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Synaptic and Behavioral Interactions of Oseltamivir (Tamiflu) with Neurostimulants

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Abstract

Oseltamivir (Tamiflu), a neuraminidase inhibitor, is widely used for treatment of influenza. Because abnormal behaviors have been observed in some Japanese teenagers following oseltamivir use, its safety has been questioned. Oseltamivir is known to alter neuronal function and behavior in animals, particularly when administered in combination with ethanol. Based on this, we hypothesized that interactions of oseltamivir with other drugs may result in altered CNS excitability. We found that injection of ephedrine and caffeine overcame inactivity induced by oseltamivir and ethanol but did not alter changes in novelty seeking behavior in a Y-maze test. In *ex vivo* hippocampal slices, oseltamivir carboxylate (OTC), an active form of oseltamivir, alters excitability in the absence of ethanol. In slices pretreated with OTC, long-term depression (LTD), a form of synaptic plasticity that is correlated with Y-maze performance was not altered if caffeine or ephedrine was administered individually. However, LTD could not be induced in slices pretreated with OTC if caffeine and ephedrine were administered simultaneously. These observations suggest that combination of oseltamivir with other neurostimulants may alter synaptic plasticity and this may contribute to behavioral changes associated with the drug.

Keywords

Tamiflu; oseltamivir; long-term depression; ephedrine; caffeine; avian influenza

Introduction

The outbreak of bird flu (highly pathogenic avian influenza A caused by H5N1 and H9 N2 strains) may kill millions of people worldwide if the infection spreads by human-to-human contact.¹⁻² Because oseltamivir (Tamiflu®), an antiviral agent that acts as a neuraminidase inhibitor, may be effective in treating avian influenza,³⁻⁴ governments of multiple countries are storing the drug to minimize the risk of outbreak. However, the safety of oseltamivir has been questioned based on accidental deaths and behavioral changes following its use by young people including teenagers in Japan.⁵⁻⁶ Sudden death of infants has also been reported after use of oseltamivir. These deaths may have resulted from influenza-associated encephalopathy but questions about the safety of oseltamivir have arisen in Japan where oseltamivir was commonly prescribed until last year. Although there are a few reports to conclude that a causal relationship between oseltamivir use and the abnormal behaviors or accidental deaths are less

likely,⁷⁻⁸ the United States Food and Drug Administration (FDA) issued a stronger psychiatric warning about oseltamivir in 2008.⁹

Oseltamivir is metabolized to oseltamivir carboxylate (OTC)¹⁰ and other metabolites in the body.¹¹ Sialic acid, which may inhibit cellular adhesion, is cleaved by neuraminidase. Thus, it is speculated that neuraminidase, which is blocked by OTC, may play important roles in the central nervous system (CNS) function, including neuronal development and impulse conduction.¹²⁻¹⁴ Whereas treatment of hippocampal neurons with neuraminidase increases seizure threshold, its blockade decreases seizure threshold, suggesting that endogenous neuraminidase participates in the regulation of neuronal activity.¹⁵ Furthermore, it has been shown that neuraminidase activity in the hippocampus is increased during seizures.¹⁶ Taken together, these results suggest that OTC could have effects on the CNS and thus play a role in behavioral changes.

We previously examined behavioral and neurophysiological effects of oseltamivir and OTC in rat hippocampal slices and found that propagation of excitatory synaptic inputs from dendrites to cell bodies is enhanced by oseltamivir and OTC.¹⁷ In hippocampal slices, it has also been shown that oseltamivir and OTC induced spike bursts through neuronal synchronization.¹⁸ Although it has been claimed that oseltamivir does not enter the brain, high doses cause brain damage in animals,¹⁹ and a recent study has shown that oseltamivir does cross the blood brain barrier (BBB).²⁰ It has been shown that systemic administration of oseltamivir causes hypothermia in mice²¹ and increases dopamine release from the prefrontal cortex in rats,²² indicating that systemically administered oseltamivir reaches the CNS to alter neuronal function. We previously observed that loss of righting reflex (LORR) in rats following injection of ethanol was diminished by pretreatment with oseltamivir, suggesting that oseltamivir has neurological actions when administered with other agents.¹⁷

