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Effect of a neuraminidase inhibitor (oseltamivir) on mouse jump-down behavior via stimulation of dopamine receptors

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ABSTRACT

Oseltamivir (Tamiflu®, Roche Laboratories, Inc.) is a neuraminidase inhibitor that can cause jump-down behaviors in children. There is a mouse slip-down model, in which the dopamine D2 receptor activity is increased by serum sialoglycolipids and the mouse jump-down behavior appears in response to the dopamine D2 receptor agonist, PPHT. The present study examined the effect of oseltamivir on jump-down behavior in mice. Oseltamivir sialylates a serum glycolipid and this modified glycolipid induces jump-down behavior via the stimulation of dopamine D2 receptors. This mechanism may be involved in the abnormal behavior of children taking oseltamivir.

Oseltamivir (Tamiflu®, Roche Laboratories, Inc.) is an antiviral drug used to treat influenza. It can reduce the duration and severity of the illness if administered within 48 hours after the onset of symptoms (4, 13, 19). Recently, fatal accidents due to abnormal behaviors such as jumping from high places after taking oseltamivir have been reported in juvenile patients in Japan (7, 18, 20). These abnormal behaviors usually occur after the initial administration of oseltamivir (8). In March 2007, the Japanese Ministry of Health, Labour and Welfare announced a ban on the use of oseltamivir in patients from 10–19 years of age (12). However, little is known about either the contribution of this drug to these behaviors or the mechanism involved.

Sialic acids exist mostly in the terminal positions of biomolecules (such as glycoproteins, glycolipids and gangliosides) and cell membranes and are involved in a wide variety of physiological processes, including immune functions (24, 26). Neuraminidases are called sialidases because they hydrolyze the terminal sialic acid linkage in these biomolecules and variations in human sialidase activity have been implicated in serious diseases and symptoms including neuropsychiatric problems (1, 2, 25). Oseltamivir is a representative neuraminidase inhibitor.

Slip-down behavior in mice is induced by a comparatively low-dose of the dopamine D2 receptor agonist, PPHT (17), and that the representative dopamine D2 receptor agonists morphine and quinpirole induce jump-down behavior in mice (6, 10). In addition, we noticed that mouse jump-down behavior appeared by increased doses of PPHT during previous experiment. An increase in dopamine D2 receptor activity is closely associated with sialylation of a serum glycolipid (15). Oseltamivir might influence the dopamine D2 receptor activity through sialoglycolipids and oseltamivir might be connected to the manifestation of the jump-down behavior. An animal model is useful for understanding these behavioral phenomena. Therefore, the jump-down behavior in mice could be a model for the jump-down behavior in human children taking oseltamivir.

The present study investigated the effect of oseltamivir on jump-down behavior in mice, a serum glycolipid associated with this behavior and changes in glycosylation of this glycolipid in response to oseltamivir.

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MATERIALS AND METHODS

Animals. Eight-week-old male ddY mice that weighed 25–30 g were purchased from Japan SLC (Shizuoka, Japan). A group of 5 mice were housed in a plastic cage (338 × 140 × 225 mm) with free access to food (ED-7; Clea Japan, Tokyo, Japan) and water. The animal room was kept at 21–25°C with 50–60% humidity and was illuminated from 7 : 00 to 19 : 00. All experiments were carried out according to the guidelines of the Ethics Committee for Animal Experiments of the Akita University School of Medicine.

Dosage of PPHT inducing mouse jump-down behavior. The dopamine D2 receptor agonist 2-(*N*-phenylethyl-*N*-propyl) amino-5-hydroxytetralin hydrochloride (PPHT; Funakoshi, Tokyo, Japan) was dissolved in water. The observation of mouse jump-down behavior is the same as procedure of observation of mouse slip-down behavior which we previously reported (17). This method was established for the detection of neuronal D2 dopamine receptor activity. In brief, a raised platform measuring 10 cm in diameter and 20 cm high was prepared. One group of five mice in a cage was intraperitoneally injected with 200, 150, 100, or 50 μg/kg of PPHT, or 100 μL of physiological saline (PS) as a control. Twenty minutes after the injection, the mice were placed individually on the platform for 5 min and thereafter both slip-down behavior and jump-down behavior were investigated.

Effect of oseltamivir on jump-down behavior. From results of the above experiment, 50 μg/kg of PPHT was determined to be a suboptimal dose of mouse jump-down behavior. Other naïve mice groups were prepared. Two trials separated by a 20-min interval were observed to confirm that all of the mice remained on the platform for 5 min without jumping-down and slipping-down before the administration of drugs. Then the mice were injected intraperitoneally with oseltamivir solution or with PS as a control (5 mice each). Oseltamivir was dissolved in water and 25 mg/kg, 50 mg/kg, or 100 mg/kg was injected intraperitoneally in mice (5 mice each). Two hours after injection with oseltamivir solution or with PS, each mouse was placed individually on the platform and jump-down behavior was examined for 5 min. These mice were also injected intraperitoneally with PPHT at the suboptimal dose of 50 μg/kg. Twenty minutes after injection with PPHT of 50 μg/kg, jump-down behavior was again examined for 5 min.

