM was stimulated. The present results suggest that NBM-originating vasodilative activation can protect the ischemia-induced delayed death of cortical neurons by preventing a blood flow decrease in widespread cortices. [Japanese Journal of Physiology, 52, 383–393, 2002]

Key words: cerebral cortex, transient ischemia, regional cerebral blood flow, delayed neuronal death, nucleus basalis of Meynert.

In Alzheimer's disease accompanying dementia, the degeneration of cells in the basal forebrain is quite striking [1, 2]. Cholinergic neurons originating in the nucleus basalis of Meynert (NBM) and in the septal complex of the basal forebrain project to the cerebral cortex and hippocampus. Therefore there is a possible link between the cholinergic system and the cognitive mechanism. It was recently demonstrated that the cholinergic system had vasodilative action in the cerebral cortex and hippocampus (see reviews by Sato and Sato [3, 4]). These vasodilative responses are independent of both systemic blood pressure and regional cerebral metabolism. The physiological relevance of these responses, however, has not been completely understood. Neurons in the cerebral cortex and hippocampus are degenerative in Alzheimer's disease [5, 6], and they are also quite vulnerable to ischemia. The late death of neurons following transient ischemia,

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termed "delayed neuronal death," has been demonstrated to occur in the hippocampus and cerebral cortex since Kirino [7] and Pulsinelli *et al.* [8] originally found it.

From these findings, we reached a hypothesis that increases in the cortical and hippocampal blood flow induced by the activation of the basal-forebrain–originating cholinergic neural vasodilative system could protect the delayed death of cortical and hippocampal neurons because of ischemia in the cerebral cortex and hippocampus. We preliminarily stimulated the cholinergic vasodilative system by an I.V. injection of nicotine, a nicotinic acetylcholine receptor (nAChR) agonist, and produced an increase in the hippocampal blood flow by activating nAChRs of the cholinergic vasodilative system. We showed that an increase in the hippocampal blood flow following stimulation of the nAChRs reduced the delayed death of the hippocampal neurons produced by the intermittent transient occlusions of bilateral carotid arteries, besides the continuous ligation of bilateral vertebral arteries in rats [9]. Thus in the present study, we aimed to examine if an increase in the regional cerebral blood flow (CBF) in the cortex produced by electrical stimulation of the cholinergic vasodilative system of NBM could protect the delayed death of cortical neurons. Since the vasodilative response as a result of a stimulation of the unilateral NBM was elicited only in the cerebral cortex ipsilateral to the site of stimulation [10, 11], it was necessary to develop a model of cerebral ischemia that can produce the deleyed neuronal death in the cerebral hemisphere of the cortex. Thus the first purpose of the present study was to develop a Wistar rat model producing a moderate delayed neuronal death in the ipsilateral cerebral cortex by intermittent transient occlusions of the unilateral carotid artery. The second and main purpose was to clarify whether an NBM stimulation–induced increase in CBF protects the delayed neuronal death in the cortex following intermittent transient ischemia.

MATERIALS AND METHODS

The experiments were performed on 45 adult (4–6 months old) male Wistar rats (BW, 300–410 g). They were provided by the animal-breeding farm of the Tokyo Metropolitan Institute of Gerontology. The rats were maintained on a 12-h light/12-h dark photoperiod in an ambient temperature of 22±2°C. The animals were fed laboratory food with water ad libitum. Of the 45 rats, 19 rats were used for the measurement of CBF and 26 for the histological study. The rats used for the histological study were divided into 5 groups that were perfused with fixative at 5 different time points during the experiments, i.e., with no operation as the controls, at 10 min, on the 2nd day (48 h), 5th day (120 h), and 7th day (168 h) after the end of intermittent occlusions of the unilateral common carotid artery. CBF was continuously recorded before (control), during, and after the intermittent occlusions in each rat. The histological studies on the 2nd, 5th, or 7th days after the intermittent occlusions were chronic experiments; all the other histological studies and measurement of CBF were acute experiments (Fig. 1).

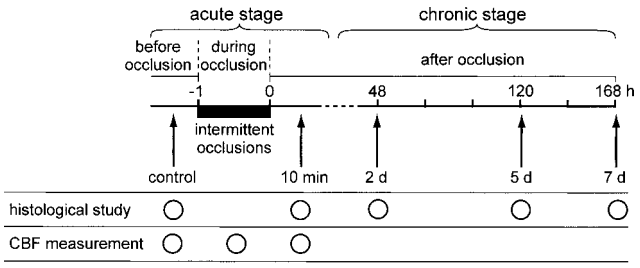

Fig. 1. Diagram showing the time schedule of the present experiments for histological study and cerebral blood flow (CBF) measurement.

General surgery and anesthesia. The animals were anesthetized with halothane, and a transient occlusion of the unilateral carotid artery was performed under halothane anesthesia. The concentration of halothane was 3.5% during the induction of anesthesia, and it was then reduced to 1.5–1.0% during the surgery and fixed at 1.0% after surgery. The trachea was intubated, and respiration was maintained with an artificial respirator (SN-480-7, Shinano, Tokyo) with an inhalation of halothane mixed in a gas composed of 30% O_2 and 70% N_2. The end-tidal CO_2 concentration, monitored with a gas monitor (1H26, NEC Sanei, Tokyo), was kept at about 4% by changing the ventilatory frequency (80–95 times/min) and volume (2.5–2.8 ml). Rectal temperature, monitored by a thermistor, was maintained at close to 37.5°C with a feedback-controlled heating pad and lamp (ATB-1100, Nihon Kohden, Tokyo). The temperature of the left lateral cranial muscle monitored by a needle-type thermistor (MGA-III, Nihon Kohden) was maintained at close to 37.5°C with another feedback controller. In experiments for the measurement of CBF, a femoral artery was cannulated for measuring the systemic mean arterial pressure (MAP).

Transient occlusion of the unilateral carotid artery. After a ventral midline cervical incision, the left common carotid artery was exposed. A small cuff (vascular occluder OC2, *In Vivo* Metric Systems, CA, USA) made of compliant silicon rubber was placed around the left common carotid artery. This apparatus

was filled with water and connected to a syringe. An occlusion of the artery was performed by inflating the cuff with water by pushing the syringe to produce ischemia. Two different types of transient occlusion were performed: (1) continuous occlusion for 10 min, which was performed in some experiments of the measurement of CBF; (2) intermittent occlusions for 60 min, which were performed in most of the experiments. In the latter, occlusions of 5 s duration were repeated 360 times with an interval of 5 s.