In light of this, it is important to note that patients typically take antiviral agents with other CNS-active drugs, including stimulants like caffeine and ephedrine, as well as alcohol. In Japan, where most problems with oseltamivir have been reported, alcohol use among teenagers is relatively common, though use of other abused drugs is less frequent.²³ Furthermore, BBB permeability to OTC could be enhanced by the presence of alcohol, a solvent that is known to increase BBB permeability to other agents.²⁴ Caffeine is also routinely ingested and Ephedra (Mahuang) is often prescribed to treat flu-like symptoms in Japan. Thus, it is possible that the putative neuropsychiatric effects of oseltamivir occur as a result of interactions with other CNS-active agents.

The primary aim of the present study is to determine whether oseltamivir has adverse CNS interactions when administered with other agents used to treat flu-like symptoms. Such findings could help to establish a safety profile for using oseltamivir and other neuraminidase inhibitors to manage viral infections. In this study, we examined interactions of oseltamivir and ethanol in *combination with* caffeine and ephedrine in a rat behavioral test using a Y-maze. Because prior studies have indicated that Y-maze performance is correlated with synaptic long-term depression (LTD),²⁵⁻²⁷ we also examined drug interactions on LTD in rat hippocampal slices, a preparation that allows direct examination of how drugs influence neuronal function. In this *ex vivo* study where we can apply drugs directly at known concentrations, we used OTC instead of oseltamivir, because we previously observed that in hippocampal slices OTC is more potent than its prodrug oseltamivir.¹⁷ Because OTC has effects in slices in the absence of ethanol, we specifically focused on the interactions of OTC with ephedrine and caffeine.

Materials and Methods

Animals

All experiments were performed in accordance with the guidelines of the Washington University Animal Study Committee. Every effort was made to minimize the number of animals used and their suffering in all experimental procedures. Male Spague-Dawley rats obtained from Harlan (Indianapolis, IN, USA) at postnatal date (PND) 23 were reared with a cycle of 12 hours white light and 12 hours dim light until experiments.

Behavioral studies and drug injections

The first trial experiment was done to determine the effects of treating rats (postnatal day 28-33) with a combination of oseltamivir, ephedrine and caffeine. In this experiment oseltamivir (2% volume of body weight, 50 mg/kg, i.p.) or the same volume of saline was followed in 2 hours by simultaneous intraperitoneal injection (0.3% volume of body weight) of caffeine (30 mg/kg) and ephedrine (30 mg/kg) in saline at an interval of 2 hours.

In subsequent studies, spontaneous alternation behavior was examined using a Y-maze as previously described.²⁶⁻²⁷ In this test, a rat was placed in the center of a maze with three arms that were 95 mm wide, 636 mm long and 240 mm deep at angles of 120° with respect to each other. Rats were allowed to explore the apparatus for up to 10 min and entry into an arm was counted only when the hind limbs completely entered the arm. An alternation was defined as any three consecutive choices of three different arms without re-exploration of a previously visited arm. The percentage of alternations was determined by dividing the total number of alternations by the total number of choices minus 2.²⁷ The number of completed alternations was determined by counting the number of times that the rats successively entered each of the three arms of the maze without reentering a previously visited arm in first 12 entries or in 10 min, whichever came first. Thus, the highest score possible on this measure is 10. Y-maze tests were video-taped.

The initial Y-maze test was performed 1-2 hours after transfer of rats from the animal care facility. After the initial Y-maze test, ethanol (1.0 g/kg, i.p. as 26% v/v in saline) or ethanol then oseltamivir in saline (2% volume of body weight, 45 min apart) was administered (i.p.) to albino rats (postnatal day 30 ± 2) at an interval of 2 hours. After these injections, the Y-maze test was repeated. The third Y-maze test was done 20 min after simultaneous intraperitoneal injection (0.3% volume of body weight) of caffeine (30 mg/kg) and ephedrine (30 mg/kg).

