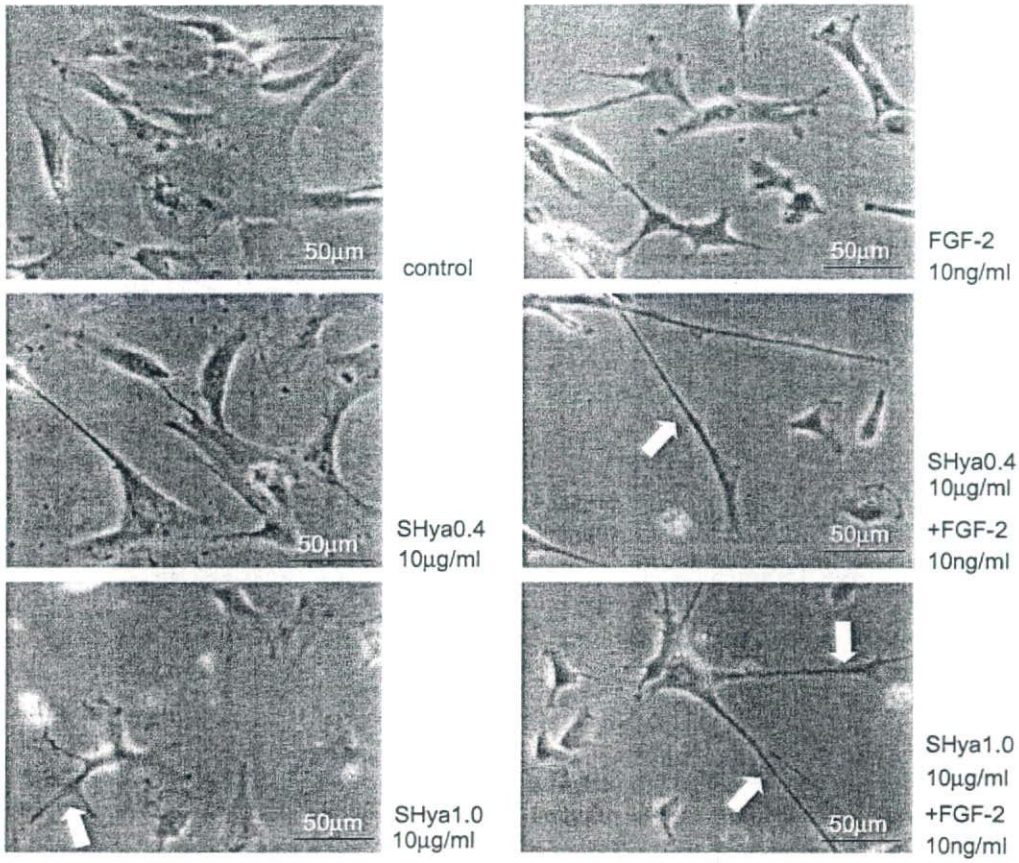

Fig. 7. Effects of SHya, FGF-2, and SHya with FGF-2 on morphological changes of cultured NHA over 20 days. The concentrations of each regent were 10 μg/ml SHya0.4, 10 μg/ml SHya1.0, and 10 ng/ml FGF-2. The arrow shows stellation of NHA.

[9,10]. Previous studies suggested that heparin enhances the effects of FGF-2 on NSC proliferation and neural network regeneration and that SHyas also enhanced FGF-2 activity and affected cell proliferation because SHya binds to FGF-2 [17,18]. Therefore, we examined the effects of 10 μg/ml SHya and SHya plus 10 ng/ml FGF-2 on astrocyte cultures. NHA proliferation increased smoothly until 30 days in in vitro culture, but cell proliferation virtually stopped after 30 days with NHA cell body enlargement (Figs. 4–7, control). The cell body enlargement stopped cell proliferation and caused cell senescence. Senescence in cells arrests cell growth in the G1 phase and changes the morphology and metabolism. Some of the senescence-associated changes that are common to many different cell types include cellular enlargement, increased lysosome biogenesis, and expression of a β-galactosidase that has a pH optimum of six [24]. Some reports suggested that 10 ng/ml FGF-2 increased cell proliferation of rat mesencephalic glia and human astrocytes [25,26]. In this study, treatment with 10 ng/ml FGF-2 promoted NHA proliferation and prevented cell enlargement (Figs. 4–7), and this result was consistent with previous reports. Other reports suggested that FGF-2 enhances growth and maintains the potential for multi-differentiation in human mesenchymal stem cells [27,28]. Old astrocytes have reduced neurotrophic effects compared to young astrocytes, and treatment of old astrocytes with FGF-2 recovered their neurotrophic effects on neurons [29]. These results suggest that treatment with FGF-2 maintained NHA cell proliferation and prevented NHA cell senescence.

Treatment with SHya0.4 or SHya1.0 did not affect NHA proliferation. On the other hand, the combination of one of the SHyas and FGF-2 increased NHA proliferation, although the increase was lower than after treatment of FGF-2 only (Fig. 4). We demonstrated that treatment with heparin had no effect on NHA cell proliferation and on FGF-2 activity in astrocyte proliferation (unpublished results). Sulfated groups are necessary to bind FGF-2 to sulfated glucosaminoglycans, and it was considered that the FGF-2 binding activity of sulfated glucosaminoglycans is proportional to the number of sulfated groups. However, the effects on NHA proliferation did not differ with the combination treatment of FGF-2 plus SHya0.4 or SHya1.0. These results suggested that the effects of SHya on astrocyte proliferation do not involve FGF-2 activity or reduce the FGF-2 activity in NHA proliferation.

The change of normal resting astrocytes into reactive astrocytes enhances neurotrophic factor production and synaptic plastic activities. Reactive astrocytes increase their levels of GFAP, an astrocyte marker protein, by themselves and transform the cell shape into a stella [7,30]. This cell transformation is promoted by phosphorylation of protein kinase K [7]. In this study, treatment with FGF-2 caused transformation of NHA. This is consistent with previously reported evidence that FGF-2 causes the stellation of astrocytes [30]. The use of SHyas for NHA culture promoted distinct stellation, and they were more effective in promoting NHA stellation than FGF-2. The combination of FGF-2 and SHya0.4 or SHya1.0 promoted distinct NHA stellation more than treatment of FGF-2 alone, and, in particular, FGF-2 and SHya1.0 caused marked stellation (Figs. 5–7). In addition, treatment with SHya1.0 increased mRNA expressions of GFAP and nestin, a neuron specific maker, and GFAP protein expression in astrocytes (Fig. 8). An increase in these protein expressions produced activation of astrocytes in some reports [1,7]. This result showed that NHA stellation was strongly

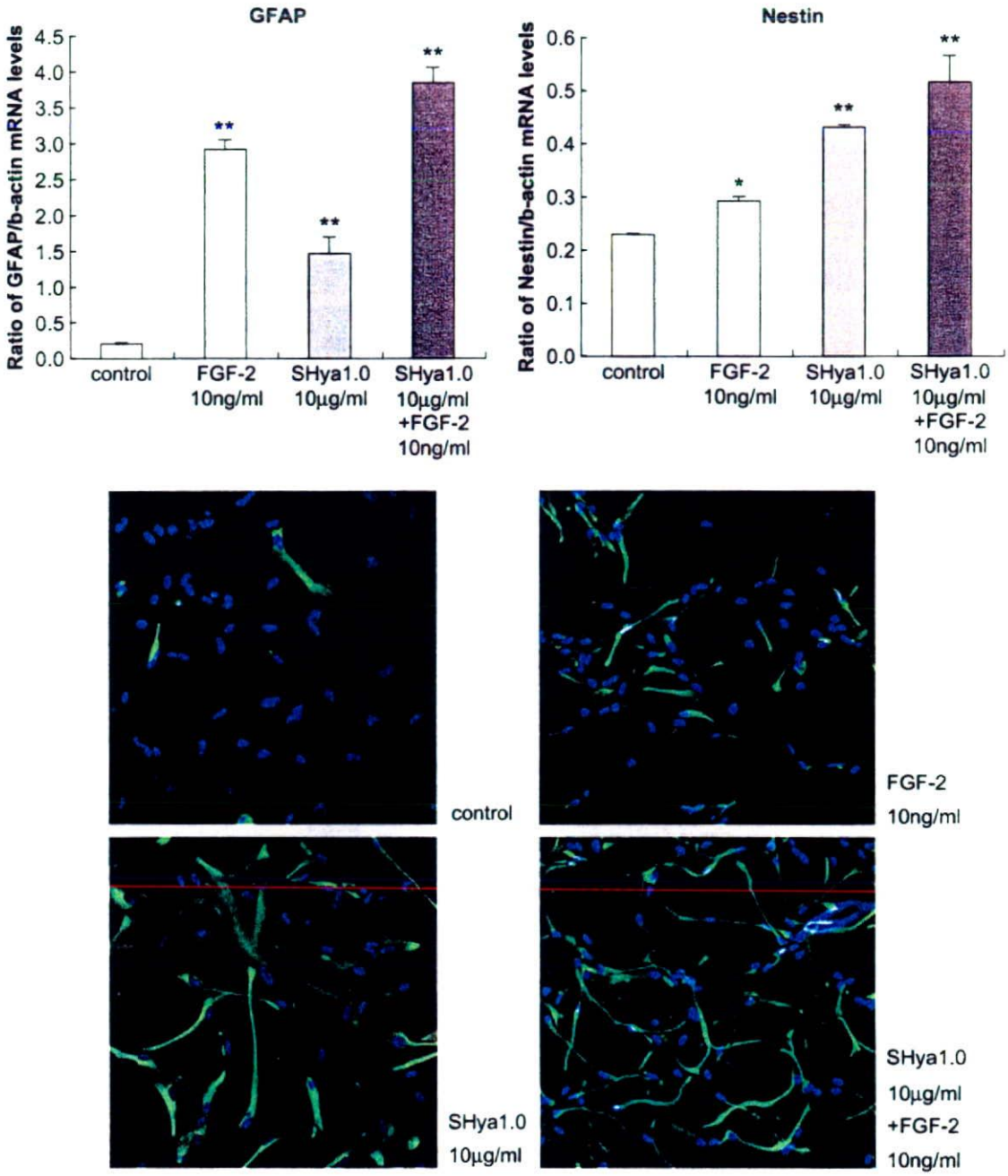

Fig. 8. Effect of SHya on GFAP and nestin mRNA expressions of astrocytes and immunostaining of GFAP. The concentrations of each regent were 10 μg/ml SHya1.0 and 10 ng/ml FGF-2. The arrow shows stellation of NHA. Values are mean ± SD ($n = 4$). Experimental data were significantly different from that of the control group (*, $p < 0.05$; **, $p < 0.01$).

enhanced by treatment with SHya1.0 and that FGF-2 with SHya transformed normal resting astrocytes to activating astrocytes depending on the D.S. of SHya but did not without FGF-2. Based on the assumption that transformation of NHA would increase connections between the NHA themselves, and we measured a cell–cell adhesion and gap-junctional intercellular communication. The SLDT assay showed that treatment with 10 μg/ml SHya1.0 increased the dye influx into NHA, indicating that gap junctions of astrocytes were increased and that cell–cell adhesions were intensified. It was previously shown that SHya increased the mRNA levels of the adhesion molecules *N*-cadherin and connexin43 in cultured rat calvarial osteoblasts [17]. We hypothesized that SHya increased a cell–cell adhesion by promoting NHA stellation and might increase adhesion molecules in NHA similar to that in calvarial osteoblast cells. Heparin, the average D.S. of which was 0.6, also promoted NHA stellation with FGF-2 [31]. The changes in NHA morphology via FGF-2 system depended on the D.S. of SHya, and the NHA transformations may have intensified the cells' crosstalk with neurons.

