Biochemical Determinations of Cholinergic Innervation in Cerebral Arteries

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SUMMARY Endogenous choline acetyltransferase (ChAT) activity and high affinity (HA) $^3$H-choline uptake can be assayed specifically and used to quantify cholinergic neuronal mechanisms. In general, arteries at the base of the brain in the cat and rabbit were distinguished by high and in the dog by lower ChAT activity and HA $^3$H-choline levels of uptake. There was a linear relationship between the mean values of the observations from each of the two assays on given cerebral arteries from the three species ($r = 0.871$, $P < 0.001$). Since ChAT activity and HA $^3$H-choline uptake essentially are confined to cholinergic nerve terminals, our results imply that the Circle of Willis and its main branches in the cat, rabbit, and dog are cholinergically innervated. Circ Res 45:217-218, 1979

THE NATURE of the innervation of cerebral arteries and its physiological function has been of interest since the early anatomical studies of Willis in 1664 (Purves, 1972). Adrenergic nerve fibers supplying pial vessels have been demonstrated most convincingly by the highly specific and sensitive Falck histofluorescence technique (Falck et al., 1965). Further supportive evidence has been derived from electron microscopy (Iwayama et al., 1970), in vitro studies of isolated cerebral vessels (Lee et al., 1976), and biochemical investigations (Berkowitz et al., 1970; Su et al., 1977). In comparison, the mapping of the putative cholinergic innervation has been more difficult due to a lack of specific methods. An attempt has been made to use the histochemical technique of staining for acetylcholinesterase (AchE) to localize cholinergic nerve fibers (Borodulya and Pletchkova, 1976; Derm and Stone, 1976; Edvinsson et al., 1972), and confirmation has been sought with electron microscopy (Iwayama et al., 1970; Edvinsson et al., 1972), by pharmacological studies on isolated vessels (Edvinsson et al., 1972; Lee et al., 1976, 1978), and by measurement of cerebral blood flow (D'Alecy and Rose, 1977). However, the experimental evidence in favor of a cholinergic innervation is equivocal.

A more direct approach to demonstrate a cholinergic innervation in cerebral blood vessels would be the application of specific biochemical methods. Fonnum (1969, 1975) developed a highly sensitive radioenzymatic assay for endogenous choline acetyltransferase (ChAT) activity. A second method, which has been successfully used in research on cholinergic innervation, is high affinity (HA) choline uptake (Haga and Noda, 1973; Yamamura and Snyder, 1973). ChAT, the synthesizing enzyme, as well as the HA choline uptake mechanism, is confined to cholinergic systems in both vertebrates and invertebrates. In contrast, AchE is found consistently in noncholinergic structures (Eranko et al., 1970; Hume and Waterson, 1978). ChAT activity (Fonnum et al., 1976) and the HA choline uptake system (Suszkiw and Pilar, 1976) are localized principally in cholinergic nerve terminals, with comparatively little activity in cell bodies. Lesions in cholinergic brain regions (Kuhar et al., 1973; McGreer et al., 1971) and in peripheral tissues (Pert and Snyder, 1974; Suszkiw and Pilar, 1976) have resulted in loss of ChAT and HA choline uptake. In the brain, there is good correlation between regional ChAT, $V_{max}$ for HA choline uptake, and acetylcholine (Ach) content (Yamamura and Snyder, 1973). The highest choline uptake is present in the striatum, an area distinguished by a high Ach content, Ach turnover rate, and ChAT activity.

In the present study, the radioenzymatic assay for ChAT and the HA choline uptake method were used to quantify the cholinergic innervation in the main cerebral arteries in the dog, cat, and rabbit. The positive identification of cholinergic innervation and a knowledge of its regional variation in the cerebral vasculature are prerequisites to understanding its physiological function.

