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ORIGINAL PAPER

Effect of acupuncture-like stimulation on cortical cerebral blood flow in aged rats

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Abstract This study aimed to examine the effect of acupuncture-like stimulation on cortical cerebral blood flow (CBF) in aged rats and the contribution of the intracranial cholinergic vasodilatory system on its response. In urethane-anesthetized rats of 30–37 months of age, manual acupuncture-like stimulation of a forepaw produced an increase in the CBF, independent of systemic arterial pressure. The increase in the CBF induced by forepaw stimulation was abolished by intravenous administration of cholinergic receptor antagonists. Manual acupuncture-like stimulation of a forepaw increased extracellular acetylcholine release in the cerebral cortex. These results suggest that natural somatic afferent stimulation, such as acupuncture-like stimulation, activates the intracranial — most likely, basal forebrain — cholinergic vasodilatory system in the cerebral cortex, even in extremely aged rats.

Keywords Acupuncture · Cortical cerebral blood flow · Cortical acetylcholine release · Cholinergic vasodilation · Aging · Rat

Introduction

Cerebral blood flow (CBF) disturbances that increase with advancing age may impair nervous functions such as consciousness, motor and visceral functions. Acupuncture has been used to improve the dysfunctions caused by disturbances in the CBF, such as stroke [1–5], but the mechanisms of this improvement have not yet been clarified.

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Our previous study using adult 4- to 9-month-old rats showed that acupuncture-like stimulation of a forepaw produced an increase in the CBF in the cortex as a reflex response, independent of emotional responses, under anesthesia [6]. In that study, we further demonstrated that the increase in the CBF following manual acupuncture-like stimulation of a forepaw was independent of the pressor response and occurred in the bilateral parietal cortices similarly. The CBF response was proven to be a reflex whose afferent pathways consisted of somatic afferents innervating the skin and underlying muscles, and efferent pathways consisted of the intracranial cholinergic vasodilator originating in the basal forebrain [6].

A cholinergic neural vasodilative response in the cortex, independent of systemic blood pressure and metabolic vasodilation, has been reported to occur when cholinergic neurons originating in the magnocellular nucleus of the basal forebrain [the nucleus basalis of Meynert (NBM)] and projecting to the cortex were activated [7, 8]. This cholinergic vasodilative system, which operates by increasing acetylcholine (ACh) release [9], relies upon the activation of both muscarinic and nicotinic ACh receptors in the parenchyma of the cortex [7].

Cholinergic neurons in the basal forebrain show degeneration in patients with Alzheimer's disease [10] and in healthy aged people [11, 12]. Previously, we reported that the vasodilation response of the cortex by stimulation of the NBM declined in extremely old rats above 30 months [13, 14]. In the extremely old rats of 32–42 months, the increased CBF response produced by direct electrical stimulation of the NBM at low intensity (50 μA) was not significantly different from that in adult rats, but the response at high intensity (100–200 μA) was significantly lower than that in adult rats [13]. These findings suggest the possibility that the CBF response

elicited by acupuncture-like stimulation may also decline in extremely old rats. Since disturbances of the CBF occur more commonly among older people, it is necessary to study the effect of acupuncture on the CBF in relation to the intracranial cholinergic vasodilative system in old animals.

Therefore, the present study aimed to clarify whether the intracranial cholinergic vasodilative system in the cortex is functional during an acupuncture-like stimulation in extremely aged rats. For this purpose, we examined the effect of acupuncture-like stimulation on the CBF and extracellular ACh release in the cortex in aged (30- to 37-month-old) rats.

Materials and methods

Animals

The experiments were performed on 7 male and female Wistar rats, aged 30–37 months. All animals were obtained from the animal farm of Tokyo Metropolitan Institute of Gerontology. This study was approved by the Animal Committee of our Institution.

General experimental conditions

The responses of CBF and ACh release in the cortex to acupuncture-like stimulation of a forepaw were examined under general anesthesia, as described previously [6]. Briefly, the rats were anesthetized with urethane (0.8–1.1 g/kg, i.p.). Respiration was maintained by means of an artificial respirator (model 683, Harvard, USA) through a tracheal cannula. End-tidal CO_2 concentration was maintained at 3.0–4.0 % by monitoring with a respiratory gas monitor (Microcap, Oridion Medical, Jerusalem, Israel). Arterial blood pressure was measured through a catheter with a pressure transducer (TP-400T, Nihon Kohden, Tokyo, Japan) inserted into a femoral artery. Body temperature was measured rectally and continuously using a thermistor and maintained at approximately 37.5 °C by means of an infrared lamp and a heater system (ATB-1100, Nihon Kohden, Tokyo, Japan). The depth of anesthesia was adjusted by additional urethane doses (100 mg/kg, i.v. via a catheter inserted into a femoral vein) when necessary and by monitoring body movement, stability of blood pressure, and respiratory movement. The cortical cerebral blood flow was measured in all 7 rats. In 4 of 7 rats, extracellular ACh release in the cerebral cortex was also measured. At the end of the experiment, each rat was killed by a lethal dose of sodium pentobarbital.