In the following study, we measured mRNA expressions of astrocyte-producing neurotrophic factors in NHA. Treatment with SHya1.0 increased mRNA expressions of FGF-2, midkine, BDNF, NGF, IGF-1 of astrocytes. These results also strongly suggest that SHya1.0 enhances astrocyte activity and supports neurocytes

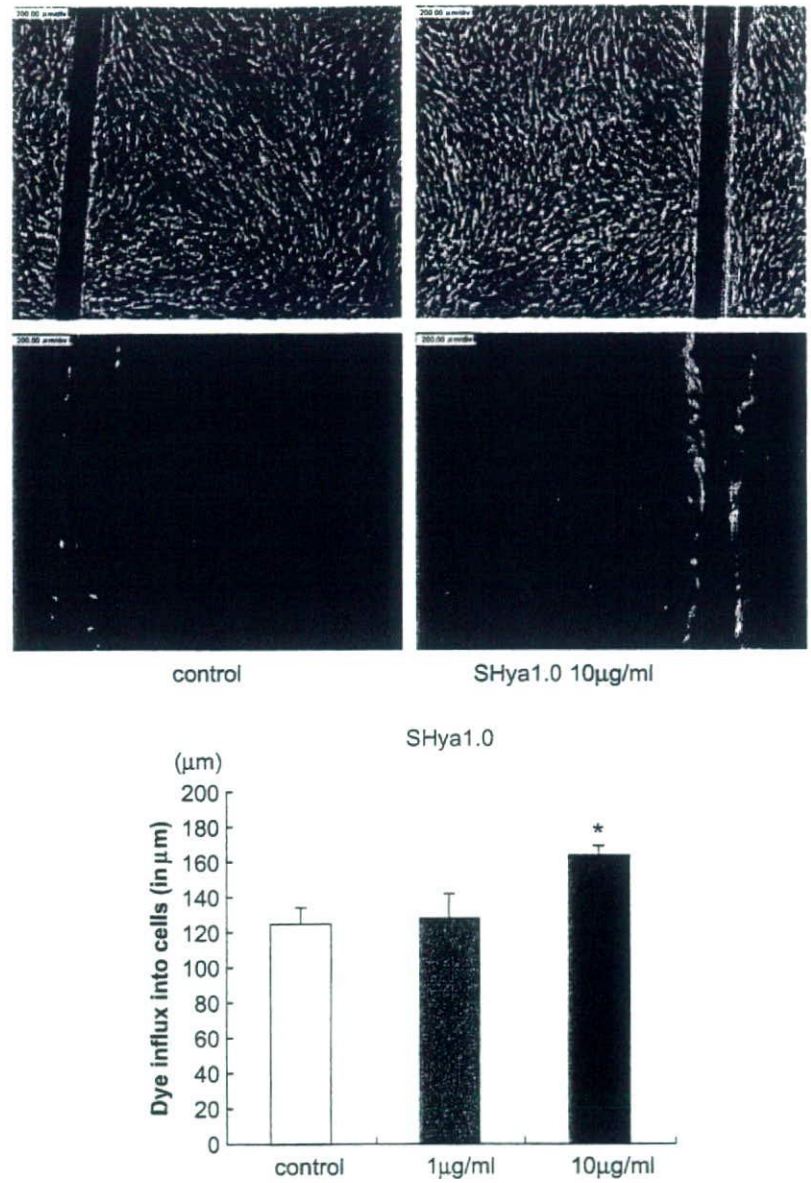

Fig. 9. Estimation of cell–cell adhesion of NHA by SLDT assay. The extent of dye influx into NHA cells was measured by the SLDT assay. NHA were cultured in normal NHA culture medium with 1, 10 μg/ml SHya1.0 or without (control). Values are mean ± SD ($n = 4$). Experimental data were significantly different from the control group (*, $p < 0.05$).

functionally and structurally. In particular, FGF-2 performs some functions of neurocytes, and together with EGF, which is needed for survival of neural stem cells. Glial cells cultured in the presence of FGF-2 and EGF expressed nestin in the culture dish [6,28,29]. These reports show that these trophic factors closely participate in proliferation and differentiation of neural stem cells and neurons. In addition, FGF-2 and midkine bind heparan sulfates and enhance neurotrophic activity by themselves. A combination of FGF-2 and heparin promoted astrocyte stellation [31]. Midkine and BDNF are also involved in neuronal growth, and interaction of these neurotrophic factors with chondroitin sulfate caused the expansion of dendrites [11]. That is, the effects of sulfated glucosaminoglycans on the CNS involve heparin-binding neurotrophic factors such as FGF-2, midkine, and others. Our study showed that the FGF-2, midkine, and BDNF levels were increased significantly by SHya treatment. FGF-2 bound to SHya and activated itself [17,18]. Midkine also binds to heparan sulfates and chondroitin sulfates, like FGF-2, and we hypothesized that SHya interacts with midkine [30,31]. Another report indicated that direct interaction of chondroitin sulfate oligosaccharides, which have installed sulfate groups, with midkine and BDNF promoted neuronal growth in cultured hippocampal neurons [11]. Thus, FGF-2, midkine, and BDNF are involved in the stellation of astrocytes by SHya. On the other hand, some neurotrophic factors, such as NGF, FGFs, and BDNF, are secreted for neurogenesis and neuroprotection in the injured brain. IGF-1 is also involved in neuroprotection, neurogenesis, and glucose utilization [32,33]. A recent report showed that IGF-1 contributed to the maintenance of youthful levels of cognition during aging in mammals [34]. These results suggested that IGF-1 might prevent a decline in the activity of neurocytes in the CNS. These trophic factors work together or independently, and their expressions may be mutually regulated [35–37]. In this study, treatment with FGF-2 increased the midkine mRNA level, but it did not change the BDNF and NGF mRNA levels. Apparently, SHya itself

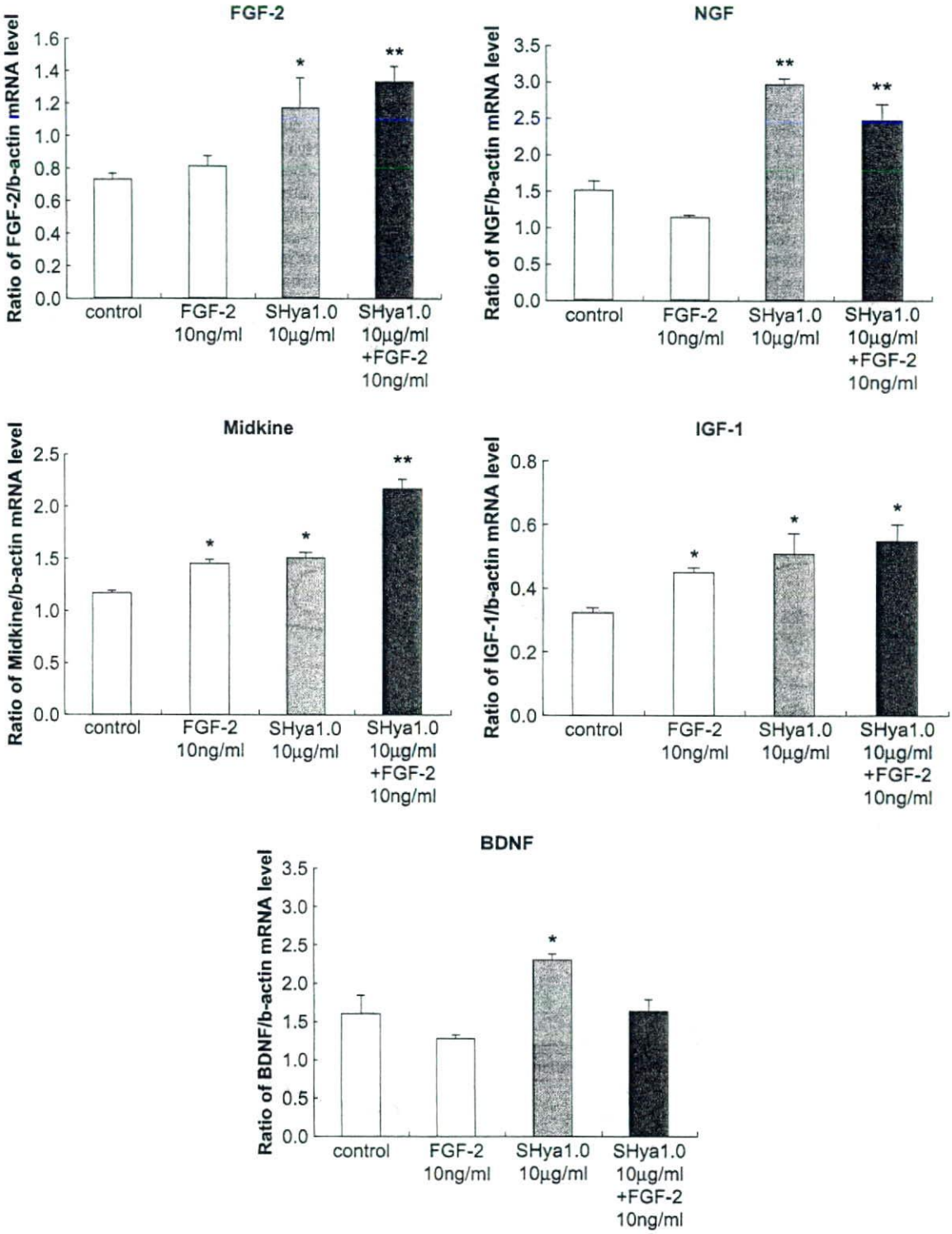

Fig. 10. Effects of SHya on mRNA expressions of FGF-2, midkine, and BDNF in astrocytes. NHA were cultured in normal NHA culture medium (control) or with 10 μg/ml SHya, 10 ng/ml FGF-2, or SHya with FGF-2. Values are mean ± SD ($n = 3$). Experimental data were significantly different from the control group (*, $p < 0.05$; **, $p < 0.01$).

and activation of these neurotrophic factors by SHya promoted the mRNA expression of these factors.

Heparin has a similar effect to that of SHya, and heparin transformed normal astrocytes to the stellar morphology [30]. Neurocan, a brain specific chondroitin sulfate, interacted with FGF-2 and caused the multiplication of neural stem cells in the neonatal rat brain [38]. Chondroitinase ABC, a digestive enzyme of chondroitin sulfate proteoglycan, enhanced outgrowth-associated protein-43-positive fibers after rat spinal cord injury [39]. Midkine, a heparin-binding growth factor, also promoted neural precursor

cell growth [31]. These reports suggest that sulfated glucosaminoglycans enhanced the activity of the heparin-binding growth factor midkine and FGF-2. However, we hypothesized that the effects of SHya on astrocytes were not limited to enhancement of neurotrophic factor activities. In our study, the effect of 20 ng/ml FGF-2 on stellation did not differ from that of 10 ng/ml FGF-2 (data not shown), but a combination of 10 ng/ml FGF-2 and SHya0.4 or SHya1.0 promoted more distinct NHA stellation than treatment with 10 or 20 ng/ml FGF-2, and, in particular, the combination of 10 ng/ml FGF-2 and SHya1.0 caused marked stellation (Figs. 4–6). In addition, BDNF and NGF mRNA levels also were increased by SHya, but FGF-2 reduced this increase. Therefore, SHya not only activates neurotrophic factors but also affects NHA stellation and enhances the neurotrophic factor-producing activity of astrocytes. That is, SHya is an efficient inducer of activation of astrocytes (Fig. 11).

