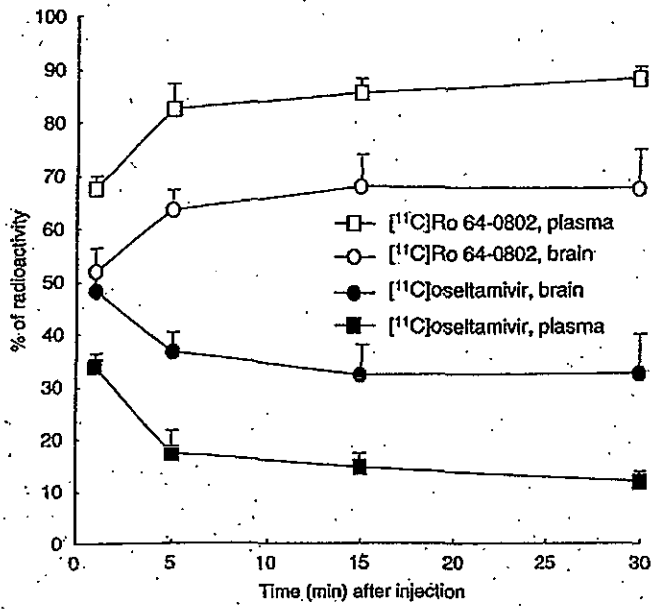

Fig. 7. Percentage (mean±S.D., n=3) of [^{11}C]oseltamivir and [^{11}C]Ro 64-0802 in the plasma and brain of mice at four time points after injection of [^{11}C]oseltamivir (30–44 MBq) into mice (n=3).

Fig. 7 shows the in vivo metabolic results of [^{11}C] oseltamivir in the plasma and brain. After injection into the mouse, [^{11}C]oseltamivir in the plasma decreased to 34% at 1 min and to 17% at 5 min and maintained this level until 30 min. In the brain, [^{11}C]oseltamivir decreased to 48% at 1 min and to 37% at 5 min. [^{11}C]Ro 64-0802 was observed as another main radioactive component on HPLC charts. Within this experiment, 66–88% and 52–68% of total radioactivity in the plasma and brain were assigned to [^{11}C] Ro 64-0802, respectively.

4. Discussion

This study describes the biodistribution and metabolism of the anti-influenza drug [^{11}C]oseltamivir and its active metabolite [^{11}C]Ro 64-0802 in mice. In these experiments, we determined the presence and amounts of the two PET ligands in the living brains of mice. These findings are useful in elucidating the cause of the side effect of Tamiflu in the CNS.

As can be seen from the distribution data (Fig. 2), at 1 min after tracer injection, [^{11}C]Ro 64-0802 displayed higher initial uptakes in the brain (0.39% ID/g) and blood (6.69% ID/g) than [^{11}C]oseltamivir in the brain (0.20% ID/g) and blood (2.79% ID/g). At 15 min, the radioactivity of [^{11}C]oseltamivir (0.14% ID/g) in the brain became higher than that of [^{11}C]Ro 64-0802 (0.08% ID/g). From that point, the two ligands displayed a slow decline of radioactivity in the brain. At 60 min, the level of [^{11}C] oseltamivir (0.06% ID/g) was twofold higher than that of [^{11}C]Ro 64-0802 (0.03% ID/g) in the brain. The ratio of brain/blood radioactivity was calculated to examine the characteristics of the ligands passing through the BBB and accumulating in the brain. The ratios increased from 0.07 to 0.14 for [^{11}C]oseltamivir and from 0.06 to 0.13 for [^{11}C]Ro 64-0802 during this experiment. By analyzing the micro-PET data and time–activity curves (Figs. 3 and 4), SUVs of the two ligands in the living brains could be obtained with statistical significance. The radioactivity concentration of [^{11}C]oseltamivir in the brain was quantified at SUV 0.25–0.05 (0.72–0.15% ID/g) between 0 and 90 min after injection, while that of [^{11}C]Ro 64-0802 was at SUV 0.38–0.02 (1.15–0.07% ID/g). The uptake difference between the two ligands persisted until the end of PET experiments. These results obtained by the dissection method and micro-PET demonstrated that a small amount of the two PET ligands passed the BBB and entered the brain.

