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Modulation of cholinergic synaptic functions by sialylcholesterol

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The effects of sialylcholesterol, a synthetic ganglioside analogue, on cholinergic synaptic functions were investigated using synaptosomes prepared from C57BL/6 mouse brain cortices. Addition of α-sialylcholesterol stimulated high K (50 mM)-evoked acetylcholine (ACh) release from synaptosomes at concentrations ranging from 1 to 5 μM. The β-anomer of the sialyl compound also increased the neurotransmitter release at 5 μM, but the effect was much smaller than that of the α-anomer. Beta-sialylcholesterol appeared to increase high-affinity choline uptake and ACh synthesis, resulting in an increment in the release of ACh. On the other hand, α-sialylcholesterol did not change the synthetic rate of ACh, and instead it increased the depolarization-induced influx of calcium ions into synaptosomes, while the β-anomer did not affect the divalent cation influx. The enhanced calcium influx is thought to increase ACh release from synaptosomes treated with α-sialylcholesterol. These results imply that the two anomers of sialylcholesterol may modulate the synaptic membrane machinery differently, that is, the α-anomer may activate voltage-dependent calcium channels and the β-anomer may facilitate high-affinity choline uptake.

In order to evaluate the ameliorating effect of sialylcholesterol, α-sialylcholesterol was applied to the synaptosomes from aged mice (34 months old), which have been shown to have a decreased ACh release (Tanaka *et al.*, 1995, *J Neurosci Res*, in press [1]). The reduced neurotransmitter release recovered to the levels of younger animals, suggesting that sialylcholesterol might have a potential therapeutic use for restoring synaptic function that occurs in aged brains.

Keywords: sialylcholesterol, synaptosomes, acetylcholine, choline uptake, calcium ions

Introduction

During the aging process, deficits in cognitive functions such as memory and learning occur not only in humans but also in experimental small animals [2]. Failure of the cholinergic neurons has been shown to be responsible for such age-related impairments of brain function [2–7]. However, the results of neurochemical studies of aged synapses do not seem to be in agreement. Among studies of aged brains, many have reported some decreased activities of choline acetyltransferase [8, 9] or low production rates of acetylcholine [10–12]. Other studies, by contrast, have demonstrated insignificant changes or no changes in some of these parameters [2]. We have recently found that brain ageing reduces ACh release from synapses without affecting the synthetic rate or levels of the neurotransmitter, and that the reduced ACh release may be due to a decreased influx of calcium ions through voltage-dependent calcium channels [1].

It has been known that gangliosides (sialic acid-containing glycosphingolipids) play important roles in neuronal events, such as synaptic transmission, cell differentiation and recognition [13]. Gangliosides are a family of naturally occurring sialyl compounds and are abundant in neural plasma membranes [14, 15]. There are many reports indicating that gangliosides are involved in the modulation of synaptic transmission [16–20]. Sialylcholesterol, a synthetic amphipathic, low-molecular-weight sialyl compound that is used as a ganglioside analogue, has been shown to stimulate the differentiation of neuroblastoma cells [21] and stimulate transglutaminase activity in superior cervical and nodose ganglia [22]. These results raise the possibility that sialylcholesterol may have the ability to modulate neuronal activities and

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to restore the decreased synaptic transmission in aged brains.

In the present study, we have evaluated the effects of sialylcholesterol on cholinergic synaptic functions using synaptosomes from mouse cerebral cortices. Both α- and β-sialylcholesterols stimulated high K-evoked ACh release. Different modes of action are discussed for the α- and β-anomers.

Materials and methods

Chemicals

Alpha-sialylcholesterol (Fig. 1, A) and β-sialylcholesterol (Fig. 1, B) were generous gifts from Nissin Food Products Co. Ltd (Osaka, Japan). Fura-2 AM was purchased from Wako Pure Chemical Co. (Tokyo, Japan) and Ficoll (type 400-DL) was from Sigma Chemical Co. (St Louis, MO, USA). [^{3}H]Choline chloride (specific activity, 69 Ci mmol^{-1}) was purchased from Amersham Life Science (Buckinghamshire, England). All other chemicals used were of analytical grade.

