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Middle Cerebral Artery Occlusion Increases the Sensitivity of Cortical Neurons to Acetylcholine and Impairs Cognitive Function in Rats

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Abstract

The goal of the present study was to relate the degree of cortical cholinergic deafferentation induced by middle cerebral artery (MCA) occlusion to changes in the sensitivity of frontal cortical neurons to iontophoretic administration of acetylcholine (ACh) and to changes in cognitive performance in the Morris water maze in rats. In Wistar rats, MCA occlusion reduced the density of acetylcholinesterase (AChE)-positive fibers in the frontal cortex by 57% (n = 8) and the activity of cholineacetyl-transferase (ChAT) by 59% (n = 5). The MCA occluded rats took significantly longer to locate the submerged platform in the water maze than sham-operated rats, although swim speeds were similar, at a time when neurological deficits were minimal. Basal neuronal firing rates were reduced after MCA occlusion. The percentage of neurons responding to ACh was increased but responses to carbachol and glutamate were unaffected. The increased sensitivity of cortical neurons to ACh correlated positively with the loss of AChE activity and with the impaired performance of the MCA-occluded rats in the water maze. The increased size of the responses to the cholinomimetics is probably due to increased sensitivity of post-synaptic cholinoceptors. These data confirm that MCA occlusion in the rat causes a loss of cortical AChE-positive fibers and behavioral effects which are suggestive of memory disruption. The increased proportion of neurons responding to ACh is likely to result from the loss of AChE activity. Loss of cholinergic neurons may contribute to the cognitive impairment seen in patients with cerebrovascular accidents or stroke.

الملخص

هدفت هذه الدراسية لمعرفة درجة علاقة الموصلات العصبية المخية عن طريق عمل غلق للشريان المخي الأوسط إلى التغيرات التي تحدث في حساسية الأعصاب المخية الأمامية لإفراز الموصلات الكيميائية العصبية والوظائف المعرفية لحيوان مائي (مورز). فئران (وستر) غلق الشريان المخي الأوسط خفض من كثافة الإنزيم الاستيليكولين (الالياف الموجبة) في الفص الأمامي للمخ بحوالي 57% ونسبة 59% الإنزيم الناقل للإستيليكولين في 5 فئران وكان مهارة العوم لم تتغير في المجموعتين. وكان معدل نشاط الألياف القاعدية للمخ انخفض بعد غلق الشريان. وكانت نسبة الألياف التي استجابت للاستيليكولين قد زادت ولكن الاستجابة بالنسبة للكارباكول والجلوتاميد لم تتأثر. وزيادة حساسية الألياف المخية للاستيليكولين أثبت زيادة ضرورية ايجابية لنقص انزيم الاستيليكولين قد زادت ولكن الاستجابة بالنسبة للكارباكول والجلوتاميد لم تتأثر. وزيادة حساسية الألياف المخية للاستيليكولين أثبتت زيادة ضرورية ايجابية لنقص انزيم الاستيليكولين. ونقص الافراز الوظيفي للفئران في الماء ويعزى سبب الزيادة في الاستجابة على الحساسية في العقد المستقبلة للكولين. وعلى هذا أثبتت هذه الدراسية أن غلق الشريان المخي الأوسط للفئران قد تسبب في نقصان انزيم الاستيليكولين وتغير سلوك الفئران يعزى الى اضطرابات الذاكرة. وأن زيادة نسبة الألياف العصبية التي تستجيب الى الاستايل كولين يمكن ان تنتج عن فقدان نشاط انزيم الأستايل كولين. الخلل المعرفي الذي يرى في مرض بعد السكتة الدماغية قد يؤدي على فقدان ألياف كولنيرجية.

Keywords: Ischemia, McAocclusion, Ach, Stroke, Cognitive impairment.

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1. Introduction

Stroke is the third most common cause of death and the most common cause of functional disability in adults (American Heart Association, 2003). In addition to the many physical signs and symptoms, acute stroke and other forms of cerebrovascular diseases are well-recognized causes of cognitive impairment (Nas et al., 2004; Srikanth et al., 2004; Talell et al., 2004; Werring et al., 2004; Zhou et al. 2004). Personality changes had also been reported after stroke (Stone et al., 2006).

