Impaired hippocampal long-term potentiation and failure of learning in β1,4-N-acetylgalactosaminyltransferase gene transgenic mice

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Gangliosides (sialic acid-containing glycosphingolipids) play important roles in many physiological functions, including synaptic plasticity in the hippocampus, which is considered as a cellular mechanism of learning and memory. In the present study, three types of synaptic plasticity, long-term potentiation (LTP), long-term depression (LTD) and reversal of LTD (depotentiation, DP), in the field excitatory post-synaptic potential in CA1 hippocampal neurons and learning behavior were examined in β1,4-N-acetylgalactosaminyltransferase (β1,4 GalNAc-T; GM2/GD2 synthase) gene transgenic (TG) mice, which showed a marked decrease in b-pathway gangliosides (GQ1b, GT1b and GD1b) in the brain and isolated hippocampus compared with wild-type (WT) mice. The magnitude of the LTP induced by tetanus (100 pulses at 100 Hz) in TG mice was significantly smaller than that in control WT mice, whereas there was no difference in the magnitude of the LTD induced by three short trains of low-frequency stimulation (LFS) (200 pulses at 1 Hz) at 20 min intervals between the two groups of mice. The reduction in the LTP produced by delivering three trains of LFS (200 pulses at 1 Hz, 20 min intervals) was significantly greater in the TG mice than in the WT mice. Learning was impaired in the four-pellet taking test (4PTT) in TG mice, with no significant difference in daily activity or activity during the 4PTT between TG and WT mice. These results suggest that the overexpression of β1,4 GalNAc-T resulted in altered synaptic plasticity of LTP and DP in hippocampal CA1 neurons and learning in the 4PTT, and this is attributable to the shift from b-pathway gangliosides to a-pathway gangliosides.

Keywords: β1,4-N-acetylgalactosaminyltransferase / depotentiation / hippocampus / learning and memory / long-term potentiation

Introduction

Synaptic plasticity is considered to be the cellular basis of learning and memory in the brain (Bliss and Collingridge 1993). In rodent CA1 hippocampal neurons, three types of synaptic plasticity, long-term potentiation (LTP), long-term depression (LTD) and reversal of LTD (depotentiation, DP), have been investigated in detail (Bliss and Lømo 1973; Fujii et al. 1991; Dudek and Bear 1992). LTP is a state of persistent synaptic enhancement induced by a brief period of high-frequency electrical stimulation (HFS or tetanus) of afferents (Bliss and Gardner-Medwin 1973; Bliss and Collingridge 1993). LTD is also an activity-dependent synaptic phenomenon, in which low-frequency afferent stimulation (LFS) depresses the synaptic response in a naive pathway (Dudek and Bear 1992; Mulkey et al. 1994; Linden and Connor 1995). DP is another type of synaptic plasticity in which LFS reduces the potentiated response in a pathway in which LTP has been pre-established (Barrionuevo et al. 1980; Staubli and Lynch 1990; Bashir and Collingridge 1994).