Isolation of the glycolipid fraction from sera. The effect of oseltamivir was thought to be related to serum glycolipid. Oseltamivir solution (100 mg/kg) was injected intraperitoneally in 5 groups of mice and PS (100 μL) was injected into another 5 groups. Sera were collected 2 h after this treatment. Glycolipids were separated from the sera by the methanol-chloroform method, as reported previously (16). In brief, 1 mL of sera was added to 2.5 mL methanol and 1.25 mL chloroform. The fluid was agitated for 2 min and left at room temperature (RT) for 10 min. Then another 1.25 mL chloroform was added and the mixture was agitated for 30 s. After addition of 1.25 mL water and agitation for another 30 s, the mixture was centrifuged at 150 × *g* for 5 min at RT, thus resulting in an upper methanol-water layer containing proteins and a lower chloroform layer containing lipids and glycolipids. The lower layer was collected and evaporated and the lipids and glycolipids were redissolved in 2 mL water. The solution was applied to an ion-exchange DE-52 column (Whatman International, Maidstone, UK) saturated with 10 mM $NaHCO_3$, pH 8.3 and eluted with 50, 100, 150, 200, 250 and 300 mM NaCl in stages. The eluted fractions were refined to less than 3 kDa with the use of an ultrafiltration membrane (Centricon; Amicon, Tokyo, Japan). Two hundred microliters of each fraction was injected intraperitoneally into the naïve group of 5 mice and then the mice were additionally injected with 50 μg/kg PPHT 20 min after the fraction injection. The effect on neural D2 receptor activity was investigated by the jump-down method.

Detection of the sugar chain structures of effective glycolipids. To detect the sugar chain structures of the glycolipids, a 50% ethanol lectin-enzyme-linked immunosorbent assay (lectin-ELISA) was performed, as reported previously (16). In brief, 50 μL of the lipid fraction eluted with 250 mM NaCl in the serum of 5 mice treated with 100 mg/kg oseltamivir or 5 mice treated with PS was mixed with 50 μL ethanol and poured into one well of a 96-well plate (Sumitomo Bakelite, Tokyo, Japan). After 2 h, the well was washed three times with a washing solution (PS containing 0.005% Tween 20; Seikagaku Co., Tokyo, Japan). After a 30-min block with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), the well was washed again three times with the same washing solution. Five different biotinylated lectins that recognize specific sugar

chain structures (ABA, Galβ_{1-3}GalNAc; DBA, GalNAcα_{1-3}GalNAc; SSA, Sialα_{2-6}Gal; AAL, Fucα_{1-2}Gal; MAM, Sialα_{2-3}Gal) were prepared at 2 μg/mL in PS and 100 μL of the mixture was added to the well. After 1 h incubation at RT, the well was washed three times with the washing solution. One hundred microliters of peroxidase-conjugated avidin (Seikagaku Co.) prepared at 0.1 μg/mL in PS was then added to the well. After 15-min incubation at RT, the well was washed four times with PS. Development of the color reaction was performed with a developing kit (Sumitomo Bakelite) and absorbance was measured at 455 nm and 650 nm.

Confirmation of the glycolipid effect. The glycolipid fraction eluted with 250 mM NaCl from the sera of mice treated with PS or the sera of mice treated with 100 mg/kg oseltamivir was applied to a Maackia amurensis agglutinin (MAM) affinity column (Seikagaku Co.). MAM is the specific lectin for Sialα_{2-3}Gal and this affinity column was used to refine the glycolipid that has Sialα_{2-3}Gal in the sugar chain terminal. Fractions eluted with 6 M urea were desalted over a CD-50 desalting column (Pharmacia, Uppsala, Sweden) and freeze-dried. Glycolipids were redissolved in water to a concentration of 10 μg/mL. A concentration of 25, 50, or 100 μg/kg of glycolipid solution was injected intraperitoneally into mice (5 each) and the effect on jump-down behavior was investigated after treatment with the suboptimal dose of PPHT.

Statistical analysis. The Kruskal-Wallis rank test was used to determine significant differences among groups. The Mann-Whitney U test was used for further analysis. $P < 0.01$ was considered to be statistically significant.