Despite their presence, the radioactivity levels of [^{11}C] oseltamivir and [^{11}C]Ro 64-0802 in the mouse brains were much lower than some useful PET probes [25–27] developed by us for clinical brain imaging. Ro 64-0802 is a potent inhibitor of the influenza virus. However, this acidic compound has low lipophilicity, with a clog$P$ (P: octanol/water partition coefficient) value of −0.97 calculated with a Pallas 3.4 software. The low lipophilicity of [^{11}C]Ro 64-0802 may restrict its passage through the BBB at a high concentration. On the other hand, oseltamivir, an ester prodrug of Ro 64-0802, becomes more lipophilic (clog$P$: 1.29) than Ro 64-0802. This improved lipophilicity increased the uptake of [^{11}C]oseltamivir through various peripheral organs, as reflected in Fig. 3A. By contrast, [^{11}C]Ro 64-0802 was not distributed into various organs efficiently (Fig. 3B). The present PET study supported that oseltamivir is a successful prodrug of Ro 64-0802. However, although the uptake of [^{11}C]oseltamivir into the peripheral organs was significantly improved, the radioactivity level of [^{11}C] oseltamivir in the brain was limited to only twofold higher than that of [^{11}C]Ro 64-0802 (Fig. 4). This result could be explained as oseltamivir being a substrate of P-glycoprotein (P-gp) [28,29]. The high density of P-gp at the BBB may restrict the entrance of [^{11}C]oseltamivir into the brain.

In the PET study, radioactive metabolites of a radioligand in the ROI can confound the imaging and measurement, regardless of whether the metabolite binds to the target sites. To elucidate the putative influence of radioactive metabolites on PET experiments, we first performed an in vitro metabolite assay of the two ligands in the brain homogenate and plasma of mice. As shown in Fig. 5, [^{11}C]oseltamivir was metabolized to [^{11}C]Ro 64-0802 in the plasma. In the brain homogenate, [^{11}C]Ro 64-0802 was also identified, although its formation rate (0.02 pmol/min/mg protein) was much slower than that (5.1 pmol/min/mg protein) in the plasma. In vivo metabolite analysis of [^{11}C]oseltamivir demonstrated the presence of [^{11}C]oseltamivir and [^{11}C]Ro 64-0802 in the plasma as well as the brain, respectively (Fig. 7). The presence of [^{11}C]Ro 64-0802 in the brain may be because (a) [^{11}C]Ro 64-0802 itself entered the brain from the plasma or (b) [^{11}C]Ro 64-0802 was yielded by the

hydrolysis of [^{11}C]oseltamivir with esterase in the brain. This result could be supported by the following: (a) 0.39% ID/g was measured in the brain at 1 min after [^{11}C]Ro 64-0802 injection; (b) [^{11}C]Ro 64-0802 of 0.5 pmol/mg brain protein was measured after exposing [^{11}C]oseltamivir to the brain homogenate for 30 min (Fig. 6). Thus, while determining [^{11}C]oseltamivir in the brain with PET, the influence of [^{11}C]Ro 64-0802 on the result may be considered. On the other hand, we tried to perform an in vivo metabolite assay of [^{11}C]Ro 64-0802 in the brain. Unfortunately, due to the low radioactivity in the brain, we failed to obtain statistically significant results. We assumed that the in vitro stability of [^{11}C]Ro 64-0802 in the plasma and brain homogenate may guarantee that the PET experiment with [^{11}C]Ro 64-0802 is not influenced by its putative radioactive metabolites.

Our in vivo result differs from the previous report in the metabolism of oseltamivir. It was reported that this agent was not metabolized in the rodent brain [4,30]. The difference could be explained by the characteristic of a PET ligand with high specific activity. Since the carrier mass contained in the injected [^{11}C]oseltamivir was only 0.01–0.1 mg/kg, the carrier might be easily hydrolyzed by esterase present in the brain. However, the amount of nonradioactive oseltamivir used for the previous analysis was 1000 mg/kg by oral administration [29]. In that case, even trace oseltamivir was hydrolyzed, and the resulting Ro 64-0802, compared to the high concentration of unmetabolized oseltamivir, might be easily ignored. On the other hand, it was reported that oseltamivir was not metabolized in human plasma but was metabolized in the liver fraction in humans [31]. In contrast to humans, this drug was metabolized in the plasma but was stable in the liver fraction of rats [31]. This result may be due to the species difference of esterase between primates and rodents. Thus, for clinical PET investigation of [^{11}C]oseltamivir and [^{11}C]Ro 64-0802, a radioactive metabolite assay should be performed using human plasma.