Methods

Mongrel dogs (12-20 kg) and cats (2-4 kg) were anesthetized with sodium pentobarbital (30 mg/kg, iv and 50 mg/kg, ip respectively), and adult white rabbits (2-3 kg) were stunned prior to exsanguination. The entire brain with pial vessels attached, as well as the common carotid artery, was removed rapidly and placed in gassed Krebs phosphate solution on ice for dissection with the aid of a dissecting microscope. The composition of the Krebs solution was: NaCl, 115 mM; KCl, 4.75 mM; CaCl$_2$, 1.3

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Since the cerebral arteries of the dog were of sufficient size, the anterior, middle, and posterior cerebral arteries, including their branches to a vessel diameter of 0.5 mm, the communicating phosphate buffer, 15.6 mM (pH 7.4); and glucose, 12 mM (pH 7.4).

Assays were standardized with rabbit iris, a tissue with a known cholinergic innervation (Suszkiw and Pilar, 1976; Chiappinelli et al., 1976), which poses similar problems in tissue handling as blood vessels, and is available readily in quantities sufficient to permit kinetic studies.

ChAT Assay

Iris and blood vessels were homogenized in Potter-Elvehjem glass grinders (Kontes Glass Co.) in a homogenization solution containing: NaCl, 300 mM; sodium phosphate buffer, 10 mM (pH 7.4); EDTA, 1 mM; and Triton X-100, 0.5% (vol/vol) (pH 7.4). The volume of the homogenization solution was adjusted to give a tissue concentration of 1.5 mg/40 μl.

The radioenzymatic assay was adapted largely from Fonnum’s method (1969, 1975). The procedure was carried out on ice. The ChAT activity was determined in 6-ml centrifuge tubes (with ground-glass stoppers). To the homogenate (40 μl), the following substrate mixture was added (final concentration per 60 μl): [1-C]acetyl-coenzyme A (58-59.5 mCi/mmol, Amersham/Searle Corp.), 0.05 mM; NaCl, 300 mM; sodium phosphate buffer, 50 mM (pH 7.4); choline chloride, 8 mM; EDTA, 10 mM; and eserine sulfate, 0.1 mM (pH 7.4). The reaction mixture contained 10 μl of [14C]-acetyl-coenzyme A and 10 μl of the buffered choline substrate. The radiochemical was diluted with unlabeled acetyl-coenzyme A (Sigma Chemical Co.). The mixture was incubated for 12 minutes at 37°C, and the reaction was stopped by the addition of 5 ml of 10 mM sodium phosphate buffer (pH 7.4). Furthermore, 1 ml of 3-heptanone containing 15 mg sodium tetrphenyl boron (Sigma Chemical Co.) was added to the tubes and the contents were lightly shaken for 1 minute. After centrifugation at 3000 rpm for 3 minutes, 0.5 ml of the organic phase was transferred into vials containing 10 ml toluene scintillation cocktail and 2 ml acetonitrile. The radioactivity was determined in the liquid-scintillation counter (Mark III, Searle Analytic, Inc.) at a counting efficiency of 82%.

Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. ChAT activity was expressed as nmol Ach/g wet weight per hour and as nmol Ach/mg protein per hour.

3H-Choline Uptake

Dissected iris and blood vessels were preincubated in 5 ml Krebs phosphate buffer at 37°C for 10 minutes. Following preincubation, each tissue was transferred to a vial containing 2 ml choline chloride, 5 μM (final concentration); [methyl-3H]-choline chloride (10.1 Ci/mmol, Amersham/Searle), 2 μCi. The incubation was carried out for 4 minutes at 37°C. The uptake reaction was stopped by washing the tissue in 100 ml cold Krebs solution for 15 seconds, whereupon it was blotted and weighed. Immediately thereafter, each tissue was placed in a scintillation vial for digestion in Soluene-100 (0.5 ml, Packard Instrument Corp.). Scintillation cocktail (10 ml) and ethanol (1 ml) were added, and the radioactivity was assayed in the scintillation counter. Counting efficiency was 30-40% as determined by an internal standard.

Results

Standardization of CHAT Activity in Rabbit Iris

The time course of activity in homogenized iris was followed from zero to 30 minutes and was linear up to 12 minutes (Fig. 1A). The assay was standardized for an incubation time of 12 minutes at 37°C.