Animals and preparation of synaptosomes

Adult and aged mice (female C57BL/6) were supplied by the Department of Animal Science at our Institute. Synaptosomes were prepared by Ficoll discontinuous gradient centrifugation according to the method of Booth and Clark [23] with a minor modification. Briefly, cerebral cortices of mice were homogenized with a Dunce-type glass-Teflon homogenizer (total clearance 0.1 mm) in an isotonic solution consisting of 10 mM Tris-HCl-buffered 0.32 M sucrose (pH 7.4), containing 1 mM K-EDTA. The homogenates were centrifuged at 1300 × **g** for 3 min and the resulting supernatant was subjected to a further centrifugation at 17 000 × **g** for 10 min. The precipitates were suspended in 12% (w/w) Ficoll in 0.32 M sucrose solution with a gentle homogenization (five up and down strokes with a 0.5 mm-clearance homogenizer). The suspension was transferred into Ultra-Clear ultracentrifuge tubes, and 7.5% (w/w) Ficoll in 0.32 M sucrose was carefully layered above this suspension. A layer of 0.32 M sucrose was then placed on top of this layer. The tubes were centrifuged at 99 000 × **g** for 60 min. The interface between the 7.5% and 12% Ficoll was collected and was washed twice with Krebs-Ringer salt solution. The Krebs-Ringer salt solution contained 143 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.5 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES sodium salt in order to maintain pH at 7.4.

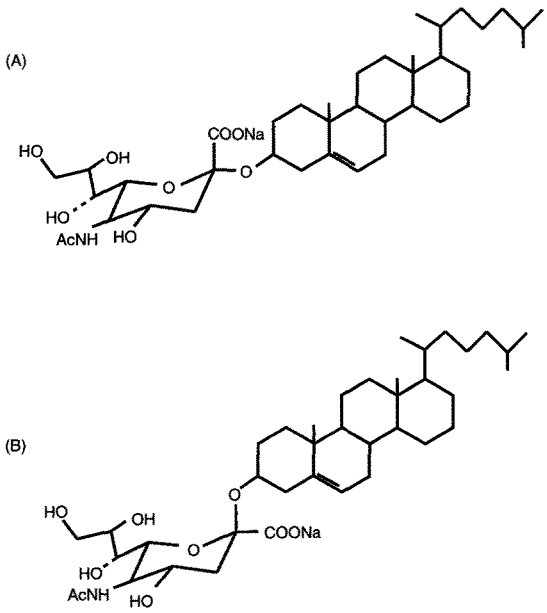

Figure 1. Structures of sialylcholesterols. (A) α-sialylcholesterol; (B) β-sialylcholesterol.

Release of ACh

Synaptosomes were preincubated in Krebs-Ringer solution containing 150 µM eserine at 37 °C for 30 min with or without sialylcholesterol. The synaptosomes were washed and resuspended in ice-cold Krebs-Ringer solution. Fifty µl of Krebs-Ringer solution containing 140 mM KCl was added to 100 µl of the synaptosomal suspension. After a 5 min incubation at 37 °C, the sample was immediately cooled by placing it on ice to terminate the ACh release processes. Ethylhomocholine (EHC, 150 pmol) was added to the sample as an internal standard, and the sample was centrifuged at 2000 × **g** for 10 min at 4 °C. The supernatant was introduced into a high-performance liquid chromatograph (HPLC) equipped with an electrochemical detector (LC-4B, BAS, Japan) to quantify the ACh released.

Synthesis of ACh

Synaptosomes were incubated in an eserine-containing Krebs-Ringer solution at 37 °C for 30 min with or without sialylcholesterol. At the end of the incubation, the reaction was stopped by adding 0.1 N perchloric acid. After the addition of 200 pmol of EHC, the mixture was centrifuged and the resultant supernatant was subjected to the same HPLC analysis used to determine the release of ACh.