5. Conclusion

This study demonstrated the effects of sulfated hyaluronan on proliferation, morphological transformation, and mRNA expression of neurotrophic factors in astrocytes. SHya promoted the stellation and neurotrophic factor-producing activities of astrocytes. The combination of SHya and FGF-2 promoted stellation of astrocytes more than SHya alone and increased astrocyte proliferation. The effect of SHya on astrocytes depended on the D.S. of SHya and involved FGF-2 activity. SHya alone promoted astrocytes stellation and increased cell–cell adhesion. These results suggest that SHya supports restructuring of the network between astrocytes or astrocytes and neurons. SHya increased FGF-2, midkine, and BDNF mRNA expressions. These neurotrophic factors are involved in neurogenesis, neuroprotection, and neural dendrite expansion. These neurotrophic factors interact with SHya functions and

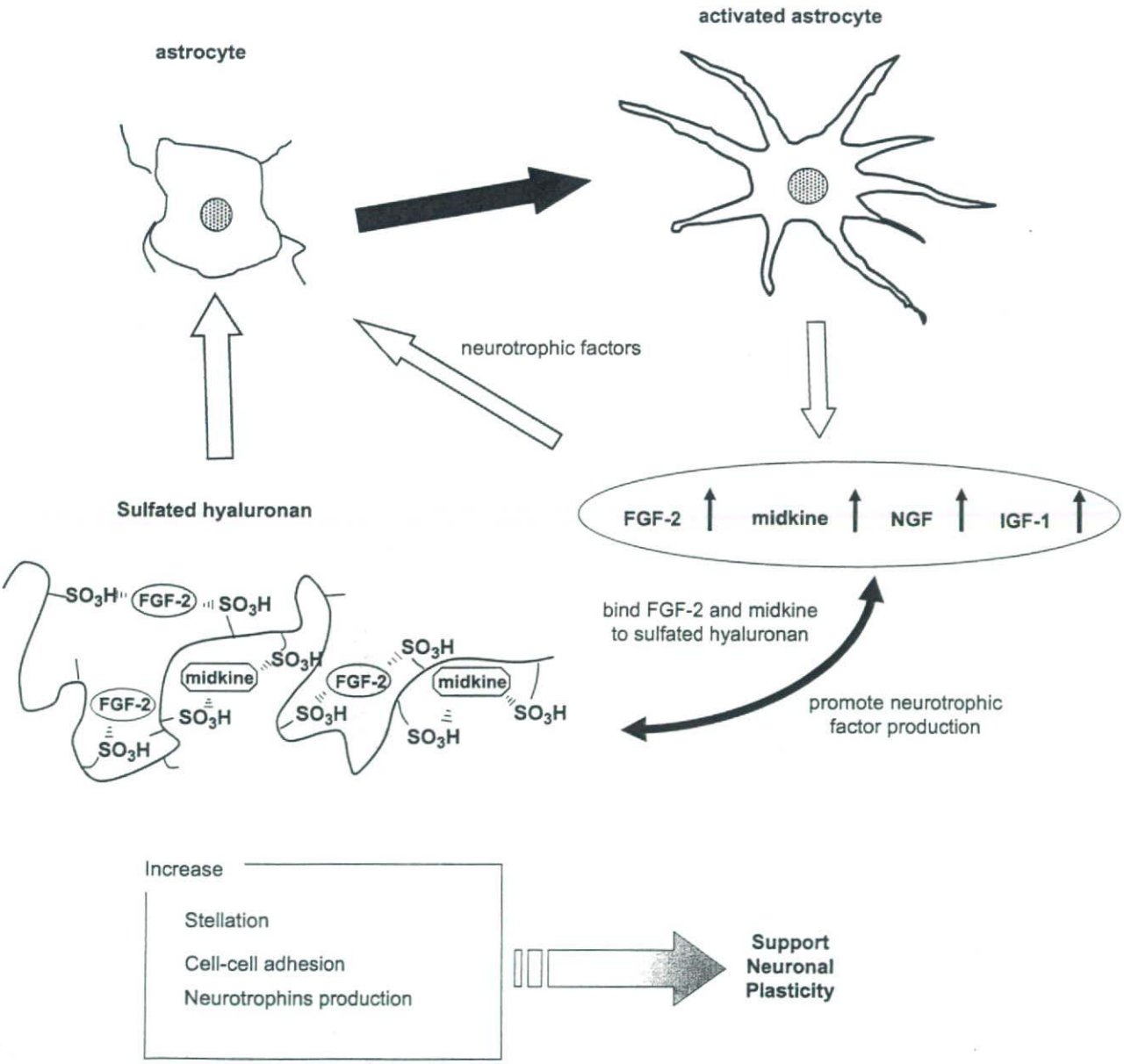

Fig. 11. Proposed mechanism of the effect of SHya on human astrocyte activities.

involve astrocyte stellation. Finally, astrocyte activities are required for the survival of neurons and regeneration of the neural network after brain injury, surgery, and neural stem cell transplantation. Synthesized SHya may enhance the survival of neurons and neural stem cells and affect neural plasticity by activating astrocytes. We expect that SHya will be applicable to safe and reliable medical biomaterials for neuroprotection and neurogenesis.

Acknowledgments

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Effects Intracerebral Microinjection and Intraperitoneal Injection of [60]Fullerene on Brain Functions Differ in Rats

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Fullerenes are condensed ring aromatic compounds with extended π systems and unique cage structures. Fullerenes are used for medical devices such as carbon nanotubes because they are very flexible and suitable for drug delivery systems. Recently, fullerene derivatives and tube-shaped materials have been used for neuroregeneration studies, and we expect that fullerenes and carbon nanotubes have potential uses as materials in novel medical devices targeting the brain. However, little information on the effects of fullerenes on brain function is available; thus, we examined the effects of [60]fullerene (C_{60}) on the central nervous system in this study. In a V79 cell colony Asia, the IC_{50} of C_{60} was 1620 μg/ml. In an *in vivo* study, 0.25 mg/kg B.W. of C_{60} was injected into the lateral brain ventricle or abdominal cavity of rats. The intracerebral injection of C_{60} increased the locomotor behavior of the rats on days 1 and 30 after the injection. The intraperitoneal injection of C_{60} did not change the locomotor behavior of rats acutely, but it was decreased on day 30. The intracerebral injection of C_{60} affected monoamine concentrations in the rat brain. In particular, serotonin turnover rates were increased in the hypothalamus, cerebral cortex, striatum, and hippocampus, and dopamine turnover rates were increased in the hypothalamus, cerebral cortex, and striatum. The intraperitoneal injection of C_{60} decreased only the dopamine turnover rate in the hippocampus. These results suggest that intracerebral injection of C_{60} had different effects on the central nervous system than intraperitoneal injection. In conclusion, it was suggested that fullerene did not cross the blood-brain barrier. The intracerebral injection of C_{60} affected neurotransmission in the brain widely, and the monoamine dysbolism might be related to changes in locomotor activity.

Keywords: [60]Fullerene, Neurotransmitter, Monoamine, Behavior, Intracerebral Injection.

RESEARCH ARTICLE

1. INTRODUCTION

Fullerenes are condensed ring aromatic compounds with extended π systems that have unique cage structures. Recently, fullerenes have been used for medical devices such as carbon nanotubes because they have useful properties for biomedical applications.[1] The biological attributes of fullerenes include photocleavage,[2] antiapoptotic activity,[3] drug and gene delivery,[4,5] antioxidation,[6] chemotaxis,[7] antibacterial activity,[8] and enzyme inhibition.[9] In addition, there are reports of neuroprotective effects of fullerenes. The ability of polyhydroxylated [60]fullerene (C_{60}) to prevent hydrogen peroxide- and cumene hydroperoxide-induced damage in rat hippocampal slices suggested its neuroprotective potency.[10] Hexasulfobutylated [60]fullerene had a neuroprotective effect on focal cerebral ischemia in rats *in vivo*.[11] These results suggest that fullerenes have neuroprotective effects against oxidative stress. However, examinations of the effects in normal rats and normal condition *in vitro* are few. Tsuchiya et al. evaluated the effects of C_{60} on normal mouse embryonic midbrain cells *in vitro* and mouse embryos *in vivo*. They reported that intraperitoneal (i.p.) injection of C_{60} into pregnant mice at 50 mg/kg exhibited a harmful effect on the embryo head region and tail.[12] It has been suggested that different C_{60} derivatives have different biological actions. Thus, C_{60} derivatives have different cytotoxicity potentials depending on the chemical structure of the derivative, but all C_{60} derivatives have same cage structure and this affects the cytotoxicity of fullerenes.[13] In this study, we used unmodified C_{60} and demonstrated its effects on normal Wistar rats, especially on locomotor behavior and concentrations of brain neurotransmitters.

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2. MATERIALS AND METHODS

2.1. V79 Colony Assay

The cytotoxicity of C_{60} was examined by a colony assay using V79 cells as described in the "Guidelines for Basic Biological Tests of Medical Materials and Devices-Part III: Cytotoxicity tests." Chinese hamster fibroblast V79 cells were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan) and grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 37 °C humidified atmosphere of 5% CO_2 and 95% air. For the assay, suspensions of 50 V79 cells/ml were seeded on 24-well plates, and the medium was changed to Eagle's MEM supplemented with 5% FBS and 1% penicillin-streptomycin. After 24 incubation in a 37 °C humidified atmosphere of 5% CO_2 and 95% air, 1 ml medium with C_{60} solution (5% C_{60}, 2% Tween 80, 93% sterile water) (Frontier Carbon Co., Tokyo, Japan), 2% Tween 80 without C_{60}, or physiolgical saline solution alone (control) was added to each well, and the cells were cultured for 7 days. The cell cultures were fixed with 10% formalin solution, and the colonies formed were stained with 5% Giemsa solution. The number of colonies in each well was counted, and the efficiency of the culture material was calculated as a ratio of the number of colonies in the sample to that in the control.

2.2. Animals

Male Wistar rats (270 g weight; SLC, Shizuoka, Japan) were kept in individual wire cages in a temperature- and humidity-controlled room (24 °C and 55% relative humidity) under regular lighting conditions (12 h light: dark cycle), and given food and water *ad libitum*. This experiment was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health Sciences (NIHS).