Gangliosides (sialic acid-containing glycosphingolipids) are abundant in the plasma membrane, especially in the termini and synapses of neural tissue (Ando 1983). Ganglioside biosynthesis occurs by sequential glycosylation reactions via two major pathways, designated the “a-pathway” (GM2, GM1a and GD1a) and the “b-pathway” (GD3, GD2, GD1b, GT1b and GQ1b), with a common precursor, GM3, in which analogous steps in the two pathways are catalyzed by the same glycosyltransferases (Ledeen and Yu 1982; Hettmer et al. 2003). Several reports have suggested that gangliosides play many physiological roles in synaptic transmission in the nervous system (Romer and Rahmann 1979; Carpenter et al. 1988;
In CA1 neurons in rat hippocampal slices, Wieraszko and Seifert (1985) demonstrated that enhancement of LTP can be produced either by addition of the monosialoganglioside GM1 or by an increase in endogenous GM1 as a result of enzymatic treatment. Hwang et al. (1992) also showed that pre-incubation or by an increase in endogenous GM1 as a result of enzymatic treatment. Seifert (1985) demonstrated that enhancement of LTP can be induced by HFS in a medium with a normal Ca\(^{2+}\) concentration (2.5 mM) or a low (1.0 mM) or high (5.0 mM) Ca\(^{2+}\) concentration. In addition, Li and Mei (1994) reported that a larger LTP is induced in rat hippocampal CA1 neurons by incubating slices with GM1. In contrast to these positive modulatory effects of GM1 on LTP, Okada et al. (1994) reported that exogenously applied GM1 and/or GD1a fails to enhance LTP in hippocampal CA1 neurons. We have previously studied the effects of GM1 and GQ1b on the induction of LTP and showed that LTP is enhanced in the medium with a normal Ca\(^{2+}\) concentration (2.5 mM) by addition of either GM1 or GQ1b to the perfusion medium or by bath incubation of the slices with either GM1 or GQ1b in low (1.0–1.1 mM) Ca\(^{2+}\) medium (Furuse et al. 1998). In this previous study, we reported that GQ1b has a significantly stronger effect than GM1 on LTP induction. Thus, the role of each ganglioside in the induction of synaptic plasticity should be explored in more detail. \(\beta\)1,4-N-acetylgalactosaminyltransferase (\(\beta\)1,4 GalNAc-T) transgenic (TG) mice have been established, in which the conversion of GM3 to GM2 (a-pathway) is markedly increased and that of GD3 to GD2 (b-pathway) markedly decreased in the skin (Fukumoto et al. 1997). In the present study, we examined whether a similar shift from b-pathway to a-pathway occurred in the whole brain and isolated hippocampus. We then studied three types of synaptic plasticity, LTP, LTD and DP, in the hippocampal CA1 region using hippocampal slices from \(\beta\)1,4 GalNAc-T TG and wild-type (WT) mice to clarify the effects of gangliosides, especially those of GM1 and GQ1b, on the induction of synaptic plasticity. We also tested learning behavior in the four-pellet taking test (4PTT) in TG and WT mice to investigate whether the shift in expression of gangliosides resulted in changes in learning behavior.

**Results**

*Ganglioside patterns in the brain of TG and WT mice*

To examine the ganglioside patterns in the brain of WT and TG mice, we isolated gangliosides from the whole brain of 12-week-old male mice and analyzed them by thin-layer chromatography (TLC). As shown in Figure 1, in the two types of gangliosides, GM1 and GD1a levels were increased and GD1b, GT1b and GQ1b levels decreased compared with the WT sample. We also examined the amount of total ganglioside in, and the ganglioside composition of, the isolated hippocampus. As summarized in Table I, a-pathway gangliosides were significantly increased and b-pathway gangliosides decreased in TG mice compared with WT mice, with no significant difference in the total amount of ganglioside. The weight of the hippocampus in 12-week-old male TG mice (20.7 ± 0.3 mg, \(n = 4\)) was also not significantly different from that in sex- and age-matched WT mice (21.6 ± 0.6 mg, \(n = 4\)). These results show that levels of b-pathway gangliosides, including GQ1b, are markedly decreased in the hippocampus of TG mice.

**LTP, LTD and DP in TG and WT mice**

**LTP in TG and WT mice.** At hippocampal CA1 neuron synapses, the mean value of the S-EPSP (slope of the excitatory post-synaptic potential) in response to test stimuli at an intensity eliciting the half-maximum value was \(-1.11 \pm 0.14 \text{mV/ ms (} n = 18\text{) in TG mice and} -1.14 \pm 0.15 \text{mV/ ms in WT mice (} n = 19\text{; data not shown). There was no significant difference in basic synaptic transmission between the TG and WT mice. As shown in Figure 2 (left panel), a tetanus of 100 pulses at 100 Hz induced LTP in hippocampal CA1 neurons in both types of mice. The mean magnitude of the short-term potentiation (STP) of the S-EPSP at 1–5 min after tetanus was 166.9 ± 12.3% (\(n = 7\)) of the pre-tetanus control level in TG mice and 222.6 ± 17.5% in WT mice (\(n = 8\)).**