Two to 24 h after inducing permanent focal cerebral ischaemia (MCA occlusion), edema and pan-necrosis evolves, affecting neurons and glia, in the frontoparietal cortex and lateral striatum of rats. Neurological and behavioral effects are apparent including forelimb flexion, unilateral circling (Materossi et al., 1982; Bederson et al., 1986; Persson et al., 1989), decreased locomotor performance, and decreased performance in the step-through passive avoidance procedure (Yamamoto et al., 1988; Togashi et al., 1996). After 1 week, there is a loss of AChE-positive fibers from the frontoparietal cortex, probably because of subcortical damage to the cholinergic projection from the nucleus basalis magnocellularis (Kataoka et al., 1991; Togashi et al., 1996).

The causes of post-stroke cognitive impairment are not fully understood (Zhou et al. 2004). The present study attempts to correlate the loss of AChE-positive fibers produced in Wistar rats by combined permanent left MCA and left common carotid artery (CCA) occlusion with two functional measures, sensitivity of cortical neurons to iontophoreticalIy administered ACh and performance of rats in 2 tasks of cognitive ability, the Morris water maze and the step-through passive avoidance procedure.

2. Materials and methods

2.1. Animals and housing

Adult male Wistar rats weighing 280-330 g were housed in groups of 4-6 in PVC cages (350 x 530 mm long x 180 mm high) in an environment maintained at 19-22 °C and a relative humidity of 55% respectively with a 14 h/l0 h light/dark cycle (light on from 06.00 to 20.00 h). Food and water were available *ad libitum.*

2.2. Permanent MCA occlusion surgery

The operative procedures were undertaken using halothane anesthesia (4% in a mixture of 70% N_2O and 30% O_2 for induction reducing to 2% for maintenance) in 13 rats. Each rat was allowed to breathe spontaneously. Cranial and rectal temperatures were maintained between 36.5 and 38 ^oC.

Unilateral left CCA occlusion was followed by subtemporal, subperiosteal craniectomy (with intact zygoma) and exposure of the main trunk of the left MCA under 25-fold magnification of an operating stereomicroscope (Tamura et al., 1981; Shigeno et al., 1985). The MCA was electrocauterised from a point proximal to the lenticulostriate artery to the level of the MCA with the inferior cerebral vein (n = 13). In sham-operated rats, both vessels were exposed but not occluded (n = 5). The ipsilateral CCA occlusion was carried out to reduce variability of the infarct volume.

2.3. Neurological effects of MCA occlusion

The rats were assessed for neurological deficits using the following rating scale: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resistance to lateral push; 3, forelimb flexion, decreased resistance to lateral push and unilateral circling in three successive trials (Bederson et al., 1986).

2.4. Behavioral effects of MCA occlusion

2.4.1. Locomotor performance

Three weeks after MCA occlusion locomotor performance of the rats was assessed on three consecutive trials at 15 min intervals using a rotarod (12 rpm, 9 cm diameter, 2 mm grooves, 60 s cut-off).

2.4.2. Step-through passive avoidance

The day after evaluating the locomotor performance of the rats on the rotarod, they were tested in a step-through passive avoidance procedure. On the first day, each rat was placed in the illuminated chamber and was allowed free access to the darkened chamber. The latency to enter the darkened chamber was recorded and on entering, the rat was exposed to foot-shock (0.8 mA, 1 s). On the second day, the rat was again placed in the illuminated chamber and the latency for it to enter the darkened chamber was recorded (cut-off 300 s).

2.4.3. Morris water maze

Over the next two weeks the rats were evaluated for their ability to locate a hidden platform in the Morris water maze. Each rat was placed in the maze for two trials a day over 12 days and the latency for each rat to swim to the submerged escape platform was recorded (cut-off 60 s).

On the 13th-15th days, a visible cue was placed on the platform. On the 16th and 17th days, this cue was removed and the latency for each rat to swim to the platform was recorded.

