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L-*Threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol stimulates ganglioside biosynthesis, neurite outgrowth and synapse formation in cultured cortical neurons, and ameliorates memory deficits in ischemic rats*

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To address the role of brain gangliosides in synaptic plasticity, the synthetic ceramide analog, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) was used to manipulate the biosynthesis of gangliosides in cultured cortical neurons. Spontaneous synchronized oscillatory activity of intracellular Ca^{2+} between the neurons, which represents synapse formation, was suppressed by the depletion of endogenous gangliosides by D-*threo*-PDMP, an inhibitor of glucosylceramide synthase. The decreased functional synapse formation was normalized by supplementation of G_{Q1b} but not by the other gangliosides, suggesting that *de novo* synthesis of ganglioside G_{Q1b} is essential for the synaptic activity (Mizutani A. *et al.*, *Biochem. Biophys. Res. Commun.* 222, 494–498, 1996). On the other hand, the enantiomer of the inhibitor, L-*threo*-PDMP, could elevate cellular levels of glycosphingolipids including gangliosides. This paper presents our recent findings on the neurotrophic actions of L-*threo*-PDMP *in vitro* and *in vivo*. We found that L-PDMP could up-regulate neurite outgrowth, functional synapse formation and ganglioside biosynthesis through activating G_{M3}, G_{D3} and G_{Q1b}

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Abbreviations: DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's minimum essential medium; GlcCer, glucosylceramide; GSL, glycosphingolipid; LacCer, lactosylceramide; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; MAPK, mitogen activated protein kinase; NGF, nerve growth factor.

synthases. Simultaneously, the activity of p42 mitogen-activated protein kinase was also facilitated by L-PDMP. To evaluate the efficacy of this drug on long term memory, rats were trained for 2 weeks using an 8-arm radial maze task, and then forebrain ischemia was induced by 4-vessel occlusion (for 10 min × 2 with a 60 min interval). Repeated treatment of L-*threo*-PDMP (40 mg/kg, i.p. for 6 days, twice a day) starting 24 h after the ischemia, improved the deficit of the well-learned spatial memory, demonstrating the potential therapeutic use of the ceramide analog for treatment of neurodegenerative disorders.

PDMP (1-phenyl-2-decanoylamino-3-morpholino-1-propanol) is a synthetic analog of ceramide and possesses two chiral centers at the C1 and C2 positions, to which hydroxyl and aminoacyl groups are attached. It thus forms four isomers (Fig. 1). The stereo-specific action of PDMP isomers on UDP-glucose:*N*-acylsphingosine glucosyltransferase (GlcCer synthase) has been clearly demonstrated, since only D-*threo*-PDMP was able to inhibit GlcCer synthase and the other isomers including the L-*threo* and *erythro* forms could not affect this enzyme activity at all *in vitro* (Inokuchi & Radin, 1987). D-*threo*-PDMP (D-PDMP) leads to extensive depletion of endogenous glycosphingolipids (GSLs) including gangliosides biosynthesized from GlcCer and causes accumulation of ceramide, and it has proved useful as a tool for studying various functional roles of endogenous GSLs (Radin *et al.*, 1993; Inokuchi, 1997). Previously, we unexpectedly found that, when B16 melanoma cells were incubated with L-PDMP, the cellular levels of the major GSLs, GlcCer, lactosylceramide (LacCer), and G_{M3}, became significantly elevated (Inokuchi *et al.*, 1989). In addition, it was demonstrated that L-PDMP could enhance the activities of glycosyltransferases including GlcCer synthase, LacCer synthase, G_{M3} synthase and G_{D3} synthase, and led to elevated GSL levels (Chatterjee, 1991; Inokuchi *et al.*, 1995; Usuki *et al.*, 1996).

Neuronal cell differentiation and development are known to be closely correlated to the biosynthesis and expression of gangliosides (Varon *et al.*, 1988). It has been reported that exogenous gangliosides can elicit neurite outgrowth and neural repair *in vitro* and *in vivo* (Pepeu *et al.*, 1994). In particular, G_{M1} and G_{Q1b} have been found not only to enhance the nerve growth factor (NGF)-induced neurite outgrowth, but also to display NGF-like activities (Tsuji *et al.*, 1986; Di Patre *et al.*, 1989; Ferrari *et al.*, 1991; Maysinger *et al.*, 1993). We demonstrated previously that the functional synapse formation, measured by spontaneous synchronized oscillatory activity of intracellular Ca^{2+} in cultured rat cerebral cortical neurons, was inhibited by depletion of the endogenous gangliosides on addition of a glucosylceramide synthase inhibitor, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP, Fig. 1) into the culture medium (Mizutani *et al.*, 1996). This suppressed synaptic activity was normalized by supplementation with ganglioside G_{Q1b}, but not with other gangliosides. The importance of polysialylated gangliosides, including G_{Q1b}, in the central nervous system was further indicated by the observations that mice deficient in β1,4-*N*-acetylgalactosaminyltransferase (G_{M2}/G_{D2} synthase), exhibited a reduction in both conduction velocity of somatosensory nerves and performance in a passive avoidance task test (Takamiya *et al.*, 1996; Furukawa *et al.*, 1997). These lines of evidence suggest that *de novo* synthesis of gangliosides (probably of G_{Q1b}) is essential for synaptic function and even for learning and memory.

Therefore, it seemed interesting to apply PDMP to the cultured neuronal cells in an attempt to modulate ganglioside biosynthesis. Specifically, L-PDMP might possess an ability to increase the ganglioside levels in neuronal cells, and thereby produce beneficial effects. Here, we report our recent studies on the neurotrophic actions of L-PDMP *in vitro* and *in vivo*.