Good correlation was found between the amount of Ach synthesized and the amount of tissue homogenate (Fig. 1B; r = 0.998, P < 0.01). Tissue concentrations of up to 3.0 mg iris/40 μl were evaluated. Because of the small amounts of blood vessel available and the importance of optimizing the detection, we selected tissue concentrations of 1.0-2.0 mg/40 μl homogenate. Since only small amounts of [14C]-Ach were expected to be synthesized, no attempt was made to determine the optimal concentration of acetyl-coenzyme A (acetyl-CoA). To maximize the specific activity of the assay, a concentration of 0.06 mM of acetyl-CoA was chosen.

Blank values comprised approximately 0.5% of the total radioactivity added. Incubation of tissue without choline substrate and heat-inactivated tissue produced comparable low blank values. To ensure further the reliability of the assay, samples were incubated in a medium without eserine but containing AchE (electrophorus electricus, 3.11.7). When incubated, ChAT activity in the rabbit iris was decreased by 92-94%.

Fonnum (1975) previously has shown that this radioenzymatic assay can distinguish [14C]-Ach from
$^{14}$C-acetylcarnitine because Ach is extracted at neutral pH from the aqueous into the organic phase. Most of the acetylated contaminants, in particular acetylcarnitine, are not sufficiently basic to be extracted under these conditions.

ChAT activity in the rabbit iris was found to be $1129.4 \pm 36.2$ (mean $\pm$ se; $n = 14$) nmol/g per wet weight per hour. When we used the method of Lowry et al. (1951) for protein determination, corresponding values of $11.54 \pm 0.43$ nmol/mg protein per hour were obtained. Chiappinelli et al. (1976) determined a ChAT activity of 10 nmol/mg per hour in the chick iris.

**ChAT Activity in Cerebral Blood Vessels**

(Fig. 2)

ChAT activity was present in all dog cerebral arteries studied. Mean values were highest in the anterior cerebral artery and the Circle of Willis (135.4 ± 39.9 and 136.2 ± 15.6 nmol/g per wet weight per hour, respectively). The middle cerebral and posterior cerebral arteries exhibited lower mean values of ChAT activities (91.8 ± 5.0 and 80.9 ± 10.6 nmol/g per hour, respectively), although only that of the Circle of Willis differed significantly from these latter two measurements ($P < 0.05$). ChAT activity in the basilar artery was 35.2 ± 7.8 nmol/g per hour, which was less than the four previously cited measurements ($P < 0.05$). Thus, in the dog, the ChAT activity is greater in the anterior compared with posterior vessels at the base of the brain. All dog cerebral vessels exhibited markedly greater ChAT activity than did the common carotid artery ($3.9 \pm 1.2$ nmol/g per hour, $P < 0.05$).

Activity in cat cerebral vessels tended to be higher than in the dog, and mean values suggest a reversed pattern of ChAT density. The basilar artery exhibited a ChAT activity of 270.3 ± 60 nmol/g per hour; the anterior and posterior cerebral arteries included with the Circle of Willis and the middle cerebral arteries were 211.0 ± 22.5 and 162.3 ± 27.9 nmol/g per hour, respectively. None of these values is significantly different from each other. Collectively, the cat cerebral vessels exhibited greater ChAT activity than did the common carotid artery ($P < 0.05$).

The ChAT activity in the rabbit Circle of Willis was $199.0 \pm 25.6$ nmol/g per hour compared to $144.9 \pm 15.7$ nmol/g per hour in the basilar artery. As in the previous two species, the cerebral vessels demonstrated a greater activity than did the common carotid artery of the rabbit ($P < 0.05$).

There was an apparent interspecies difference in ChAT activity in some blood vessels from comparable anatomical locations. For instance, the mean values for the basilar artery in the three species differed significantly from each other ($P < 0.05$).

When ChAT activity was expressed per mg protein, comparable results were obtained.
Standardization of HA $^3$H-Choline Uptake in Rabbit Iris

$^3$H-Choline uptake was examined at two temperatures at times between 1 and 12 minutes (Fig. 3A). At 37°C, $^3$H-choline uptake was linear between 1 and 6 minutes, and at 4°C the small accumulation also was linear over the same time interval. On this basis, the incubation time for $^3$H-choline uptake was selected at 4 minutes. Yamamura and Snyder (1973) concluded that the limited uptake at 4°C may represent some process other than diffusion. In our study, we considered uptake at 4°C to represent low affinity (LA) uptake. A good correlation was found between $^3$H-choline uptake and tissue weight ($r = 0.977$, $P < 0.001$ for 37°C; $r = 0.949$, $P < 0.001$ for 4°C; Fig. 3B), which ranged from 2 to 11 mg of intact tissue.

