Impairment of hippocampal long-term potentiation and failure of learning in mice treated with \(d\)-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol

Hiroki Fujiwara\(^1\), Kotaro Ikarashi\(^1\), Yoshihiko Yamazaki\(^1\), Jun-Ichi Goto\(^1\), Kenya Kaneko\(^1\), Makoto Sugita\(^1\), Hiroshi Kato\(^1\), Hiroshi Sasaki\(^2\), Jin-ichi Inokuchi\(^3\), Koichi Furukawa\(^4\), and Satoshi Fujii\(^1\)

\(^1\)Department of Physiology, Yamagata University School of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan; \(^2\)Department of Software Science, Tamagawa University College of Engineering, Machida 194-8610, Japan; \(^3\)Division of Glycopathology, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan; and \(^4\)Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan

(Received 9 July 2012; and accepted 24 July 2012)

ABSTRACT

Gangliosides (sialic acid-containing glycosphingolipids) play important roles in many physiological functions, including synaptic plasticity in the hippocampus, which has been suggested as the basal cellular process of learning and memory in the brain. In the present study, long-term potentiation (LTP) and long-term depression (LTD) in CA1 hippocampal neurons and learning behavior were examined in mice treated with \(d\)-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (\(d\)-PDMP), an inhibitor of ganglioside biosynthesis. Mice treated with \(d\)-PDMP, but not those treated with \(l\)-PDMP, showed impairment of LTP induction in hippocampal CA1 neurons without any significant change in LTD formation and also showed a failure of learning in the 4-pellet taking test. These results indicate that de novo synthesis of gangliosides in the brain is involved in synaptic plasticity of LTP in mouse hippocampal CA1 neurons and plays important roles in learning and memory.

In rodent CA1 hippocampal neurons, long-term potentiation (LTP) and long-term depression (LTD) are two types of synaptic plasticity, considered to be the cellular basis of learning and memory in the brain (6, 21). LTP is a state of persistent synaptic enhancement induced by a brief period of high frequency electrical stimulation (HFS) of afferents (4, 5), while LTD is another activity-dependent synaptic phenomenon in which low-frequency afferent stimulation (LFS) depresses a synaptic response in a naive pathway (8, 19).

Gangliosides (sialic acid-containing glycosphingolipids) are abundant in the plasma membrane, especially in the termini and synapses of neural tissue (1). Ganglioside biosynthesis occurs by sequential glycosylation reactions via two major pathways designated the “a-pathway” (GM2, GM1a, and GD1a) and “b-pathway” (GD3, GD2, GD1b, GT1b, and GQ1b), with a common precursor, GM3. The analogous steps in the two pathways are catalyzed by the same glycosyltransferases (13, 17).

We previously studied the effects of GM1 and GQ1b on induction of LTP in CA1 neurons in rat hippocampal slices and showed that LTP is enhanced by bath application of either ganglioside, with GQ1b having a significantly greater effect than GM1 (12). In our recent study (14), we investigated LTP and LTD in the field excitatory post-synaptic potential (EPSP) in CA1 hippocampal neurons and learning behavior in \(\beta_1,4\)-N-acetylgalactosaminyltransferase (\(\beta_1,4\) GalNAc-T; GM2/GD2 synthase) gene transgenic (TG) mice (10), which showed a significant decrease in levels of b-pathway gangliosides (GQ1b, GT1b, and GD1b) and a significant increase in lev-
els of a-pathway gangliosides (GT1a, GD1a, and GM1) in the brain and isolated hippocampus compared to wild-type (WT) mice. This previous study demonstrated attenuation of LTP induction in the hippocampal CA1 neurons of TG mice, with no significant difference in LTD formation, and also showed impairment of learning in the 4-pellet taking test (4PTT) in TG mice, with no significant difference in daily activity or activity during the 4PTT between TG and WT mice.

In cultured cortical neurons, the biosynthesis of b-pathway gangliosides, including GG1b, can be inhibited by the ceramide analog d-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propranol (d-PDMP) or stimulated by its l-enantiomer, l-PDMP (22, 25). Inokuchi et al. (15) reported that prolonged treatment of rats with the two PDMP isomers had opposite effects on functional synapse formation measured by spontaneous synchronized oscillatory activity of intracellular Ca\(^{2+}\) between the neurons, suggesting that upregulation of synaptic activity by l-PDMP was correlated with stimulation of ganglioside biosynthesis by activation of b-pathway ganglioside synthases. In addition, intraperitoneal injection of l-PDMP twice a day for 6 days after induction of forebrain ischemia stimulates ganglioside biosynthesis in the rat brain and ameliorates spatial cognition deficits (26).