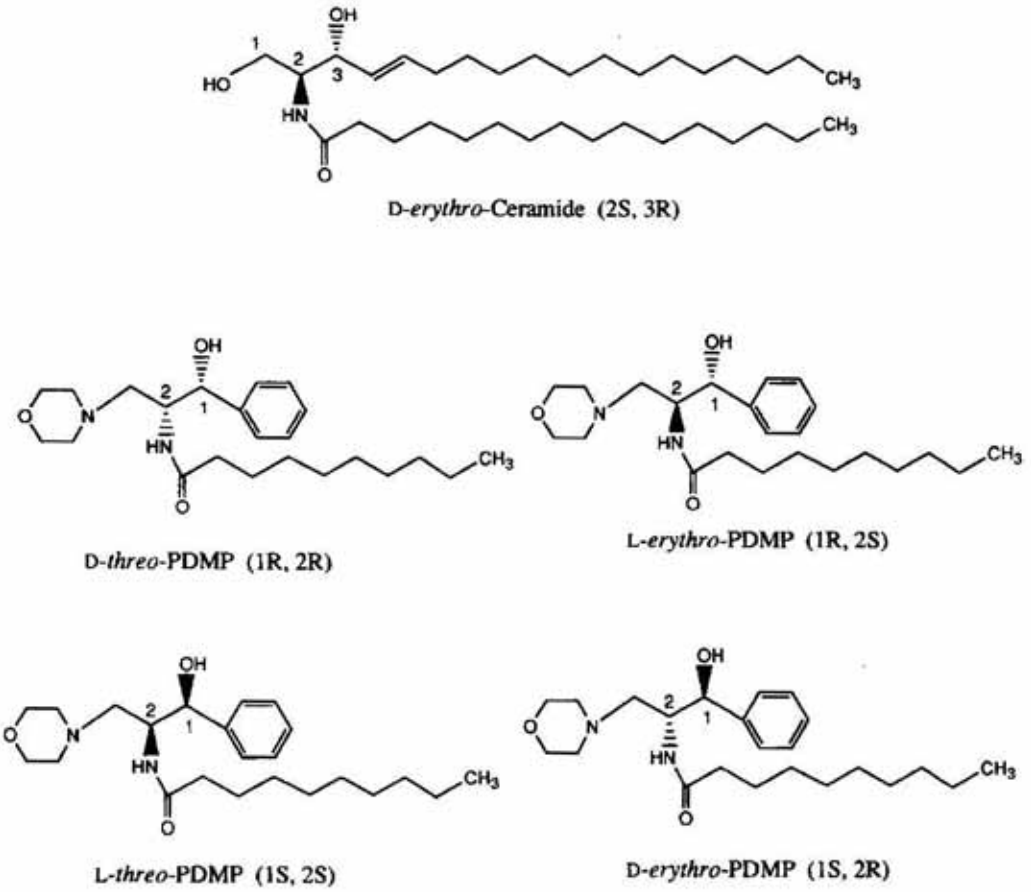

Figure 1. Structural comparison of ceramide and PDMP isomers.

MATERIALS AND METHODS

Explant culture of rat neocortical neurons and assay of neurite outgrowth and the application of PDMP isomers. Rat neocortical explants were prepared by the method described previously (Nagata *et al.*, 1993). In brief, the neocortical tissues were dissected from embryonic 17-day-old rat brain. The tissues were cut into small pieces of various sizes from 50 to 200 μm, and then suspended in serum-free DMEM, supplemented with 3.7 g of $NaHCO_3$ per liter. Twenty four-well polystyrene plates were coated with 0.1% polyethylenimine in 0.15 M sodium borate buffer (pH 8.4) for several hours, washed twice with distilled water and with serum-free medium once, and were filled with serum-free medium. Around 200 explants per 0.5 ml of serum-free medium were seeded into each well. Two hours later, 50 μl of the medium of explant culture was replaced with a PDMP containing medium and incubated at 37°C for 2 days in a 5% CO_2-air incubator. Explants were fixed with 500 μl of freshly prepared 1% glutaraldehyde in phosphate buffered saline (PBS) per well for 20 min, and stained with 0.2 to 0.3 ml of 0.1% Coomassie Brilliant Blue R-250 to visualize the neurite outgrowth. The extent of neurite outgrowth was evaluated by phase-contrast microscopy and quantified by counting the numbers of explants having a neurite which was more than one somal diameter long (50–200 μm). At least 150 explants/well were counted and percentage of explants having neurites matching with the above criteria was calculated. The assay was performed with more than 3 wells per each experimental group and the control for statistical analysis. Both stereoisomers (D-*threo*- and L-*threo*-,

PDMP) were synthesized as described previously (Inokuchi & Radin, 1987) and dissolved in H_2O to make a 4 mM stock solution. The stock solutions were diluted with the culture medium and applied at the appropriate concentrations.

Primary culture of rat cerebral cortical cells. Primary cultures of dissociated cerebral cortical neurons from 18-day fetal rats (Wistar) were prepared as described by Muramoto *et al.* (1988). In brief, after dissociation of brain tissue with papain (Worthington Biochemical Co.), cells were mechanically dissociated and plated on 5% polyethyleneimine (Sigma) coated coverslips. The cultures were maintained in a 7% CO_2 atmosphere at 37°C. The culture medium was Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 5% new born calf serum (Nakashibetsu Serum Center), 5% heat-inactivated horse serum (Gibco) and 1 mM sodium pyruvate (Sigma), 50 units/ml penicillin G (Meiji Seika Co.), and 0.025 mg/ml streptomycin sulfate (Meiji Seika Co.) and 1.2 mg/ml $NaHCO_3$.