Sodium-dependent high affinity uptake of choline

Synaptosomes were incubated in Krebs-Ringer solution with or without sialylcholesterol (5 µM) at 37 °C for 30 min. After washing the synaptosomes with Krebs-Ringer solution, they were resuspended in the same isotonic salt solution. An aliquot of the synaptosomes (200–300 µg protein) was incubated in 0.5 ml of

Krebs-Ringer solution containing 1 μM [^{3}H]choline chloride (0.25 μCi) at 37 °C for 4 min in a shaking water bath. The reaction was stopped by the addition of 3 ml ice-cold Krebs-Ringer solution and the samples were centrifuged at 2000 × **g** for 5 min at 4 °C. The resultant synaptosomal pellets were further washed with the isotonic buffer to remove the remaining [^{3}H]choline chloride in the fluid. The synaptosomes were dissolved in a small amount of 1% SDS and were transferred into counting vials. The radioactivity taken-up by synaptosomes was determined by liquid scintillation counting in 5 ml of Aquasol-2 scintillation fluid. The sodium-dependent high-affinity uptake was determined by subtracting the sodium-independent uptake obtained with samples suspended in a modified Krebs-Ringer solution in which the sodium was sodium replaced with 0.25 M sucrose.

Determination of intrasynaptosomal calcium ion concentration

Synaptosomes were incubated for 20 min at 37 °C in Krebs-Ringer solution with or without sialylchoresterol. The incubation mixture was further incubated with 5 μM Fura 2-AM and 0.25% bovine serum albumin for 20 min at 37 °C. The synaptosomes were washed twice by centrifugation (3000 × **g**, 3 min). The resulting pellets were resuspended in Krebs-Ringer solution and kept in an ice bath in the dark. Just before each measurement, an aliquot of the synaptosomal sample was centrifuged with 0.5 ml Krebs-Ringer solution at 12 800 × **g** for 10 s in a bench-top centrifuge (model 5410, Eppendorf). The synaptosomal pellets were resuspended in 2 ml of the same isotonic solution, prewarmed to 37 °C. Fluorescence measurements were carried out with a Shimadzu-RF 1500 spectrofluorophotometer (Shimadzu Co., Japan) with excitation wavelengths at 335 and 375 nm and emission at 510 nm. Data obtained during the first 3 s following the application of high-K^+ stimuli were employed to calculate the changes in $[Ca^{2+}]_i$. Calculation of $[Ca^{2+}]_i$ was carried out by the ratio method principally according to Grynkiewicz *et al.* [24] as follows:

$$[Ca^{2+}]_i = [(fR-R_{min})/(R_{max}-fR)](S_{f2}/S_{b2})Kd,$$

where R is the ratio of the fluorescence intensities at the excitation wavelengths. The value of R_{max} was obtained by solubilization of the synaptosomes with 1% SDS, and R_{min} was determined by adding 5 mM EGTA at pH 8.5. S_{f2} and S_{b2} showed fluorescence of Fura 2 at the excitation wavelength of 375 nm at zero calcium and full calcium saturation, respectively. For K_d, the dissociation constant of the Fura 2-Ca^{2+} complex, a value of 224 nM was used [24]. To compensate for the stray light due to light scattering caused by synaptosomal particles, we introduced a correction factor, f, which was obtained from the ratio of the fluorescence intensities at 375 nm to 335 nm using synaptosomes without Fura 2 [1].

Determination of protein concentrations

Protein concentrations in the synaptosomal samples were determined according to the colorimetric method of Lowry *et al.* [25] using bovine serum albumin as a standard. Since we have found no significant differences between adult and aged mice in protein yields of synaptosomal preparations [26], all neurochemical data obtained were expressed per mg protein.

Results and discussion

Effects of sialylcholesterol on ACh release

We examined the effects of exogenous sialylcholesterol on the release of ACh in synaptosomes prepared from mouse cerebral cortices. As shown in Fig. 2, both α- and β-sialylcholesterol significantly increased the high K-evoked ACh release at low concentration (5 μM). The enhancing effect on ACh release by α-sialylcholesterol was more prominent (>160% of control) than that by the β-anomer (120% of control). However, neither the α- nor the β-sialyl compound facilitated the neurotransmitter release at high concentration (150 μM). We then determined the dose-dependency of the ACh release on α-sialylcholesterol. As shown in Fig. 3, α-sialylcholesterol increased the high K-evoked ACh release in a concentration range between 1 and 5 μM. Spontaneous release of ACh was not affected by the sialyl compounds at any of the concentrations tested.