$^3$H-Choline accumulation in the rabbit iris, like other cholinergically innervated tissue, was characterized by two uptake systems (Pert and Snyder, 1974; Suszkiw and Pilar, 1976; Yamamura and Snyder, 1973). The Na$^+$-dependent, HA uptake system has been reported to have a $K_m$ of 1-10 µM; $K_m$ values for the LA system were 10-100 µM (Attweh et al., 1975). Both the Lineweaver-Burk and bivariate linear regression analysis revealed an HA uptake system with a $K_m$ of 7.7 ± 0.76 µM and a $V_{max}$ of 24.1 ± 1.3 nmol/g per 4 minutes, values which fall within the range of reported findings. No apparent $K_m$ for the LA uptake was determined since, even at a 100 µM external choline concentration, saturation for the LA system was not reached. From these kinetic studies we concluded that for incubation a concentration of 5 µM of nonradioactive choline would be appropriate.

$^3$H-Choline Uptake in Cerebral Blood Vessels (Fig. 4)

In the dog, values for $^3$H-choline uptake into the three main cerebral arteries and the Circle were not significantly different and ranged from 3.49 ± 0.35 to 4.33 ± 0.25 nmol/g per 4 minutes. As with the ChAT results, the dog basilar artery tended to show a lower choline accumulation than did other vessels. Uptake into all cerebral vessels tested was significantly greater than that in the common carotid artery ($P < 0.05$).

Although the mean value for uptake into the basilar artery of the cat was higher (7.68 ± 0.31

![Figure 3](http://circres.ahajournals.org/)

Figure 3 Standardization of $^3$H-choline uptake in rabbit iris. A: Time course of $^3$H-choline uptake at △ (37°C) and ● (4°C). Values represent means of three determinations. B: Correlation between variable wet weights and $^3$H-choline uptake at △ (37°C) and ○ (4°C). Lines were fitted by linear regression. Incubation conditions are described under Methods.

![Figure 4](http://circres.ahajournals.org/)

Figure 4 $^3$H-Choline uptake in cerebral arteries in dog, cat, and rabbit. Incubation was carried out for 4 minutes at 37°C (clear bars) and 4°C (shaded bars). Vertical lines indicate mean ± SE; in parentheses are the number of determinations. A—anterior cerebral artery; M—middle cerebral artery; P—posterior cerebral artery; C—Circle of Willis; B—basilar artery; CC—common carotid artery. In the rabbit, C represents the Circle of Willis plus major branches.
nmol/g per 4 minutes) than that of the middle cerebral artery and the Circle of Willis (6.83 ± 0.37 and 6.67 ± 0.35 nmol/g per 4 minutes, respectively), no significant difference was observed between these three vessels. They were higher than those in the dog. However, all cat cerebral vessels were distinguished by a markedly higher choline accumulation than that of the common carotid artery (P < 0.05).

Uptake of 3H-choline in the rabbit basilar artery (8.00 ± 0.60 nmol/g per 4 minutes) could not be distinguished statistically from that into the vessels of the Circle (7.03 ± 0.46 nmol/g per 4 minutes). Again, uptake into the common carotid artery of the rabbit was lower in comparison (P < 0.05). In general, uptake values into the cerebral arteries in the cat and rabbit were similar and greater than for the dog.

**Correlation between ChAT Activity and 3H-Choline Uptake Measurements**

ChAT activity and choline accumulation are both confined to cholinergic nerve terminals. If both parameters are related to the number of terminals, it might be hypothesized that a correlation would be found between the two assays. The means of the individual ChAT plus 3H-choline uptake values for given vessels from the three species were linearly related to each other (Fig. 5; r = 0.871, P < 0.001).