Assay for functional synapse formation. Functional synapse formation was observed by means of optical monitoring of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of cultured neurons loaded with the fluorescent Ca^{2+} indicator, fura-2 (Muramoto *et al.*, 1988). Optical monitoring of $[Ca^{2+}]_i$ was carried out following the method of Ogura *et al.* (1987), using video-assisted multi-site fluorometry equipment (Kudo & Ogura, 1986).

Mitogen activated protein kinase activity. Immunoprecipitation of MAPK-cultured cells were rinsed with ice-cold phosphate buffered saline (PBS) and immediately scraped into homogenization buffer (25 mM Hepes, pH 7.4, 25 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate, 25 mM *p*-nitrophenylphosphate, 25 mM β-glycerophosphate, 1 mM EGTA, 2 mM dithiothreitol, 10 mM NaF, 0.1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cells were homogenized using a Potter-Elvehjem homogenizer and centrifuged to remove insoluble cell components. Protein concentration in the supernatants was measured by the method of Smith *et al.* (1985) using bovine serum albumin as a standard. The cell extracts were adjusted to equal protein concentrations (1 mg/ml) and incubated with anti-MAPK antibody that specifically recognizes p42 MAPK (Santa Cruz Biotechnology), for 2 h at 4°C. Protein A-Sepharose (Pharmacia) was added to each sample, and incubation was continued for an additional 1 h. Immune complexes were washed three times with cold homogenization buffer and once with PBS. SDS-/PAGE buffer was added to the immune complexes and heated at 100°C for 5 min. The supernatants were removed and subjected to the in-gel kinase assay and Western blot analysis to measure the amount of immunoprecipitated MAPK.

In-gel kinase assay. MAPK activity was measured using the in-gel kinase assay method. Immunoprecipitated MAPK was run on 10% SDS/polyacrylamide gels containing 0.5 mg/ml myelin basic protein as a substrate, and renatured according to the method of Kameshita & Fujisawa (1989). Phosphorylation was performed as described by Gotoh *et al.* (1990). Incorporation of ^{32}P was measured using the BAS 2000 system.

Western blotting. Cell extracts or immunoprecipitated MAPK was separated on 7.5% SDS/polyacrylamide gels, transferred electrophoretically to Immobilon-P (Millipore), and blocked in PBS containing 0.1% Tween-20 and 5% skim milk. Blots were incubated with an anti-ERK1 antibody that recognizes both p44 and p42 MAPK (Transduction Laboratories). The anti-ERK1 antibody was detected using the Phototope-Star Western blot detection kit (BioLabs) according to the protocol provided by the manufacturer. Chemiluminescence image on X-ray film was analysed by Flying-spot scanner CS9000 (Shimadzu).

Eight-arm radial maze task. Male Wistar rats (200–250 g), supplied by Kyu-Do, Japan, were placed on a platform in the center of an

8-arm radial maze. Each rat visited each arm and ate the food pellet at the end of each arm. They learned not to re-enter an arm that they had previously depleted of its pellet. Out of eight trials, the fully trained rats made less than 1 error, on the average. They utilized the spatial relations between their own location and the various objects in the background environment and used their natural ability (a win-shift search performance). A video image motion analyzer system was used to quantify the task performance (Iwasaki *et al.*, 1996). Behavioral observation was discontinued after 10 min even if the animal did not finish the task. After training to criterion and a 24 h rest period, 4-vessel occlusion was performed period (Pulsinelli & Brierley, 1979) for a 10 min period. An hour later, the occlusion was repeated. Rats failing to demonstrate the loss of righting reflex were excluded from further study. Rats which only underwent cauterization of the vertebral arteries were used as sham-operated controls. The PDMP was dissolved in 5% Tween 80 in saline at a concentration of 20 mg/ml and injected i.p. twice a day for 6 days. The dosage was chosen on the basis of a study with labeled D-PDMP (Shukla & Radin, 1991) which showed that a single injection of 40 mg/kg could be expected to yield an initial brain concentration of about 25 μM; radioactivity could be detected for < 8 h.

RESULTS

Effects of L- and D-PDMP on the neurite extension in neocortical explants in serum free medium

When explants from embryonic neocortical neurons were cultured with L-PDMP for 2 days in serum-free medium, marked increases in dense neurite outgrowth and the numbers of neurite branches were observed. On the other hand, treatment with D-PDMP resulted in an inhibition of neurite outgrowth. A typical microphotograph is presented in Fig. 2A. The effects of the two isomers on the extent of neurite outgrowth were quantified by counting the numbers of explants having a neurite more than one somal diameter long (Fig. 2B). D-PDMP showed a dose-dependent inhibitory effect from 5 to 20 μM. On the other hand, L-PDMP enhanced the neurite outgrowth over the same concentration range and the maximal stimulatory activity was obtained at 15 μM (34% *vs.* 18% in the control, $P < 0.05$). At 20 μM L-PDMP, the stimulatory effect was marginal (25% *vs.* 18% in the control). The treatment of explants with either PDMP isomer at higher concentrations caused detachment of explants from the culture plates probably due to a detergent-like non specific effect.

Facilitation of functional synapse formation and ganglioside synthesis

L-PDMP at 20 μM and 40 μM in primary culture of cortical neurons increased the frequency of spontaneous synchronous oscillations between the neurons on day 9 of *in vitro* culture (DIV), a 50% increase being achieved compared to controls with no L-PDMP added (Fig. 3A). D-PDMP, which blocks GSL biosynthesis, showed the opposite effect as reported by Mizutani *et al.* (1996). The time course of the stimulatory effect of 20 μM L-PDMP indicated that a prolonged exposure to L-PDMP for at least 8 days is required to achieve this effect (Fig. 3B).