In the chronic experiments, immediately after the end of the intermittent occlusions, the halothane anesthesia was removed. The animals resumed spontaneous respiration and were disconnected from the respirator. Antibiotic (viccillin 50 mg/kg, I.M.) was administered. After awakening, the animals were housed at an ambient temperature of 24–26°C and fed laboratory food with water ad libitum. No motor abnormalities, such as seizures, rolling, or circling, were noted during the 2- to 7-d survival period following the ischemic insults.

Measurement of cortical cerebral blood flow. The cortical CBF was measured with a laser Doppler flowmeter (LDF), as described by Adachi *et al.* [11]. The rat's head was fixed on a stereotaxic instrument (SR-5, Narishige, Tokyo) in a prone position. After craniotomy, recording probes (diameter, 0.8 mm) of LDF (ALF 21D, Advance, Tokyo) were placed extradurally on the cortices at three different coronal levels, as shown in Fig. 2A: at (1) the anterior level, 2–4 mm anterior to the bregma and 2–4 mm lateral to the midline; B2–4, L2–4 (the frontal cortex); (2) the middle level; B−1–1, L5–7 (the parietal cortex); (3) the posterior level; B−3.5 to −5.5, L2–4 (the occipital cortex) [12, 13]. Care was taken to avoid placing the probe on a large pial artery. The flow signal was averaged with a 1 s time constant. The CBF, expressed in mV from the output of the laser Doppler flowmeter, was continuously recorded on a polygraph (RM-6000, Nihon Kohden) and on Chart software of MacLab system (MacLab/8s, ADInstruments, Sydney, Australia).

Histological study. Rats without occlusion (control) and at various times after occlusion were deeply anesthetized with pentobarbital and perfused transcardially with heparinized saline followed by 10% formalin in 0.1 M phosphate buffer at pH 7.4. The brains were left *in situ* for at least 2 h at 4°C, then removed and cut in a coronal direction into slices 3 mm thick and stored in the fixative at a room temperature of 24–26°C for about 4 h. The specimens were then embedded in paraffin.

Cross sections of the brain were cut at a thickness of 6 μm. These sections were stained with hematoxylin and eosin. The cortices at three different coronal levels were selected for counting cortical neurons, as shown in Fig. 2B–D: at (1) the anterior level, 3.2 mm anterior to the bregma, (2) the middle level, 0.26 mm posterior to the bregma, and (3) the posterior level, 4.3 mm posterior to the bregma.

The 6 μm-thick coronal slices were used for counting neurons with a microscope (AH-2, Olympus, Tokyo), using an ocular grid. We classified and counted all the neurons damaged histologically, at a magnification of 400×, in the cortical areas at each hemisphere of the 3 coronal levels shown by hatched areas in Fig. 2B–D. Neurons in which cell bodies were shrunken and the nuclei were darkly stained, and the nucleoli became difficult to distinguish (as reported by

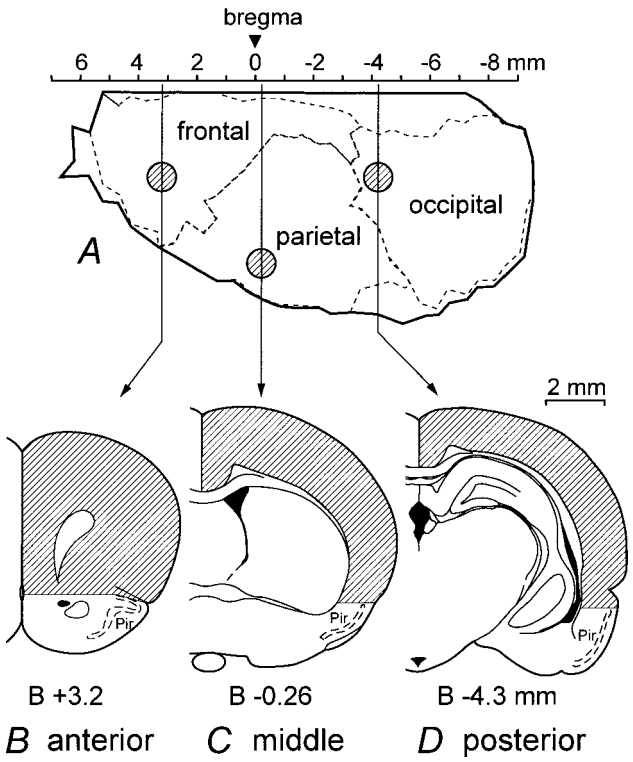

Fig. 2. Diagram showing the cortices at three different coronal levels at the anterior, middle, and posterior levels, used for CBF measurement (A) and histological study (B–D). Only the left hemisphere is shown representatively. A: Dorsal view of the rat cerebral cortex in stereotaxic coordinates [12]. Each circle represents a position of the recording probe of the laser Doppler flowmeter. B–D: Coronal planes of the brain atlas [13]. Hatched area represents the cortical region used for counting neurons at each level. The vertical border is the midline, and the horizontal border is the dorsal edge of the piriform cortex (Pir) at each of the 3 coronal levels. The area was mainly composed of the frontal cortex at the anterior level, the parietal cortex at the middle level, and the occipital, parietal, and temporal cortices at the posterior level.

Brown and Brierley [14]) were counted as damaged neurons. In 5 control rats we additionally counted all the neurons undamaged histologically, at a magnification of 200×, in the same cortical areas.

NBM stimulation. A rat was fixed in a prone position on a stereotaxic instrument (SR-5, Narishige), and the skull was partially removed. A coaxial metal electrode of 0.2 mm outer diameter (TN200-173, Unique Medical, Tokyo) was inserted into the unilateral NBM on the left side, positioned 2.3 mm posterior to the bregma, 3.7 mm lateral to the midline, and 7.6 mm vertical under the bregma height, according to the Paxinos and Watson's atlas [13] and Biesold *et al.* [10]. We performed NBM stimulation by passing a rectangular pulse current with a stimulator (SEN-7203, isolator; SS-202J, Nihon Kohden). Repetitive electrical stimulation for 65–66 min was delivered to the NBM, ipsilateral to the occluded artery, starting 5 min before the intermittent occlusions and finishing around the end of them.

In the chronic experiments for the histological study, the electrode was removed immediately after the end of stimulation, and the hole in the skull was covered with dental cement.

Statistical analysis. All values are given as means±SE. We made comparisons by using the Mann-Whitney *U*-test, the paired *t*-test, or one-way analysis of variance (ANOVA), followed by post hoc comparisons by using the Newman-Keuls test. The differences were considered significant if $p<0.05$.