**Table I. Altered ganglioside profile in the hippocampus of TG mice**

<table>
<thead>
<tr>
<th>Gangliosides (sialic acid µg/mg protein)</th>
<th>WT mice ((n = 6))</th>
<th>TG mice ((n = 6))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM1</td>
<td>6.13 ± 0.68</td>
<td>5.88 ± 0.30</td>
</tr>
<tr>
<td>GD1a</td>
<td>31.0 ± 2.5</td>
<td>58.7 ± 1.4*</td>
</tr>
<tr>
<td>GT1a</td>
<td>4.1 ± 0.2</td>
<td>11.0 ± 0.5*</td>
</tr>
<tr>
<td>GD1b</td>
<td>8.1 ± 0.3</td>
<td>2.7 ± 0.3*</td>
</tr>
<tr>
<td>GT1b</td>
<td>35.7 ± 1.2</td>
<td>10.9 ± 0.8*</td>
</tr>
<tr>
<td>GQ1b</td>
<td>11.7 ± 1.0</td>
<td>4.3 ± 0.6*</td>
</tr>
</tbody>
</table>

\(\ast P < 0.001\) compared with WT mice.
STP and LTP was significantly smaller in TG mice than in WT mice ($P<0.05$), indicating that induction of LTP at CA1 synapses was attenuated in TG mice.

**LTD in TG and WT mice.** Three trains of LFS (200 pulses at 1 Hz, 20 min intervals) induced LTD in hippocampal CA1 neurons in both TG ($n=11$) and WT ($n=10$) mice (Figure 3A). The mean magnitude of the LTD of the S-EPSP at 15–20 min after the end of the first LFS ("1st" in the right panel of Figure 3A) was 85.6 ± 5.7% of the pre-LFS control level in TG mice and 94.2 ± 4.3% in WT mice, whereas the corresponding values for the mean magnitude of the LTD of the S-EPSP after the second LFS ("2nd" in the right panel of Figure 3A) were 78.0 ± 4.9 and 87.4 ± 4.0% of the control level and those after the end of the third LFS ("3rd" in the right panel of Figure 3A) 74.4 ± 5.2 and 80.0 ± 5.6%. The magnitude of each LTD in TG mice tended to be larger than in WT mice, but the difference was not statistically significant (right panel in Figure 3A). Thus, we conclude that LTD formation in hippocampal CA1 neurons was not affected in TG mice.

**DP in TG and WT mice.** To produce DP, a train of LFS (200 pulses at 1 Hz) was given once at 30 min after tetanus, then a second and third time at 20 min intervals (Figure 3B, top left panel). As shown in the top right panel of Figure 3B, the mean magnitude of the LTP of the S-EPSP at 25–30 min after the tetanus (indicated as “T”) was 145.7 ± 8.5% of the pre-tetanus control level in TG mice ($n=6$), significantly smaller ($P<0.05$) than the value of 170.1 ± 5.8% of the pre-tetanus control level in WT mice ($n=6$). The first LFS train reduced the S-EPSP to 115.6 ± 7.2% of the pre-tetanus control level in TG mice and 150.3 ± 5.9% of the pre-tetanus control level in WT mice (indicated as “1st”), the corresponding values for the second LFS being 98.7 ± 9.2 and 140.0 ± 0.6% of the pre-tetanus control level (“2nd”) and those for the third train 92.8 ± 11.6 and 147.1 ± 12.7% of the pre-tetanus control level (“3rd”). The magnitude of the S-EPSP responses after the first, second and third LFS in TG mice was significantly ($P<0.01$) smaller than in WT mice (top right panel in Figure 3B). The reduction in the LTP of the S-EPSP measured after the third LFS in the TG or WT mice was, respectively, 133.2 ± 28.3 or 45.6 ± 13.4% of the averaged S-EPSP at 25–30 min after the end of the tetanus (bottom panel of Figure 3B, see Materials and methods). Thus, the magnitude of DP (the reduction in the LTP) of the S-EPSP measured after the third LFS in TG mice was significantly greater ($P<0.01$) than in WT mice, showing that LFS reduces LTP in hippocampal CA1 neurons and that the process is facilitated in TG mice.