2.3. Intracerebral and Intraperitoneal Injections of C_{60}

The rats were anesthetized, and a hole was drilled in the skull using a stereotaxic high-speed drill (Muromachi Kikai Co., Ltd., Tokyo, Japan). A microliter syringe was stereotaxically inserted into the right lateral ventricle (0.6 mm posterior to the bregma, 1.6 mm lateral to the midline, and 3.5 mm ventral to the cortical surface) according to the atlas of Paxinos and Watson.[14] C_{60} (0.25 mg/100 g B.W.) dissolved in physiolgical saline solution containing 2% Tween 80 (0.1 μl/100 g B.W.) or physiolgical saline solution containing 2% Tween 80 (0.1 μl/100 g B.W.) was injected at the rate of 1 μl/min using a brain infusion injector (Muromachi Kikai Co., Ltd.). Rats of the control group were injected with the same volume of physiolgical saline solution. The same volume (0.25 mg/100 g B.W.) of C_{60} was injected intraperitoneally (i.p.) into other rats, and control rats were injected i.p. with the same volume of physiolgical saline.

2.4. Open Field Test

Motor activities (total locomotor distance, average locomotor speed, number of sections crossed, and opening latency) of rats were measured by an open field test. The open field box was 70 × 70 × 70 cm. Tests were performed at 1, 7, and 30 days after brain injection. The rat was placed in the open field box for 10 minutes, and the behavior was recorded using a DV-Track video tracking system CompACT VAS/DV (Muromachi Kikai Co., Ltd.). The floor of the open field box was divided into 16 squares, and one crossing was counted when the rat stepped over a division.

2.5. Neurotransmitter Concentrations in Rat Brain Tissue

After the behavioral test, rats were decapitated, and brain tissues were removed. Wet brain tissue was homogenized in two times the volume of 0.2 M perchloric acid buffer (pH 2) and kept on the ice for 1 hour. Homogenates

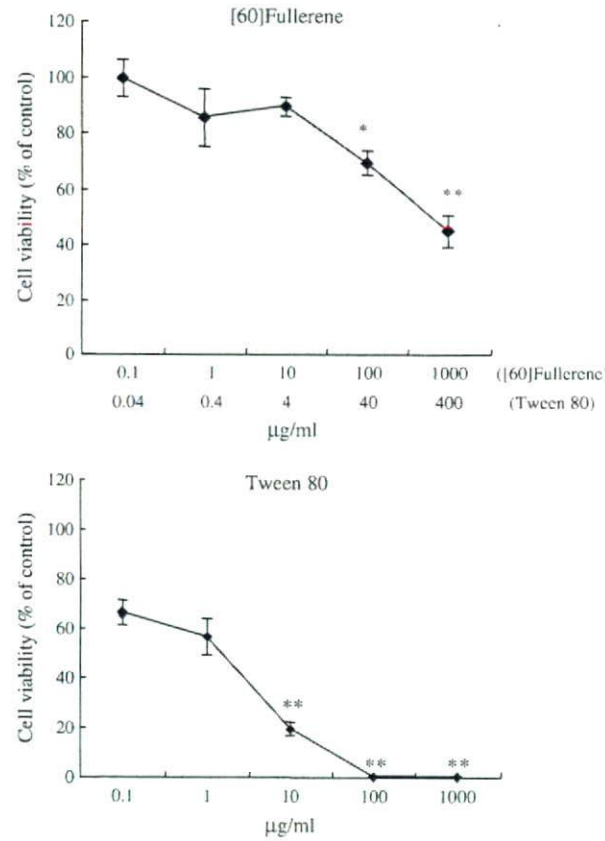

Fig. 1. V79 colony assay. V79 cells were treated with C_{60} and cultured for 7 days. Data are expressed as mean ± SD ($n = 4$). Experimental data showed significant differences from the control group (*; $p < 0.05$ **; $p < 0.01$).

Table I. Effects of C_{60} and Tween 80 on the survival of rats. C_{60} (0.25 mg/100 g B.W.), Tween 80 (0.1 μl/100 g B.W.), or physiolgical saline (control) was injected into the right lateral ventricle or the abdominal cavity. Surviving rats were counted 24 h after injection.

	Alive (%)	Number of rats
Control	100	(10/10)
[60]Fullerene	100	(8/8)
Tween 80	25	(2/8)

C_{60} (0.25 mg/100 g B.W.), Tween 80 (0.1 μl/100 g B.W.), or physiolgical saline (control) was injected into the right lateral ventricle or the abdominal cavity. Surviving rats were counted 24 h after injection.

were centrifuged at 20,000 g for 15 min at 0 °C, then filtered through a 0.45 μm cellulose acetate membrane filter. Neurotransmitters were detected with an HPLC system under the following conditions: the mobile phase was 0.1 M sodium acetate citric acid buffer and 15% methanol containing EDTA-2Na and 1-octanesulfonic acid sodium salt. The HPLC system was equipped with a reversed-phase column (MA-5ODS, 4.6 × 150 mm; Eicom, Kyoto, Japan) and an electrochemical detector (HTEC-500; Eicom,). Chromatograms were recorded and all calculations were performed using an integrator (EPC-500IS; Eicom). We measured dopamine (DA), serotonin (5-HT), and norepinephrine (NE), and the metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine hydrochloride (3MT), and 5-hydroxyindole-3-methoxyphenylacetic acid (5HIAA).

3. STATISTICAL ANALYSIS

A Tukey-Kramer test was used to analyze differences in cell viability between the control and the other groups in the V79 colony assay. In the *in vivo* studies, data for the individual groups are expressed as means, and one-way ANOVA was performed for statistical analysis. Student's *t*-test was used to assess differences between the two groups. In all cases, $P < 0.05$ was considered significant. Results are expressed as mean ± SD.

4. RESULTS

4.1. V79 Colony Assay

Treatment with C_{60} decreased V79 colony formation in a dose-dependent manner. The IC_{50} of C_{60} in V79 cells was

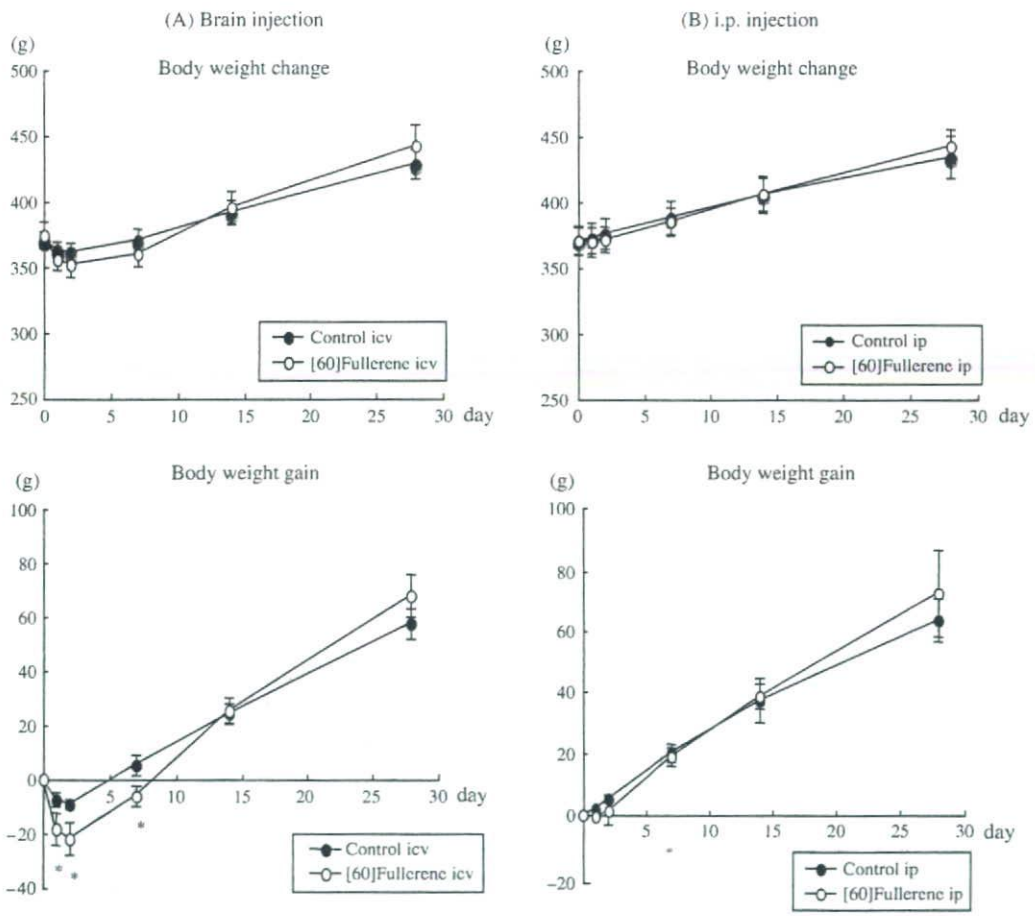

Fig. 2. Body weight change and body weight gain due to C_{60} injection. C_{60} (0.25 mg/100 g B.W.) was injected into the right lateral ventricle (A) or i.p. (B). Control group rats were injected with the same amount of physiolgical saline. Data are expressed as mean ± SD ($n = 6$). Experimental data showed a significant difference from the control group (*; $p < 0.05$).

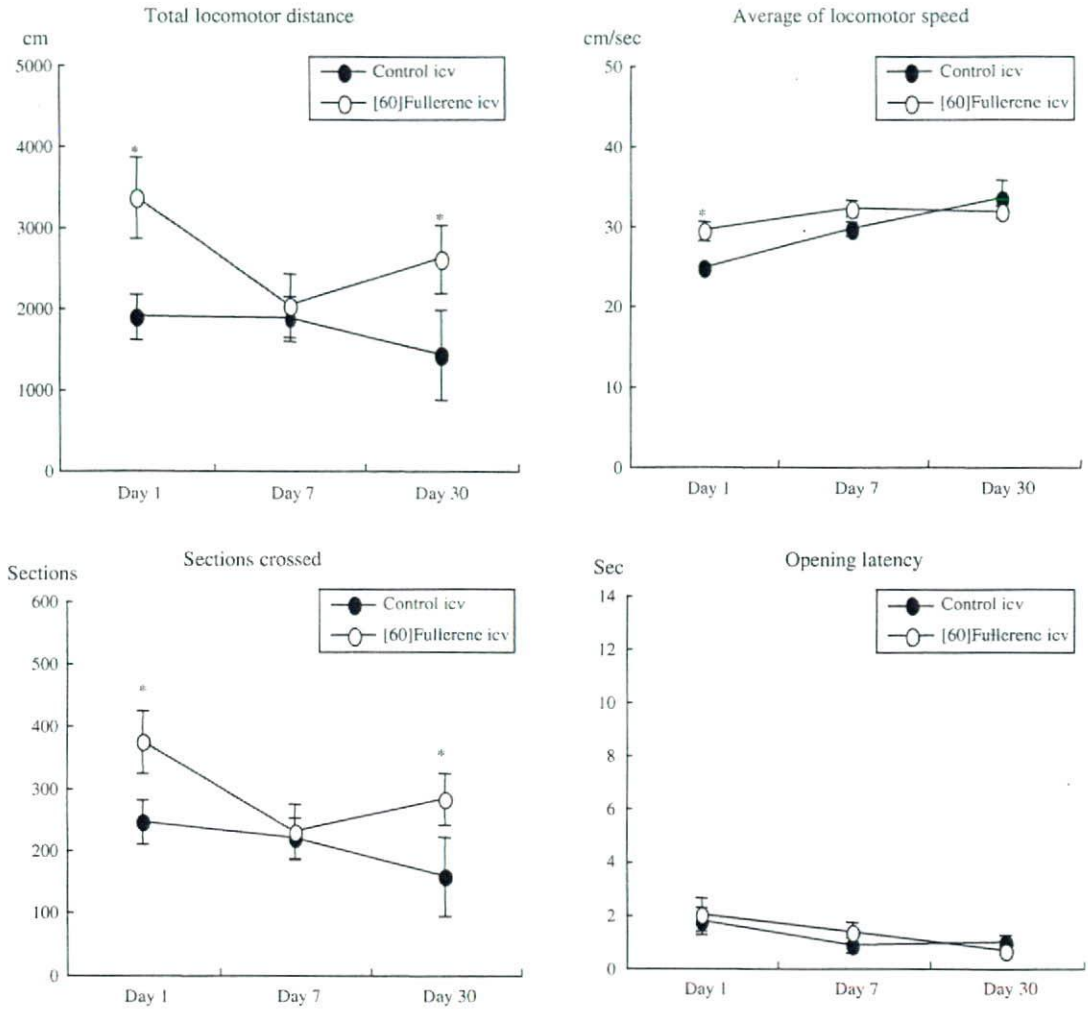

Fig. 3. Effect of intracerebral injection of C_{60} on locomotor activity of rats. C_{60} (0.25 mg/100 g B.W.) was injected into the right lateral ventricle. Control group rats were injected with same amount of physiolgical saline. Open field tests were performed 1, 7, and 30 days after C_{60} brain injection. Data are expressed as mean $\pm$ SD ($n = 6$). Experimental data showed significant difference from the control group (*; $p < 0.05$).