Measurement of cortical cerebral blood flow

The head of each rat was fixed on a stereotaxic instrument (SR-5, Narisige, Tokyo, Japan) in the prone position. After craniotomy, two probes (diameter, 0.8 mm) of a laser Doppler flowmeter (ALF21D, Advance Co., Tokyo, Japan) were placed on the surface of bilateral parietal lobes (AP = 0 to −3 mm, L = +2 to +5 mm). The flowmeter probe was fixed using a balancing holder (ALF-B, Advance Co., Tokyo, Japan). The output of the laser Doppler flowmeter was expressed in mV and recorded on a polygraph.

Measurement of acetylcholine release in the cerebral cortex

Extracellular ACh in the parietal cortex was collected by a microdialysis technique in 4 rats. A microdialysis probe (outer diameter, 0.5 mm; length of perfusion, 3 mm; CMA/12, CMA/Microdialysis AB, Sweden) was inserted in the right parietal cortex at an angle of 30° to the vertical line, to a depth of 3.5 mm from the cortical surface at AP = +0.2, L = +4, as described originally by Kurosawa et al. [9]. The microdialysis probe was perfused at a speed of 2 μl/min with artificial cerebral spinal fluid (aCSF) containing (in mM) NaCl (122.7), KCl (2.4), $CaCl_2$ (1.5), $MgCl_2$ (1.1), $NaHCO_3$ (27.5), KH_2PO_4 (0.6), Na_2SO4 (0.5), glucose (6), and the acetylcholinesterase inhibitor physostigmine (5 μM). The aCSF was bubbled with 95 % O_2/5 % CO_2 to adjust the pH to 7.4. The recovery rate of ACh by the microdialysis probe in vitro at room temperature was 16–21 %. The perfused fluid was collected every 3 min in a sample cup kept on ice. The perfusate in each sample (6 μl) was mixed with 6 μl (120 fmol) of isopropylhomocholine (an internal standard), dissolved in aCSF. ACh was measured by high-performance liquid chromatography (HPLC) using an electrochemical detector (HTEC-500, Eicom, Kyoto, Japan). The mobile phase, consisting of 50 mM $KHCO_3$, 400 mg/l sodium 1-decanesulfonate (Tokyo Kasei Kogyo, Japan), and 50 mg/l EDTA 2Na, was pumped at a rate of 150 μl/min through a microbore separation column (AC-GEL, 2 × 150 mm). The ACh was converted to hydrogen peroxide and betaine by immobilized acetylcholinesterase and choline oxidase packed into a column (AC-ENZYMEPAKII, 1.0 × 4 mm). Both the separation column and the enzyme column were maintained at 33 °C. Hydrogen peroxide was measured using an electrochemical detector, and ACh was calculated by hydrogen peroxide measurement. The platinum working electrode was held at 0.45 V vs Ag/AgCl.

Acupuncture-like stimulation including manual and electrical stimulation

Manual stimulation In 7 rats, a stainless steel acupuncture needle with a diameter of 340 μm (No. 10, Seirin, Shizuoka, Japan) was inserted into the skin and underlying muscles of a forepaw at a depth of about 5 mm. Manual stimulation was performed by rotating the needle to the right and left at a frequency of about 1 Hz for 1 or 3 min.

Electrical stimulation In 6 rats, 2 needles of 200 μm in diameter (No. 3, Seirin, Shizuoka, Japan) were inserted into the interdigit forepaw of the 2nd and 3rd digits 5 mm apart, and 5 mm deep, and an electric square wave current was passed between these 2 needles using an electrical stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan). The parameters of electric stimulation were 0.5 ms duration, 20 Hz frequency, and 0.2–10 mA intensity for 30 s.

Drugs

Atropine (5 mg/kg, atropine sulfate; Sigma, USA) and mecamylamine (20 mg/kg, mecamylamine hydrochloride; Sigma, USA) were administered intravenously, to block muscarinic and nicotinic ACh receptors, respectively.