RESULTS

The effect of continuous occlusion of the unilateral common carotid artery on cortical cerebral blood flow

Figure 3 shows typical sample recordings of responses of CBF at the middle level, ipsilateral (A and B) and contralateral (C) to the artery occluded, and MAP (D) during continuous occlusion of the unilateral common carotid artery for 10 min. MAP was decreased by about 10 mmHg during occlusion, as seen in Fig. 3D. In the contralateral side, CBF was not influenced during the occlusion, as seen in Fig. 3C. In the ipsilateral side, CBF was decreased during the occlusion, as seen in Fig. 3 A and B. The decrease was composed of two phases, i.e., an initial short-lasting moderately decreasing phase and a late long-lasting marginally decreasing phase. During the initial decreasing phase, CBF started to decrease immediately after the onset of occlusion, and it decreased maximally up to about 60% of the control within a few seconds; it then started to recover toward the control CBF within 15 s, as seen in Fig. 3A. During the late decreasing phase, CBF started to decrease within 15 s after the onset of occlusion, reaching the maximum decrease of 85% of the control within 2 min, and the maximum decrease lasted during the occlusion, as seen in Fig. 3B.

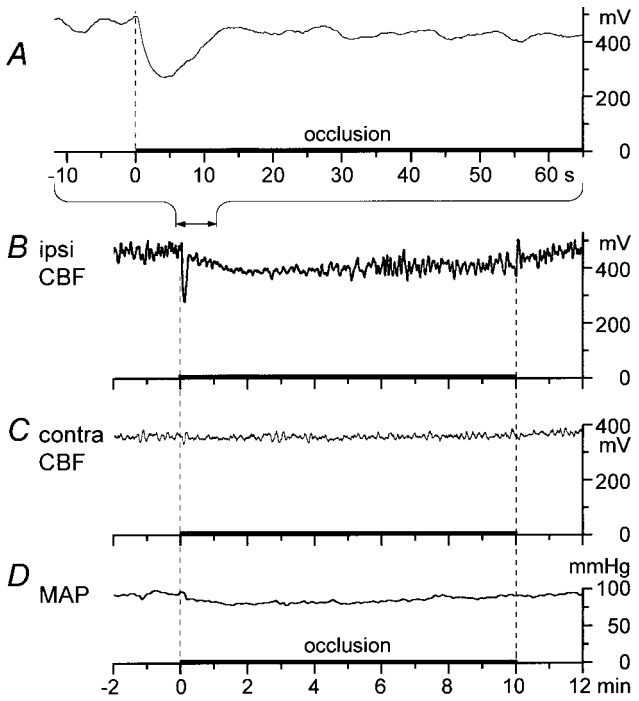

Fig. 3. Effects of continuous occlusion of the unilateral common carotid artery for 10 min on CBF at the middle level, ipsilateral (A, B) and contralateral (C) to the artery occluded, and mean arterial pressure (MAP; D). Typical sample recordings in one rat. The horizontal heavy bar on the bottom abscissa indicates the occlusion.

Essentially the same responses of CBF and MAP were both obtained in all 3 rats tested.

The effect of intermittent occlusions of the unilateral common carotid artery on cortical cerebral blood flow

Figure 4A–C shows typical sample recordings of responses of CBF at the middle level ipsilateral to the artery occluded (A and B) and MAP (C) during intermittent occlusions of the unilateral common carotid artery of every 5 s, repeated 360 times for 60 min. The ipsilateral CBF was decreased during each occlusion of the unilateral common carotid artery for 5 s (Fig. 4A). During the first 5 s occlusion, CBF started to decrease immediately after the onset of occlusion and reached the maximum decrease within a few seconds; the maximum decrease lasted until the occlusion was removed. This decrease in CBF for the first 5 s occlusion was reproducible when the following occlusion for 5 s was intermittently repeated 360 times every 5 s,

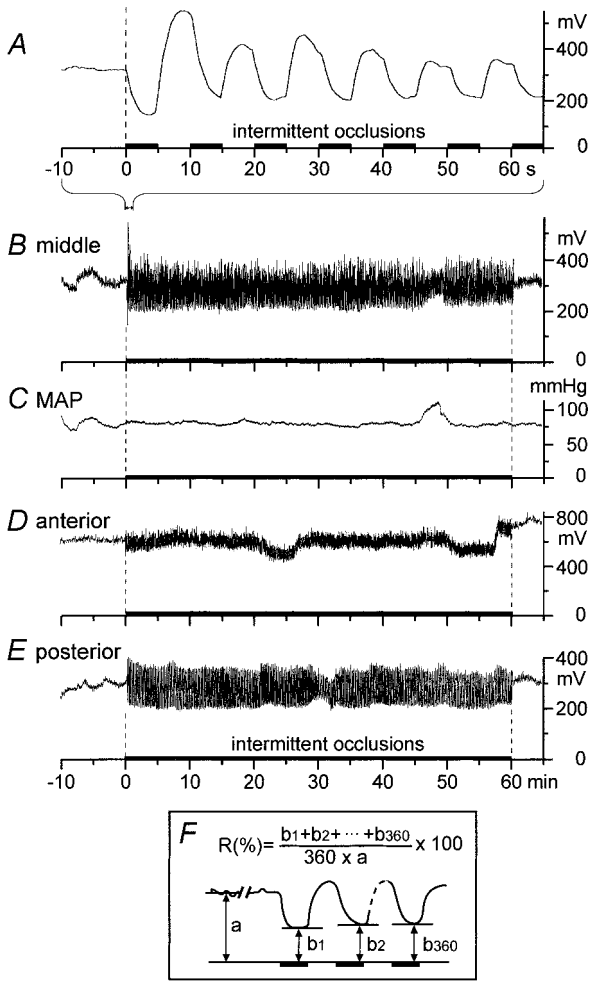

Fig. 4. Effects of intermittent occlusions of the unilateral common carotid artery for 60 min on CBF ipsilateral to the artery occluded and MAP. A–E: Typical sample recordings of CBF at the middle level (**A**, **B**) and MAP (**C**) in one rat, and those of CBF at the anterior (**D**) and posterior (**E**) levels in 2 other rats. The horizontal heavy bar on the bottom abscissa indicates the intermittent occlusions of every 5 s. **F**: Diagram showing how the response of CBF during intermittent occlusions for 60 min was calculated. CBF was measured as the mean of the minimum CBF during each 5 s occlusion of 360 times, $(b_1+b_2+\ldots+b_{360})/360$, and the response ($R$) is expressed as a percentage of the control CBF (a) 5–10 min before occlusion.

as seen in Fig. 4B. MAP was not affected during these intermittent occlusions, as seen in Fig. 4C.

Figure 4D and E shows typical sample recordings of CBF responses at the anterior (D) and posterior (E) levels, ipsilateral to the artery occluded, during intermittent occlusions of the unilateral common carotid artery. CBF at the anterior and posterior levels decreased during occlusions essentially in a manner similar to that at the middle level.