**Four-pellet taking test**

We next examined whether the attenuation of LTP induction observed in TG mice resulted in a change in learning behavior in the 4PTT, performed as shown in Figure 4A. Figure 4B shows the mean time from the start of the 4PTT until all four pellets were taken for each of 10 successive days for TG mice (filled circles, $n=7$) or WT mice (open circles, $n=7$). On average, the WT mice took all four pellets within 4 min after the 7th trial, whereas TG mice failed to achieve the learning criterion of 4 min even after the 10th trial. The mean time for taking all four pellets was significantly longer in TG mice than in WT mice ($F_{1,12}=12.4$, $P<0.001$, two-way repeated-measures analysis of variance (ANOVA)). When a mouse succeeded in taking all four pellets in 4 min in three successive trials, the first day was defined as completion of learning in the 4PTT and the results were plotted as a cumulative curve. As shown in Figure 4C, using seven mice per group, learning was complete in WT mice on day 3 (one mouse) to day 8 (all seven mice), whereas it was complete in TG mice from day 2 (one mouse) to day 15 (six mice). One TG mouse failed to learn after more than 18 trials, the maximum number of trials. The day of establishment of learning was significantly increased in TG mice (Kolmogorov–Smirnov test, $P<0.05$). These results demonstrate that TG mice show a learning impairment in the 4PTT.

**Behavioral observations**

Figure 5A shows examples of the daily behavior of WT mice (left panel) and TG mice (right panel). Activity was measured as the mean time spent accessing the feeder box between 00:00 and 05:00 in the home cage (Figure 5B, left panel) and
the mean number of times the mouse crossed the lines between the four sectors of the floor in 1 min during the 4PTT (Figure 5B, right panel). 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. As shown in Figure 5B, there was no significant difference in activity between TG and WT mice in the home cage or during the 4PTT.

Discussion
In the present study, we demonstrated an increase in a-pathway gangliosides, including GM1, and a decrease in b-pathway gangliosides, including GQ1b, in the whole brain and isolated hippocampus of β1,4 GalNAc-T TG mice and examined the in vitro effects of genetic modification of ganglioside biosynthesis on neural plasticity in hippocampal CA1 neurons by comparing LTD, LTD and DP in β1,4 GalNAc-T TG and WT mice. We found that LTP induction was attenuated, DP was facilitated and LTD produced by three LFS unaffected in TG mice (Figures 2 and 3).

Positive effects of GM1 (Wieraszko and Seifert 1985; Hwang et al. 1992; Li and Mei 1994) and GQ1b (Furuse et al. 1998) on the induction of activity-dependent LTP in hippocampal CA1 neurons have been reported. More recently, She et al. (2005) demonstrated that GM1 reverses lead-induced
deficits in LTP in the hippocampal dentate gyrus area in vivo in rats. As the TG mice showed increased levels of a-pathway gangliosides, including GM1, and decreased levels of b-pathway gangliosides, including GQ1b (Figure 1 and Table I), and because both gangliosides are reported to have positive effects on LTP, the smaller LTP induction in the TG mice may be due to the decrease in b-pathway gangliosides. This interpretation is consistent with our previous observation that GQ1b has a stronger effect than GM1 on the induction of LTP (Furuse et al. 1998; Fujii, Igarashi, et al. 2000).

The attenuation of LTP induction and the facilitation of DP seen in the β1,4 GaINAc-T TG mice suggest that b-pathway gangliosides, including GQ1b, play an important role in enhancing synaptic potentiation in hippocampal CA1 neurons. One specific effect of GQ1b on synaptic activity has been demonstrated using cultured rat cerebral cortical neurons (Mizutani et al. 1996). In that study, the synchronous oscillatory activity between neurons, monitored using fura-2 calcium imaging, was suppressed after total ganglioside depletion of neurons using an inhibitor of glucosylceramide synthesis and, when GM3, GM1, GD3, GD1b or GQ1b was added back to the ganglioside-depleted cells, only GQ1b was able to normalize the reduced synaptic activity. These results are further evidence that GQ1b is essential for synaptic activity.