Spinal transection

The spinal cord was transected at the 1st and 2nd thoracic (T1–2) level in 7 rats. After transection of the spinal cord, 4 % Ficoll (Ficoll 70, Pharmacia, Uppsala, Sweden) was intravenously injected or dopamine was continuously infused intravenously to maintain systolic blood pressure above 60 mmHg.

Statistical analysis

All values are presented as mean ± standard error of the mean (SEM). Statistical comparisons were carried out by means of a paired *t*-test, a Student's *t*-test, and a one-way repeated-measures ANOVA, followed by a Dunnett's multiple comparison test or by a Bonferroni multiple comparison test. A p value of < 0.05 was considered to be statistically significant.

Results

Cerebral blood flow responses in the parietal cortex to manual acupuncture-like stimulation to a forepaw

In aged rats, the CBF in the parietal cortex and mean arterial pressure (MAP) measured under resting conditions were 306 ± 34 mV and 78 ± 4 mmHg, respectively ($n = 7$, mean ± SEM).

As shown in Fig. 1b, manual acupuncture-like stimulation delivered to a forepaw for 1 min produced increases in the CBF in the parietal cortex ipsilateral to the stimulating site, and MAP, in a 37-month-old rat. Both the CBF and MAP usually began to increase within 10 s of the onset of stimulation, reaching their maximum during stimulation. The increased CBF and MAP gradually returned to the control level after the stimulation had ceased. Figure 1c summarizes the maximum responses of CBF and MAP during the stimulation of a forepaw. Increases in CBF in ipsilateral and contralateral cortices reached 116 ±1 % and 119 ± 2 %, respectively (Fig. 1c). The ipsilateral and contralateral CBF responses were almost identical, and no significant differences were found. The maximum MAP response was 116 ± 1 %.

Responses of cerebral blood flow and mean arterial pressure after spinal transection at the T1–2 level

The spinal cord was transected at the T1–2 level to eliminate any influence of pressor responses following stimulation of a forepaw on the CBF (Fig. 1a). In 7 spinalized, aged rats, CBF and MAP measured under resting conditions were 296 ± 28 mV and 61 ± 3 mmHg, respectively. In this spinal preparation, manual acupuncture-like stimulation of a forepaw for 1 min no longer produced MAP responses, but still produced an increase in the CBF, as shown in a sample recording obtained from a 37-month-old rat (Fig. 1d). Figure 1e summarizes the maximum responses of both CBF and MAP to stimulation of a forepaw in spinalized rats. The summarized results demonstrate that in aged rats, following the stimulation of a forepaw, no MAP responses were observed, but significant increases in the CBF were produced both in the ipsilateral (111 ± 2 %) and the contralateral (114 ± 2 %) cortices. The ipsilateral and contralateral CBF responses were almost identical, and no significant differences were found.

Cerebral blood flow responses to electro-acupuncture stimulation of a forepaw

Figure 2a shows sample records of CBF responses evoked by electro-acupuncture stimulation of a forepaw via 2 acupuncture needles at different intensities in a 35-month-old spinalized rat. The CBF response was elicited by stimulation at intensities of 2.0, 5.0, and 10.0 mA, but not at 0.5 mA. Figure 2b summarizes the maximum CBF response at different stimulus intensities. Electro-acupuncture stimulation at less than 1.0 mA had no significant effect on the CBF. When the stimulus intensity was above 2.0 mA, the CBF was significantly increased. Increasing