The responses of CBF during intermittent occlusions for 60 min were calculated and expressed as % of the preocclusion control CBF, as shown in Fig. 4F. The hatched columns of Fig. 8 summarize the responses of ipsilateral CBF at the anterior (A), middle (B), and posterior (C) levels during the intermittent occlusions of the unilateral common carotid artery for 60 min. The occlusions produced significant decreases in CBF in these 3 cortices in comparison to the preocclusion control CBF. The CBF during the occlusions at the anterior, middle, and posterior levels decreased to 87.0±2.1, 77.6±3.6, and 67.8±4.9% of the preocclusion control CBF, respectively (mean±SE, $n=5$).

In the contralateral side, CBF was not significantly influenced during the intermittent occlusions in these 3 cortices, although a marginal decrease was occasionally noted at the anterior and posterior levels (data not shown).

Cortical neurons at three different coronal levels in the control preparation

The cortices at three different coronal levels were selected for measuring the cortical neurons, as shown in Fig. 2B–D: at (1) the anterior level, 3.2 mm anterior to the bregma; (2) the middle level, 0.26 mm posterior to the bregma; and (3) the posterior level, 4.3 mm posterior to the bregma. The cortical region used for counting neurons at each level is shown by a hatched area in Fig 2B–D.

There were several thousand neurons in the cortical hemisphere at each coronal level. In the control rats, almost all cortical neurons showed undamaged shapes histologically, except for a small number (less than 28) of neurons showing damaged shapes, as described in the following section. The numbers of undamaged neurons at the anterior, middle, and posterior levels were 7,786±369, 6,168±304, and 5,932±290, and those of damaged neurons were 18.8±2.4, 19.2±4.3, and 9.0±3.2, respectively, as shown in Table 1A. The numbers of damaged neurons at the middle level are also shown in Fig. 6A.

The effect of intermittent occlusions of the unilateral common carotid artery on cortical neurons

Figure 5A shows an example histological picture of the cortical neurons at the middle level ipsilateral to the artery occluded by a coronal section taken on the 5th day after the intermittent occlusions of the unilateral common carotid artery of every 5 s, repeated 360 times for 60 min. Neurons showing damaged shapes were increased. The damaged shape means that the cell bodies were shrunken and the nuclei were darkly stained, and the nucleoli became difficult to distinguish (as reported by Brown and Brierley [14]), as shown by arrowheads in Fig. 5Ab. The damaged neu-

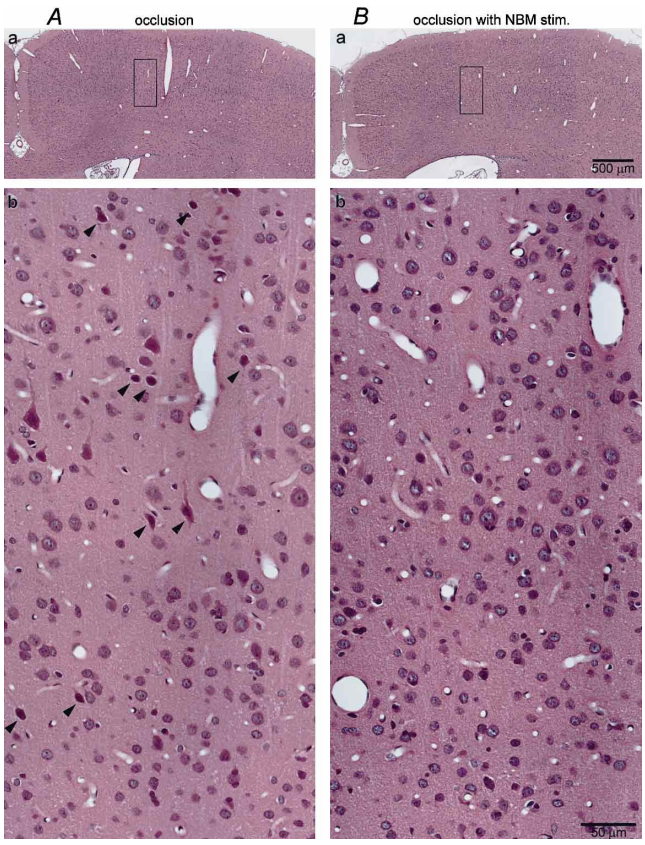

Fig. 5. Cortical neurons at the middle level ipsilateral to the artery occluded. Examples of histological pictures by coronal section (6 μm thick) taken on the 5th day after intermittent occlusions of the unilateral common carotid artery without (**A**) and with (**B**) NBM stimulation, stained with hematoxylin and eosin. Arrowheads in Ab represent damaged neurons.

rons were either scattered or clustered in the cortical region.

Table 1B summarizes the numbers of damaged neurons in one coronal section of 6 μm thickness of the cortices ipsilateral to the artery occluded at the anterior, middle, and posterior levels in rats prepared at 10 min and on the 2nd day, 5th day, and 7th day after the intermittent occlusions of a common carotid artery every 5 s, repeated 360 times for 60 min. The numbers of damaged neurons at the middle level (Table 1B) are plotted in Fig. 6B–E. The numbers of damaged neurons at the middle level did not significantly change at 10 min (Fig. 6B) and on the 2nd day (Fig. 6C) after the occlusions, whereas they were significantly increased to 104.0±29.0 on the 5th day (Fig. 6D). On the 7th day, the damaged neurons were 61.3±9.6, not significantly increased (Fig. 6E).

The time courses of increases in the numbers of damaged neurons at the anterior and posterior levels following the intermittent occlusions were essentially similar to that at the middle level, as shown in Table 1B. On the 7th day, only the numbers of damaged neurons at the posterior level remained significantly increased. The numbers of damaged neurons in the 3 cortices ipsilateral to the artery occluded in rats prepared on the 5th day after the intermittent occlusions are plotted as hatched columns in Fig. 9. In subsequent experiments, the effect of NBM stimulation was examined on neuronal damage on the 5th day after the intermittent occlusions.

In the contralateral side, the numbers of damaged

Table 1. The numbers of undamaged and damaged neurons in one coronal section of 6 μm thick in the left cortices at 3 different coronal levels in the control rats without occlusion (A) and in rats prepared at various times after intermittent occlusions of the unilateral common carotid artery for 60 min (B).

	A. Control (n=5)		B. Following occlusion (damaged)			
	Undamaged	Damaged	10 min (n=4)	2 d (n=4)	5 d (n=5)	7 d (n=4)
Anterior	7,786±369	18.8±2.4	15.0±3.8	17.3±4.5	75.2±9.7**	46.5±13.3
Middle	6,168±304	19.2±4.3	18.8±1.5	17.0±5.6	104.0±29.0**	61.3±9.6
Posterior	5,932±290	9.0±3.2	51.5±14.0	55.8±17.4	181.0±21.6**	92.3±20.0**

Values are mean±SE. ** p<0.01; significantly different from the control group using ANOVA followed by Newman-Keuls multiple comparison tests.