One explanation for the different effects of GQ1b and GM1 on activity-dependent LTP may be the manner in which Ca2+ is bound by these two molecules, as GM1 has only one negatively charged sialic acid group, while GQ1b has four, each of which can bind to positively charged molecules outside the cell, such as Ca2+ (Ando, 1983). In a previous study (Furuse et al. 1998), we measured the activity-dependent LTP of the population spikes in CA1 neurons when slices were incubated in the low Ca2+ (1.0–1.1 mM) medium containing either GM1 or GQ1b and found that the LTP in the treated slices was significantly increased compared with controls and that this effect was especially apparent in GQ1b-treated slices. Since, in hippocampal CA1 neurons, Ca2+ influx through N-methyl-d-aspartate (NMDA) receptors/Ca2+ channels plays a key role in the formation of LTP (Ascher and Nowak 1988; Bliss and Collingridge 1993; Lisman 1994), it is possible that, acting as Ca2+ donors, GQ1b–Ca2+ complexes may provide more Ca2+ release and/or Ca2+ influx through NMDA receptors than GM1–Ca2+ complexes, resulting in the facilitation of LTP induction.

Another possible explanation for the different effects of GQ1b and GM1 on HFS-induced LTP may be specific properties of GQ1b, which facilitates synaptic NMDA receptor activation in the hippocampus (Jung et al. 2010). In the presence of ATP, GQ1b stimulates ecto-protein kinase, enhancing the phosphorylation of the extracellular domains of synaptic membrane proteins, one of which could be the NMDA receptor/Ca2+ channel (Fujii, Kato, et al. 1995, 1999), and triggering the biological process resulting in ATP-induced LTP. We have previously shown that HFS-induced LTP also involves the phosphorylation of extracellular domains of synaptic membrane proteins (one of which could be the NMDA receptor/Ca2+ channel) and the modulation of NMDA receptors/Ca2+ channels during LTP induction (Fujii, Ito, et al. 1995; Fujii, Kuroda, et al. 2000). Thus, it is possible that, in hippocampal CA1 neurons, b-pathway gangliosides, including GQ1b, are involved in the formation of LTP through the modulation of NMDA receptors/Ca2+ channels. The possibility that a change in the number and/or subunit composition of NMDA receptors in TG mice results in the reduction in LTP induction cannot be excluded. Whether GQ1b is involved in determining NMDA receptor number and/or function remains to be investigated.

Recently, it is suggested that glial cells participate and regulate the induction of various forms of synaptic plasticity through the communication with neurons (Ben Achour and Pascual 2010; Perea and Araque 2010). Although we demonstrated the alteration of the ganglioside profile in TG mice from isolated hippocampus, it is unknown whether these gangliosides changes are CA1 neuron-specific or are also occurred in glial cells. The roles of gangliosides expressed on glial cells in the synaptic plasticity should be clarified.

Previous studies have shown that LTD and DP have different properties and induction mechanisms (Fujii, Matsumoto, et al. 2000; Lee et al. 2000). A summary of the results of the present study (Table II) shows that the change of ganglioside...
content affects the DP, but not LTD, again suggesting that LTD and DP in hippocampal CA1 neurons have different induction mechanisms. However, the exact mechanisms underlying the effects of gangliosides on DP are still unknown.

In addition to the changes in ganglioside composition, it is possible that the structure of other sugar chains, such as N-glycans or O-glycans on glycoproteins, is also altered in GalNAc-T TG mice. We cannot rule out the possibility that alterations in these sugar chains may affect induction of synaptic plasticity, but, in our unpublished studies, β1,4 GalNAc-T was found to selectively catalyze glycosylation of glycolipids, but not glycoproteins. Thus, the phenotype of the TG mice observed in this study should be due to the change in the ganglioside composition.