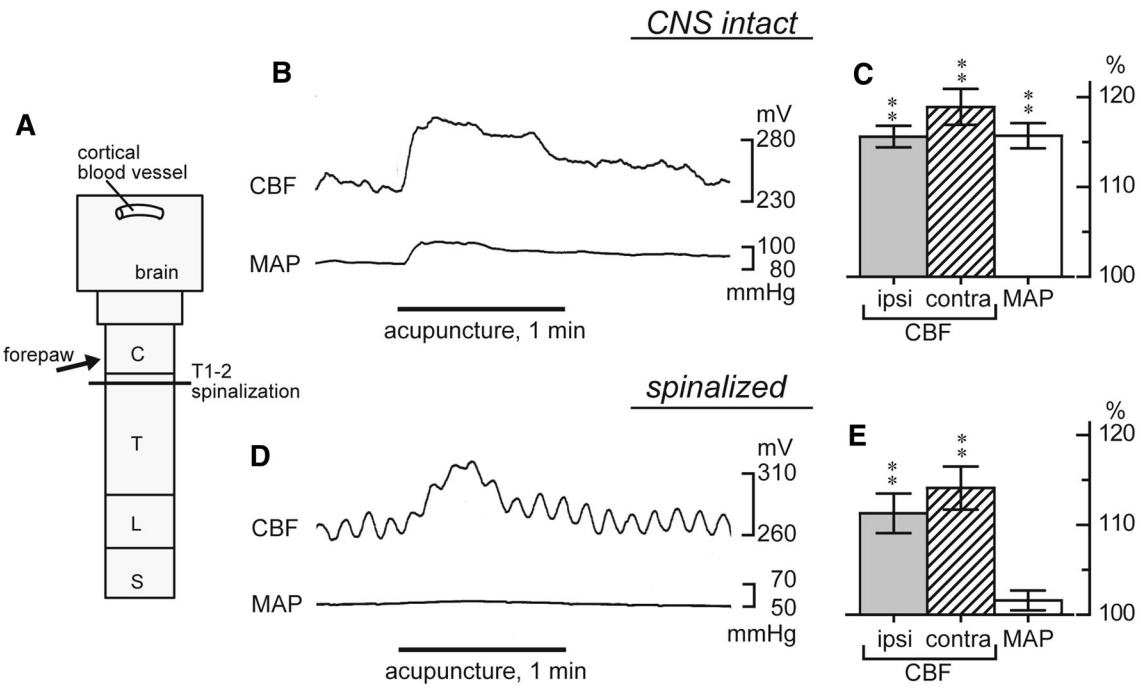

Fig. 1 The effect of manual acupuncture-like stimulation of a forepaw on the CBF in the parietal cortex, and on MAP, in aged rats. **a** Schematic diagram of experimental preparation. **b**, **c** CNS-intact condition. **d**, **e** Spinalized condition. **b**, **d** Sample recordings of the CBF in the parietal cortex ipsilateral to the stimulating site, and MAP, following acupuncture-like stimulation of a forepaw in a 37-month-old rat. The *bars* indicate stimulation period of 1 min. **c**, **e** Summary of responses of the CBF (*shaded column*: ipsilateral to the stimulated site, *hatched column*: contralateral to the stimulated site) and MAP. Maximum responses of CBF and MAP were expressed as percentages of the pre-stimulus control values. *Each column* and *vertical bar* represents a mean ± SEM ($n = 14$ in 7 rats, 2 trials per rat). $*p < 0.05$; $**p < 0.01$; significantly different from pre-stimulus control values using paired *t*-test. There were no significant differences between ipsilateral and contralateral CBF responses (paired *t*-test)

the stimulus intensity to 5.0 mA or 10.0 mA augmented the increased response of the CBF.

Effect of cholinergic receptor blockades on cerebral blood flow response

The basal CBF level was not changed following the administration of cholinergic receptor blockers. The CBF responses elicited by electro-acupuncture stimulation of a forepaw in the spinalized rats were attenuated after the intravenous injection of atropine, a muscarinic ACh receptor antagonist, and were further attenuated by an additional injection of mecamylamine, a nicotinic ACh receptor antagonist (Fig. 3a). The increase in the CBF during stimulation in the control before the injection of drugs was 122 ± 3 %, attenuating to 108 ± 4 % following atropine injection, and to 102 ± 1 % following an additional mecamylamine injection (Fig. 3b).

Responses of cortical acetylcholine release to manual acupuncture-like stimulation to a forepaw

Extracellular ACh release in the parietal cortex (Fig. 4a), measured every 3 min under resting conditions in 4 aged rats, was 65 ± 12 fmol/3 min at the beginning of the experiment. For each animal, the value of cortical ACh release was stable in the resting condition.

Extracellular ACh release in the parietal cortex was significantly increased during manual acupuncture-like stimulation to a forepaw for 3 min (Fig. 4b). During stimulation, extracellular ACh release in the parietal cortex ipsilateral to the stimulating site reached 84 ± 6 fmol/3 min. The increased cortical ACh release returned to the pre-stimulus control level during the 3 min after the end of stimulation. Increase in ACh release in the right parietal cortex was elicited by stimulation of not only ipsilateral (right) but also contralateral (left) forepaw (Fig. 4c). There were no significant differences between the ipsilateral and contralateral responses.