Hippocampal Slice Electrophysiology

Naïve rats (postnatal date 28-35) were anesthetized with isoflurane and decapitated. Hippocampi were rapidly dissected, placed in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 5 KCl, 2 MgSO₄, 2 CaCl₂, 1.25 NaH₂PO₄, 22 NaHCO₃, 10 glucose, gassed with 95% O₂-5% CO at 4-6°C, and cut transversely into 400 µm slices using a vibratome. Slices were prepared from the septal half of the hippocampus and were placed in an incubation chamber containing gassed ACSF for 1 hr at 30 °C. ACSF was perfused at 2 ml/min. At the time of experiment, slices were transferred individually to a submersion recording chamber. Experiments were done at 30 °C.

Extracellular recordings were obtained from the apical dendritic region for analysis of population excitatory postsynaptic potentials (EPSPs) using 2 M NaCl glass electrodes with resistances of 5-10 MΩ. Evoked synaptic responses were elicited with 0.2 msec constant current pulses through a bipolar electrode placed in the Schaffer collateral-commissural pathway. Synaptic responses in CA1 were monitored by applying single stimuli to the Schaffer

collateral pathway every 60 sec at an intensity sufficient to elicit 50% maximal EPSPs. After establishing a stable baseline for at least 10 min and a control input-output (IO) curve, LTD or LTP was induced by applying low frequency stimulation (LFS) consisting of 900 individual pulses at 1 Hz (LTD) or high frequency stimulation (HFS) consisting of a single 100 Hz $\times$ 1 sec stimulus train (LTP) using pulses of the same amplitude. Following LFS and HFS, responses were monitored every 60 sec for 60 min.

Chemicals

The test solution of oseltamivir was prepared by dissolving a Tamiflu tablet (75 mg) in saline. OTC was obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Statistics

Statistical analyses were done in SigmaStat (Jandel Scientific Software, San Rafael, CA, USA). ANOVA test was used for analysis of results from the Y-maze test. Results from LTD studies were analyzed with Student's *t*-test or Mann Whitney U-test where appropriate. Chi-square test was used for analysis of occurrence of odd behaviors.

Results

As previously reported, injection of oseltamivir alone in rats did not induce abnormal patterns of behavior.¹⁷ Because CNS stimulants such as ephedra and caffeine are often taken by flu patients in Japan, ephedrine (30 mg/kg) and caffeine (30 mg/kg) were injected simultaneously into 12 rats housed in four separate cages. Administration of both agents caused hyperactivity, including hopping, darting and sweating lasting over 30 min. While sweating may be unusual in rodents, we observed that drug-treated animals developed a wet appearance of their fur beginning in the neck region and eventually covering their entire body. No abnormal behaviors were noticed subsequently. Another group of 12 rats was pretreated with oseltamivir (50 mg/kg) and showed similar hyperactivity immediately after injection of ephedrine and caffeine. Interestingly, two of these 12 rats attempted to mount other cage mates 2-3 hours after injection. This behavior was observed even though other hyperactive behaviors had diminished. However, no significant difference in the number of affected animals was detected with a Chi-square test compared to 12 control rats. Mounting was not observed in rats treated with ephedrine alone (N=11) or caffeine alone (N=11) after oseltamivir injection.

In subsequent studies, we examined spontaneous alternation behavior in a Y-maze. For these studies, rats were studied individually. The Y-maze test provides a measure of novelty seeking and exploratory behavior.²⁷ When placed at the center of the Y-maze, control rats typically checked the arms of the maze in an alternating fashion without re-exploring previously visited arms, and routinely entered the arms of the maze 12 times within a 10 min observation period. Y-maze performance was not altered when the test was repeated 2 to 3 hours after injection of oseltamivir (50 mg/kg) alone. Similarly, the number of arm entries and the alternation score were not altered after simultaneous injection of ephedrine and caffeine (30 mg/kg each) after oseltamivir treatment.