RESULTS

Suboptimal dose of the dopamine D2 receptor agonist PPHT

A concentration of 100 μg/kg of PPHT induced slip-down behavior, but 50 μg/kg did not. On the other hand, concentrations of 200 and 150 μg/kg induced jump-down behavior, but 100 and 50 μg/kg did not (Table 1).

Effect of oseltamivir on jump-down behavior

To determine whether oseltamivir induces jump-down behavior in mice, the behavior of the mice 2 h were examined after the injection of various concentrations of oseltamivir or PS and then at 20 min after the injection of 50 μg/kg PPHT. Mice treated with oseltamivir or PS showed no jump-down behavior, but after being treated with D2 receptor agonist, the mice treated with 50 or 100 mg/kg oseltamivir showed jump-down behavior (Table 2). The number of mice exhibiting jump-down behavior increased dose-dependently.

Effects of serum glycolipid fractions on jump-down behavior

To determine whether glycolipid fractions from 100 mg/kg oseltamivir-treated mice induce jump-down behavior, the mice were injected intraperitone-

Table 1 *Jump-down or slip-down behavior induced by the dopamine D2 receptor agonist PPHT*

	Dose of PPHT (μg/kg)				PS
	200	150	100	50	(control)
Jump-down behavior	5	5	0	0	0
Slip-down behavior	0	0	5	0	0

Values in this table indicate the number of mice that showed jump-down or slip-down behavior among the 5 treated mice. PPHT: 2-(*N*-phenylethyl-*N*-propyl) amino-5-hydroxytetralin hydrochloride. PS: physiologic saline.

Table 2 *Number of mice exhibiting jump-down behavior*

	Dose of oseltamivir solution (mg/kg)			PS
	100	50	25	(control)
2 h after oseltamivir injection	0	0	0	0
20 min after further D2 receptor agonist injection	5*	2*	0	0

Values in this table indicate the number of mice that showed jump-down behavior among the 5 treated mice. The D2 receptor agonist was used at 50 μg/kg. In the lower data, a statistically significant difference was found ($k = 3$, $n_1 = n_2 = n_3 = 5$, $n = 15$, $H = 12.5$, $P < 0.009$; Kruskal-Wallis rank test). *$P < 0.01$ vs. control (Mann-Whitney U test). PS: physiologic saline.

ally with serum glycolipid fractions eluted with various concentrations of NaCl and evaluated their behavior. The glycolipid fraction from 100 mg/kg oseltamivir-treated mice eluted with 250 mM NaCl induced jump-down behavior (Table 3). None of the other fractions from 100 mg/kg oseltamivir- or PS-treated mice induced this behavior.

Sugar chain reactivities of glycolipid eluted with 250 mM NaCl

To identify the sugar chain structures of the effective glycolipid eluted with 250 mM NaCl, the reactivity of five kinds of sugar chain structures were investigated using by the 50% ethanol lectin-ELISA method. The sugar chain structures $Gal\beta_{1-3}GalNAc$, $GalNAc\alpha_{1-3}GalNAc$, $Sial\alpha_{2-6}Gal$ and $Sial\alpha_{2-3}Gal$ were found in the glycolipid fractions of mice treated with 100 mg/kg oseltamivir or PS (Table 4). No $Fuc\alpha_{1-2}Gal$ reactivity was found. The $Gal\beta_{1-3}GalNAc$, $GalNAc\alpha_{1-3}GalNAc$ and $Sial\alpha_{2-6}Gal$ reactivity was similar in both groups, but the $Sial\alpha_{2-3}Gal$ reactivity was greater in the mice treated with 100 mg/kg oseltamivir than in the mice treated with PS.

Confirmation of the glycolipid effect

To confirm the glycolipid effect, three concentrations of the glycolipid fraction or PS were each injected intraperitoneally into 5 mice and the effects on jump-down behavior were investigated. After refinement with a MAM column, the glycolipid from mice treated with oseltamivir dose-dependently induced jump-down behavior after treatment with 50 μg/kg PPHT. The glycolipid from mice treated with PS did not induce this behavior, even at the dose of 100 μg/kg and after treatment with 50 μg/kg PPHT (Table 5).

DISCUSSION

Oseltamivir is a sialic acid analogue that inhibits influenza type A and type B neuraminidase, the viral enzyme that allows the release of virus from infected cells. In addition, oseltamivir is an ester prodrug activated by hepatic carboxylesterases. It is thought that the sudden onset of reactions such as abnormal behaviors and sudden death during sleep are caused by the prodrug of oseltamivir, oseltamivir phosphate. On the other hand, adverse reactions such as

Table 3 *Jump-down behavior in mice treated with glycolipids isolated from sera*

Injected fraction	Serum glycolipids from mice treated with PS	Serum glycolipids from mice treated with 100 mg/kg oseltamivir
Eluted with 50 mM NaCl	0	0
100	0	0
150	0	0
200	0	0
250	0	5
300	0	0

Values in this table indicate the number of mice that showed jump-down behavior among the 5 injected mice. All mice were also treated with 50 μg/kg dopamine D2 receptor agonist PPHT 20 min after the fraction injection. PS: physiologic saline.