In four aged, spinalized rats, the basal level of extracellular ACh release in the parietal cortex was 46 ± 3 fmol/3 min. As shown in Fig. 4d, manual acupuncture-like stimulation of a forepaw in these rats produced a significant increase in ACh release in the parietal cortex ipsilateral to the stimulating site, reaching 81 ± 11 fmol/3 min. Increase in ACh release in the right parietal cortex was elicited by stimulation of not only ipsilateral (right) but also contralateral (left) forepaw (Fig. 4e). There were no

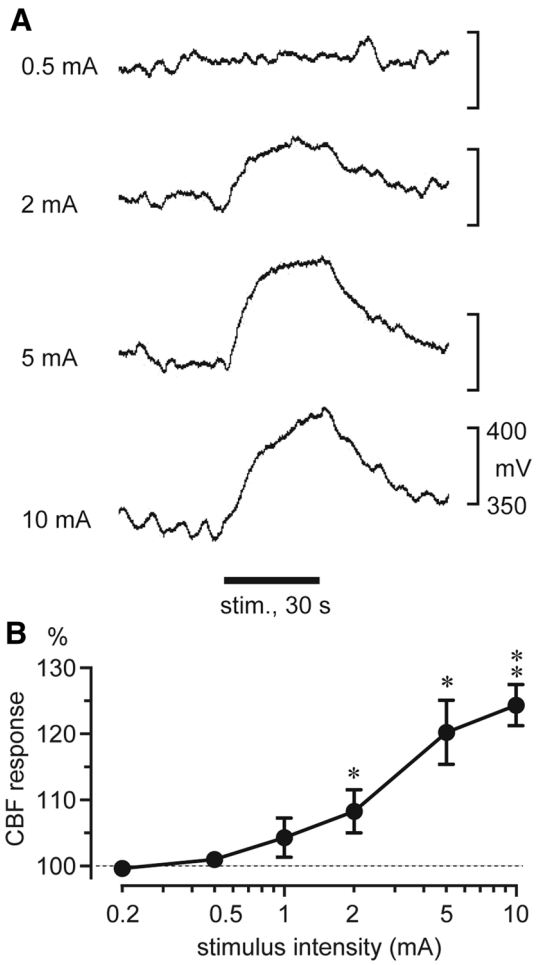

Fig. 2 Relationships between intensities of electro-acupuncture stimulation of a forepaw and magnitude of increase in the CBF in aged rats. **a** Sample recordings of CBF in a 35-month-old rat. **b** Summarized graph of relationships between stimulus intensity and magnitude of increase in the CBF ($n = 6$ in 6 rats). Other details are the same as in Fig. 1

significant differences between the ipsilateral and contralateral responses.

Discussion

The present study demonstrated for the first time that manual acupuncture-like stimulation of a forepaw increases extracellular ACh release in the parietal cortex in aged (30- to 37-month-old) rats under anesthesia, and thereby increases the CBF in the parietal cortex via activation of cholinergic receptors (Fig. 5). This response was proven to be a reflex independent of the emotional responses that might arise due to somatic sensory stimulation by acupuncture, because the present study was performed using rats in an anesthetized, unconscious condition in which emotional responses were eliminated.

Increased cerebral blood flow response independent of blood pressure response

In the present study, manual acupuncture-like stimulation of a forepaw for 1 min increased the CBF in the parietal cortex and MAP during the stimulation in central nervous system (CNS)-intact rats. The CBF can be influenced passively in a time period of seconds by MAP responses, since the autoregulation of the CBF is insufficient to work against the MAP response during a short time period of few seconds [15]. In the present study, the spinal cord was transected at the first to second thoracic level (T1–2), to prevent an increase in MAP following an acupuncture-like stimulation of a forepaw (Fig. 5). After the spinal transection, sensory information from the forepaw could ascend via the normal circuitry to the brain through the cervical spinal cord, propagate to the cardiovascular center in the brainstem, and be integrated into the brainstem; however, the information integrated into the brainstem could not descend to the preganglionic sympathetic neurons in the thoracic and lumbar spinal cord because of the transection of the descending pathways at the top of the T1–2 spinal cord. After spinal transection at T1–2 level, manual acupuncture-like stimulation to the forepaw for 1 min no longer produced MAP responses, but still produced an increase in CBF responses. Thus, the CBF response in aged rats is not completely dependent on an increase in MAP. The magnitudes of increase in the CBF independent of MAP observed in extremely aged, spinalized rats (111 ± 2 %) was similar to that observed in adult spinalized rats (112 ± 3 %, [6]). These results suggest that, during acupuncture-like stimulation, the aspect of the vasodilative response of cortical blood vessels that is independent of pressor response is well maintained in extremely aged rats.