**Discussion**

Numerous attempts have been made to demonstrate the presence and nature of the transmitter involved in the dilator innervation of cerebral blood vessels. Although this transmitter has been assumed to be cholinergic, studies have not demonstrated the criteria generally required for transmitter identification (Lee et al., 1975, 1978). The origin of the dilator fibers innervating cerebral arteries has not been established satisfactorily. Chorobski and Penfield (1932) concluded from stimulation experiments that cholinergic fibers reach intracranial arteries via the facial nerve, the great superficial petrosal nerve, and the internal carotid plexus. Certainly, surgical removal of the superior cervical ganglia will lead to a degeneration of sympathetic but not dilator innervation of cerebral vessels (Lee et al., 1978). However, analogous sectioning of facial or petrosal nerves has not yet been carried out.

Many studies of the nonsympathetic cholinergic innervation of cerebral blood vessels have employed the histochemical technique of cholinesterase staining. However, this method is valid only in conjunction with other assays for a cholinergic system, because AchE may be present in noncholinergically innervated tissue (Eranko et al., 1970; Hume and Waterson, 1978). As we have shown that both HA choline uptake and the acetylating enzyme for the transmitter in cholinergic nerves are present in cerebral arteries, the evidence obtained by the cholinesterase staining technique acquires added weight. In the cat, Edvinsson et al. (1972) demonstrated AchE-containing nerve fibers in the wall of vessels at the base of the brain. The pattern of these fibers closely resembled those of the adrenergic system. Denn and Stone (1976) found in the macaque monkey a rich supply of AchE-containing fibers surrounding the arteries of the Circle of Willis. Similar results were obtained by Borodulya and Pletchkova (1976) in studies on dog cerebral arteries. The AchE in cerebral vessels in the cat, a species with a dominant dilator innervation, persisted after chronic bilateral cervical sympathectomy (Lee et al., 1978). Fifty percent of the axon terminals in the anterior cerebral artery of the rat contained electron-translucent vesicles, which is consistent with but not indicative of cholinergic nerves (Iwayama et al., 1970). Axon terminals with similar profiles have been described in other species (Edvinsson et al., 1972; Edvinsson, 1975).

Even though measurements of ChAT activity and choline uptake do not distinguish between afferent or efferent neurons, it is unlikely that the former contributed significantly, as there are comparatively few afferent fibers in blood vessels (Abraham, 1969). Only 18% of the fibers in the facial nerves of humans, dogs, and cats are afferent (Van Buskiik, 1945). Although intrinsic neurons have been described in blood vessels of other regional beds (Honig and Frierson, 1976), they have not been observed in the cerebral vasculature.

Cholinergic nerve terminals are characterized biochemically by their high content of Ach, ChAT, and a HA choline uptake system. ChAT, the synthesizing enzyme for Ach, catalyzes the acetylation of choline by acetyl-CoA. The distribution of ChAT and its product, Ach, are similar in cholinergic systems (Yamamura and Snyder, 1973). ChAT is present in excess in the cholinergic neuron and, therefore, is believed to play no role in the regulation of Ach synthesis. The HA choline uptake system currently is believed to be the rate-limiting step (Jenden et al., 1976).

Choline is accumulated intraneuronally by an HA and LA uptake system (Haga and Noda, 1973;
Pert and Snyder, 1974; Simon and Kuhar, 1976; Suszkiw and Pilar, 1976; Yamamura and Snyder, 1973). All cell membranes seem to possess an LA choline uptake (Diamond and Milfay, 1972), but the specialized HA system is considered to be unique to the cholinergic nerve terminal. HA uptake is strongly dependent on temperature and Na ions (Suszkiw and Pilar, 1976). Approximately 90% of the choline accumulated via the HA system is acetylated, in contrast to the limited acetylation of choline entering via the LA uptake (Yamamura and Snyder, 1973). Further support for the relationship between ChAT, HA choline uptake, and cholinergic nerve terminals is based on studies employing lesions in different brain regions (Kuhar and Sethy, 1973). Further support for the relationship between ChAT, HA choline uptake, and cholinergic nerve terminals is based on studies employing lesions in different brain regions (Kuhar and Sethy, 1973; McGeer et al., 1971) and peripheral structures (Pert and Snyder, 1974; Suszkiw and Pilar, 1976).