2.5. Electrophysiological effects of MCA occlusion

After completing the behavioral experiments (6 weeks after MCA occlusion), the rats were anaesthetized with urethane (1.5 g/kg i.p.), placed in a stereotaxic frame and the cranium overlying forelimb representation areas (Hall and Lindholm, 1974) in the frontal cortex (motor area 4, Lysakowski et aI., 1989) was removed. The cortex was exposed by a narrow slit in the dura and covered with 4% agar in 0.9% w/v NaCl solution. Rectal temperature was maintained at 37 ± 1°C. Spontaneously active neurons were recorded from the central barrel (containing 2 M NaCl) of a 6-barrelled glass microelectrode (tip diameter 6-8 µm) during vertical penetrations of the frontal cortex (4 µm steps). Records were kept of recording depth

relative to cortical surface. Unit activity was amplified, filtered (500 Hz-l0 KHz) and displayed on a digitizing oscilloscope to facilitate discrimination and quantification (see below).

Drugs were applied iontophoretically through the outer barrels of the micro-electrode. These contained: ACh chloride (0.2 M, pH 4.0, Sigma), carbamylcholine chloride (carbachol, 0.2 M, pH 4.0, BDH), Na L-glutamate (0.2 M, pH 8, Sigma) and atropine sulphate (50 mM, pH 5.0, Sigma). A 5-9 nA backing current was applied to each drug-containing barrel and compensated through a barrel filled with 2 M NaCl.

All drugs were applied with a current of 30 nA for 20 s and current compensation was routinely used. Each drug was applied 3 times (separated by recovery periods of 1 min) to each neuron and the average of the three applications was stored for analysis. The interval between ejections of two different drugs was at least 2 min. The firing rate before drug application was compared with the firing rate during, and for the 20 s period immediately following, drug application. If ACh or carbachol produced significant effects (see Abdulla et al., 1994 for details), atropine was applied for 25 s commencing 5 s before the agonist to confirm the muscarinic nature of the response. Responses which were not blocked by atropine were not included in the analyses.

2.6. Histological verification and AChE staining

At the end of each experiment, the rats were deeply anaesthetized and perfused by transcardiac infusion of 300 ml of 4% paraformaldehyde and 15% saturated picric acid in phosphate buffer (0.1 M, pH 7.4) by means of a peristaltic pump. The brains were removed and kept in 4% paraformaldehyde for 3-4 h, and then repeatedly washed with phosphate buffer at 4 °C to remove colouration due to picric acid. AChE staining was visualized on 30 µm sections with acetylthiocholine iodide as substrate and iso-OMPA (Sigma) as an inhibitor of nonspecific esterase and with 3,3' -diaminobenzidine intensification (Geula and Mesulam, 1989). The total length of AChE-staining axons per unit area was determined using an IBAS 2000 image analyzer. Briefly, the AChE-containing axons and cell bodies were discriminated from background, converted to a binary image and the cell bodies and any artifacts were eliminated interactively. The axon images were thinned to 1 pixel and their total length expressed as a proportion of the field area (Abdulla et al., 1994).

2.7. Choline acetyltransferase activity

Frontal cortical ChAT activity was measured in a separate group of 5 sham-operated and 5 MC A-occluded rats. The rats were killed by cervical dislocation, their brains rapidly removed and tissue (about 30 mg) was dissected from the frontal cortex of each hemisphere and stored at -70 ^{o}C until assay. ChAT activity was calculated from the formation rate of [^{14}C]ACh from [^{14}C]acetylcoenzyme A by a method derived from Fonnum (1975). Briefly, samples were homogenised in NP-40 (1 % phosphate buffer, Sigma) using 8.3 µl/mg tissue wet weight. Ten µl aliquots of tissue homogenate were incubated, in duplicate, at 37 ^{o}C for 5 min with 10 µl incubation medium at pH 7.4 containing NaCl (0.75 M), NaH_2PO_4 (135 mM), choline (20 mM), EDTA (50 mM), acetylcoenzyme A (1 mM), physostigmine sulphate (0.4 mM) and 2 µCi, in 10 µl of [14C]-acetylcoenzyme A (New England Nuclear). Incubation was stopped with 5 ml of cold NaH_2PO_4 (10 mM, pH 7.4). [^{14}C]ACh was extracted with acetonitrile containing tetraphenylboron (20 mgl ml) and counted in a PPO-POPOP toluene scintillant.

2.8. Statistical analysis

Unit responses to drugs were recorded as the average firing rate of 3 drug applications, and consisted of three 20 s blocks (each of eighty 250 ms epochs) obtained immediately before (control), during and for 20 s after drug application as described above. Epochs recorded during drug application were compared to control activity using Wilcoxon tests for each neuron. Zones which differed significantly from control activity were automatically detected and used to calculate response latency and duration.