The present evaluation of adult mice showed that microPET could be used to measure the amount of [^{11}C]oseltamivir and [^{11}C]Ro 64-0802 in the living brain with a mature BBB. Although radioactivity levels in the brain were not so high, their uptake may be increased if the BBB is immature or impaired. In fact, the amount of nonradioactive oseltamivir in the brain was reported to be significantly high in newborn rats [29]. On the other hand, organic solvents such as alcohol sometimes enhance BBB permeability [32]. Interaction with other CNS-active drugs may also raise the possibility of the two ligands entering brains. Therefore, PET could be used to elucidate the difference between mature and immature (adult and young) or normal and abnormal states of living brains. Although PET scan on young patients is not easy, a PET study on human adults may be helpful to estimate the amounts of [^{11}C]oseltamivir and [^{11}C]Ro 64-0802 in the brains of young patients. Now, we are comparing the uptake of the two ligands into the brain using rodents of different ages and are searching for their binding sites in animal brains. The present results provide evidence that the two PET ligands are worth investigating in human brains.

5. Conclusions

This study determined the distribution and metabolism of [^{11}C]oseltamivir and [^{11}C]Ro 64-0802 in mice. The two promising PET ligands could be used to measure their amounts in living brains and to elucidate the relationship between their presence and amounts in the brain and the side effects of Tamiflu on the CNS.

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References

[1] Kim CU, Lew W, Williams MA, Liu H, Zhang L, Swaminathan S, et al. Influenza neuraminidase inhibitor possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. J Am Chem Soc 1997;119:681–90.

[2] Calfee DP, Hayden FG. New approaches to influenza chemotherapy. Neuraminidase inhibitors. Drugs 1998;56:537–53.

[3] Kim CU, Chen X, Mendel DB. Neuraminidase inhibitors as anti-influenza virus agents. Antivir Chem Chemother 1999;10:141–54.

[4] Eisenberg EJ, Bidgood A, Cundy KC. Penetration of GS4071, a novel influenza neuraminidase inhibitor, into rat bronchoalveolar lining fluid following oral administration of the prodrug GS4104. Antimicrob Agents Chemother 1997;41:1949–52.

[5] Li W, Escarpe PA, Eisenberg EJ, Cundy KC, Sweet C, Kakeman KJ, et al. Identification of GS 4104 as an orally bioavailable prodrug of the influenza virus neuraminidase inhibitor GS 4071. Antimicrob Agents Chemother 1998;42:647–53.

[6] He J, Massarella P, Ward P. Clinical pharmacokinetics of the prodrug oseltamivir and its active metabolite Ro 64-0802. Clin Pharmacokinet 1999;37:471–84.

[7] (a) http://www.nature.com/news/2007/070319/full/446358a.html. (b) http://www.forbes.com/feeds/ap/2007/04/04/ap3582952.html.

[8] http://www.mhlw.go.jp/shingi/2007/12/2/1225-7.html.

[9] Rodriguez JA, Piddini E, Hasegawa T, Miyagi T, Dotti CG. Plasma membrane ganglioside sialidase regulates axonal growth and regeneration in hippocampal neurons in culture. J Neurosci 2001; 21:8387–95.

[10] Crain SM, Shen KF. Neuraminidase inhibitor, oseltamivir blocks GM1 ganglioside-regulated excitatory opioid receptor-mediated hyperalgesia, enhances opioid analgesia and attenuates tolerance in mice. Brain Res 2004;995:260–6.

[11] Ono H, Nagano Y, Matsunami N, Sugiyama S, Yamamoto S, Tanabe M. Oseltamivir, an anti-influenza virus drug, produces hypothermia in mice. Biol Pharm Bull 2008;31:638–42.