Table 4 *Sugar chain reactivities of the serum glycolipids eluted with 250 mM NaCl*

	Serum glycolipid from mice treated with PS	Serum glycolipid from mice treated with 100 mg/kg oseltamivir	PS (negative control)
$Gal\beta_{1-3}GalNAc$	0.126	0.127	0.036
$GalNAc\alpha_{1-3}GalNAc$	0.116	0.118	0.050
$Sial\alpha_{2-6}Gal$	0.091	0.088	0.045
$Sial\alpha_{2-3}Gal$	0.086	0.153	0.043
$Fuc\alpha_{1-2}Gal$	0.037	0.035	0.036

Values indicate absorbance at dual wavelengths of 455 nm and 650 nm.
Serum glycolipid was obtained from the 5 mice treated with PS or the 5 mice treated with 100 mg/kg oseltamivir.
PS: physiologic saline.

Table 5 *Mice jumping-down after treatment with refined glycolipid*

Glycolipid dose (μg/kg)	Glycolipid from mice treated with PS	Glycolipid from mice treated with 100 mg/kg oseltamivir
100	0	5*
50	0	3
25	0	0
PS (control)	0	

Values in this table indicate the number of mice that showed jump-down behavior among 5 treated mice. All mice were treated with 50 μg/kg D2 receptor agonist. In the right column data, statistical differences were analyzed by the Kruskal-Wallis rank test ($k = 3$, $n_1 = n_2 = n_3 = 5$, $n = 15$, $H = 12.5$, $P < 0.009$).

*$P < 0.01$ vs. control (Mann-Whitney U test). PS: physiologic saline.

pneumonia, sepsis, hyperglycemia and gastrointestinal bleeding are thought to be delayed reactions induced by oseltamivir and the active metabolite of oseltamivir, oseltamivir carboxylate (OC), is thought to be the cause of delayed reactions (9). Recently, OC has been described to have an inhibitory action on human cytosolic sialidase. It was hypothesized that this effect might be a mechanism by which oseltamivir induces adverse neuropsychiatric reactions (14). Since OC inhibits the human cytosolic sialidase and it can also damage the cell functions in various human tissues, this may explain the delayed reactions induced by oseltamivir. However, abnormal behaviors occur after the initial administration of oseltamivir (8), therefore, it is supposed that the mechanism of such abnormal behavior might be different from the hypothesis (9).

There were two novel findings in the present study: 1) oseltamivir induced jump-down behavior in mice in association with stimulated neuronal D2 receptor activity and 2) this effect of oseltamivir was associated with the Sialα_{2-3}Gal form of sialylation of serum glycolipid. Neither oseltamivir nor its carboxylic acid metabolite, GS4071, influence the re-uptake/release of three monoamines (dopamine, serotonin and norepinephrine) or GTP binding in postsynapses (23). However, another investigation using rats indicates the possibility that oseltamivir has effects on the central nervous system, especially when combined with other agents (11). Serum glycolipid would be expected to be sialylated following oseltamivir treatment; however, the mechanism of sialylation with the Sialα_{2-3}Gal form is not clear. Lipids can pass through the blood-brain barrier and dopaminergic neurons possess glycoside receptors (21). Serum glycolipid regulates the dopaminergic neuron activity, while sialylation enhances the effect of increased D2 receptor activity by agonists. A previous study reported that oseltamivir enhances the effect of ganglioside on opioid receptors (5).

Hyperthermia excites the hypothalamus, which decreases the plasma sialic acid level (3). Decreased sialylation of serum glycolipids regulates dopaminergic neuron activity relating to hyperthermia. With regard to the jump-down behavior of children taking oseltamivir, oseltamivir may block glycolipid desialylation and neuronal D2 receptor activity in children infected with influenza may be increased. Slip-down behavior in mice is caused by dopaminergic hyperactivity to escape from an uneasy situation (15). Jump-down behavior in mice is also caused by dopaminergic hyperactivity and could also indicate behavior to escape from an uneasy situation. This is a basic adaptative behavior in animals and experience refines the behavior. Some children may not be able to handle this type of hyperactive D2 activity.

The results of the present study shed light on the mechanism underlying abnormal behaviors of some children in response to oseltamivir. It would be useful to examine the oseltamivir-induced jump-down behavior in influenza-infected mice. Human studies such as a sugar-chain analysis of blood phospholipids of patients who take oseltamivir are also needed.

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