1620 μg/ml, and the IC_{50} of Tween 80 in V79 cells was 1.45 μg/ml (Fig. 1). All data are shown as the average of four samples.

4.2. Open Field Test

The intracerebral (i.c.v.) injection of B.W. C_{60} (0.25 mg/100 g containing 0.1 μl Tween 80) did not cause acute toxicity, and all animals survived (Table I). However, injection of Tween 80 (0.1 μl/100 g B.W.) produced acute toxicity, and 75% of the animals died. The i.c.v. injection of C_{60} decreased the postoperative body weight significantly, but the i.p. injection of C_{60} did not change the rat body weight (Fig. 2). In the open field test, the brain C_{60}-injected group ($n = 6$) showed higher total locomotor distance, average locomotor speed, and number of sections crossed compared with control group ($n = 6$) on day 1. On day 7, there was no difference between the two groups. On day 30, total locomotor distance and number of sections crossed of the intracerebral C_{60}-injected group was increased compared with control group (Fig. 3). In contrast to the brain-injected group, the i.p. C_{60}-injected group had significantly decreased total locomotor distance and number of sections crossed compared with the control group on day 30 (Fig. 4).

4.3. Neurotransmitter Concentrations in Rat Brain Tissue

The intracerebral injection of C_{60} (0.25 mg/100 g containing 0.1 μl Tween 80) decreased NE concentrations in the hypothalamus and hippocampus. Concentrations of serotonin or its metabolite 5HIAA were changed in the hypothalamus, cerebral cortex, striatum, and hippocampus. Concentrations of dopamine or its metabolites DOPAC, HVA, and 3MT were changed in the hypothalamus, cerebral cortex, and striatum. The turnover rates of serotonin were increased in the hypothalamus, cerebral cortex,

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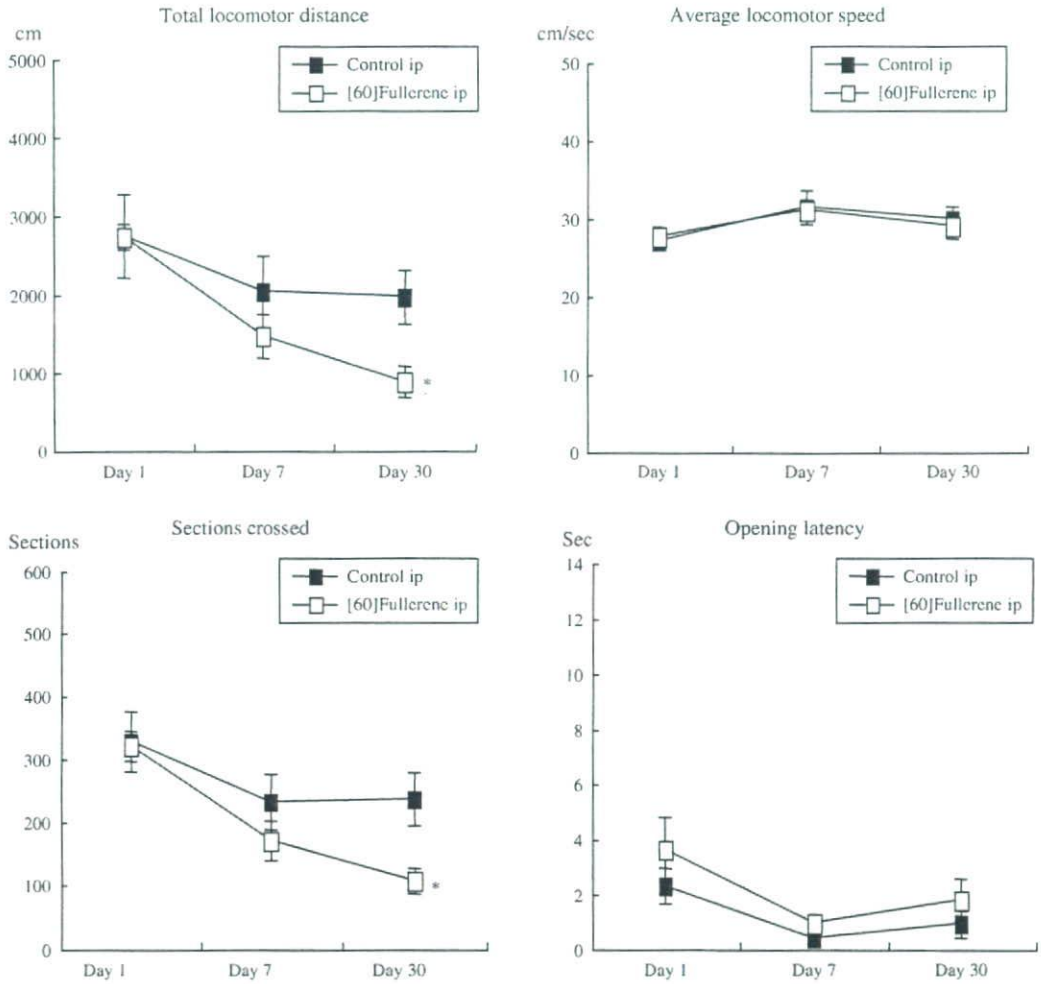

Fig. 4. Effect of i.p. injection of C_{60} on locomotor activity of rats. C_{60} (0.25 mg/100 g B.W.) was injected into the abdominal cavity. Control group rats were injected with the same amount of physiolgical saline. Open field tests were performed 1, 7, and 30 days after i.p. C_{60} injection. Data are expressed as mean $\pm$ SD ($n = 6$). Experimental data showed significant difference from the control group (*; $p < 0.05$).

striatum and hippocampus. The turnover rates of dopamine were increased in the hypothalamus, cerebral cortex, and striatum (Table II). The intraperitoneal injection of C_{60} increased the NE concentration in the hippocampus and the serotonin concentration in the striatum. The turnover rates of dopamine were decreased in the hippocampus. 5HIAA, dopamine, DOPAC, HVA, and 3MT concentrations and the turnover rates of serotonin were not significantly different from the control in any region (Table III).

5. DISCUSSION

NOVEL synthesized biomaterials are attractive candidates for medical devices. In particular, clinical brain and neural treatments, including implantation techniques, have been rapidly advancing, and novel medical devices for the brain are being developed every day. However, adverse events such as foreign-body reaction, inflammation, and tumor formation have been reported in clinical human and animal studies. In addition, because the blood-brain barrier (BBB) usually protects the central nervous system (CNS) from harmful materials,[15] we consider that the currently available reports about oral or i.p. injection studies and *in vivo* studies are insufficient to establish the effect of the novel materials on brain function. Therefore, investigation of the direct effects of these biomaterials on the CNS before application as medical devices is necessary.

In this study, we demonstrated effects of C_{60} on the CNS and behavior of rats. First, we examined the cytotoxicity of C_{60} and its catalyst Tween 80 by a V79 colony assay. The IC_{50} of Tween 80 in the V79 cells was 1.45 μg/ml. However, IC_{50} of C_{60} in the V79 cells was 1620 μg/ml, and this result indicated that the cytotoxicity of C_{60} on V79 cells is very weak. In this study, C_{60} was dissolved in the culture medium or physiolgical saline solution with 2% Tween 80. Tween 80 is a common detergent, and it has acute cytotoxicity in high doses. Treatment with Tween 80 presented strong cytotoxicity in the V79 colony assay treatment, but C_{60} did not have distinct cytotoxicity in

Table II. Effect of intracerebral C_{60} injection on brain monoamine neurotransmission. C_{60} (0.25 mg/100 g B.W.) was injected into the right lateral ventricle, and rats were decapitated after 30 days. Control group rats were injected with the same amount of physiolgical saline. Data are expressed as mean ± SD ($n = 6$). Experimental data showed a significant difference from the control group (*; $p < 0.05$).

		NE	5HT	5HIAA	DA	DOPAC	HVA	3MT	5HIAA/5HT	(DOPAC + HVA (+3MT))/DA
Hypothalamus	Control	27.51 ± 3.97	9.98 ± 1.38	12.79 ± 1.15	6.21 ± 0.53	2.57 ± 0.23	0.55 ± 0.04		0.91 ± 0.05	0.51 ± 0.03
	[60]fullerene	33.26 ± 2.78	11.68 ± 0.88	16.00 ± 0.88*	7.10 ± 0.64	3.68 ± 0.44*	0.79 ± 0.07*		1.24 ± 0.08*	0.64 ± 0.06*
Cerebral cortex	Control	2.67 ± 0.06	1.35 ± 0.08	1.56 ± 0.08	0.30 ± 0.04	0.14 ± 0.02	0.08 ± 0.01		0.96 ± 0.05	0.57 ± 0.05
	[60]fullerene	2.36 ± 0.14*	1.02 ± 0.06*	1.57 ± 0.12	0.29 ± 0.01	0.20 ± 0.02*	0.11 ± 0.03		1.25 ± 0.08*	0.87 ± 0.01*
Striatum	Control	0.34 ± 0.04	0.59 ± 0.01	1.27 ± 0.04	14.2 ± 0.96	5.62 ± 0.55	1.26 ± 0.10	0.68 ± 0.07	1.77 ± 0.08	0.44 ± 0.02
	[60]fullerene	0.26 ± 0.04	0.54 ± 0.05	1.88 ± 0.25*	18.2 ± 1.86	10.57 ± 1.45*	2.10 ± 0.21*	0.93 ± 0.09*	2.93 ± 0.41*	0.61 ± 0.04*
Hippocampus	Control	2.46 ± 0.08	1.39 ± 0.11	2.25 ± 0.13	0.17 ± 0.02	0.06 ± 0.00	0.03 ± 0.01		1.33 ± 0.04	0.44 ± 0.04
	[60]fullerene	2.08 ± 0.13*	0.87 ± 0.04*	2.00 ± 0.09	0.20 ± 0.02	0.07 ± 0.02	0.04 ± 0.03		1.89 ± 0.07*	0.52 ± 0.28
										ng/mg tissue

C_{60} (0.25 mg/100 g B.W.) was injected into the right lateral ventricle, and rats were decapitated after 30 days. Control group rats were injected with the same amount of physiolgical saline. Data are expressed as mean ± SD (n=6). Experimental data showed a significant difference from the control group (*; $p < 0.05$).