The change in *de novo* synthesis of gangliosides produced by this treatment was analyzed by metabolic labeling of GSLs with [^{14}C]galactose for 6 h before each harvest (Fig. 4A). The slow but clear elevation of *de novo* synthesis of GSLs became clearly evident by 6 days of exposure to L-PDMP. Metabolic labeling of gangliosides in cortical neurons cultured with 20 μM L-PDMP for 8 days pointed to an elevation in the activity of biosynthetic pathways leading to the gangliosides, particularly G_{M3}, G_{D3} and G_{Q1b} (Fig. 4B). The elevation of ganglioside synthesis

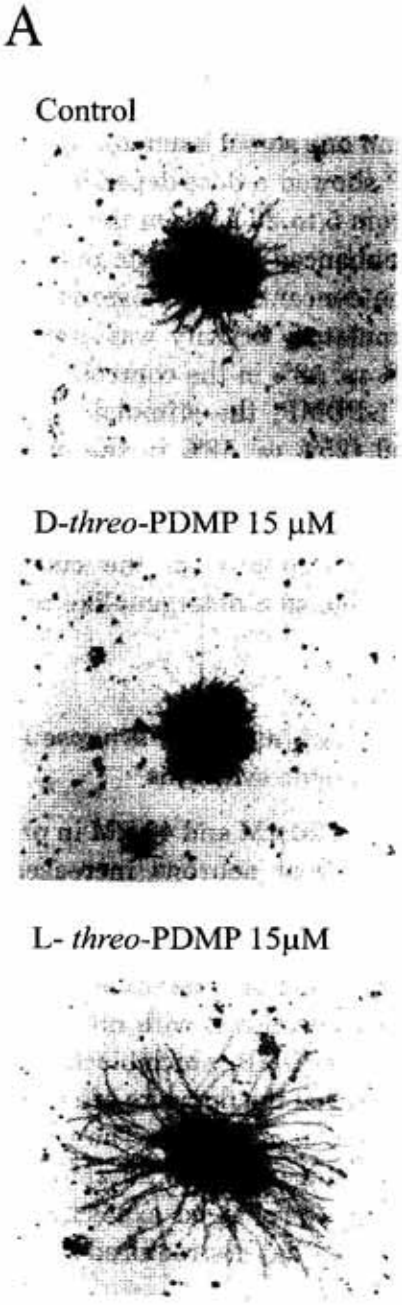

Figure 2. Effects of PDMP isomers on neurite extension of rat neocortical explants.

(A) The explants were incubated for two days without (control) or with 15 μM D-PDMP or 15 μM L-PDMP in the absence of serum. The procedure is given in Materials and Methods. (B) The explants having neurites greater than one somal diameter were counted, and the results expressed as their ratio to the total number of explants. The data are means of more than triplicate determinations. Statistical significance between the treated *versus* the control is indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$. —— Bar = 100 μm.

was confirmed to be due, in part at least, to increased activity of three ganglioside synthases, as assayed with lysates prepared from the cells pretreated with L-PDMP as described above. G_{M3} synthase was increased by 100%, G_{D3} by 200%, and G_{Q1b} synthase by 340% (Fig. 4C). Analysis of the time course of G_{Q1b} enzymatic synthesis showed that treatment with 20 μM L-PDMP had no effect by 10 h but by 2 days the activity had already risen about 265% over the control activity (Fig. 4D). This delayed type of activation was also observed in our previous experiments (Inokuchi *et al.*, 1989; 1990; 1995; and Usuki *et al.*, 1996). In other assay conditions in which cell lysates prepared from non-pretreated cortical cells were treated with 20 μM L-PDMP during the enzyme assays, none of the above enzyme activities was affected (not shown) and similar results were also obtained previously (Inokuchi *et al.*, 1989; 1990; 1995). It has been also demonstrated that, when intracellular distribution of the fluorescent analog of PDMP was examined, the fluorescence could

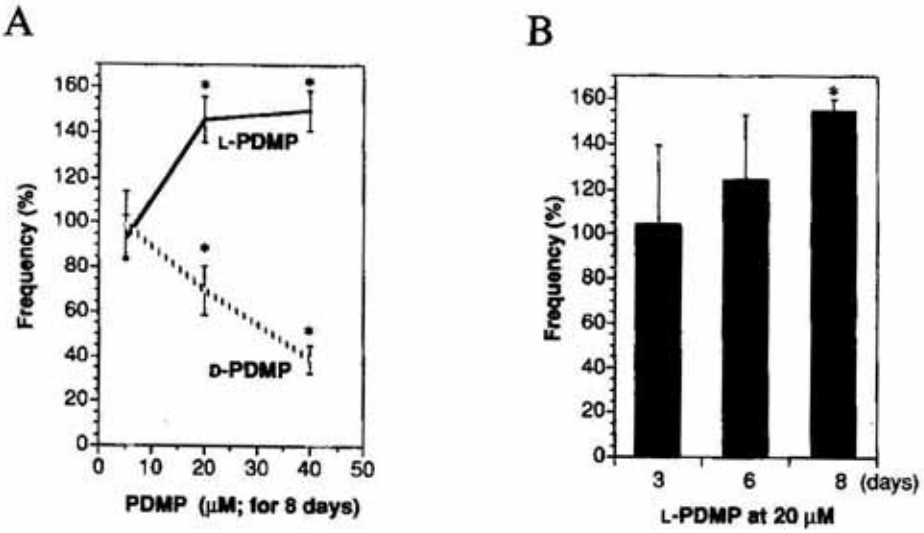

Figure 3. (A) The dose-dependent effects of PDMP isomers on the frequencies of synchronous oscillations of the cortical neurons. (B) Time-dependence of the stimulatory effect of L-PDMP on functional synapse formation.