Independent t-tests were used to compare the averaged firing rates, latencies to onset and durations of drug action and the lengths of AChE-containing axons of the control and ischemic rats. A $\chi 2$ test was used to compare the sensitivity of different drugs between the control group and the ischemic groups. The regressions of the ACh-induced changes in firing rate vs. baseline rate for the sham-operated and MCA-occluded rats were compared using MANOVA.

3. Results

3.1. Neurological effects of MCA occlusion

One day after permanent proximal MCA occlusion, 4/11 animals exhibited unilateral circling (neurological score of 3). The following day 9/11 animals had neurological scores of 2. After 7 days the majority of the animals (8/11) had neurological scores less than 2 and exhibited mainly forelimb flexion (6/11) or no apparent neurological deficit (2/11). After 19 days only two animals had a neurological score of 2.

3.2. Behavioral effects of MCA occlusion

The performance of MCA-occluded rats on the rotarod 3 weeks after occlusion was not significantly different from that of sham-operated rats over three consecutive trials (sham-operated 50 ± 6 s, 38 ± 6 s, 37 ± 6 s, MCA-occluded 42 ± 6 s, 49 ± 6 s, 37 ± 6 s). However, MCA occluded rats performed less well than sham operated rats in the step-through passive avoidance procedure with a mean latency to enter the darkened chamber on the test day of 210.7 ± 34.7 s compared with 300s (cut-off time) for the sham-operated rats (Figure 1). There were no significant differences between the 2 groups on the training session (26.7 ± 4.0 s for the control group and 28.1 ± 4.8 s for the MCA-occluded rats).

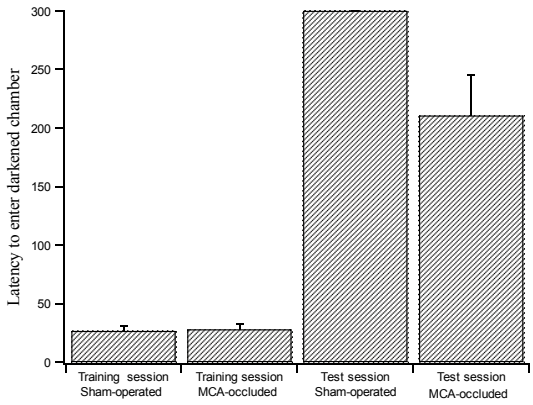

Figure 1. Histogram of mean latencies ± s.e.m. of sham-operated and MCA-occluded rats to enter the darkened chamber in the step-through avoidance procedure.

On the final two training days in the water maze (days 11 and 12), MCA-occluded rats took longer to find the submerged platform than sham-operated rats, 40.0 ± 7.0 s compared with 8.3 ± 1.0 s ($P<0.001$). When the cue was placed on the submerged platform, the MCA-occluded rats located the submerged platform more rapidly and by the third day (day 15), performed as well as sham-operated rats (Figure 2). As can be seen from the figure, the MCA-occluded rats continued to locate the hidden submerged platform with similar latencies to sham-operated rats when the cue was removed (days 16 and 17).

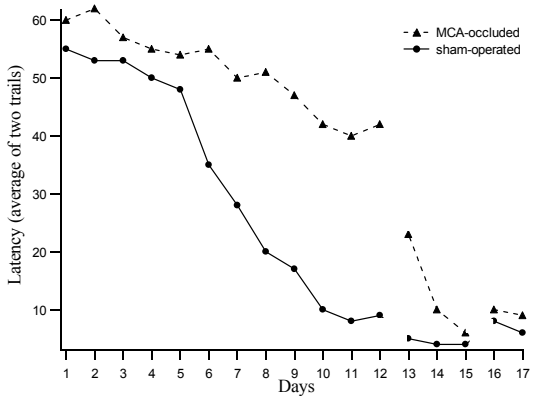

Figure 2. Mean latencies (2 trials/day) of rats to locate the hidden escape platform in the Morris water maze. MCA-occluded rats took longer to locate the platform than sham-operated rats. On days 13-15, when platform position was indicated by a visible cue, latenies of MCA-occluded rats approximated that of sham-operated rats and remained similar when the cue was removed (days 16 and 17).