Table III. Effect of i.p. C_{60} injection on brain monoamine neurotransmission. C_{60} (0.25 mg/100 g B.W.) was injected into the abdominal cavity, and rats were decapitated after 30 days. Control group rats were injected with the same amount of physiolgical saline. Data are expressed as mean ± SD ($n = 6$). Experimental data showed significant difference from the control group (*; $p < 0.05$).

		NE	5HT	5HIAA	DA	DOPAC	HVA	3MT	5HIAA/5HT	(DOPAC + HVA (+3MT))/DA
Hypothalamus	Control	31.93 ± 2.08	14.07 ± 0.89	12.09 ± 1.72	5.74 ± 0.75	3.58 ± 0.23	0.97 ± 0.18		1.23 ± 0.10	0.83 ± 0.10
	[60]fullerene	32.98 ± 1.89	13.18 ± 1.22	18.73 ± 2.32	6.19 ± 0.33	3.70 ± 0.51	0.75 ± 0.51		1.62 ± 0.18	0.71 ± 0.05
Cerebral cortex	Control	2.60 ± 0.19	1.21 ± 0.06	1.70 ± 0.16	0.31 ± 0.02	0.17 ± 0.01	0.07 ± 0.01		1.15 ± 0.07	0.65 ± 0.08
	[60]fullerene	2.75 ± 0.17	1.25 ± 0.09	1.98 ± 0.14	0.29 ± 0.03	0.21 ± 0.03	0.09 ± 0.02		1.30 ± 0.08	0.81 ± 0.06
Striatum	Control	0.41 ± 0.14	0.58 ± 0.05	1.31 ± 0.07	16.5 ± 0.58	8.10 ± 0.31	1.64 ± 0.06	0.76 ± 0.03	1.86 ± 0.09	0.52 ± 0.03
	[60]fullerene	0.31 ± 0.05	0.87 ± 0.09*	1.47 ± 0.10	15.5 ± 1.28	8.37 ± 0.49	1.62 ± 0.11	0.71 ± 0.04	1.49 ± 0.25	0.57 ± 0.02
Hippocampus	Control	2.09 ± 0.17	1.16 ± 0.11	2.28 ± 0.21	0.10 ± 0.03	0.08 ± 0.01	0.19 ± 0.01		1.61 ± 0.10	2.62 ± 0.37
	[60]fullerene	2.49 ± 0.15*	1.08 ± 0.07	2.20 ± 0.10	0.19 ± 0.04	0.11 ± 0.04	0.16 ± 0.03		1.67 ± 0.04	1.31 ± 0.27*
										tissue ng/mg

C_{60} (0.25 mg/100 g B.W.) was injected into the abdominal cavity, and rats were decapitated after 30 days. Control group rats were injected with the same amount of physiolgical saline. Data are expressed as mean ± SD ($n = 6$). Experimental data showed significant difference from the control group (*; $p < 0.05$).

the *in vitro* study. In addition, intracerebral injection of 0.1 μl/100 g B.W. Tween 80 alone caused acute toxicity and death of injected rats, but intracerebral injection of C_{60} with 0.1 μl/100 g B.W. Tween 80 into the rat brain and intraperitoneal injection did not produce acute toxicity *in vivo*. These results suggest that C_{60} prevented the acute cytotoxicity of Tween 80. Some reports have shown that C_{60} has a cytoprotective effect against some toxicants and oxidative stress *in vitro* and *in vivo*, and we assumed that Tween 80 does not remain long-term *in vivo*.[16,17] These results suggest that the co-injection of C_{60} and Tween 80 did not have cytotoxicity or that it was very weak in this study.

Intracerebral injection of C_{60} decreased the postoperative body weight of rats, but i.p. injection did not. The body weight loss was not fatal, but the result suggested that brain injection of C_{60} decreased food intake. However, in the open field test, total locomotor distance, locomotor speed, and numbers of sections crossed were increased significantly by C_{60} on the day after operation. In addition, the body weight gain of C_{60} injected rats tended to have increased more than that of control rats by day 30. These results suggest that brain injection of C_{60} affected neurotransmission-related feeding behavior. Thus, we measured serotonin (5HT) and norepinephrine (NE), and the metabolite 5HIAA. 5HT and NE are found abundantly in the hypothalamus, which is the main source of neurotransmission. 5HT and NE neurotransmissions relate to feeding, emotion, emotional behavior, stress, hormone secretions; dysbolisms of these neurotransmitters cause cibophobia, hyperphagia, dystrophy, or depression-like behavior.[18,19] Intracerebral injection of C_{60} changed the serotonin and 5HIAA concentrations in the hypothalamus, cerebral cortex, striatum, and hippocampus, and 5HT turnover rates were increased in these four regions. The results suggest that C_{60} increased 5HT neurotransmission. NE concentrations also changed in the cerebral cortex and hippocampus. It is known that 5HT and NE neurotransmissions are enhanced when homeostasis is in disorder, for example, due to stress or inflammation and a variety of other changes in peripheral conditions.[18,19] However, i.p. injection of C_{60} increased the 5HT concentration only in the striatum, and did not affect 5HT turnover rates in the entire region. These results suggest that C_{60} did not pass the BBB to affect brain neurotransmission directly. On the other hand, some substances, for example, the glutamate receptor agonist kainic acid,[20] the gamma-aminobutyric acid (GABA) receptor antagonist strychnine,[21] and other neurotoxins[22] express neurotoxicity at lower doses than the C_{60} concentration used in this study and cause vital body weight loss and locomotor difficulty or death. These results suggested that intracerebral injection of C_{60} did not cause acute harmful neurotoxicity, but it did cause inflammation or neurotransmission disability of monoamines in the brain. The intracerebral injection of C_{60} also increased dopamine turnover rates in the hypothalamus, cerebral cortex, and striatum. Dopamine is an important neurotransmitter for behavior in the brain striatum, and dopamine neurotransmission promoted ambulation or decreased anxiety in several rat studies.[23,24] Dopamine dysbolism is related to depression, and depletion of dopamine causes Parkinson's disease.[25,26] Infusion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a dopaminergic neuron-specific neurotoxin, into the brain reduced cognitive and motor functions in the rat and decreased dopamine concentration in the prefrontal cortex and striatum.[27] Rats that exhibit depressive-like behavior due to intraperitoneal administration of the kappa-opinion receptor agonist salvinorin A had reduced extracellular concentrations of dopamine within the nucleus accumbens.[28] In contrast, an excess of dopamine neurotransmission induces locomotor hyperactivity.[29,30] Dopaminergic neurotransmission is closely involved in motor activity and emotional behavior. In this study, we showed an increase of dopamine turnover rates that was consistent with the increase of locomotion on day 30. On the other hand, i.p. injection of C_{60} did not change concentrations of dopamine and its metabolites in all brain regions examined or the dopamine turnover rate in the striatum. These results suggest that brain injection of C_{60} increased dopamine neurotransmission similar to serotonin in the brain and that the dopamine neurotransmission might induce locomotor hyperactivity behaviors.

In conclusion, we investigated the neurotoxicity of intracerebral injection of C_{60} and compared it with i.p. injection of C_{60}. In the *in vitro* study, we showed that the cytotoxicity of C_{60} was about 1620 μg/ml (10 ppm) in V79 cells. In an *in vivo* study, intracerebral injection of 0.25 mg/ml C_{60} did not cause acute vital neurotoxicity. However, some neurotransmitter concentrations were changed in several brain regions. We suggest that chronic expose of the brain to C_{60} may cause serotoninergic and dopaminergic neurotransmission abnormalities and change behavior and homeostasis. Recently, medical progress had produced novel medical devices targeting the brain. We recommend reexamination of the materials used for medical devices targeting on brain.

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RESEARCH ARTICLE

Intracerebral Microinjection of Stannous 2-Ethylhexanoate Affects Dopamine Turnover in Cerebral Cortex and Locomotor Activity in Rats

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Abstract: Stannous 2-ethylhexanoate [$Sn(Oct)_2$] is used as a catalyst for production of poly-L-lactic acid and copolymers that are implanted in cranial surgery, but reports on its effects on the central nervous system are few. We examined the effects of $Sn(Oct)_2$ on cell viability *in vitro* and on neurotransmission and behavior in the rat. Treatment of normal human astrocytes with 10 mg/mL $Sn(Oct)_2$ reduced mitochondrial activity to 16% of the control. Injection of $Sn(Oct)_2$ at 6.28 mg/kg BW (2 mg/kg BW Sn) into right lateral ventricle of the rat brain tended to increase the ambulation distance after 30 days when compared with the control group. The turnover of dopamine neurotransmission was increased in the cerebral cortex. These results suggest that $Sn(Oct)_2$ is cytotoxic to astrocytes *in vitro*. Injection of $Sn(Oct)_2$ into the brain had no or very weak immediate neurotoxicity, but long-term exposure to $Sn(Oct)_2$ increased dopamine neurotransmission turnover. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 87B: 381–386, 2008

Keywords: stannous 2-ethylhexanoate; open field test; dopamine, serotonin, cytotoxicity

INTRODUCTION

Biodegradable polymers are attractive candidates for scaffolding materials because they degrade as new tissues are formed. However, adverse events, such as foreign-body reaction, inflammation, and tumor formation, have been reported in human clinical and animal studies. Therefore, confirmation that these polymers and related materials used as catalysts lack toxicity is required.

Stannous 2-ethylhexanoate [$Sn(Oct)_2$] is commonly used as a catalyst in the production of poly-L-lactic acid (PLLA) and other copolymers that are implanted in cranial surgery.[1] Food dishes and trays are also made of PLLA. The toxicity of $Sn(Oct)_2$ has been examined in *in vitro* and *in vivo* studies.[2,3] $Sn(Oct)_2$ is resolved into Sn and 2-ethylhexanoic acid in the stomach.[4] Therefore, the toxicity of $Sn(Oct)_2$ depends on the toxicities of Sn and 2-ethylhexanoic acid.[5] It is known that 2-ethylhexanoic acid is metabolized from 2-ethylhexanol in the body and that the acceptable daily intake (ADI) of 2-ethylhexanoic acid is 0.5 mg/kg BW/day.[6,7] The provisional tolerable weekly intake (PTWI) of tin is 14 mg/kg BW/week.[8,9]

$Sn(Oct)_2$ has recently been used as a catalyst in the production of medical devices. In these cases, $Sn(Oct)_2$ does not degrade to Sn and 2-ethylhexanoic acid because it does not pass through the acidic conditions of the stomach. However, little information about the effects of the combination of Sn and 2-ethylhexanoic acid on mammals is available. It was reported that a combination of harmful materials increases the materials' toxicity.[10] Based on these reports, we considered that further examination is required before $Sn(Oct)_2$ is used as a catalyst in the production of medical device materials.