Jung et al. (2008) reported that GQ1b-treated rats show highly increased memory performance in the Morris water maze test. In our study, TG mice, with an altered ganglioside profile in the hippocampus, exhibited learning impairment in the 4PTT compared with control mice (Figure 4), and we have also observed (unpublished data) that learning in the 4PTT in the gerbil is impaired by damage to the hippocampal CA1 region by transient ischemia. Although the possibility that 4PTT is also dependent on other brain regions cannot be

Table II. Summary of changes in synaptic plasticity and learning behavior in TG mice

<table>
<thead>
<tr>
<th>Gangliosides</th>
<th>Synaptic plasticity</th>
<th>Learning</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-pathway</td>
<td>b-pathway</td>
<td>LTP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LTD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4PTT</td>
</tr>
</tbody>
</table>

TG mice  ↑  ↓  ↓  ±  ↑  ↓

Note that GM1 and GQ1b are components of the a-pathway and b-pathway, respectively. An upward arrow indicates an increase or facilitation compared with the control, a downward arrow indicates a decrease or attenuation, and ± indicates no significant change. TG, transgenic; LTP, long-term potentiation; LTD, long-term depression; DP, depotentiation; 4PTT, four-pellet taking test.
completely eliminated, our results suggest that a decrease in GQ1b in hippocampal neurons leads to learning impairment.

To arrive at the conclusion that learning behavior is based on a positive relationship between LTP and learning, careful observation of the animal’s behavior was essential. We therefore checked sensory and motor functions and activity during daily life and during the 4PTT in TG mice and found no changes compared with WT mice (Figure 5). Thus, the present results considerably strengthen the hypothesis that synaptic plasticity, especially LTP and DP, is the cellular basis of learning and memory (Morris et al. 1986; Silva et al. 1992; Bliss and Collingridge 1993; Xu et al. 1998; Rioult-Pedotti et al. 2000).

Materials and methods

Animals

Male β1,4GalNAc-T TG mice and WT mice with body weights of 15.8–36.0 g and ages ranging from 6 to 22 weeks 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-week-old at the start of the 4PTT. Ganglionic levels were determined in 12-week-old mice. All mice were housed singly with food and water freely available and maintained on a natural light/dark cycle for more than 3 days before the experiments. The animal experiments adhered to Yamagata University Guidelines for Animal Experimentation.

TG mice

β1,4 GalNAc-T TG mice were generated by transfection of a transgene into fertilized mouse eggs (Fukumoto et al. 1997). The β1,4 GalNAc-T transgene was constructed by inserting the AhoI fragment of mouse cDNA clone pTM3-5 into the EcoRI site of the pCAGGS vector after ligation of an EcoRI linker (Takamiya et al. 1995). In TG mice, β1,4 GalNAc-T mRNA levels in the brain are ~3-fold higher than in WT mice and β1,4 GalNAc-T enzyme activity is also higher than in WT mice (Fukumoto et al. 1997).

Isolation of gangliosides and TLC

Gangliosides were prepared using a modified procedure based on a previous method (Yamashiro et al. 1995). Briefly, glycolipids were extracted from sliced brain tissues with chloroform/methanol (2:1, 1:1 and 1:2, sequentially) and the acidic fractions isolated by ion-exchange column chromatography. TLC was performed as described previously (Furukawa et al. 1985) and the bands analyzed using a flying spot scanner (CS-9000, Shimadzu, Kyoto, Japan).