Last, our findings in cerebral arteries demonstrate an exceedingly good correlation between ChAT activity and the HA choline uptake system. If we consider the above studies collectively, there is good reason to conclude that both ChAT activity and HA choline uptake are acceptable indices of cholinergic innervation. This implies that the values measured have quantitative significance.

In each of the three species surveyed, ChAT activity was observed in the main cerebral arteries and in the Circle of Willis. Mean ChAT activity was highest in the cat cerebral arteries and lowest in those of the dog. The lower ChAT activity in the dog was paralleled by a low choline uptake. Some regional differences related to species were encountered. In the cat, the basilar artery tended to have the highest ChAT and choline uptake measurements. These findings contrast particularly with those of the dog. Our results are not consistent with those of Edvinsson et al. (1972), who determined by AChE staining that the cat basilar artery was innervated by fewer cholinergic fibers than was the anterior part of the Circle.

Lee et al. (1978) carried out a detailed pharmacological study of the neurogenic dilator response in the cat basilar artery. Vasodilation induced by exogenous Ach, but not neurogenic dilation, could be counteracted by atropine, anticholinesterases, and hemicholinium—observations that do not support the cholinergic hypothesis. However, the results of our present study in the same species show the presence of cholinergic mechanisms. In the rabbit, only a token neurogenic vasodilator response was obtained in vitro (Lee et al., 1976), yet biochemical parameters are as high in this species as in the cat. In the dog, a distinct neurogenic vasodilation was observed (Duckles et al., 1977), but biochemical parameters were low.

ChAT activity and HA choline uptake are both components of the synthetic machinery necessary for a cholinergic transmitter. Our results demonstrate the presence of such parameters in cerebral vessels, and this implies a cholinergic innervation of the arteries at the base of the brain. Only further studies will resolve the apparent inconsistencies between our biochemical results and the functional pharmacological findings.

References


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choline synthesis: Does cytoplasmic acetylcholine control high affinity choline uptake? Science 194: 635-637

IMMEDIATELY following the onset of ischemia, a number of functional, metabolic, and morphological changes occur within the myocardial cell (Jennings et al., 1969). Following short periods of ischemia, the restoration of normal blood flow results in the resumption of normal cardiac function and metabolism. However, as the duration of ischemia increases, irreversible damage occurs. The minimum time necessary to induce irreversible damage is determined by a number of factors, including the severity of coronary flow reduction, the delivery of nutrients, tissue high energy phosphate and substrate content, the contractile state, and the rate at which undesirable metabolic waste products are removed from the heart. The extent to which each of these variables contributes to the transformation from viable to necrotic tissue remains unclear.

Of the factors involved in the onset of irreversible damage, ATP is of particular interest since it appears to be the direct energy donor for the contrac-

Relationship between Adenine Nucleotide Metabolism and Irreversible Ischemic Tissue Damage in Isolated Perfused Rat Heart

THOMAS C. VARY, EVANGELOS T. ANGELAKOS, AND STEPHEN W. SCHAFFER

SUMMARY The relationship between energy metabolism and the extent of irreversible ischemic damage was examined in an isolated perfused working rat heart. The amount of cardiac work recovered after reperfusion of hearts exposed to severe global ischemia was dependent upon both the duration of ischemia and the type of substrate provided (either 5 mM glucose or 5 mM glucose + acetate). There appear to be two distinct phases in the ability to recover mechanical function in the reperfused ischemic heart. The second phase corresponds to the onset of severe irreversible tissue damage. Irreversible mitochondrial damage was not found to correspond with the onset of heart failure since the ATP/ADP ratio remained constant in the reperfused myocardium. Furthermore, there does not appear to be a direct correlation between the total ATP content and the extent of irreversible damage, either during ischemia or following reperfusion. However, the total adenine nucleotide content during ischemia showed dramatic changes which correspond temporally with the initiation of the second phase of damage. The observation that the adenine nucleotide pool becomes further depleted during reperfusion suggests that alterations in the salvage pathway for adenine nucleotide synthesis have occurred. Loss of adenine nucleotides appears to be an excellent marker for irreversible heart failure. Acetate provides some protection to the ischemic myocardium. The mechanism by which acetate mediates this protective effect is discussed. Circ Res 45: 218-225, 1979

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