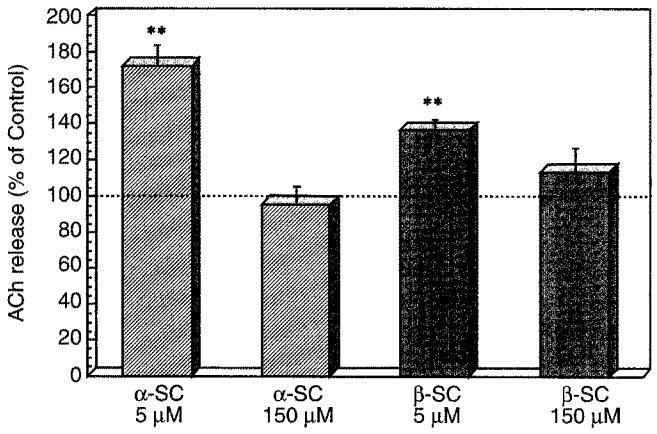

Figure 2. Effect of α- and β-sialylcholesterol on ACh release from synaptosomes isolated from C57BL/6 mouse brain cortices. Synaptosomes were pre-incubated in eserine-containing Krebs-Ringer solution with or without sialylcholesterol at 37 °C for 30 min. After washing the synaptosomes, they were further incubated with 50 mM KCl-containing Krebs-Ringer solution for 5 min at 37 °C. ACh released was quantified by HPLC. α-SC, α-sialylcholesterol; β-SC, β-sialylcholesterol. Values are the means ±SD ($n = 3$). **, $p < 0.01$ (vs control, two-tailed Student's t-test).

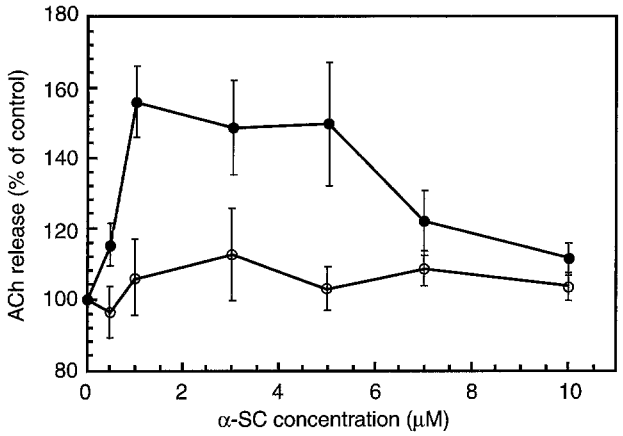

Figure 3. Dose-dependency of ACh release from mouse brain synaptosomes on α-sialylcholesterol. Values are the means ±SD of five determinations. Closed circle, ACh released by the stimulation of high K (50 mM); open circle, ACh released spontaneously (at 5 mM KCl).

Effects of sialylcholesterol on ACh synthesis and high-affinity choline uptake

Figure 4A depicts the effect of sialylcholesterol on ACh synthesis in synaptosomes. Addition of 5 μM β-sialylcholesterol moderately but significantly increased ACh synthesis. On the other hand, α-sialylcholesterol did not change the synthetic rate of the neurotransmitter at the same concentration. Since the transport of choline by sodium-dependent high-affinity choline uptake has been identified as the rate-limiting step for ACh synthesis [27], the transporter might be modulated by β-sialylcholesterol. Thus, we examined the effect of sialylcholesterol on sodium-dependent high-affinity choline uptake. As shown in Fig. 4B, β-sialylcholesterol-treated synaptosomes showed a 30% increase in high-affinity choline uptake. The α-anomer, however, did not have any effect on the uptake of choline. It is conceivable from these results that the increase in ACh release by β-sialylcholesterol may be due to the enhanced production of ACh that was caused by an increase in the uptake of choline by the high-affinity uptake system. The increased release of ACh by α-sialylcholesterol may be due to a different mechanism.