Increased acetylcholine release in the cortex

The present study demonstrated that manual acupuncture-like stimulation of a forepaw increased extracellular ACh release in the parietal cortex of both CNS-intact and T1–2 spinalized, extremely aged rats. It has been reported that (1) extracellular ACh release in the cortex is independent of increase in systemic blood pressure [16], (2) the majority of cholinergic fibers in the cortex originate in the magnocellular nucleus of the basal forebrain (the NBM) [17–19], and (3) cortical ACh is released by stimulation of the NBM [9, 20]. Therefore, the present results suggest that, even in extremely aged rats, manual acupuncture-like stimulation of a forepaw activated basal forebrain cholinergic fibers which project to the cortex (Fig. 5).

The observed increase of ACh in the cortex by acupuncture-like stimulation of a forepaw in aged rats in the

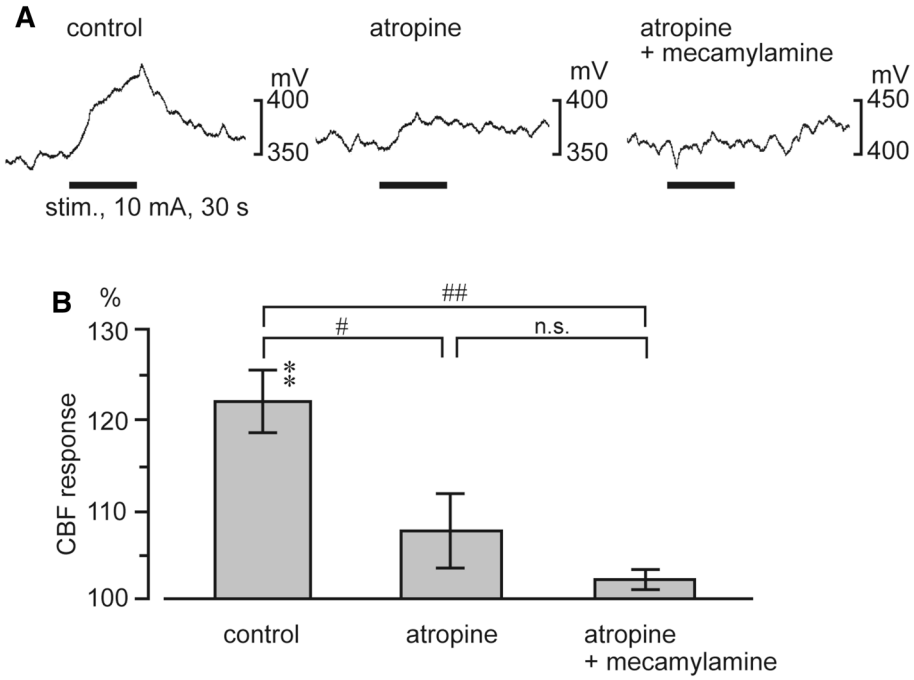

Fig. 3 Effects of cholinergic receptor antagonists on the CBF responses induced by electro-acupuncture stimulation of a forepaw in aged rats. **a** Sample recordings of the CBF in a 35-month-old rat. **b** Summary of CBF responses ($n = 4$ in 4 rats). Data for cholinergic receptor antagonists were taken 10–15 min after administration of the drugs. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; significant difference between the responses by one-way repeated-measures ANOVA followed by a Bonferroni multiple comparison test. Other details are the same as in Fig. 1