In the present study, we examined LTP and LTD in the field EPSP in CA1 hippocampal neurons and learning in the 4PTT in mice treated with d-PDMP or l-PDMP.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice aged 12- to 22-weeks-old with body weights of 20.6–30.0 g were used for the electrophysiology studies, in which the age of the mouse did not affect the results (data not shown). The behavioral tests were performed on mice that were 12- to 15-weeks-old when the 4PTT trials were started. The mice were housed singly with free access to food and water and maintained on a natural light/dark cycle for more than 3 days before experiments. The animal experiments adhered to the Yamagata University Guidelines for Animal Experimentation.

PDMP treatment. The ceramide analog d-PDMP or its enantiomer, l-PDMP, was dissolved in 5% Tween 80 in saline and injected intraperitoneally twice a day for 6 days at a dose of 40 mg/kg as described previously (15). Control mice were injected with 5% Tween 80 in saline (vehicle).

Electrophysiology. Hippocampal slice experiments were carried out one day after the end of treatment with d-PDMP, l-PDMP, or Tween 80 (control) after observation of behavior for at least 3 days. The mice were anesthetized with diethyl ether, the hippocampus was dissected from the right hemisphere, and transverse slices (500 μm thickness) were prepared using a rotor slicer (Dosaka DK-7700, Kyoto, Japan). After incubation for more than one hour in artificial cerebrospinal fluid (aCSF) consisting of (in mM) 124 NaCl, 5.0 KCl, 2.5 CaCl\(_2\), 1.3 MgSO\(_4\), 1.25 NaH\(_2\)PO\(_4\), and 10 glucose, aerated with a gas mixture of 95% O\(_2\) and 5% CO\(_2\) and maintained at 30 ± 1°C, the test slice was transferred to a recording chamber, where it was continuously perfused with aCSF at 30 ± 1°C.

As shown in the upper panel of Fig. 1A, a bipolar tungsten stimulating electrode (Stim) was placed in the stratum radiatum in the CA1 region close to CA2 to stimulate the Schaffer collateral/commissural fibers. A recording electrode (Rec) was placed in the stratum radiatum in the CA1 region to measure the initial slope of the field EPSP (S-EPSP) (lower panel in Fig. 1A). At the beginning of each experiment, a stimulus-response curve was established by increasing the stimulus intensity and measuring the S-EPSP, then the strength of the stimulus pulse (50 μs duration) was adjusted to elicit an S-EPSP 40–60% of maximal and was fixed at this level.

After checking the stability of responses to a test stimulus given every 30 s, a train of HFS (100 Hz, 15 pulses) or LFS (1 Hz, 200 pulses) was delivered to induce, respectively, LTP or LTD. In the LTP experiments, the mean S-EPSP measured over the 10 min period at 50–60 min after tetanus was expressed as a percentage of the control value measured in the 10 min period immediately before HFS delivery. LTP in the S-EPSP was defined as a value greater than 120% of the baseline value. In the LTD experiments, three trains of LFS were applied successively at 20 min intervals and the mean magnitude of the S-EPSP during the 5 min period at 15–20 min after the end of each LFS measured.

Behavioral observations. The behavior of mice housed singly in a cage (35 cm wide, 30 cm long, and 18 cm high) was monitored and recorded for 4 mice simultaneously. The time spent removing food from a feeder box and the amount of food taken were recorded by monitoring the movement and weight of the feeder box suspended from the roof of the cage.
the entrance to each feeder box and a small feeder dish (4 cm diameter) was placed on the floor of the feeder box. One small round food pellet (9–10 mg) was placed in each of the feeder boxes and a packet of 4 pellets hidden beneath each feeder dish in a small space between the floor and the bottom plate of the feeder box in order to retain the odor of the pellets even after they had been removed by the mouse. The floor of the large box and feeder boxes was made from a thin punched stainless-steel sheet. The apparatus was placed on a desk in an dimly lit experimental room with background noise (fan) and a gentle breeze applied from 1 m above the 4PTT apparatus during the learning sessions.

Learning test using the 4PTT apparatus. After monitoring behavior for 3–10 days, the 4PTT learning test was started. On the day of the learning test, the feeder box was removed from the home cage at 09:00 and the experiment was started at 21:00–22:00, usually with 4 mice being tested sequentially. After the learning test, the mouse was returned to its home cage and the feeder box replaced, allowing the mouse access to food and water ad libitum until the next morning. Each mouse was subjected to one 4PTT trial a day for at least 8 consecutive days.