Because we previously observed additive effects of ethanol and oseltamivir in an animal behavioral study,¹⁷ we treated rats with oseltamivir and ethanol. When oseltamivir was administered 40 min after injection of ethanol (1.0 g/kg), rats exhibited diminished overall activity, resulting in a decreased number of arm entries in the Y-maze (Fig. 1A, 6.8 ± 1.8 vs 12 times, only 2 out of 6 rats achieved 12 arm entries within 10 min). Although the four rats that failed to achieve 12 arm entries initially groomed themselves when they were put in the Y-maze, they subsequently became immobile with widely opened ears and ceased self

grooming. Because of this limited activity, the number of alternating arm entries was also reduced (Fig. 1B). However, the Y-maze score, the ratio of alternative arm entries compared to the total number of entries of active rats, was not reduced (triangles in Fig. 1E, only two of six rats entered the arms 12 times within 10 minutes, so the last Y-maze score of 7 comes from these 2 rats). In rats pretreated with ethanol and oseltamivir, subsequent injection of ephedrine and caffeine resulted in no decrease in activity and restored the number of arm entries (12 times in 10 min for all 6 rats) (right bar in the right cluster of Fig. 1A). Interestingly, these rats entered the arms of the maze randomly and the Y-maze score remained low (Fig. 1B and squares in Fig 1E; Y-maze score; 7.5 ± 0.3 before and 3.8 ± 0.7 after treatment). In rats treated with ethanol alone, the number of arm entries and the Y-maze score were not altered (left cluster of histograms in Fig. 1A and B, triangles in Fig. 1C). In these rats, the Y-maze parameters were not altered following injection of ephedrine and caffeine (Squares in Fig. 1C).

Because Y maze scores are correlated with LTD in the hippocampus,²⁵⁻²⁷ we examined the effects of OTC, an active metabolite of oseltamivir, on LTD induction in the CA1 region. Oseltamivir is metabolized to OTC in the brain after passing the BBB,²⁰ and we previously found that OTC is more potent than oseltamivir in causing paired pulse facilitation of population spikes. This enhanced excitability results from altered dendritic propagation in hippocampal slices and does not require the presence of ethanol.¹⁷ In the following study, therefore, slices were pretreated with 3 μ M OTC for 2 hours prior to delivery of low frequency stimulation (LFS) consisting of 900 pulses at 1Hz. In the pretreated slices, LTD was successfully induced without any significant difference from LTD in naïve slices (Fig. 2A, EPSP slope 60 min after LFS; $71.0 \pm 4.7\%$ of control in pretreated slices, N=5, and $67.1 \pm 1.5\%$ in naïve slice, N=5). In pretreated slices, LTD was also induced when LFS was delivered in the presence of 100 μ M caffeine (squares in Fig. 2C, EPSP slope 60 min after LFS; $80.7 \pm 3.9\%$ in caffeine, N=5) or 100 μ M ephedrine (circles in Fig. 2C, EPSP slope; $60.4 \pm 9.3\%$ in ephedrine, N=5). However, LTD was not induced in OTC pretreated slices when LFS was delivered in the presence of both caffeine and ephedrine (Fig. 2B, EPSP slope 60 min after LFS; $100.9 \pm 3.6\%$, N=5, $P < 0.01$ vs. control LTD in both naïve and pretreated slices). In contrast, LTD was induced in the presence of both agents in naïve slices (Fig. 2B, EPSP slope 60 min after LFS; $76.9 \pm 5.4\%$, N=5). Neither OTC alone nor the combination of OTC, caffeine and ephedrine had an effect on induction of LTP induced by a single 100 Hz $\times$ 1 sec high frequency stimulation (HFS) (EPSP slope 60 min after HFS; $140.1 \pm 4.5\%$ in slices treated with OTC alone, N=5; $138.8 \pm 1.0\%$, N=3, in the presence of OTC, caffeine and ephedrine, data not shown).