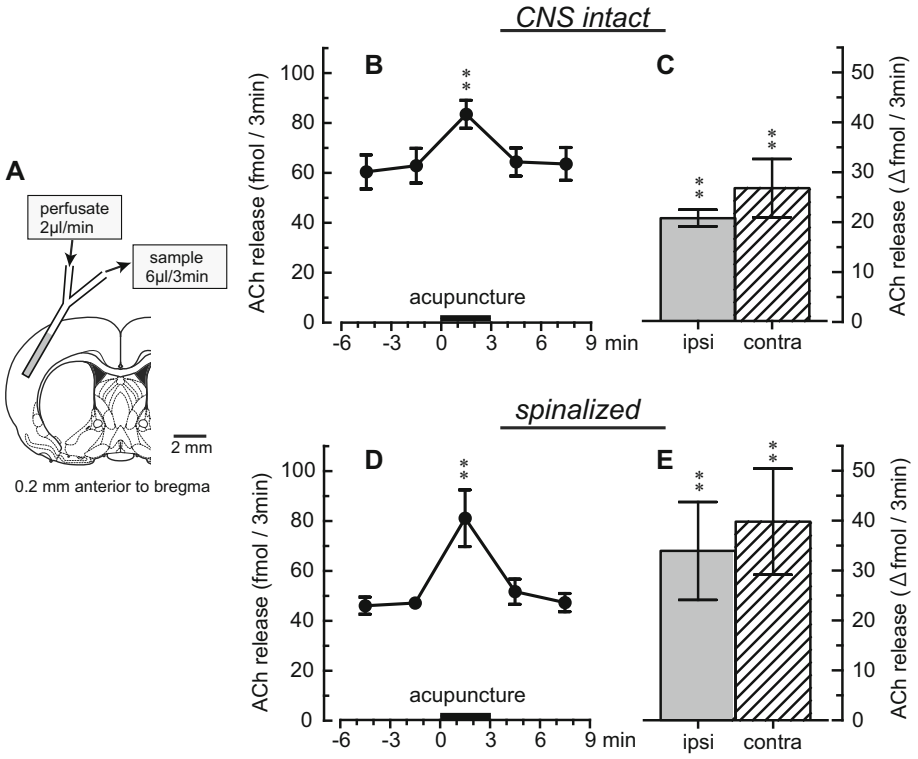

Fig. 4 Effect of manual acupuncture-like stimulation of a forepaw on extracellular ACh release in the parietal cortex in aged rats. **a** Diagram of the experiment demonstrating the microdialysis probe in the cerebral parietal cortex. **b**, **c** CNS-intact condition ($n = 8$ in 4 rats, 2 trials per rat). **d**, **e** Spinalized condition ($n = 4$ in 4 rats). **b**, **d** Temporal changes in ACh release in the parietal cortex ipsilateral to the stimulation site. Cortical ACh release measured every 3 min is plotted on the ordinate. Onset of the acupuncture-like stimulation is expressed as time zero on the abscissa. **c**, **e** Changes in ACh release in the parietal cortex ipsilateral (*shaded column*) or contralateral (*hatched column*) to the stimulation site during the stimulation. *Each column* and *vertical bar* shows mean ± SEM. $^{**}p < 0.01$; significantly different from the pre-stimulus control value using one-way repeated-measures ANOVA, followed by Dunnett's multiple comparison test. There were no significant differences between ipsilateral and contralateral cortical ACh release responses (**c** Student's *t*-test, **e** paired *t*-test)

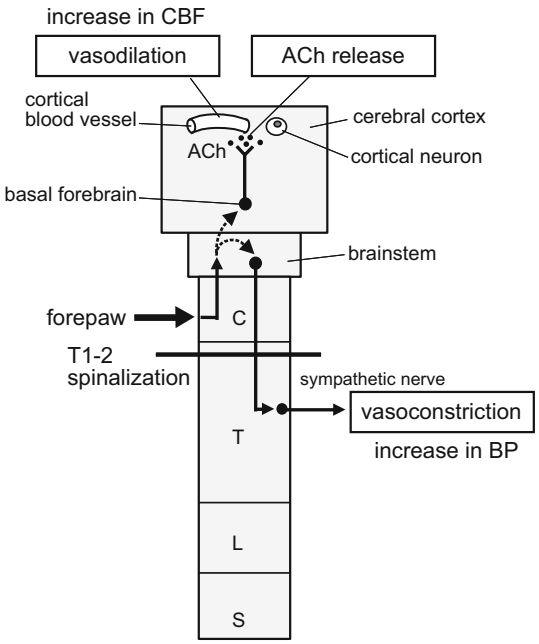

Fig. 5 Schematic diagram of a possible mechanism for cerebral cortical vasodilation by acupuncture-like stimulation of a forepaw

present results may contribute to an increase in the CBF. The present study showed that the well-maintained CBF response produced by forepaw stimulation in aged rats was blocked by the administration of muscarinic and nicotinic cholinergic receptor antagonists. Regional CBF increases following the activation of the NBM [7]. Cholinergic receptor antagonists can attenuate the increase in the CBF following stimulation of the NBM [7]. This evidence suggests that, in aged rats, the activation of the cholinergic vasodilative system in the cortex by acupuncture-like stimulation originates in the basal forebrain.

In the present study, unilateral acupuncture-like stimulation of a forepaw increased ACh release and CBF in the parietal cortex bilaterally with no statistical differences between the ipsilateral and contralateral responses, although the trend was in favor of stronger contralateral response. Since unilateral stimulation of the NBM increases CBF in the cortex ipsilateral to the stimulation [7], unilateral acupuncture-like stimulation of a forepaw may activate bilateral NBM neurons. In fact, Akaishi et al. [21] demonstrated that unilateral cutaneous pinching stimulation of a forepaw in anesthetized rats increased bilateral NBM neuronal activity, with a trend of stronger contralateral response.