Brain and clinical neurology sciences are advancing rapidly, and implantation techniques and novel medical devices for the brain are being developed every day. A synthetic biodegradable dura mater has recently been developed using PLLA and a copolymer of polyglycolic acid or polycaprolactic acid as the major components. The blood–brain barrier normally protects the central nervous system (CNS) from harmful materials,[11,12] but implantation of the synthetic dura mater directly exposes the CNS to unknown conditions and necessitates investigation of the direct effects of biomaterials used as medical devices on the CNS.

In this study, we examined the effects of $Sn(Oct)_2$ on cell viability in normal human astrocytes (NHA) *in vitro* and the effects of direct injection of $Sn(Oct)_2$ into the rat brain on neurotransmitters and behavior *in vivo*.

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MATERIALS AND METHODS

Astrocyte Microtiter Tetrazolium Assay

The effects of $Sn(Oct)_2$ on mitochondrial activity of NHA were measured using a microtiter tetrazolium (MTT) assay. NHA were seeded into 24-well plates at a density of 1×10^4/well in ABM medium supplemented with 5% FBS and recombinant human epidermal growth factor, insulin, GA-1000, ascorbic acid, L-glutamate (ANG bred kit; Sanko Junyaku, Tokyo, Japan) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. $Sn(Oct)_2$ were added directly to the ABM medium. After 1-week culture with 2.5, 5, or 10 μg/mL of $Sn(Oct)_2$, the medium in each well was replaced with 300 mL of fresh medium containing 6 mL TetraColor ONE reagent (Seikagaku, Tokyo, Japan). After 2 h, the absorbance at 450/630 nm was measured using a plate reader. The data were expressed as averages of five wells, and the procedures were performed in triplicate.

Animals

Male Wistar rats (270 g weight; SLC, Shizuoka, Japan) were kept in individual wire cages in a temperature- and humidity-controlled room (24°C and 55% relative humidity) under regular light conditions (12 h light:dark cycle) and given food and water *ad libitum*. Six rats were used in the experimental group, and six rats were used as controls. This experiment was carried out in accordance with the guidelines for the care and use of laboratory animals of the National Institutes of Health Sciences, which refer to standards of the American Association for Laboratory Animal Science.

Intracerebral Injection of $Sn(Oct)_2$

Rats were anesthetized, and a hole was drilled in the skull using a high-speed drill (Muromachi Kikai, Tokyo, Japan). A microliter syringe was stereotaxically inserted into the right lateral ventricle (0.6 mm posterior to the bregma, 1.6 mm lateral to the midline, and 3.5 mm ventral to the cortical surface) according to the atlas of Paxinos and Watson,[13] and 6.28 mg/kg BW $Sn(Oct)_2$ (2 mg/kg BW tin) was injected at the rate of 1 mL/min, using a brain infusion stereotaxic injector (Muromachi Kikai, Tokyo, Japan). Control rats were injected with the same volume of Ringer's solution.

Open Field Test

Motor activities (total locomotion distance, average locomotion speed, number of sections crossed, and beginning latency) of rats were measured by an open field test. The open field box was 70 cm × 70 cm × 70 cm. Tests were performed 1, 7, and 30 days after $Sn(Oct)_2$ injection. The rat was placed in the open field box, and its behavior was recorded using a DV-Track video tracking system CompACT VAS/DV (Muromachi Kikai, Tokyo, Japan). The floor of the open field box was divided into 16 squares, and one crossing was counted when the rat stepped over the divisions.

Neurotransmitter Concentrations in Brain Tissue

After the last behavioral test, the rat was decapitated, and brain tissues were removed. Wet brain tissues were homogenized in two volumes of 0.2*M* perchloric acid buffer (pH 2) and kept on ice for 1 h. Homogenates were centrifuged at 20,000*g* for 15 min at 0°C, then filtered through a 0.45 μm cellulose acetate membrane filter. Neurotransmitters were detected with an HPLC system under the following conditions: the mobile phase was 0.1*M* sodium acetate citric acid buffer and 15% methanol containing EDTA-2Na and 1-octanesulfonic acid sodium salt. The HPLC system was equipped with a reversed-phase column (MA-5ODS, 4.6 × 150 mm^2: Eicom, Kyoto, Japan) and an electrochemical detector (HTEC-500: Eicom, Kyoto, Japan). Recording of chromatograms and all calculations were performed using an integrator (EPC-500IS: Eicom, Kyoto, Japan). We measured dopamine (DA), serotonin (5-HT), and norepinephrine (NE), and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine hydrochloride, and 5-hydroxyindole-3-methoxyphenylacetic acid (5HIAA).

Statistical Analysis

The Tukey–Kramer test was used to analyze differences in cell viability between the control group and the test groups in the NHA MTT assay. *In vivo* data were expressed as means for individual groups, and one-way ANOVA was performed for statistical analysis. Student's *t*-test was used to assess differences between the two groups. In all cases, $p < 0.05$ was considered significant. Results were expressed as mean ± SD.

RESULTS

Astrocyte Microtiter Tetrazolium Assay

Treatment of $Sn(Oct)_2$ decreased NHA mitochondrial activity in a dose-dependent manner (Figure 1). Treatment with 10 mg/mL $Sn(Oct)_2$ decreased NHA mitochondrial activity to 16% of the control. All data shown were the average of five samples.

Open Field Test

The injection of 6.28 mg/kg BW $Sn(Oct)_2$ (2 mg/kg BW tin) into the brain did not significantly change the average body weight, but did reduce the body weight gain (Figure 2). In the open field test, the total locomotion distance and average locomotion speed were decreased in the rats ($n = 6$) injected with $Sn(Oct)_2$, and the beginning latency was

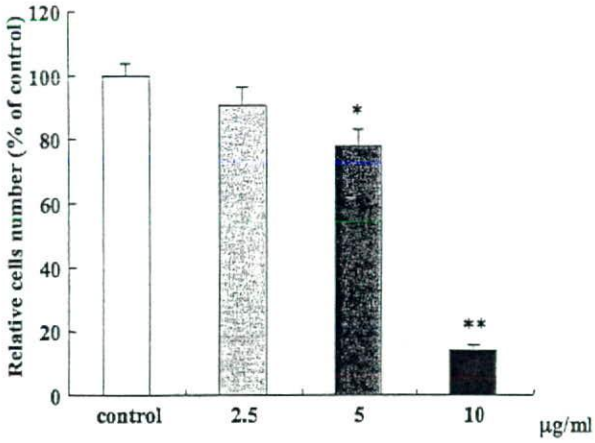

Figure 1. The effect of $Sn(Oct)_2$ on astrocytes. Astrocytes were cultured with 10 mg/mL $Sn(Oct)_2$ for 7 days, and mitochondrial activity of NHA was measured by MTT assay. Data are expressed as mean ± SD (n = 5). Experimental data showed significant difference from the control group (*$p < 0.05$; **$p < 0.01$).

increased compared with the control group (n = 6) on day 1. On day 7, there was no difference between the two groups. On day 30, the total locomotion distance and crossing sections tended to increase in the $Sn(Oct)_2$-injected group, and the beginning latency was decreased. Ambulation decreased in the control group with the repetition of tests, in contrast with the $Sn(Oct)_2$-injected group, in which it increased (Figure 3).

Neurotransmitters in Rat Brain Tissue

Injection of $Sn(Oct)_2$ into the rat brain decreased NE concentrations in the rat hypothalamus (Table I) and decreased the DA concentration in the cerebral cortex. Concentrations of DOPAC and HVA, which are DA metabolites, and the turnover rate of DA were increased in the cerebral cortex (Table I). Concentrations of 5HT and its metabolite 5HIAA were increased in the striatum by $Sn(Oct)_2$ injection. In the hippocampus, the turnover rate of 5HT was increased by $Sn(Oct)_2$ injection.

DISCUSSION

There are many reports of synthesis of novel polymers and biomaterials using $Sn(Oct)_2$ as a catalyst, but few studies of the toxicity of $Sn(Oct)_2$. $Sn(Oct)_2$ is used as a catalyst in food dish production, but its safety has been examined only in the case of oral consumption. Almost all $Sn(Oct)_2$ is resolved into Sn and 2-ethylhexanoic acid in the stomach.[4] It is known that 2-ethylhexanoic acid is metabolized from 2-ethylhexanol in the body and that the ADI for rats is 0.5 mg/kg BW/day.[6,7] The PTWI for humans of tin is 14 mg/kg BW/week (~2 mg/kg BW/day).[8,9] However, the effects of injection of $Sn(Oct)_2$ into the brain have not been examined.

In this study, we demonstrated the effects of $Sn(Oct)_2$ on the CNS and behavior of rats. Treatment of NHA *in vitro* with 10 mg/mL $Sn(Oct)_2$ decreased mitochondrial activity by 16% when compared with the control. It was suggested that the reduction of mitochondrial activity decreases ATP production. ATP not only drives energy-dependent reactions inside cells, but also functions as a potent signaling molecule in the CNS. Therefore, this result suggests that $Sn(Oct)_2$ is cytotoxic to CNS cells[14] and that specific verification of the safety of $Sn(Oct)_2$ is required before it is used as a catalyst in the synthesis of medical devices, especially in the cases of biodegradable and bioabsorbable polymers.

Intracranial injection of $Sn(Oct)_2$ did not decrease the body weight of rats, but it did decrease the weight gain. In the open field test, total locomotion distance and locomotion speed decreased, and beginning latency was increased due to $Sn(Oct)_2$ injection on day 1. On day 30, in contrast to day 1, the $Sn(Oct)_2$-injected group tended to exhibit increased total locomotion distance and crossing frequency and decreased beginning latency. Ambulation decreased in

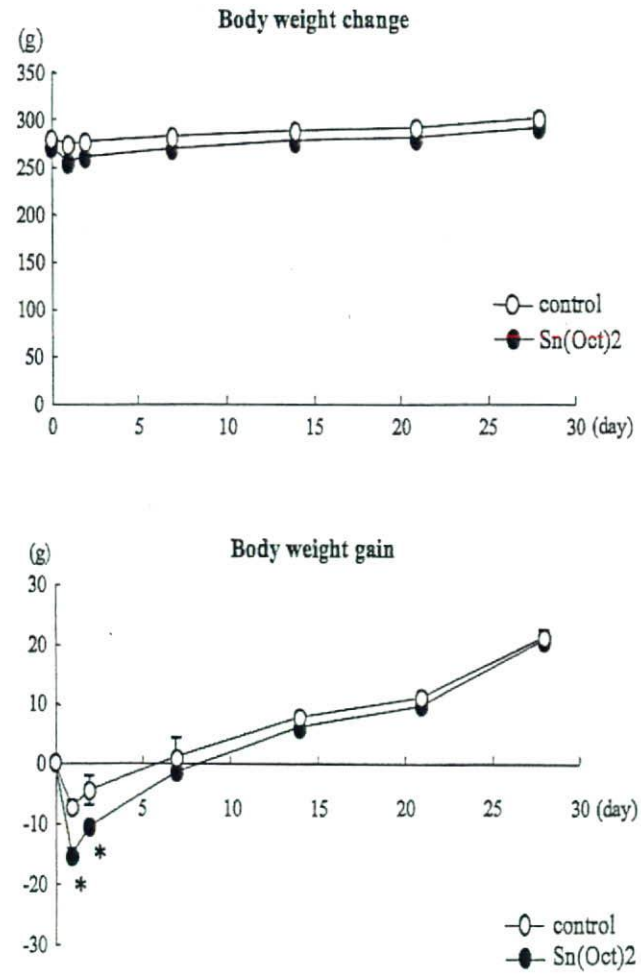

Figure 2. Changes in body weight and body weight gain because of intracranial injection of $Sn(Oct)_2$. $Sn(Oct)_2$ at 6.28 mg/kg BW (2 mg/kg BW tin) was injected into the right lateral ventricle. Control group rats were injected with the same amount of Ringer's solution. Data are expressed as mean ± SD (n = 6). *Experimental data showed a significant difference from the value of day 0 ($p < 0.05$).