[12] Izumi Y, Tokuda K, O'dell KA, Zorumski CF, Narahashi T. Neuroexcitatory actions of Tamiflu and its carboxylate metabolite. Neurosci Lett 2007;426:54–8.
[13] Usami A, Sasaki T, Satoh N, Akiba T, Yokoshima S, Fukuyama T, et al. Oseltamivir enhances hippocampal network synchronization. J Pharmacol Sci 2008;106:659–62.
[14] http://www.fda.gov/cder/foi/label/2006/021087s033lbl.pdf.
[15] Dutkowski R, Thakrar B, Froehlich E, Suter P, Oo C, Ward P. Safety and pharmacology of oseltamivir in clinical use. Drug Saf 2003;26: 787–801.
[16] Blumentals WA, Song X. The safety of oseltamivir in patients with influenza: analysis of healthcare claims data from six influenza seasons. Med GenMed 2007;9:23.
[17] Okamoto S, Kamiya M, Kishida K, Shimakawa T, Fukui T, Morimoto T. Experience with oseltamivir for infants younger than 1 year old in Japan. Pediatr Infect Dis J 2005;24:575–6.
[18] Hama R. Oseltamivir's adverse reactions: fifty sudden deaths may be related to central suppression. BMJ 2007;335:59.
[19] Fowler JS, Volkow ND, Wang GJ, Ding YS, Dewey SL. PET and drug research and development. J Nucl Med 1999;4:1154–63.
[20] Whitley RJ. The role of oseltamivir in the treatment and prevention of influenza in children. Expert Opin Drug Metab Toxicol 2007;3: 755–67.
[21] Crusat M, de Jong MD. Neuraminidase inhibitors and their role in avian and pandemic influenza. Antivir Ther 2007;12:593–602.
[22] Konno F, Arai T, Zhang MR, Hatori A, Yanamoto K, Ogawa M, et al. Radiosynthesis of two positron emission tomography probes: [^{11}C] oseltamivir and its active metabolite [^{11}C]Ro 64-0802. Bioorg Med Chem Lett 2008;18:1260–3.
[23] Le Bars D, Luthra SK, Pike VW, Luu Duc C. The preparation of a carbon-11 labelled neurohormone—[^{11}C]melatonin. Appl Radiat Isot 1987;38:1073–7.
[24] Arai T, Zhang MR, Ogawa M, Fukumura T, Kato K, Suzuki K. Efficient and reproducible synthesis of [1-^{11}C]acetyl chloride using the loop method. Appl Rad Isot 2008 [doi:10.1016/j.apradiso.2008.09.013].
[25] Zhang MR, Kida T, Noguchi J, Furutsuka K, Maeda M, Suhara T, et al. [^{11}C]DAA1106: radiosynthesis and in vivo binding to peripheral benzodiazepine receptors in mouse brain. Nucl Med Biol 2003;30: 513–9.
[26] Zhang MR, Maeda J, Ogawa M, Noguchi J, Ito T, Yoshida Y, et al. Development of a new radioligand, *N*-(5-fluoro-2-phenoxyphenyl)-*N*-(2-[^{18}F]fluoroethyl-5-methoxybenzyl)acetamide, for PET imaging of peripheral benzodiazepine receptor in primate brain. J Med Chem 2004;47:2228–35.
[27] Zhang MR, Kumata K, Maeda J, Yanamoto K, Hatori A, Okada M, et al. [^{11}C]AC-5216: a novel positron emission tomography ligand for peripheral-type benzodiazepine receptors in primate brain. J Nucl Med 2007;48:1853–61.
[28] Morimoto K, Nakakariya M, Shirasaka Y, Kakinuma C, Fujita T, Tamai I, et al. Oseltamivir (Tamiflu) efflux transport at the blood–brain barrier via P-glycoprotein. Drug Metab Dispos 2008;36:6–9.
[29] Ose A, Kusuhara H, Yamatsugu K, Kanai M, Shibasaki M, Fujita T, et al. P-glycoprotein restricts the penetration of oseltamivir across the blood–brain barrier. Drug Metab Dispos 2008;36:427–34.
[30] Sweeny DJ, Lynch G, Bidgood AM, Lew W, Wang KY, Cundy KC. Metabolism of the influenza neuraminidase inhibitor prodrug oseltamivir in the rat. Drug Metab Dispos 2000;28:737–41.
[31] Shi D, Yang J, Yang D, LeCluyse EL, Black C, You L, et al. Anti-influenza prodrug oseltamivir is activated by carboxylesterase human carboxylesterase 1, and the activation is inhibited by antiplatelet agent clopidogrel. J Pharmacol Exp Ther 2006;319:1477–84.
[32] Stewart PA, Hayagawa EM, Carlen PL. Ethanol and pentobarbital in combination increase blood–brain barrier permeability to horseradish peroxidase. Brain Res 1998;443:12–20.

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Synaptic and behavioral interactions of oseltamivir (Tamiflu) with neurostimulants

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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, it has been hypothesized that interactions of oseltamivir with other drugs may result in altered CNS excitability in this study. It has been 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.

Key words: avian influenza; caffeine; ephedrine; long-term depression; oseltamivir; Tamiflu

Introduction

The outbreak of bird flu (highly pathogenic avian influenza A caused by H5N1 and H9N2 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] While 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

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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 mice[21] 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 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 (ma huang) 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 on 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, Indiana, USA) at postnatal date (PND) 23 were reared with a cycle of 12 h white light and 12 h 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 h 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 h.