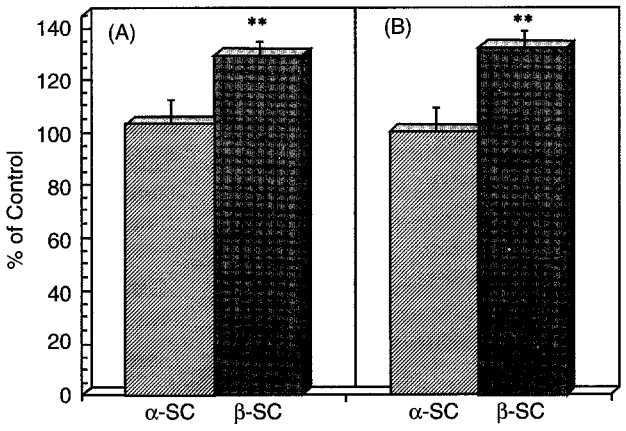

Figure 4. Effects of α- and β-sialylcholesterol on ACh synthesis (A) and sodium-dependent high-affinity choline uptake (B) in synaptosomes isolated from C57BL/6 mouse brain cortices. (A) Synaptosomes were incubated in eserine-containing Krebs-Ringer solutions with or without sialylcholesterol at 37°, 30 min. ACh synthesized was quantified by HPLC. Control value was 191.1 ± 16.4 pmol per mg protein per 30 min ($n = 5$). (B) Sodium-dependent high-affinity choline uptake was determined with [^{3}H]choline chloride as described in Materials and methods. Control value was 22.0 ± 0.8 pmol per mg protein per 4 min ($n = 4$). α-SC, α-sialylcholesterol; β-SC, β-sialylcholesterol. Values are the means ±SD. **, $p < 0.01$ (vs control, two-tailed Student's *t*-test).

Effect of sialylcholesterol on the influx of calcium ions into synaptosomes

Elevation of intrasynaptic calcium ions is a prerequisite for neurotransmission in nerve terminals [28, 29]. We postulated that the increase in ACh release by sialylcholesterol is due to the increase in the calcium ion influx which triggers exocytosis. To test whether α-sialylcholesterol affected the depolarization-induced influx of calcium ions into synaptosomes, the changes in the concentration of intra-synaptosomal free calcium ions, $[Ca^{2+}]_i$, were determined using Fura 2 as a calcium ion indicator. As shown in Fig. 5, $[Ca^{2+}]_i$ in synaptosomes was increased by 125% of control when the synaptosomes were treated with α-sialylcholesterol. On the other hand, β-sialylcholesterol had no effect on the levels of depolarization-induced calcium influx. The resting levels of $[Ca^{2+}]_i$ as measured

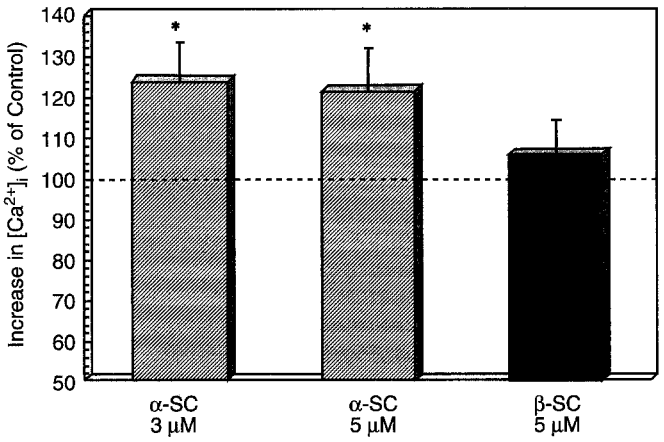

Figure 5. Effect of α- and β-sialylcholesterol on the increase in $[Ca^{2+}]_i$ in synaptosomes from C57BL/6 mouse brain cortices. Fura 2-loaded synaptosomes were stimulated with 50 mM KCl. The increase in $[Ca^{2+}]_i$ represents the levels above resting $[Ca^{2+}]_i$ at 5 mM KCl. The values are the means ±SD of three determinations. *, $p < 0.05$ (vs control, two-tailed Student's *t*-test).

at 5 mM KCl were the same for both sialylcholesterol-treated and control synaptosomes. These results imply that α-sialylcholesterol, but not β-sialylcholesterol, modulates calcium ion influx through voltage-dependent calcium channels in synapses. Therefore, it is assumed that α-sialylcholesterol increases the evoked ACh release by enhancing the calcium influx. Similar modulatory effects of sialyl compounds with calcium channel functions have been reported. Exogenous gangliosides were shown to modulate calcium flux in neuroblastoma cells and stimulate cellular events such as neuritogenesis [30, 31]. Frieder and Rapport [32] reported that gangliosides participate in calcium channel functions involved in GABA release in rat brain slices.