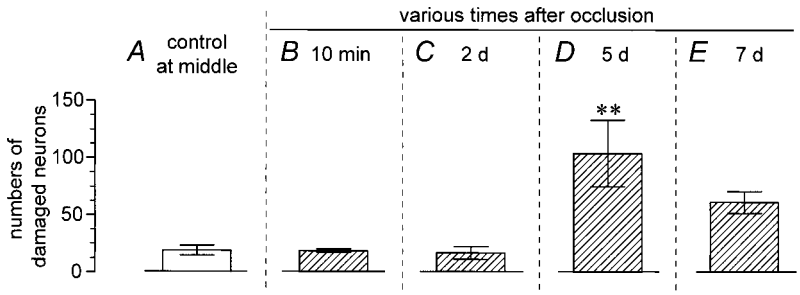

Fig. 6. The numbers of damaged neurons in one coronal section (6 μm thick) of the left cerebral cortex at the middle level in the control rats (A) and in rats prepared at 10 min (B), on the 2nd day (C), 5th day (D), and 7th day (E) after intermittent occlusions. Each column and vertical bar represents a mean±SE. ** p<0.01; significantly different from the control group using ANOVA followed by Newman-Keuls multiple comparison tests.

neurons were not significantly increased following the intermittent occlusions in these 3 cortices, though a marginal increase was noted at the anterior and posterior levels in some rats (data not shown).

At the posterior coronal level, neuronal damage similar to that in the cortex was seen in the hippocampal CA1 region, known as a most vulnerable region to ischemia [7, 8], in one rat prepared on the 5th day after the occlusions, but not in the other rats.

The effect of NBM stimulation on cortical cerebral blood flow during intermittent occlusions of the unilateral common carotid artery

We stimulated the NBM by using intermittent train pulses (1 s on/1 s off, 0.5 ms) at 50 Hz with a current intensity of 200 μA for a duration of 65–66 min. Intermittent train pulse stimulation was used because such stimulation to the NBM has been reported to be useful for inducing sustained CBF increases during long-lasting stimulation [15].

At first we observed that the increase in CBF lasted during the stimulation of the unilateral NBM up to 66 min. CBF during NBM stimulation, when measured at 5 min after the onset of stimulation, in the ipsilateral cortices at the anterior, middle, and posterior levels increased to 187.0±3.4, 210.3±14.0, and 138.0±7.5% of the prestimulus control CBF, respectively (n=5). MAP was also increased by 14.3±3.0 mmHg (n=7) when measured at 5 min after the onset of stimulation, but it returned to the control level within 15 min after the onset of stimulation.

We then examined whether this NBM stimulation affects the reduction in CBF observed during the intermittent occlusions of the unilateral common carotid artery. The occlusions were started 5 min after the onset of NBM stimulation. Figure 7A–C shows typical sample recordings of responses of CBF at the middle level ipsilateral to the artery occluded (A and B) and MAP (C) during the intermittent occlusions of the unilateral common carotid artery with NBM stimulation, which markedly increased CBF (Fig. 7B) with only a minor pressor response (Fig. 7C). When the intermittent occlusions were started 5 min after the onset of stimulation, CBF dropped during each occlusion of the unilateral common carotid artery for 5 s (Fig. 7A). However, the lowered CBF during each 5 s occlusion was still higher than the control level of CBF (Fig. 7B). That is, NBM stimulation prevented the occlusion-induced decrease in CBF.

Figure 7D and E shows typical sample recordings of CBF responses at the anterior (D) and posterior (E) levels, ipsilateral to the artery occluded during intermittent occlusions of the unilateral common carotid artery with NBM stimulation. This stimulation prevented the occlusion-induced decrease in CBF at the anterior and posterior levels essentially in a manner similar to that at the middle level.

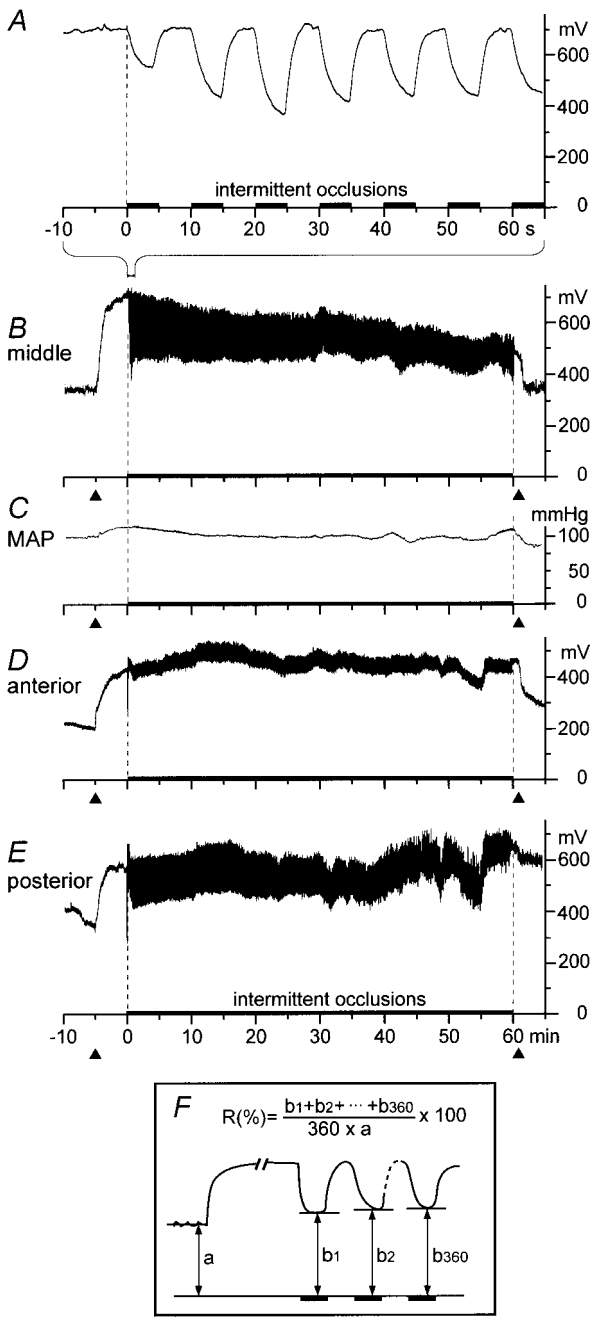

Fig. 7. Effects of intermittent occlusions of the unilateral common carotid artery for 60 min with NBM stimulation on CBF ipsilateral to the artery occluded and MAP. Typical sample recordings in one rat (**A**–**C**) and another rat (**D**, **E**). Arrows under the time scale in B–E indicate the time between which unilateral NBM ipsilateral to the artery occluded was stimulated (0.5 ms, 200 μA, 50 Hz, 1 s on/1 s off, started 5 min before the onset of occlusion and finished around the end of occlusions, for 65–66 min). See Fig. 4 for other details.