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 videotaped.

The initial Y-maze test was performed 1–2 h 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 and 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 h. 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 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 22 $NaHCO_3$, 10 glucose, gassed with 95% O_2-5% CO_2 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 h at 30 °C. Artificial cerebrospinal fluid 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 ms 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 s 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 long-term potentiation (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 × 1 s stimulus train (LTP) using pulses of the same amplitude. Following LFS and HFS, responses were monitored every 60 s for 60 min.

Chemicals

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

Statistics

Statistical analyses were done in SigmaStat (Jandel Scientific Software, San Rafael, California, 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 patients with flu 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 (Figure 1A, 6.8 ± 1.8 vs 12 times, only two of six 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

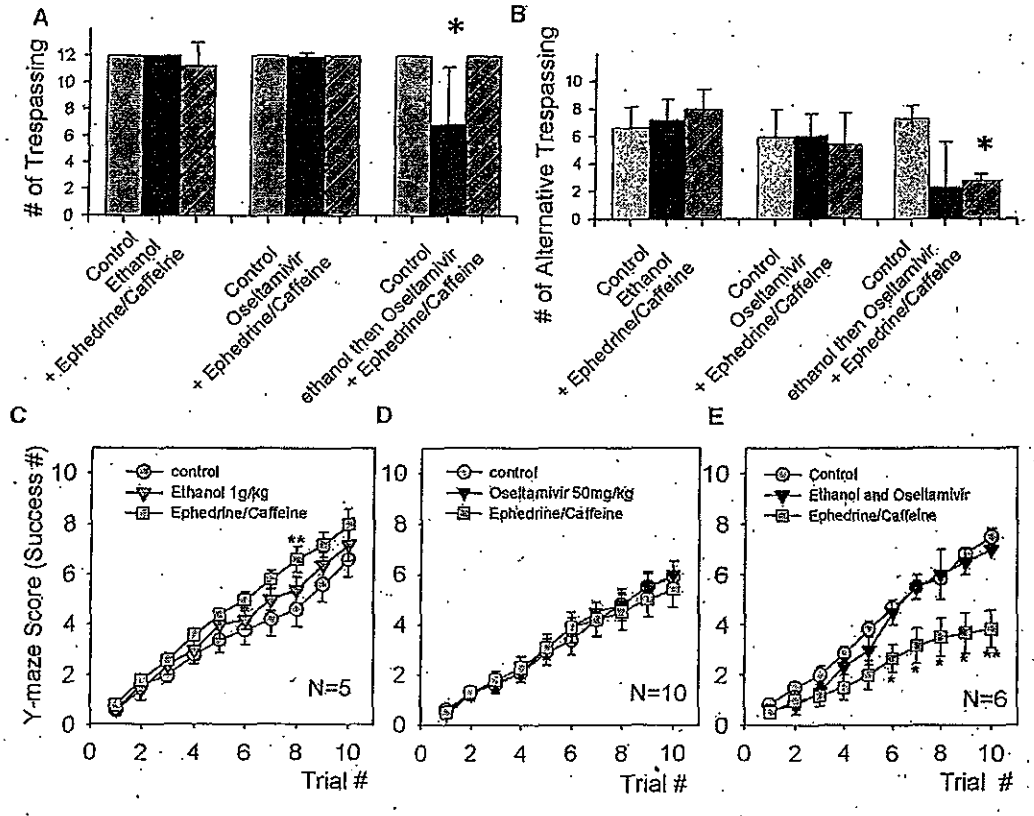

Figure 1 The graphs show effects of systemic treatment of male rats (postnatal date 29–32) with a non-sedating dose of ethanol (1.0 g/kg, i.p.) (left cluster of bars), oseltamivir (50 mg/kg, i.p.) (center) or both (right) on spontaneous alternation in a Y-maze for up to 10 min. Two hours after treatment 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 h 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 h 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.

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 (Figure 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 Figure 1E, only two of six rats entered the arms 12 times within 10 min, so the last Y-maze score of 7 comes from these two 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 six rats) (right bar in the right cluster of Figure 1A). Interestingly, these rats entered the arms of the maze randomly and the Y-maze score remained low (Figure 1B and squares in Figure 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 Figure 1A and B, triangles in Figure 1C). In these rats, the Y-maze parameters were not altered following injection of ephedrine and caffeine (Squares in Figure 1C).

Because Y-maze scores are correlated with LTD in the hippocampus,[25–27] we examined the effects of