Restoration of decreased ACh release from aged synapses by α-sialylcholesterol

We have recently reported that brain ageing reduces ACh release from synapses without affecting the synthetic rate or levels of the neurotransmitter, and that the reduction in ACh release may be due to a decreased influx of calcium ions through voltage-dependent calcium channels [1]. We then attempted to evaluate the potency of α-sialylcholesterol to recover the decreased activity of ACh release in aged synaptosomes.

Figure 6 shows the high K-evoked release of ACh from synaptosomes prepared from adult (8 months old) and aged (34 months old) mouse brains when treated with α-sialylcholesterol. A significant decrease in ACh release was found in aged synaptosomes, as previously reported [1]. Addition of α-sialylcholesterol significantly increased high K-evoked ACh release in both adult and aged synaptosomes (Fig. 6). Table 1 summarizes the ACh levels in synaptosomes and the amounts of ACh released from synaptosomes of adult and aged mouse brains. The ACh contents were nearly the same in both adult and aged synaptosomes, but the percentage release of ACh (the ratio of ACh released to ACh stored in synaptosomes) was reduced in aged mice. When synaptosomes were treated with α-sialylcholesterol, the percentage release of ACh was significantly increased in both adult and aged synaptosomes. This result suggests that α-sialylcholesterol may enhance the depolarization-induced

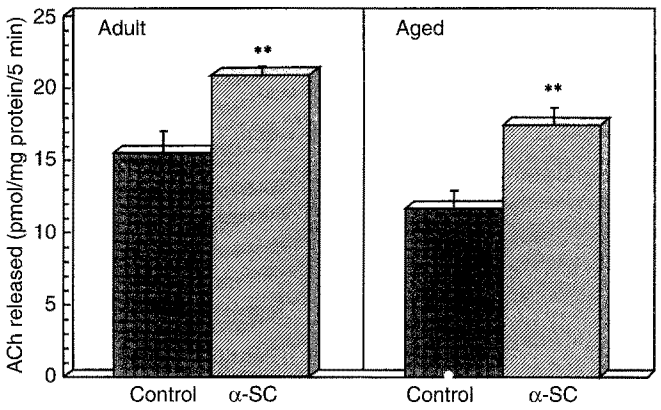

Figure 6. Effect of α-sialylcholesterol on ACh release from synaptosomes isolated from adult and aged C57BL/6 mouse brain cortices. Ages of the adult and aged groups were 8 and 34 months old, respectively. Synaptosomes were pre-incubated in eserine-containing Krebs-Ringer solutions with or without sialylcholesterol at 37 °C for 30 min. The synaptosomes were further incubated with 50 mM KCl-containing Krebs-Ringer solution for 5 min at 37 °C. ACh released was quantified by HPLC. Values are the means ±SD ($n = 3$). **, $p < 0.01$ (vs control, two-tailed Student's t-test).

release of ACh. Since α-sialylcholesterol increases the depolarization-induced influx of calcium ions into synaptosomes (Fig. 5), the augmentation in ACh release in α-sialylcholesterol-treated synaptosomes may be primarily due to the increased calcium ion influx through voltage-dependent calcium channels.

Taken together, the present findings suggest that sialylcholesterol (both α- and β-anomers) stimulates high K-evoked ACh release. However, the underlying mechanisms seem to be different for the two anomers, that is, α-sialylcholesterol increases ACh release by facilitating calcium ion influx, while the β-anomer increases the neurotransmitter release by enhancing ACh synthesis. Application of α-sialylcholesterol to aged synaptosomes was shown to be effective in restoring the decreased ACh release in ageing brains to the levels of younger animals. These actions of sialylcholesterol on synaptic functions may provide a new therapeutic tool for hypofunctions in aged brains.

Table 1. ACh contents and the amounts of ACh released from synaptosomes in adult and aged mice.