In the cells were treated with PDMP isomers at the concentrations of 5 μM and 40 μM for 8 days (1–9 DIV). In (B) the cells were treated with 20 μM L-PDMP for 1 (8–9 DIV), 3 (6–9 DIV), 6 (3–9 DIV) and 8 days (1–9 DIV). Data are mean ±S.D. values of more than triplicate determinations. $^*P < 0.01$, significant difference from the values in the absence of PDMP at each point.

be detected at Golgi apparatus within 30 min after the exogenous addition of this analog (Rosenwald *et al.*, 1992). These results suggest that L-PDMP does not activate the transferase directly but through interaction with some other factors.

Up-regulation of p42 MAPK activity

Activation of p42 mitogen-activated protein kinase (MAPK) was found to occur in response to glutamate agonist stimulation (Bading & Greenberg, 1991; English & Sweatt, 1996) and was correlated with the spontaneous synaptic activity in cortical neurons (Fiore *et al.*, 1993). Since the cortical cell culture used in this study forms glutamatergic synapses (Robinson *et al.*, 1993), we measured the content and activity of p42 MAPK in the cortical cells treated with or without 20 μM L-PDMP. As shown in Fig. 5, L-PDMP had almost no effect on the content of p42 MAPK, however, this kinase activity was elevated in a slow but long lasting manner. L-PDMP did not affect the MAPK activity in a short-term experiment (1–60 min) in the absence of serum (not shown). These results suggest that the activation profile of p42 MAPK by L-PDMP could be correlated with both increased ganglioside biosynthesis and synaptic activity.

Improvement of the spatial memory deficit in ischemic rats

In the course of trials to evaluate the efficacy of L-PDMP on memory *in vivo*, we have tested the effect of the two PDMP isomers on the deficit of previously acquired spatial memory after transient forebrain ischemia in rats. The experimental protocol is shown in Fig. 6A. Rats treated with 40 mg/kg of L-PDMP showed a significant recall of the acquired spatial memory (Fig. 6B). By contrast, D-PDMP did not help the recall and, possibly, even produced more errors than in the controls (Fig. 6B). Neither of the two PDMP isomers had any effect on heart rates, blood pressure, or body temperature (not shown).

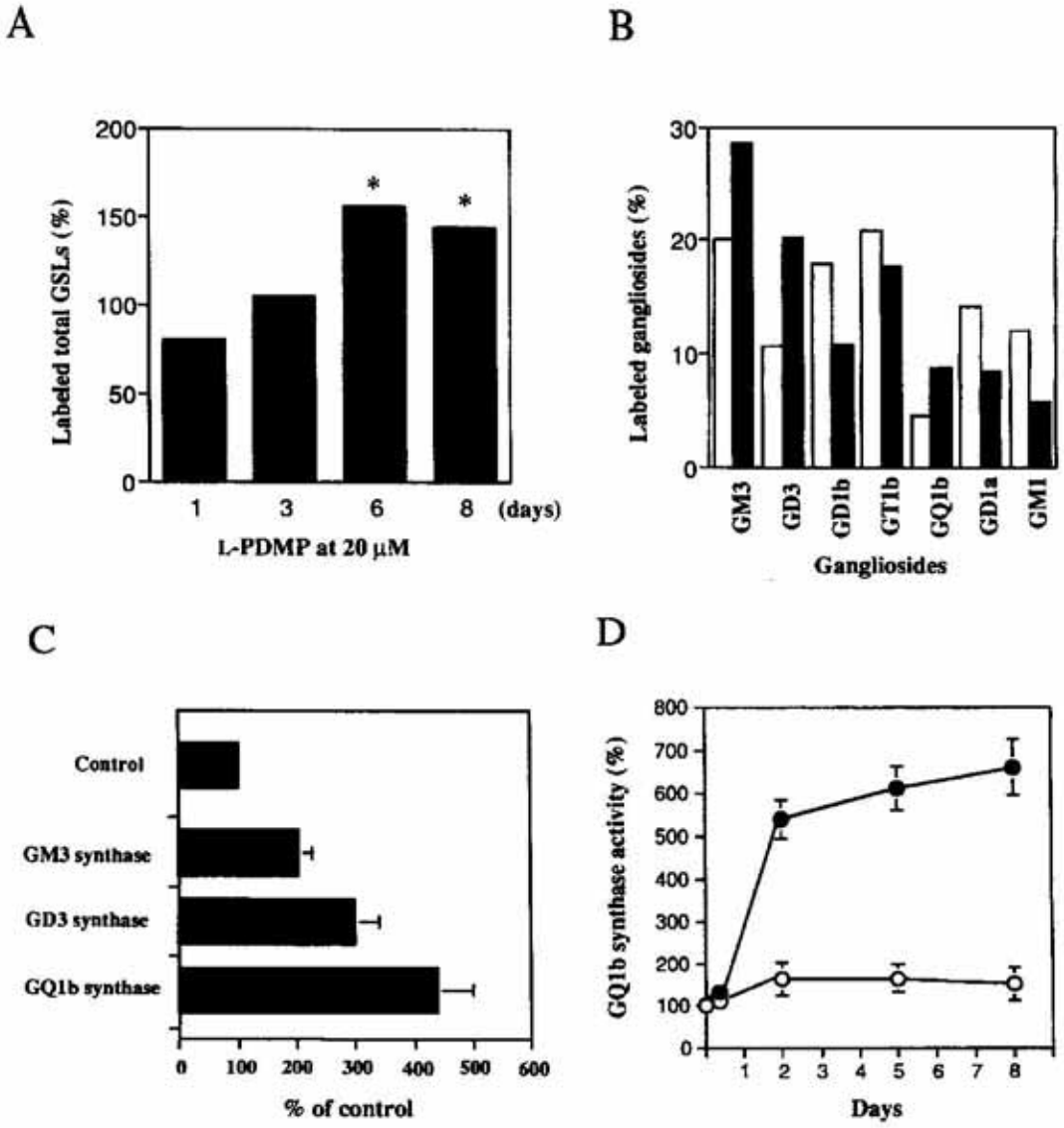

Figure 4. Stimulation by L-PDMP of biosynthesis of gangliosides.