Discussion

The present study shows that administration of a non-sedating dose of ethanol in combination with oseltamivir resulted in diminished behavioral activity and poor locomotion in rats. Evaluation of the changes in behaviors is difficult but the pattern of response in the alternation task may represent a form of anxious or fearful behavior as manifest by altered exploration.²⁸ This behavioral restraint may result from risk assessment, the first line of defense against a threat, and appears as a decrease in environmental exploration and locomotion. Some rats were simply immobile without self grooming, also suggesting an augmented risk assessing behavior.²⁹ Immobility (freezing) is also thought to be a second level of defense, and may involve assessment of a perceived threat from an undetermined source. If this is the case, random movement after injection of caffeine and ephedrine, which results in the impaired Y-maze performance, may represent a form of flight from an unknown threat as a third level of defense rather than environmental exploration. This may also reflect a form of a behavioral agitation.

It is also possible that oseltamivir alters cognitive processes maintained through synaptic plasticity. Of importance is that oseltamivir, when combined with CNS stimulants, clearly impairs Y-maze performance. Thus, the random movements observed may have features similar to abnormal and agitated behaviors reported in humans following oseltamivir ingestion. Previous studies have indicated that Y-maze scores are correlated with induction of LTD, a form of hippocampal synaptic plasticity that may contribute to novelty-seeking behaviors.²⁷ We previously showed that administration of ethanol inhibits both LTD and Y-maze performance.²⁶ Impairment of LTD induction by the combination of OTC and CNS stimulants, which was observed in the present study, suggests that the combination of drugs affects the synaptic plasticity that may underlie this form of cognitive processes. It is also possible that the combination changes synaptic function and plasticity in other than regions of the CNS resulting in more profound changes in behavior.

Intentionally or unintentionally, patients with flu may consume CNS stimulants and other drugs. Alcohol, for example, is commonly used socially and may be ingested to relieve some flu-like symptoms by young teenagers.²³ Caffeine and related compounds are often included in soft drinks, nutritional supplements and common cold regimens.³⁰ In Japan, ephedra is often taken by flu patients as part of a prescription of Chinese herbal medications that are thought to have antiviral effects.³¹ Importantly, herbs such as Mahuang (ephedra) are not free from adverse effects.³² Just as with oseltamivir, suicides have been reported with abuse of ephedra.³¹ Thus, it is possible that oseltamivir results in enhanced stimulant actions in the CNS and agitated behavior when combined with other stimulants. It is also possible that the interactions are more complicated during viral infections when there may be changes in the integrity of the BBB.³⁴⁻³⁵ It has been reported that P-glycoprotein at the BBB plays an important role in accumulation of oseltamivir in the CNS.²⁰ In P-glycoprotein knock-out mice oseltamivir concentration in the cerebrospinal fluid (CSF) is 5.5 fold higher.³⁶ Moreover, it was previously described that brain levels of oseltamivir were 1500 times those of adult animals exposed to the same dose.¹⁹ The accumulated oseltamivir is likely converted to OTC in the CNS. Although it has been reported that OTC concentrations in the CSF in adult healthy volunteers administered 150 mg oseltamivir reach only about 0.1 μM ,³⁷ much higher levels are expected if the BBB is immature or impaired.