Age (months)		*ACh content (A) (pmol per mg protein)*	*ACh released (B) (pmol per mg protein)*	*% release (B × 100/A)*
8	Control	47.0 ± 3.7	15.5 ± 1.5	33.3 ± 0.8
	α-SC	49.6 ± 0.4	20.9 ± 0.6**	42.0 ± 0.8**
34	Control	39.7 ± 3.3	11.7 ± 1.2	29.5 ± 1.2
	α-SC	44.4 ± 4.2	17.6 ± 1.1**	39.8 ± 3.2**

Values are the means ±SD ($n = 3$ in both adult and aged groups). α-SC, α-sialylcholesterol.
**, $p < 0.01$ (vs control, two-tailed Student's t-test)

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References

1. Tanaka Y, Hasegawa A, Ando S (1995) *J Neurosci Res*, in press.
2. Decker MW (1987) *Brain Res* **12**, 423–38.
3. Bartus RT, Dean RL, Beer B, Lippa AS (1981) *Science* **217**, 408–17.
4. Coyle JT, Price DL, Delong MR (1983) *Science* **219**, 1184–90.
5. Smith G (1988) *Brain Res Rev* **13**, 103–18.
6. Gage FH, Chen KS, Buzsaki J, Armstrong D (1988) *Neurobiol Aging* **9**, 645–55.
7. Hasselmo ME, Bower JM (1993) *Trends Neurosci* **16**, 218–22.
8. Luine VN, Renner KJ, Heady S, Jones KJ (1986) *Neurobiol Aging* **7**, 193–98.
9. Michalek H, Furtuna S, Pintor A (1989) *Neurobiol Aging* **10**, 143–48.
10. Gibson GE, Peterson C, Jendenm DJ (1981) *Science* **231**, 647–76.
11. Gibson GE, Peterson C (1981) *J Neurochem* **37**, 978–84.
12. Sims NR, Marek KL, Bowen DM, Davidson AN (1982) *J Neurochem* **38**, 488–92.
13. Hakomori S (1986) *Sci Am* **254** (5), 32–41.
14. Ando S (1983) *Neurochem Int* **5**, 507–37.
15. Ledeen RW (1984) *J Neurosci Res* **12**, 147–59.
16. Wieraszko A, Seifert W (1984) *Neurosci Lett* **53**, 123–28.
17. Wieraszko A, Seifert W (1985) *Brain Res* **345**, 159–64.
18. Wieraszko A, Seifert W (1986) *Brain Res* **371**, 305–13.
19. Hollmann M, Seifert W (1989) *J Neurochem* **53**, 716–23.
20. Thomas PD, Brewer G (1990) *Biochim Biophys Acta* **1031**, 277–89.
21. Tsuji S, Yamashita T, Tanaka M, Nagai Y (1988) *J Neurochem* **50**, 414–23.
22. Ando M, Nakashima Y, Nagata Y (1991) *J Neurochem* **57**, 1848–54.
23. Booth RFF, Clark JB (1978) *Biochem J* **176**, 365–70.
24. Grynkiewicz G, Poenie M, Tsien RW (1985) *J Biol Chem* **260**, 3440–50.
25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) *J Biol Chem* **193**, 265–75.
26. Tanaka Y, Ando S (1992) *J Biochem* **112**, 117–21.
27. Simon JR, Atweh S, Kuhar MJ (1975) *J Neurochem* **26**, 909–22.
28. Katz B, Miled R (1967) *J Physiol* **192**, 407–36.
29. Llinas R, Nicholson NC (1975) *Proc Natl Acad Sci USA*, **72**, 187–90.
30. Wu G, Ledeen RW (1994) In *Progress in Brain Research* vol. 101 (L. Svennerholm, A. K. Ashburg, R. A. Reisfeld, K. Landhoff, K. Suzuki, G. Tettamanti and G. Toffano, eds) pp. 101–12. Amsterdam: Elsevier Science.
31. Wu G, Vaswani KK, Lu ZH, Ledeen RW (1990) *J Neurochem* **55**, 484–91.
32. Frieder B, Rapport MM (1987) *J Neurochem* **48**, 1048–52.