In each 4PTT trial, the mouse was transferred from its home cage and placed on the central stage of the 4PTT apparatus in an opaque box with no base (9 × 9 cm sides, 7 cm high) for 30 s before starting the experiment, then the time from the start of the experiment to the ingestion of the 4th pellet from feeders placed at the four corners was measured.

Statistical analysis. All values are given as the mean ± SEM. The LTP and LTD results were analyzed for statistical significance using the unpaired two-tailed Student’s t-test, while the 4PTT data were analyzed using repeated measure ANOVA with the group as the between-subject factor and the trial day as the within-subject factor. Post hoc analyses (Scheffe’s F test) were carried out when needed. A difference was considered statistically significant if $P < 0.05$. 

4-pellet taking test

Apparatus. The apparatus for the 4PTT, shown in Fig. 1B, was a large transparent acrylic box (31 cm wide, 31 cm long, and 26 cm high) with a stage (10 cm wide, 10 cm long, and 5 cm above the floor) at the center of the floor and four removable feeder boxes (5.5 × 5.5 cm entrance, 10 cm long) fixed outside the corners of the box. An opaque push-open door (5 cm wide and 4.5 cm high) was attached at

by means of a sensor attached to the feeder box and a specially developed computer program.
RESULTS

**LTP and LTD in PDMP-treated mice**

HFS (15 pulses at 100 Hz) induced LTP of the S-EPSP in the L-PDMP-treated mice and Tween 80-treated control mice, but not in the D-PDMP-treated mice; Fig. 2A shows sample wave forms and the summarized results. As shown in Fig. 2B, the mean magnitude of LTP of the S-EPSP in D-PDMP-treated mice or Tween 80-treated mice was, respectively, $116.1 \pm 4.2\%$ (n = 6) or $132.5 \pm 6.2\%$ (n = 7) of the pre-HFS control level, the former value being significantly smaller than the latter ($P < 0.05$), whereas the corresponding value of $157.4 \pm 15.1\%$ (n = 8) in L-PDMP-treated mice was not significantly different from that in the Tween 80-treated control mice. This result shows that induction of LTP is attenuated in mice treated with D-PDMP.

Three trains of LFS (200 pulses at 1 Hz) applied successively at 20 min intervals induced LTD of the S-EPSP in all three groups of mice; Fig. 3A shows sample wave forms and the summarized results. Fig. 3B shows the mean magnitude of LTD of the S-EPSP measured in the 5 min period at 15–20 min after the first, second, or third LFS train. The three respective LTD values were $88.5 \pm 6.3\%$, $90.9 \pm 4.0\%$, and $87.1 \pm 5.9\%$ of the pre-LFS control value in the L-PDMP-treated mice (n = 7), $89.8 \pm 2.3\%$, $86.7 \pm 6.0\%$, and $80.4 \pm 7.1\%$ in the D-PDMP-treated mice (n = 6), and $88.5 \pm 4.2\%$, $81.0 \pm 5.8\%$, and $78.8 \pm 7.9\%$ in the Tween 80-treated mice (n = 6). No significant differences were found in the LTD after the first, second, or third LFS train among the three groups (Fig. 3B), showing that LTD formation in hippocampal CA1 neurons is not affected by treatment with L-PDMP or D-PDMP.

**4-pellets taking test (4PTT)**

We then investigated whether treatment with D-PDMP or L-PDMP influenced learning behavior in the 4PTT. Fig. 4 shows the mean time from the start of the 4PTT until the taking of the 4th pellet for the Tween 80-treated control mice and the PDMP-treated mice. The mice in all three groups showed a significant decrease in the time needed to complete the 4PTT with increasing number of trial days ($F_{(7, 87)} = 5.9$; $P < 0.001$). Analysis of variance showed that there was a significant main effect of PDMP treatment ($F_{(2, 7)} = 19.3$, $P < 0.0001$) and a non-significant interaction of PDMP treatment and trial day ($F_{(14, 87)} = 0.68$, $P = 0.79$). Post hoc analysis revealed that mice treated with D-PDMP required a longer time for completion of the 4PTT than mice treated with Tween 80 or L-PDMP, the results for which did not differ from one another.

Activity was measured as the mean time spent accessing the feeder box between 00 : 05 : 50 in the home cage and as the mean number of times that the mouse crossed the lines between the 4 sectors of the floor in 1 min during the 4PTT. The former measurement is based on the observation that a mouse takes food frequently after midnight and the latter measures the activity of exploring the box during the 4PTT. However, no significant difference in activity between the three groups of mice was
seen in either the home cage or during the 4PTT (data not shown).