Somatic afferent fibers

Manual acupuncture-like stimulation has been reported to excite somatic afferent fibers belonging to all four groups (I, II, III, IV) in rats [22, 23]. Our previous study in adult rats showed that both group III and IV fibers were involved in the afferent limb of the reflex elevation of the CBF by repetitive electrical stimulation (electro-acupuncture stimulation) of a forepaw [6]. In that study using adult rats, the threshold intensities of group II, III, and IV fibers in the radial nerve were 0.28 ± 0.06, 0.55 ± 0.10, and 3.25 ± 0.63 mA, respectively.

Our present study in extremely aged rats also examined CBF responses produced by electro-acupuncture stimulation of a forepaw with different intensities. When the stimulus intensity was less than 1.0 mA, no significant CBF responses were observed. Stimulation at 2.0 mA produced significant increase in the CBF. This response was further increased at 5.0 and 10.0 mA. The relation of CBF responses to different stimulus intensities and the magnitudes of CBF responses to each stimulus intensity observed in extremely aged rats were almost identical to that in adult rats reported in our previous study [6]. Therefore, we can speculate that activations of group III and IV afferents fibers are also responsible for the CBF response in extremely aged rats, although, in the current study, threshold intensities of each fiber group were not determined. This also suggests well-maintained activation of group III and IV afferent fibers by acupuncture-like stimulation of a forepaw in extremely aged rats, an idea supported by a report showing well-maintained reflex response of cardiac sympathetic nerve activity evoked by activation of group III and IV fibers due to cutaneous pinching stimulation of a hind paw in extremely aged rats of above 30 months [24].

In the present study, the magnitude of the CBF response elicited by electrical stimulation at the maximal intensity of 10 mA was larger than the response elicited by manual acupuncture-like stimulation. This result suggests that an electrical stimulation of a forepaw with the maximal intensity activates all fibers innervating the forepaw, while a manual acupuncture-like stimulation activates only some of these fibers.

Age-related changes of the basal forebrain cholinergic vasodilative system

Our previous study reported that the basal forebrain cholinergic vasodilative system in the cortex significantly declines in extremely aged rats above 30 months, but not in aged rats of 24–25 months, when compared to adult (4- to 7-month-old) rats [13]. The increases in CBF responses were stimulus intensity-dependent. The increase in the CBF response produced by direct electrical stimulation of the NBM at low intensity (50 μA) was similar between adult rats (118 ±5 %) and extremely aged rats (112 ± 4 %). However, the CBF responses at higher intensities

(100–200 μA) were significantly smaller in extremely aged rats (120 ± 3 % at 200 μA) than in adult rats (156 ± 12 % at 200 μA) [13]. The present study demonstrated well-maintained CBF responses due to increases in ACh release in the cortex produced by acupuncture-like stimulation, even in extremely aged (30- to 37-month-old) rats. The increase in the CBF produced by manual acupuncture-like stimulation of a forepaw in extremely aged rats was 111 ± 2 % in the present study, and the response was found to be similar to that in adult rats (112 ± 3 %) reported previously [6]. These findings suggest that acupuncture-like stimulation may activate a smaller number of basal forebrain cholinergic neurons which is comparable to electrical stimulation of the NBM at low intensity (50 μA). Our present findings suggest that the basal forebrain cholinergic vasodilative system functioned well — even in extremely aged rats — when it was activated by natural somatic afferent stimulation such as acupuncture.

Physiological relevance of increase in release of acetylcholine in the cortex

Our present results showed that, in extremely aged rats, an increase of ACh in the cortex produced by acupuncture-like stimulation of a forepaw contributes to an increase in the CBF. Acupuncture-induced CBF increase could result in the provision of sufficient oxygen and glucose to the cerebral cortex, protecting it against neuronal death resulting from insufficient blood supply, as reported in adult mice [25].

In addition to the regulation of the CBF, the acupuncture-induced increase of ACh release in the cerebral cortex may directly influence cortical neurons (Fig. 5), thereby elevating release of cortical nerve growth factor (NGF) promoting cholinergic neuron survival [26, 27], or enhancing the level of nervous functions such as attentiveness, waking state, and consciousness [28–31].

Our present findings in extremely aged rats may support the application of acupuncture in elderly people and in patients with disturbances in the CBF — for example, stroke [1, 5], mild cognitive impairment [32], and Alzheimer's disease [33] — because it is able to activate the intracranial — most likely, basal forebrain — cholinergic vasodilative system in the cerebral cortex.

Conflict of interest The authors have no conflicts of interest to declare.

Author contributions Both authors contributed to conception and design of research, performed experiments and analyzed data, interpreted results of experiments. S.U. drafted manuscript; both authors edited and revised manuscript and approved the final manuscript.

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