(A) Effect of L-PDMP on *de novo* synthesis of total GSLs. The cortical cells were treated with L-PDMP for different intervals as in Fig. 3B. These cells were metabolically labeled with [^{14}C]galactose for 6 h before harvest and total labeled GSLs per mg protein were counted after purification as described previously (Usuki *et al.*, 1996). The data are means of triplicate determinations. $^{*}P < 0.01$, significant difference from the control value at day 0. (B) Selective acceleration of *de novo* synthesis of gangliosides, G_{M3}, G_{D3} and G_{Q1b} by L-PDMP. The cells were treated with or without 20 μM L-PDMP for 8 days (1–9 DIV) and metabolically labeled as in Fig. 4A. Equal amounts of radioactivities of total GSLs (12000 d.p.m.) in control (open bars) and the L-PDMP treated cells (closed bars) were separated by silica gel HPTLC using chloroform/methanol/0.22% $CaCl_2$ (60:35:8, by vol.). The radioactivity of each ganglioside species was detected with the BAS 2000 imaging analyzer. Data are mean values of duplicate determinations. Reproducible results were obtained in three independent experiments and a representative result is shown here. (C) Activation of ganglioside synthases in intact cortical cells by L-PDMP. The cells were treated with or without 20 μM L-PDMP for 8 days (1–9 DIV). G_{M3} synthase and G_{D3} synthase were assayed as described previously (Usuki *et al.*, 1996). G_{Q1b} synthase assay was performed similarly except that G_{T1b} (300 nmol) was used as a substrate. The specific activities of G_{M3}, G_{D3} and G_{Q1b} synthases were 2.1 ± 0.4, 3.5 ± 1.1 and 1.4 ± 0.28 nmol/h per mg protein as the control levels, respectively. Data are mean ± S.D. values of triplicate determinations. (D) The activation profile of G_{Q1b} synthase by L-PDMP. The cells were cultured with or without 20 μM L-PDMP for 1 (1–2 DIV), 2 (1–3 DIV), 5 (1–6 DIV) and 8 days (1–9 DIV), and then the G_{Q1b} synthase activities of the cell lysates of each culture were measured. Data are mean ± S.D. values of triplicate determinations.

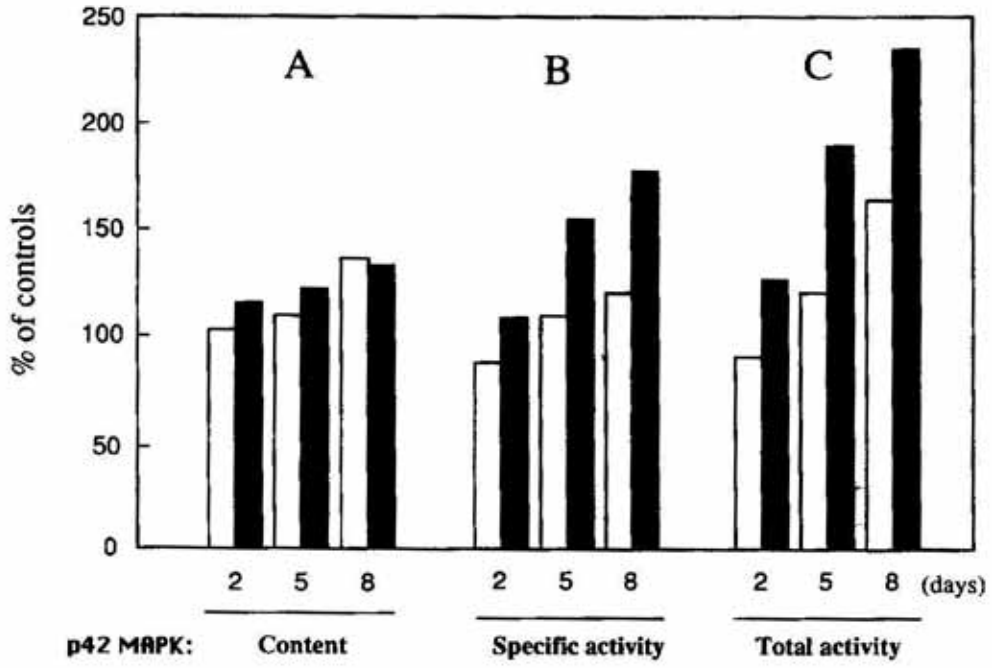

Figure 5. Effect of L-PDMP on the content and activity of p42 MAPK in the cortical cells.

Cells were treated in the absence (open bars) or presence (closed bars) of 20 μM L-PDMP for 2 (1–3 DIV), 5 (1–6 DIV) and 8 (1–9 DIV) days. (A) p42 MAPK content of the cell extract was measured by Western blotting as described in Methods. (B) Specific activity of p42 MAPK is shown. Immunoprecipitated MAPK activity was measured using the in-gel kinase assay and normalized for MAPK content measured by Western blotting as described in Methods. (C) To compare the total activity of p42 MAPK in the cortical cells, the specific activity was multiplied by MAPK content. Data are expressed as a mean percentage (duplicate) of MAPK content or activity of the cells taking each control value at 1 DIV as 100% (immediately before the addition of L-PDMP). Reproducible results were obtained in three independent experiments and representative data is shown.