The relation between use of oseltamivir and abnormal behaviors remain uncertain. It also remains unclear why these abnormal behaviors occurred primarily in Japan. This may simply reflect the frequency with which oseltamivir is used to treat influenza in Japan while oseltamivir use in the United States is relatively less common. We hypothesize that the combination of oseltamivir with CNS stimulants and/or alcohol could play a role in producing abnormal behaviors and accidental deaths. However, it is also possible that genetic variation resulting in reduced sialidase activities, which is detected in only some Asians, may account for the adverse effect of oseltamivir.³⁸ Taken together, these observations suggest that multiple factors are likely to contribute to the adverse effects of oseltamivir.³⁹ The present study suggests that oseltamivir, if combined with common neurostimulants, may alter a specific form of synaptic plasticity in the CNS; in turn, this could contribute to some of the behavior changes reported after use of oseltamivir. Further investigations, especially neurochemical analyses, will be required to elucidate the interactions of oseltamivir with other agents. This information will be important for determining the conditions under which antiviral agents can be used safely in humans given the potential need for widespread use of these drugs in event of an avian flu pandemic.

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Fig. 1.

The graphs show effects of systemic treatment of male rats (postnatal date 29-32) with a non-sedating dose of ethanol (1.0 g/kg, i.p.) (left cluster of bars), oseltamivir (50 mg/kg, i.p.) (center) or both (right) on spontaneous alternation in a Y-maze for up to 10 min. Two hours after treatment with oseltamivir and/or ethanol, ephedrine (30 mg/kg) and caffeine (30 mg/kg) were simultaneously injected (right bars in each bar cluster). Rats were exposed to the Y-maze three times; before treatment with oseltamivir and/or ethanol, and 40 min after injection of ethanol alone or 2 hours after injection of oseltamivir, and 40 min after injection of ephedrine and caffeine. Panel A depicts the total numbers of entries into arms of the Y-maze (up to 12) in 10 min and is a measure of activity in the task. Rats treated with ethanol 40 min prior to the treatment with oseltamivir show decreased activity in the maze (right purple column in A) * $P < 0.05$ against control before treatment. Panel B depicts the number of completed alternations in the Y-maze, defined as successive entry into each of the three arms of the maze without reentry into a previously visited arm. Rats treated with ethanol 40 min prior to the treatment with oseltamivir also showed impaired performance compared with ethanol alone treated rats after injection of ephedrine and caffeine (right green column in B).. ++ $P < 0.01$ against control Y-maze score obtained before treatment. Panel C-E show again the effects of systemic treatment of male rats with ethanol (1.0 g/kg, i.p.) alone (C), or oseltamivir (50 mg/kg, i.p.)

alone (D) or both (E) on spontaneous alternation in the Y-maze for up to 10 min. Rats were placed in the Y-maze three times: before treatment (open circles), 40 min after injection of ethanol alone or 2 hours after injection of oseltamivir (triangles), and 40 min after injection of ephedrine and caffeine (squares). In rats pretreated with ethanol and oseltamivir, injection of ephedrine and caffeine results in poor performance in the Y-maze (squares in E). * $P < 0.05$ by one way ANOVA test.

Fig. 2.

Impaired induction of hippocampal LTD with ephedrine and caffeine in slices pretreated with 3 μ M oseltamivir carboxylate (OTC). (A) Time courses of change in EPSPs in the CA1 region following LFS (arrows) in naïve slices (open circles) and slices treated with OTC (filled squares) ($N=5$, each) are similar, suggesting that oseltamivir alone does not alter LTD. (B) Simultaneous administration of 100 μ M ephedrine and 100 μ M caffeine inhibits LTD in slices pretreated with OTC (filled squares) but not in naïve slices (open circles) ($N=5$ in each). (C) In slices pretreated with OTC, caffeine alone (squares) or ephedrine alone (circles) failed to inhibit LTD induction. However, the inhibition was only partial with caffeine. Note that filled symbols in each graph denote pretreatment with OTC. * $P<0.05$, ** $P<0.01$ by Student's t -test

and $\#P<0.05$ by Mann Whitney U-test against oseltamivir alone. In panel B, $+P<0.05$, $++P<0.01$ by Student t -test and $\&\&P<0.01$ by Mann Whitney U-test against ephedrine plus caffeine without oseltamivir. Traces to the right depict EPSPs obtained before (solid line) and 60 min after (dotted line) LFS. Scales; 1 mV, 5 msec.