3.3. Electrophysiological effects of MCA occlusion

Basal neuronal firing rates were reduced in MCA-occluded rats from 3.1 ± 0.2 to 2.6 ± 0.2 imp S^{-1} ($P<0.05$). In sham-operated rats, 52.3% of neurons showed a muscarinic response to ACh compared with 80.3% in MCA-occluded rats (Figure 3). In contrast, the proportions of neurons responding to carbachol and glutamate were unaffected by ischemia (Figure 4). Regression analysis showed that the direction and magnitude of the response to ACh were closely correlated with the firing rate immediately before administration. The regression line obtained in MCA-occluded rats was significantly steeper than that obtained in sham-operated control rats ($P< 0.0001$, MANOVA). MCA occlusion also significantly increased the slope of the regression line for responses to carbachol.

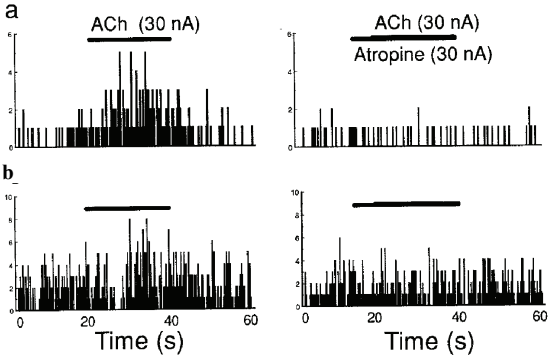

Figure 3. Spike frequency/time histograms showing the firing rates of two different frontal cortical neurons from (a) MCA occluded rat and (b) sham operated rat responding to iontophoretic application of ACh (30 nA for 20 s. The effects of ACh in both neurons were completely antagonized by iontophoretic application of atropine (30 nA for 25 s) commencing 5 s before ACh and continuing throughout the period of ACh application. Note that the base line firing rat is less in the neuron from the MCA occluded rate while the response to ACh was from such rat than the sham operated rat.

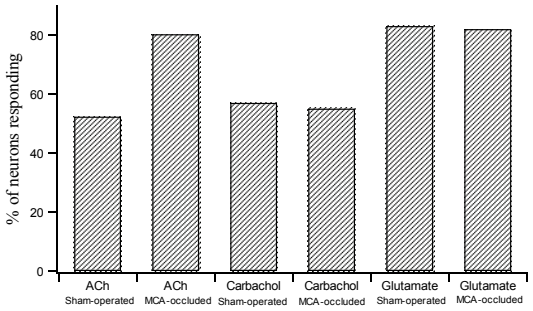

Figure 4. Histograms showing the influence of MCA occlusion on the percentages of cortical neurons responding to iontophoretic administration of Ach, carbachol and glutamate.

3.4. Histological/biochemical effects of MCA occlusion

The infarct boundaries were clearly delineated after MCA occlusion and there was a greater neuronal cell loss in the striatum than in the frontal cortex. There was a generalized loss of AChE staining from ipsilateral cortical and subcortical brain regions (Figure 5). The density of AChE-positive fibers in the frontal cortex was reduced by 57%, from 145.7 ± 6.6 mm mm^{-2} to 62.9 ± 8.2 mm mm^{-2} and this was associated with a 59% reduction in cortical ChAT activity, from 168.4 ± 14.1 to 69.2 ± 5.1 pmol ACh min^{-1} mg $tissue^{-1}$.

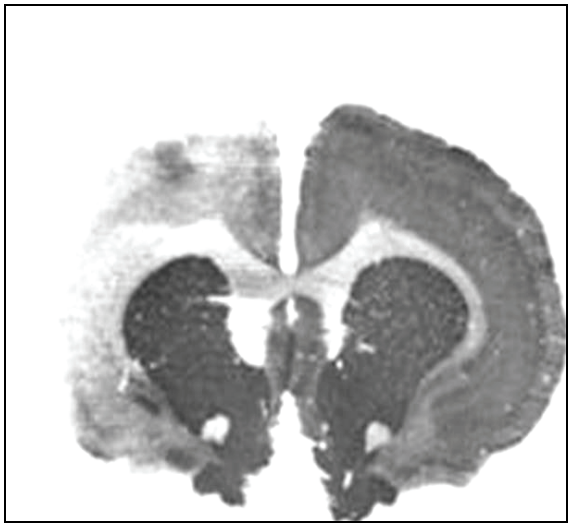

Figure 5. A 30 µm coronal brain section from an MCA-occluded rat showing loss of AChE staining in the lesioned hemisphere.