DISCUSSION

It has been clearly shown that the biosynthesis of polysialogangliosides and their expression are correlated with differentiation and synaptogenesis of neuronal cells (Ledeen, 1985; Rösner *et al.*, 1992). We demonstrated that a synthetic ceramide analog, L-PDMP, upregulates the biosynthesis of b-series gangliosides in a long-term primary culture of cortical neurons by activating G_{M3}, G_{D3} and G_{Q1b} synthases. Under the same culture conditions, L-PDMP facilitates synchronous oscillatory activity between the neurons (formation of functional synapses) in a long lasting manner. The stimulatory effect of L-PDMP on functional synapse formation and on ganglioside synthase activities could be demonstrated even in the presence of serum, suggesting that the neurotrophic action of L-PDMP does not only mimick the actions of neurotrophic factors in serum (Fig. 3 and Fig. 4).

Recently the importance of endogenous gangliosides in neuronal functions was directly evidenced by the observation that transfection of the G_{D3} synthase gene into neuroblastoma cells induced the expression of b-series gangliosides, including G_{D3} and G_{Q1b}, and also caused differentiation into cholinergic neuron-like cells (Kojima *et al.*, 1994). Nagai & Tsuji (1994) and Nagai (1995) originally demonstrated a highly specific neuritogenic effect of exogenous G_{Q1b} on human neuroblastoma cells. Interestingly, they observed the remarkable neuritogenic effect of G_{Q1b} only in the G_{Q1b}-deficient neuroblastoma cells (not in the G_{Q1b}-expressing cell lines). Their results coincided with our previous observation that the

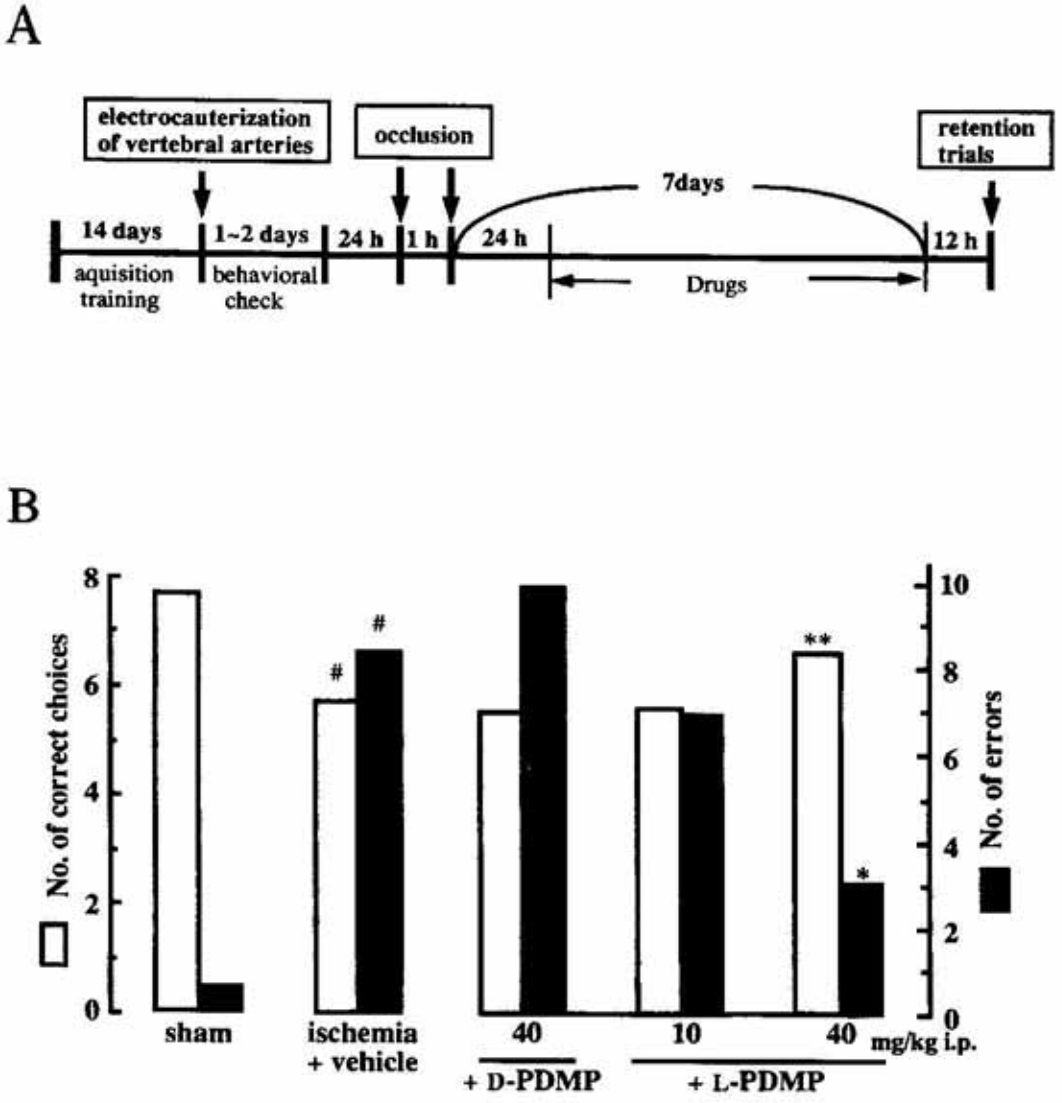

Figure 6. Effects of D- and L-PDMP on spatial memory.