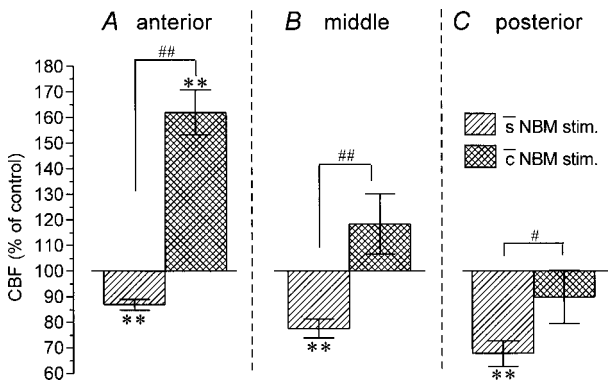

Fig. 8. Effects of NBM stimulation on the responses of CBF during intermittent occlusions of the unilateral common carotid artery at the anterior (A), middle (B), and posterior (C) levels. The responses of CBF was calculated as shown in Figs. 4F and 7F. Each column and vertical bar represents a mean±SE. # $p<0.05$, ## $p<0.01$; significantly different between responses with (c̄) and without (s̄) NBM stimulation using the Mann-Whitney *U*-test. ** $p<0.01$; significantly different from preocclusion control CBF by the paired *t*-test.

The CBF responses during intermittent occlusions for 60 min with NBM stimulation were calculated and expressed as % of the control CBF before either occlusion or stimulation, as shown in Fig. 7F. The cross-hatched columns of Fig. 8 summarize the responses of ipsilateral CBF at the anterior (A), middle (B), and posterior (C) levels during the intermittent occlusions of the unilateral common carotid artery for 60 min with NBM stimulation. CBF during the occlusions with NBM stimulation at the anterior, middle, and posterior levels was 161.9±8.8, 118.4±11.8, and 89.9±10.3% of the control CBF, respectively ($n=5$). CBF during the occlusions at the anterior level was significantly higher than the control CBF. During the occlusions at the middle and posterior levels, it was not significantly different from control CBF. The NBM stimulation prevented the occlusion-induced decrease in CBF in the 3 cortices, except that an apparent decrease in posterior CBF was obtained in 3 of the 5 rats tested. Thus the NBM stimulation completely prevented the occlusion-induced decrease in CBF at the anterior and middle levels, but not at the posterior level.

When comparing the responses of CBF during the intermittent occlusions of the unilateral common carotid artery with and without NBM stimulation at the anterior (Fig. 8A), middle (Fig. 8B), and posterior (Fig. 8C) levels, there were significant differences between the CBF responses during the intermittent occlusions with and without NBM stimulation in all 3 cortices.

The effect of NBM stimulation on cortical-delayed neuronal death following intermittent occlusions of the unilateral common carotid artery

Figure 5B shows an example histological picture of the cortical neurons at the middle level ipsilateral to the artery occluded by a coronal section taken on the 5th day after the intermittent occlusions of a common carotid artery for 60 min combined with NBM stimulation. In this picture, no damage to the cortical neurons is apparent (compare Fig. 5A and Fig. 5B).

The cross-hatched columns of Fig. 9 summarize the numbers of damaged neurons in the unilateral cortical region ipsilateral to the common carotid artery occluded at the anterior (A), middle (B), and posterior (C) levels in rats prepared on the 5th day after the intermittent occlusions for 60 min combined with NBM stimulation. The numbers of damaged neurons following the occlusions with NBM stimulation at the anterior, middle, and posterior levels were 44.5±9.1, 33.0±8.2, and 82.8±8.3, respectively ($n=4$). In the anterior and middle levels, the numbers of damaged neurons in the rats with occlusions and NBM stimulation were not significantly different from those in the control rats without occlusions. In the posterior level, the numbers of damaged neurons in the rats with occlusions and NBM stimulation were significantly higher than those in the control rats without occlusions. Thus the NBM stimulation completely prevented an occlusion-induced increase in damaged neurons at the anterior and middle levels, but not at the posterior level.

In a comparison of the numbers of damaged neurons in rats on the 5th day after the intermittent occlusions between the two groups with and without NBM stimulation in the ipsilateral cortical region, the damaged neurons following the occlusions were significantly less in the groups with NBM stimulation than in those without NBM stimulation at each level (Fig. 9).

Relationship between blood flow and the numbers of damaged neurons in the cortex

Figure 10 shows a summary of the present results described above, indicating the relationship between CBF during the occlusions (expressed as % of the preocclusion control CBF) and the numbers of damaged neurons in one coronal section (6 μm thickness) of the left cortices. This figure demonstrates that the degree of increase in the numbers of damaged neurons following the occlusions correlates with the degree of decrease in CBF during the occlusions with or without NBM stimulation. When CBF during the occlu-

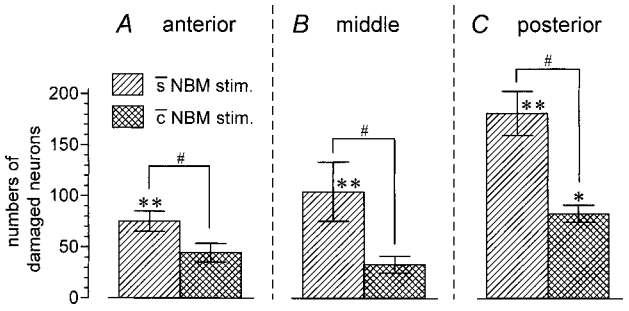

Fig. 9. Effects of NBM stimulation on the numbers of damaged neurons in one coronal section (6 μm thick) in the ipsilateral cortices on the 5th day after intermittent occlusions of the unilateral common carotid artery at the anterior (A), middle (B), and posterior (C) levels. # $p<0.05$; significant difference between the values in rats with (c̄) and without (s̄) NBM stimulation using the Mann-Whitney *U*-test. * $p<0.05$, ** $p<0.01$; significantly different from the control group using the Mann-Whitney *U*-test.