Electrophysiology

Electrophysiology was performed on hippocampal slices from TG or WT mice after observation of the animal’s behavior in a cage for at least 3 days. The mice were anesthetized with diethyl ether, the hippocampus dissected from the right hemisphere and transverse slices (500 µm thickness) prepared using a rotor slicer (Dosaka DK-7700, Kyoto, Japan). After more than 1 h incubation in artificial cerebrospinal fluid (aCSF) consisting of (in mM) 124 NaCl, 5.0 KCl, 2.5 CaCl2, 1.3 MgSO4, 1.25 NaH2PO4 and 10 glucose, aerated with a gas mixture of 95% O2 and 5% CO2 and maintained at 30 ± 1°C, the test slice was transferred to a recording chamber where it was continuously perfused with aCSF. A bipolar stimulating electrode was then placed in the stratum radiatum in the CA1 region, close to the CA2 region, to stimulate the Schaffer collateral/commissural fibers. The field EPSPs were recorded from the stratum radiatum of the CA1 region using glass electrodes and the S-EPSPs were measured. At the beginning of each experiment, the strength of the stimulus pulse (50 µs duration) was adjusted to elicit an EPSP with an initial slope of 40–60% of the maximum and fixed at this level. After checking the stability of the responses to a test stimulus given every 30 s, a train of HFS (tetanus, 100 pulses at 100 Hz) or LFS (200 pulses at 1 Hz) was delivered to induce LTP or LTD, respectively. In the LTP experiments, the mean S-EPSP measured over a 10 min period at 50–60 min after tetanus was expressed as a percentage of the control level. In the LTD experiments, three LFSs were applied successively at 20 min intervals, and the mean magnitude of S-EPSP during a 5 min period at 15–20 min after each LFS was measured. To produce DP, three trains of LFS (200 pulses at 1 Hz) at 20 min intervals were applied starting at 30 min after tetanus (100 pulses at 100 Hz), and the mean magnitude of LTP of the S-EPSP during a 5 min period at 25–30 min after tetanus and at 15–20 min after the end of each LFS was measured. Changes in the response after HFS or LTD were calculated as follows: (i) the percentage change in the response after HFS was calculated as \( (Y \times 100)/X \); (ii) the percentage change in the response after LTD was calculated as \( (Z \times 100)/X \); and (iii) the percentage reduction in LTP after LTD was calculated as \( (X - Y)/X \times 100 \), where \( X \) is the averaged value for the 10 min immediately prior to HFS or the first LFS, \( Y \) the averaged value at 25–30 or 50–60 min after the end of HFS and \( Z \) the stable level at 15–20 min after the end of the third LFS. In the equation in (iii), 100 or 0% indicate complete reduction to the pre-tetanic control level or no induction of DP, respectively.

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 four mice at once. The time spent removing food from a feeder box and the amount of food taken were recorded and continuously displayed on a screen above the home cage. This was accomplished by monitoring the movement and weight of the feeder box suspended through the roof of the cage by means of a sensor attached to the feeder box and a specially developed computer program.

Four-pellet taking test

Apparatus. The apparatus for the 4PTT 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 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 four pellets was 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 the feeder boxes was made from a thin punched stainless-steel sheet. The apparatus was placed on a desk in an experimental room and dim light, 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 a behavioral check for 3–10 days in a cage, the 4PTT 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 four 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 given 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). After leaving the mouse on the stage for 30 s, the box was removed and the 4PTT trial was started.

One food pellet was placed in each of the feeder boxes. The mouse had to jump down from the central stage onto the floor of the large box and the feeder box replaced, allowing the mouse access to food and water ad libitum until the next morning. Each mouse was given one 4PTT trial a day for at least 8 consecutive days.

During the trial, the following behaviors of the mouse were monitored: the sector in which the mouse was located and the time spent in that sector and the time spent eating a pellet, grooming the body, biting the apparatus or standing up against the wall. Each trial session was limited to 20 min. Establishment of learning was defined as the first day of 3 successive days when a mouse managed to find and eat all four pellets within 4 min. All data were stored using a specially developed computer program.

**Statistical analysis**
All values are given as the mean ± SEM. The results were analyzed for statistical significance using the unpaired two-tailed Student’s t-test, two-way repeated-measures ANOVA or the Kolmogorov–Smirnov test. A difference was considered statistically significant if P < 0.05.

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**Conflict of interest**
None declared.

**Abbreviations**
aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; β₁, 4 GalNAc-T, β₁, 4-N-acetylgalactosaminy/transferase; DP, depotentiation; 4PTT, four-pellet taking test; HFS, high-frequency stimulation; LFS, low-frequency stimulation; LTD, long-term depression; LTP, long-term potentiation; NMDA, N-methyl-d-aspartate; S-EPSP, slope of the excitatory postsynaptic potential; STP, short-term potentiation; TG, transgenic; TLC, thin-layer chromatography; WT, wild-type.

**References**