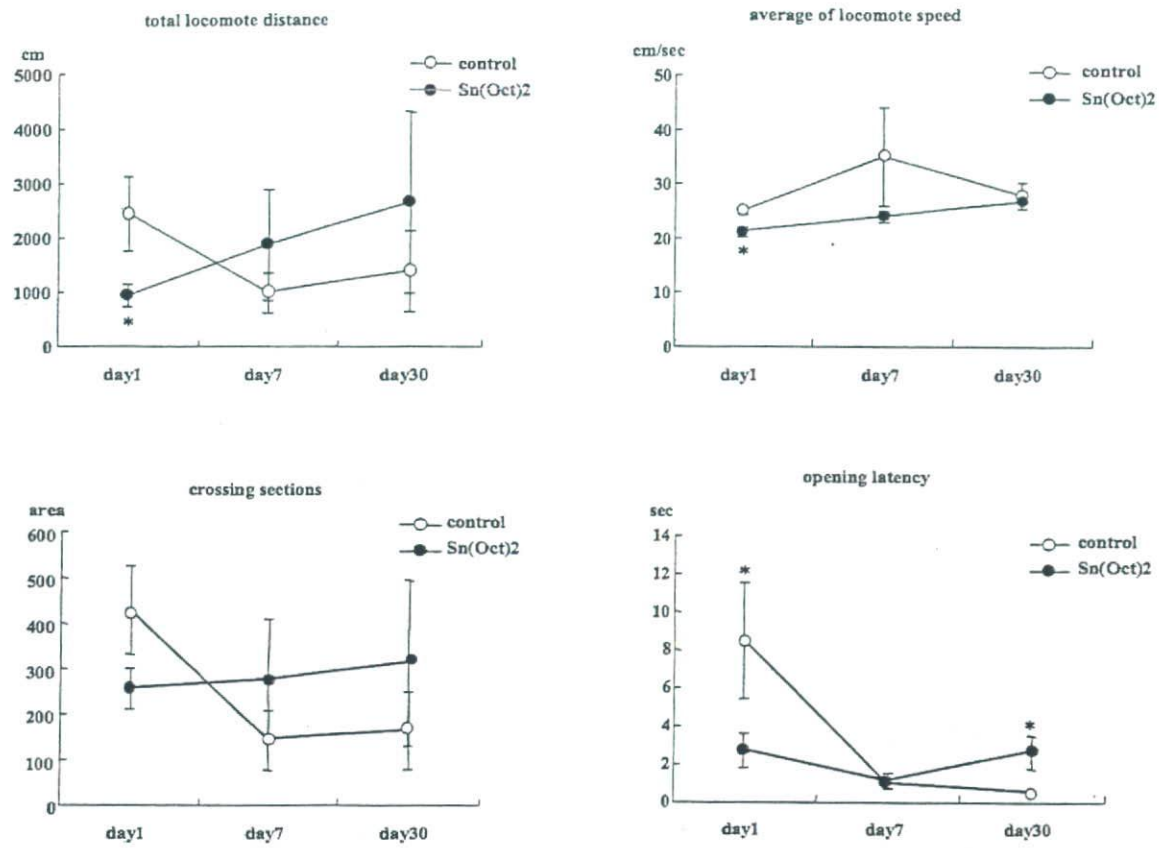

Figure 3. Effect of $Sn(Oct)_2$ on locomotion in rat. $Sn(Oct)_2$ at 6.28 mg/kg BW (2 mg/kg BW tin) was injected into the right lateral ventricle. Control rats were injected with the same amount of Ringer's solution. Open field tests were performed 1, 7, and 30 days after $Sn(Oct)_2$ injection. Data are expressed as mean ± SD (n = 6). *Experimental data showed a significant difference from the control group ($p < 0.05$).

the control group with experience in performing the test, and, in contrast, ambulation by the $Sn(Oct)_2$-injected group increased. This result suggests that intracranial injection of $Sn(Oct)_2$ decreased locomotor activity immediately but increased it afterwards. Some substances, for example, the glutamate receptor agonist kainic acid,[15] the GABA receptor antagonist strychnine,[16] and other neurotoxins[17] produce neurotoxicity at doses lower than the $Sn(Oct)_2$ concentration used in this study and cause body weight loss and locomotive difficulty or death. $Sn(Oct)_2$ did not have acute neurotoxicity or it was very weak at the $Sn(Oct)_2$ concentration used in this study. However, locomotion distance was increased by $Sn(Oct)_2$ injection after 30 days. Generally, locomotion distance in the open field test is decreased by repeated testing, and the results of our previous tests corresponded to this observation.[18] Thus, we suspect that $Sn(Oct)_2$ might produce chronic neurotoxicity.

Because intracranial injection of $Sn(Oct)_2$ tended to increase locomotion distance in repeat tests, we measured brain monoamines of $Sn(Oct)_2$-injected rats. The brain monoamines DA, 5-HT, and NE are major neurotransmitters related to emotion, stress, and several other functions.[19,20] DA is an important neurotransmitter for behavior. DA neurotransmission promotes ambulation and decreases anxiety in the rat.[21,22] DA dysfunction is related to depression, and DA depletion causes Parkinson's disease.[23,24] Infusion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which is a dopaminergic neuron-specific neurotoxin, reduced the cognitive and motor functions of the rat and decreased DA concentration in the prefrontal cortex and striatum.[25] Administration of the kappa-opioid receptor agonist salvinorin A to depressed rats reduced the extracellular concentration of DA within the nucleus accumbens.[26] Therefore, dopaminergic neurotransmission is closely involved in motor activity and emotional behavior. In this study, intracranial injection of $Sn(Oct)_2$ decreased the DA concentration in the cerebral cortex and increased concentrations of the DA metabolites DOPAC and HVA significantly. These results mean that $Sn(Oct)_2$ enhanced DA transmission and turnover. The increase of DA turnover due to $Sn(Oct)_2$ was consistent with the increase in locomotion distance observed in this study. On the other hand, the injection of $Sn(Oct)_2$ into the brain decreased the NE concentration in the rat hypothalamus, and increased concentrations of 5HT and its metabolite 5HIAA in the striatum. In the hippocampus, the rate of 5HT turnover was increased by $Sn(Oct)_2$ injection. NE and 5HT neurotransmissions are related to emotion, emotional behavior, stress,

TABLE I. Effect of $Sn(Oct)_2$ on Brain Monoamine Neurotransmission and 5HT and DA Turnover Rates

		NE	5HT	5HIAA	DA	DOPAC	HVA	3MT	5HIAA/5HT	(DOPAC+HVA (+3MT))/DA
Hypothalamus	control	78.0 ± 3.0	22.3 ± 0.9	26.9 ± 3.0	8.46 ± 0.79	6.82 ± 1.09	0.80 ± 0.06		1.12 ± 0.08	0.55 ± 0.06
	Sn(Oct)2	70.6 ± 2.3[a]	19.2 ± 1.6	27.1 ± 4.5	7.76 ± 0.66	7.98 ± 1.15	0.97 ± 0.09		1.30 ± 0.06	0.71 ± 0.03
Cerebral cortex	control	2.78 ± 0.08	1.48 ± 0.14	1.85 ± 0.14	0.21 ± 0.01	0.09 ± 0.01	0.06 ± 0.00		1.19 ± 0.09	0.52 ± 0.09
	Sn(Oct)2	2.55 ± 0.14	1.48 ± 0.08	2.12 ± 0.08	0.16 ± 0.01[a]	0.20 ± 0.05[a]	0.11 ± 0.03[a]		1.34 ± 0.15	1.34 ± 0.35[a]
Striatum	control	0.52 ± 0.05	1.21 ± 0.07	1.70 ± 0.09	19.7 ± 1.02	7.85 ± 0.62	1.58 ± 0.07	0.79 ± 0.07	1.34 ± 0.12	0.34 ± 0.03
	Sn(Oct)2	0.56 ± 0.09	1.53 ± 0.13[a]	2.12 ± 0.15[a]	19.3 ± 1.82	8.90 ± 1.11	1.87 ± 0.18	0.81 ± 0.08	1.30 ± 0.03	0.39 ± 0.03
Hippocampus	control	19.5 ± 0.74	5.57 ± 0.23	6.71 ± 0.74	2.12 ± 0.20	1.70 ± 0.27	0.20 ± 0.02		1.36 ± 0.10	0.50 ± 0.02
	Sn(Oct)2	17.7 ± 0.57	4.79 ± 0.41	6.78 ± 1.12	1.94 ± 0.16	1.99 ± 0.29	0.24 ± 0.02		1.69 ± 0.13[a]	0.64 ± 0.07

$Sn(Oct)_2$ at 6.28 mg /kg B.W. (2 mg/kg B.W. tin) was injected into the right lateral ventricle of rats, and control rats were injected same amount of Ringer's solution. Rats were decapitated after 30 days. Dates are expressed as mean ± SD (n=6).

[a] Experimental data showed a significant difference from control group data ($p < 0.05$).

and hormone secretion, and dysbolisms of these neurotransmitters can cause dystrophy or depression-like behavior.[27,28] Based on these results, we suggest that intracranial injection of $Sn(Oct)_2$ changed the neurotransmission of DA and other monoamines and that it affected the motor activity of rats. In this study, we measured DA and concentrations of its metabolites only 30 days after $Sn(Oct)_2$ injection, and all *in vivo* studies were performed after injection of 6.28 mg/kg BW $Sn(Oct)_2$ [2 mg/kg BW (2 ppm) tin]. However, these results should be compared with the clinical findings. Therefore, follow-up studies are needed to examine the effects of different concentrations of $Sn(Oct)_2$, injection times, and observation periods. In addition, it is necessary to estimate the concentration of tin degraded from a medical prosthesis, such as a dura mater.

CONCLUSIONS

We investigated the neurotoxicity of $Sn(Oct)_2$. The *in vitro* study demonstrated that $Sn(Oct)_2$ is cytotoxic to NHA at 10 mg/mL (10 ppm). The *in vivo* study showed that injection of 6.28 mg/kg BW $Sn(Oct)_2$ [2 mg/kg BW (2 ppm) tin] into rat brain did not produce remarkable acute neurotoxicity. However, we found for the first time that $Sn(Oct)_2$ caused dopaminergic neurotransmission abnormalities and altered locomotive behavior for 30 days after a single intracerebral microinjection. Recent medical progress has produced novel medical devices targeting the brain, but we recommend re-examination of medical devices targeting the brain that use these types of catalysts.

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