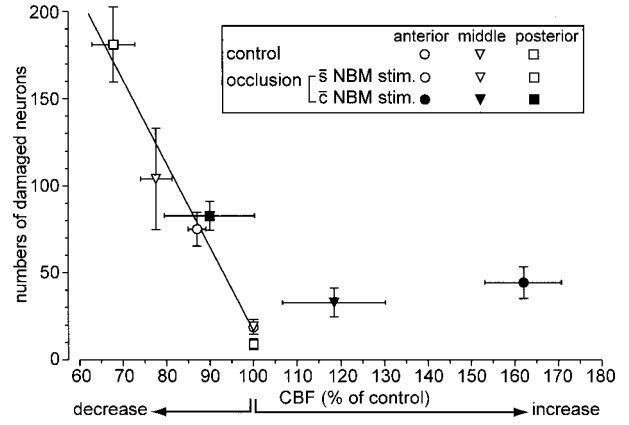

Fig. 10. Summary of the present results. Relationship between CBF during occlusion (expressed as % of the pre-occlusion control CBF) and the numbers of damaged neurons in one coronal section (6 μm thick) of the left cortices in the control rats (gray symbols) and in rats on the 5th day after intermittent occlusions for 60 min with (black symbols) and without (white symbols) NBM stimulation. Each point represents mean±SE. Linear regression analysis was performed for the data CBF≤100% ($n=7$). The equation of the regression line is $y=-4.8x+496$ ($r^2=0.96$, $p<0.0001$).

sions with or without NBM stimulation was lower than 100% of the control, the numbers of damaged neurons were correlated with the degree of decrease in CBF, i.e., the larger decrease in CBF, the more neuronal damage, as shown by a regression line in Fig. 10. On the other hand, when the degree of CBF during occlusions with NBM stimulation was higher than 100% of the control, the numbers of damaged neurons did not correlate with the degree of increase in CBF, staying approximately at the control level.

DISCUSSION

The present study is the first to demonstrate, by the use of a new rat model of ischemia producing the moderate delayed neuronal death in the unilateral cerebral cortex, that electrical stimulation of the NBM protects the ischemia-induced delayed neuronal death with a vasodilative action in widespread cortical areas. The results will be discussed in two sections.

A rat model of ischemia producing moderate delayed neuronal death in the unilateral cerebral cortex. Since the original work by Pulsinelli *et al.* [8], reports have been published on delayed neuronal death in the cerebral cortex following transient ischemia of the brain, using transient occlusion of the bilateral common carotid arteries alone or by additional permanent closure of the bilateral vertebral arteries [16, 17]. The decrease in cortical CBF during transient ischemia produced by such transient occlusion of the bilateral arteries has been measured by Pulsinelli *et al.* [18], Suzuki *et al.* [19], and Kuroiwa *et al.* [20], and in these studies CBF was reduced to 3–12% of the resting control flow. We measured cortical CBF continuously and quantitatively by using laser Doppler flowmetry during intermittent occlusions of the unilateral common carotid artery in rats, and CBF was reduced to 68–87% of the resting control flow during the occlusions. This decrease in CBF in the present study is much smaller than those reported in the previous studies [18–20]. Thus the present model producing moderate delayed neuronal death in the ipsilateral cortical neurons (in about 1–3% of total neurons) by intermittent occlusions of the unilateral carotid artery for 60 min was beneficial for testing the effects of change in the regional blood flow by vasodilative nerve stimulation on delayed cortical neuronal death.

When the unilateral carotid artery was occluded continuously for 10 min in the present study, the decrease in CBF comprised two phases, i.e., an initial short-lasting moderately decreasing phase and a late long-lasting marginally decreasing phase. When the same artery was occluded intermittently (every 5 s) for 60 min, we could repeatedly produce the initial short-lasting moderately decreasing phase of CBF, resulting in the production of the moderate delayed neuronal death. The present time course of the delayed neuronal death, reaching the maximum on the 5th day after the occlusions and decreasing on the 7th day, was in agreement with the report by Lin *et al.* [16]. Thus the effect of NBM stimulation was examined on the delayed neuronal death on the 5th day after the intermittent occlusions.

There were regional differences in the present degree of delayed neuronal death on the 5th day after the occlusions, which were correlated with the magnitude of CBF decrease during the occlusions, as shown in Fig. 10. The regional differences may be based on the morphological features of arterial anastomosis in the rat cerebral arterial circle [21].

Neuroprotective effect of NBM stimulation. In the present study, the decrease in cortical CBF during the occlusions was prevented and the delayed death of cortical neurons on the 5th day after the occlusions was reduced by repetitive electrical stimulation of the NBM ipsilateral to the occluded artery, applied during the occlusions. This result indicates that NBM stimulation had a protective effect on the delayed death of cortical neurons following transient ischemia.

The present result that focal stimulation of the NBM produced an increase in CBF of widespread cortices ipsilateral to the NBM stimulated is much in accord with the previous reports [11, 15]. Furthermore, the present study demonstrated for the first time that the same focal stimulation of the NBM prevented a decrease in CBF during cerebral arterial occlusion and protected the delayed neuronal death in widespread cortices. The effectiveness of NBM stimulation for the improvement of the degree of decrease in CBF and the delayed neuronal death were in a similar manner, i.e., the delayed neuronal death was completely protected where CBF decrease during the occlusions was completely prevented, and the delayed neuronal death was partially protected where CBF decrease was partially prevented. These results suggest that NBM-stimulation–induced prevention of the decrease in cortical CBF during the occlusions resulted in the protection against the delayed death of cortical neurons. Thus one physiological relevance of the basal forebrain–originating neural vasodilative responses, independent of regional cerebral metabolism, first found by Sato and his colleagues (see reviews by Sato and Sato [3, 4]), appears to be the protection of delayed death of cortical neurons following ischemia.

However, the present study cannot preclude the possibility of an involvement of other mechanisms, because stimulation of NBM may increase endogenous neuroprotective factors such as neurotrophic factors [22] in the cerebral cortex, and these factors may help to protect the cortical neurons.

It has been established that there is a diffuse projection of cholinergic nerve fibers to the widespread cortices originating in the NBM, the activation of which causes an increase in the extracellular release of ACh in the cortex; this consequently results in an increase in cortical CBF via activation of the nicotinic and muscarinic acetylcholine receptors [3, 4, 10, 23]. Thus acetylcholine and its receptors appear to be involved in the present NBM stimulation–induced protection of the delayed neuronal death in widespread cortices. Basal forebrain–originating cholinergic vasodilation via the activation of nAChRs has also been demonstrated in the hippocampus (see reviews by Sato and Sato [3, 4]). Our previous study showed that an increase in hippocampal blood flow following a stimulation of the nAChRs by nicotine reduced the ischemia-induced delayed death of the hippocampal neurons in rats [9]. An activation of the nAChRs in the hippocampus by endogenously released acetylcholine from cholinergic vasodilative nerve fibers originating in the septal complex of the basal forebrain and projecting to the hippocampus may protect the delayed hippocampal neuronal death produced by ischemia in a manner similar to that found for the cortical neurons in the present study. Thus increases in cortical and hippocampal blood flow induced by activation of the cholinergic neural vasodilative system originating in the basal forebrain could protect the delayed death of the cortical and hippocampal neurons because of ischemia in the cortex and hippocampus.