DISCUSSION
In the present study, we first examined the effects of gangliosides in vitro on neural plasticity in hippocampal CA1 neurons of mice treated with l-PDMP, d-PDMP, or vehicle. Examination of LTP or LTD in hippocampal CA1 neurons showed that the LTP induced by HFS was attenuated by d-PDMP treatment (Fig. 2), whereas the LTD produced by three trains of LFS was not affected (Fig. 3). Although the magnitude of LTP in l-PDMP-treated mice showed a tendency to increase, the differences between l-PDMP-treated mice and control mice did not reach statistical significance (Fig. 2). We next examined whether the attenuation of LTP induction observed in the d-PDMP-treated mice was accompanied by a change in learning behavior in the 4PTT. As shown in Fig. 4, a failure of learning in the 4PTT was observed in mice treated with d-PDMP.

Recently, we demonstrated attenuation of LTP induction in the field EPSP in CA1 hippocampal neurons and a failure of learning in the 4PTT in β1,4 GalNAc-T; GM2/GD2 synthase gene TG mice, which showed a marked decrease in levels of b-pathway gangliosides (GQ1b, GT1b, and GD1b) in the brain and isolated hippocampus compared to WT mice (14). Because de novo synthesis of b-pathway gangliosides is inhibited in d-PDMP-treated mice (26) and because bath application of GQ1b, one of the b-pathway gangliosides, is reported to increase LTP induced in hippocampal CA1 neurons (9, 12), we conclude that de novo synthesis of b-pathway gangliosides is involved in the synaptic plasticity of LTP in mouse hippocampal CA1 neurons and plays important roles in learning and memory in the 4PTT.

In hippocampal CA1 neurons, Ca^{2+} influx through
N-methyl-D-aspartate (NMDA) receptors/Ca\(^{2+}\) channels play a key role in the formation of both LTP (2, 6) and LTD (8, 19). In HFS-induced LTP at CA1 synapses, Ca\(^{2+}\) influx through NMDA receptors can stimulate the Ca\(^{2+}\)/calmodulin and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) signaling pathways and induce long-lasting enhancement of synaptic transmission mediated by postsynaptic \(\alpha\)-amino-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type glutamate receptors (6). The homosynaptic LTD induced in CA1 neurons by LFS at 1 Hz also appears to require NMDA receptor activation and an increase in the postsynaptic [Ca\(^{2+}\)]\(_d\) during 1 Hz LFS. This increase of postsynaptic [Ca\(^{2+}\)]\(_d\) activates calcineurin, which indirectly increases phosphatase 1 activity by dephosphorylating phosphatase 1 inhibitory proteins, allowing protein phosphatase 1 to act on CaMKII or AMPA receptor subunits (3, 18, 20, 24).

A possible explanation for the different effects of gangliosides on LTP induction and LTD formation might be that b-pathway gangliosides are more effective than a-pathway gangliosides in activating CaMKII in hippocampal neurons (11). In our previous studies, we suggested that it was possible that ganglioside-Ca\(^{2+}\) complexes may facilitate Ca\(^{2+}\) influx through NMDA receptors by acting as Ca\(^{2+}\) donors (9, 12). Other studies have suggested the possibility that gangliosides GD1b and GT1b activate CaMKII in hippocampal neurons (7, 11). In the present study, inhibition of \(\text{de novo}\) synthesis of b-pathway gangliosides in hippocampal neurons impaired LTP induction at CA1 synapses (Fig. 2), but did not affect LTD formation (Fig. 3). Since activation of CaMKII due to Ca\(^{2+}\) influx through NMDA receptors is involved in the mechanism of LTP in hippocampal CA1 neurons, but not in the mechanism of LTD, it is possible that inhibition of \(\text{de novo}\) synthesis of b-pathway gangliosides in \(\delta\)-PDMP-treated mice decreases CaMKII activity and attenuates LTP induction in hippocampal neurons, but does not affect LTD formation.

Jung et al. (16) reported that GQ1b-treated rats show a marked increase in memory performance in the Y maze test and Morris water maze test (23), suggesting a positive effect of GQ1b on LTP induction in hippocampal CA1 neurons (9, 12). In the present study, the \(\delta\)-PDMP-treated mice exhibited both impairment of LTP induction in hippocampal CA1 neurons (Fig. 2) and a failure of learning in 4PTT (Fig. 4), also suggesting effects of gangliosides, including GQ1b, on learning and memory in mice. Thus, the present results considerably strengthen the hypothesis that synaptic plasticity, especially LTP, is the cellular basis of learning and memory (6, 23).

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Biochem Biophys Res Commun 237, 595–600.