3.5. Relation between behavioral, iontophoretic and morphological studies

The relations between the performances of MCA - occluded rats in the water maze; the loss of cortical AChE-positive fibers and the sensitivity of cortical neurons to ACh were investigated by correlation analyses. Latency for the MCA-occluded animals to reach the platform in the water maze correlated positively with the loss of AChE ($r = 0.85$) and with the sensitization to ACh ($r = 0.90$). The increased sensitivity of cortical neurons to ACh also correlated positively among the MCA-occluded animals with the loss of AChE-positive fibers ($r = 0.81$).

4. Discussion

Middle cerebral artery occlusion produces ischemic damage which, in rats, typically affects areas of the frontoparietal cortex and lateral striatum. Susceptibility of the frontoparietal cortex varies among stains being less damaged in Wistar rats compared to the substantial damage which occurs in Sprague-Dawley and Fischer rats (Duverger and MacKenzie, 1988). For this reason, Wistar rats were used in the present study.

Middle cerebral artery occlusion increased the latency for rats to locate the submerged platform in the water maze. This could be explained by an impaired spatial ability during the acquisition phase of learning because the performance of the MCA-occluded rats equaled that of the sham-operated rats when a cue was placed on the submerged platform and because the improvement was maintained when the cue was removed. This indicates that once learnt the ability to retain information (i.e. how to locate the escape platform) was not impaired. In contrast, rats with excitotoxic lesions of the nucleus basalis had difficulty locating the platform when the cue was removed (Abdulla et al., 1994; 1997b). The ability of MCA-occluded rats to localize tactile stimuli delivered to the side of the body contralateral to the lesion (Grabowski et al., 1988) is impaired but this could not explain the behavioral deficits obtained in the present study because, when the cue was in place, the MCA-occluded rats located the platform with similar latencies to the sham-operated rats. An injection of microspheres into the internal carotid artery of rats produces a more severe focal cerebral ischemia, in terms of neuronal cell loss, than that observed in this study but the selective deficit in acquisition of spatial information rather than in retention or retrieval, is similar (Lyden et al., 1992). The deficits observed in ischemic rats are consistent with findings obtained in the step-through passive avoidance procedure (this study; Yamamoto et al., 1988; Tamura et al., 1989; Wahl et al., 1992; Togashi et al., 1996). As well as data obtained from many clinical studies performed on human after stroke (Nas et al., 2004; Srikanth et al., 2004; Talell et al., 2004; Werring et al., 2004; Zhou et al., 2004; Park et al., 2005; 2007). However, the results differ from those described in CFWl mice in which MCA occlusion did not produce a decrement in water maze performance (Stollenwerk et al., 1992).

Iontophoretic application of ACh and carbachol to frontal cortical neurons of sham-operated rats produced muscarinic responses (atropine-sensitive) in 52% and 55%, respectively, of neurons tested. These percentages are similar to those described previously in control rats (Abdulla et al., 1994; 1997a). MCA occlusion increased the percentage of neurons responding to ACh, without affecting the neuronal population responding to carbachol and glutamate. Since carbachol is not susceptible to hydrolysis by acetylcholinesterase the increase in the number of neurons responding to ACh was therefore probably due to decreased AChE activity. This is supported by the loss of AChE positive fibers from cortical regions. However, the sizes of the individual responses to both ACh and to carbachol were increased after MCA occlusion suggesting an increased sensitivity of post-synaptic muscarinic receptors.

A more selective cortical cholinergic deafferentation produced by an excitotoxic lesion of the nucleus basalis (Abdulla et al., 1994; 1997a) also produced similar changes in unit responses to iontophorised ACh and carbachol.

In conclusion Loss of AChE-positive fibers may be responsible for the cognitive impairment seen after stroke. Additionally, strategies designed to increase cortical cholinergic functions (grafting or pharmacological) may have therapeutic value in alleviating stroke-induced cognitive impairment.

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