Based on the present study and the previous study [9], it can be assumed that if the basal forebrain–originating cholinergic vasodilative function is declined, the delayed neuronal death in the cerebral cortex and hippocampus following ischemia will become more extensive. In Alzheimer's disease, the degeneration of cells in the basal forebrain is quite striking [1, 2], and neurons in the cerebral cortex and hippocampus are degenerative [5, 6]. It has been reported in rats that neuronal loss was observed in the cerebral cortex 14 months after lesions of the NBM [24]. We have reported that the vasodilative cortical CBF responses induced by electrical stimulation of the NBM declined in very old rats of 32–42 months as a result of a decreased activity of nAChRs [25, 26]. Such a decline in the cholinergic vasodilative function because of the degeneration of basal forebrain neurons and/or decreased activity of nAChRs, may cause insufficient CBF for the cortex and hippocampus when the cortical and hippocampal neurons require their regional CBF and lead to a progressive production of the cortical and hippocampal delayed neuronal death. This mechanism may be one of the possible explanations for the decline in cognitive function related to the cerebral cortex and hippocampus in Alzheimer's disease and the elderly when these intrinsic cholinergic neurons degenerate [1, 2, 27] and cortical nAChRs are reduced [28].

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REFERENCES

1. Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, and DeLong MR: Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215: 1237–1239, 1982
2. Arendt T, Bigl V, Tennstedt A, and Arendt A: Neuronal loss in different parts of the nucleus basalis is related to neuritic plaque formation in cortical target areas in Alzheimer's disease. Neuroscience 14: 1–14, 1985
3. Sato A and Sato Y: Cholinergic neural regulation of regional cerebral blood flow. Alzheimer Dis Assoc Disord 9: 28–38, 1995
4. Sato A and Sato Y: Regulation of regional cerebral blood flow by cholinergic fibers originating in the basal forebrain. Neurosci Res 14: 242–274, 1992
5. Terry RD, Peck A, DeTeresa R, Schechter R, and Horoupian DS: Some morphometric aspects of the brain in senile dementia of the Alzheimer type. Ann Neurol 10: 184–192, 1981
6. Mann DMA, Yates PO, and Marcyniuk B: A comparison of nerve cell loss in cortical and subcortical structures in Alzheimer's disease. J Neurol Neurosurg Psychiatr 49: 310–312, 1986
7. Kirino T: Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 239: 57–69, 1982
8. Pulsinelli WA, Brierley JB, and Plum F: Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol 11: 491–498, 1982
9. Kagitani F, Uchida S, Hotta H, and Sato A: Effects of nicotine on blood flow and delayed neuronal death following intermittent transient ischemia in rat hippocampus. Jpn J Physiol 50: 585–595, 2000
10. Biesold D, Inanami O, Sato A, and Sato Y: Stimulation of the nucleus basalis of Meynert increases cerebral cortical blood flow in rats. Neurosci Lett 98: 39–44, 1989
11. Adachi T, Biesold D, Inanami O, and Sato A: Stimulation of the nucleus basalis of Meynert and substantia innominata produces widespread increases in cerebral blood flow in the frontal, parietal and occipital cortices. Brain Res 514: 163–166, 1990
12. Zilles KJ: The Cortex of the Rat, Springer-Verlag, Berlin, 121 pp,1985
13. Paxinos G and Watson C: The Rat Brain in Stereotaxic Coordinates, 2nd ed, Academic Press, San Diego, 1986
14. Brown AW and Brierley JB: Anoxic-ischaemic cell change in rat brain light microscopic and fine-structural observations. J Neurol Sci 16: 59–84, 1972
15. Vaucher E, Borredon J, Seylaz J, and Lacombe P: Autoradiographic distribution of cerebral blood flow increases elicited by stimulation of the nucleus basalis magnocellularis in the unanesthetized rat. Brain Res 691: 57–68, 1995
16. Lin C-S, Polsky K, Nadler JV, and Crain BJ: Selective neocortical and thalamic cell death in the gerbil after transient ischemia. Neuroscience 35: 289–299, 1990
17. Iwasaki Y, Ito S, Suzuki M, Nagahori T, Yamamoto T, and Konno H: Forebrain ischemia induced by temporary bilateral common carotid occlusion in normotensive rats. J Neurol Sci 90: 155–165, 1989
18. Pulsinelli WA, Levy DE, and Duffy TE: Regional cerebral blood flow and glucose metabolism following transient forebrain ischemia. Ann Neurol 11: 499–509, 1982
19. Suzuki R, Yamaguchi T, Kirino T, Orzi F, and Klatzo I: The effects of 5-minute ischemia in Mongolian gerbils: I. Blood-brain barrier, cerebral blood flow, and local cerebral glucose utilization changes. Acta Neuropathol (Berl) 60: 207–216, 1983
20. Kuroiwa T, Bonnekoh P, and Hossmann K-A: Laser Doppler flowmetry in CA1 sector of hippocampus and cortex after transient forebrain ischemia in gerbils. Stroke 23: 1349–1354, 1992
21. Brown JO: The morphology of circulus arteriosus cerebri in rats. Anat Rec 156: 99–106, 1966
22. Lindvall O, Kokaia Z, Bengzon J, Elmér E, and Kokaia M: Neurotrophins and brain insults. TINS 17: 490–496, 1994
23. Uchida S, Kagitani F, Suzuki A, and Aikawa Y: Effect of acupuncture-like stimulation on cortical cerebral blood flow in anesthetized rats. Jpn J Physiol 50: 495–507, 2000
24. Arendash GW, Millard WJ, Dunn AJ, and Meyer EM: Long-term neuropathological and neurochemical effects of nucleus basalis lesions in the rat. Science 238: 952–956, 1987
25. Uchida S, Kagitani F, Nakayama H, and Sato A: Effect of stimulation of nicotinic cholinergic receptors on cortical cerebral blood flow and changes in the effect during aging in anesthetized rats. Neurosci Lett 228: 203–206, 1997
26. Uchida S, Suzuki A, Kagitani F, and Hotta H: Effects of age on cholinergic vasodilation of cortical cerebral blood vessels in rats. Neurosci Lett 294: 109–112, 2000
27. McGeer PL, McGeer EG, Suzuki J, Dolman CE, and Nagai T: Aging, Alzheimer's disease, and the cholinergic system of the basal forebrain. Neurology 34: 741–745, 1984
28. Nordberg A, Alafuzoff I, and Winblad B: Nicotinic and muscarinic subtypes in the human brain: changes with aging and dementia. J Neurosci Res 31: 103–111, 1992