(A) Diagrammed experimental protocol. For details see Materials and Methods. (B) Improvement of the memory deficit by L-PDMP. The data for the sham-operated rats (n = 10) are shown in the left side of the graph. The D-PDMP group (dose 40 mg/kg, n = 5) and the L-PDMP group (10 mg/kg, n = 8) show a relatively large number of errors, like the controls. The L-PDMP group (40 mg/kg, n = 13) shows significantly fewer errors and a higher number of correct choices. The data was analyzed by a two-way analysis of variance (with repeated measures, 2-way ANOVA), followed by Tukey's test for the differences among the groups when overall significance was observed in the 2-way ANOVA analysis. * $P < 0.05$ and ** $P < 0.01$ as compared with ischemic controls; # $P < 0.001$ as compared with sham-operated rats.

effect of exogenous G_{Q1b} on functional synapse formation could be observed only in the cortical neurons depleted of endogenous gangliosides by D-PDMP (not in the intact cells), suggesting that the pre-existing endogenous G_{Q1b} might be actually involved in neuronal functions (Mizutani *et al.*, 1996). On the other hand, the ability of gangliosides, especially G_{M1}, to exert trophic effects on neurons when applied as exogenous agents has been demonstrated with many *in vitro* and *in vivo* systems. However, exogenous G_{M1} did not facilitate functional synapse formation in our culture system (Mizutani *et al.*, 1996). Thus, exogenous gangliosides and L-PDMP differ at least in their mode of action. To sum up, it is

strongly suggested that induction of *de novo* synthesis in neurons of b-series gangliosides, particularly G_{Q1b}, can activate the neuronal functions.

Synchronization among neuronal responses occurs within cortical regions as diverse as the visual (Gray *et al.*, 1989), sensorimotor (Murthy & Fetz, 1992) and prefrontal cortex (Vaadia *et al.*, 1995). The strength of synchronous coupling between these widely spaced cortical neurons changes dynamically during task performance (Bressler *et al.*, 1993). Thus, such synaptic plasticity of cortical neurons could be a part of the mechanism in cortical memory processing (Fukai, 1994). Kuroda (1989) proposed that the plasticity of synaptic contacts in human association cortex constitutes the cellular mechanism of long-term memory in a "tracing circuit" model. Accordingly, the ability of L-PDMP to induce long lasting up-regulation of synchronous oscillatory activity in cultured cortical neurons could be expected to have a beneficial effect on learning and memory *in vivo*. We have tested the effect of L- and D-PDMP on the retention of previously acquired spatial memory after transient forebrain ischemia, since it has been reported that a well-learned spatial memory of a maze task in rats is stored mainly in the cortex area (McNaughton *et al.*, 1986; Okada *et al.*, 1996). It is notable that L-PDMP (40 mg/kg), which was administered i.p. as late as 24 h after the ischemic episode, could stereospecifically ameliorate the memory deficit in good agreement with our *in vitro* data reported here. A series of *in vivo* experiments is now under way to characterize the effects of L-PDMP in brain, including the changes in gangliosides, glutamate, monoamines, acetylcholine, neurotrophins and histology in normal and ischemic rats. Preliminary data has indicated that the i.p. administration of L-PDMP could enhance biosynthesis of brain gangliosides (unpublished observation).

Several lines of evidence point to the involvement of p42 MAPK in synaptic transmission. First of all, p42 MAPK and its substrates were localized at postsynaptic density (Suzuki *et al.*, 1995). Second, p42 MAPK but not p44 MAPK was activated in response to synaptic stimuli, such as glutamate (Bading & Greenberg, 1991), *N*-Methyl-D-aspartate and electroconvulsive stimulation inducing long-term potentiation (English & Sweatt, 1996). In addition, it has also been reported that p42 MAPK activity was increased simultaneously with up-regulation of endogenous (spontaneous) synaptic activity in cultured cortical neurons (Fiore *et al.*, 1993). Considering these observations, we investigated the content and activity of p42 MAPK in cortical culture forming glutamatergic synapses. The slow but long lasting facilitation of the synaptic activity by L-PDMP (Fig. 3A) was paralleled by the activation profile of p42 MAPK (Fig. 5). Thus, the slow activation of MAPK by L-PDMP may reflect multiple intermediate steps. Alternatively, the delayed response may reflect indirect activation of p42 MAPK by release of neurotrophins or other factors. At present, the cellular and molecular bases underlying the complex process of ganglioside biosynthesis and regulation are still being elucidated. Therefore, investigation of the possible interaction between ganglioside biosynthesis and signal transduction system including p42 MAPK in relation to their stimulation by of L-PDMP is a matter of importance.

Kuroda *et al.* (1992), using cultured cortical neurons, disclosed the importance of ectokinase in synapse formation. Furthermore, one of the substrates could be identified as the microtubule-associated protein (MAP) 1B, which may be phosphorylated when ATP is released into the synaptic cleft in an activity-dependent manner during synaptogenesis (Muramoto *et al.*, 1994). Therefore, we should at present answer the questions 1) whether or not the phosphorylation of extracellular domains of MAP1B or related proteins is correlated with the expression of G_{Q1b} on synaptic membranes, 2) the presence of a novel G_{Q1b}-dependent ectokinase, and 3) the following signal transduction system with possible in-

volvement of p42 MAPK activation. Studies along this line will be aided by the use of inhibitor and stimulator of ganglioside biosynthesis, D-PDMP and L-PDMP, respectively.

In conclusion, we have successfully demonstrated the usefulness of a new approach for treatment of memory deficit by up-regulating *de novo* synthesis of gangliosides and synaptic function by applying the synthetic ceramide analog. The results presented here open up the possibility of a new therapeutic approach for neurodegenerative disorders.

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