平成21年6月16日安全対策調査会
資料2－3

平成２１年２月６日 タミフル基礎WG
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(基礎 WG において調査検討した後に公表されたため追加するもの)

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Oseltamivir (Tamiflu®) increases dopamine levels in the rat medial prefrontal cortex

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ABSTRACT

Oseltamivir (Tamiflu®), a neuraminidase inhibitor, is effective for treating both seasonal flu and H5N1 influenza A virus infection. Oseltamivir is generally well tolerated, and its most common adverse effects are nausea and vomiting. However, neuropsychiatric behaviors including jumping and falling from balconies by young patients being treated by oseltamivir have been reported from Japan; this has led to warnings against its prescribing by many authorities. The pharmacological mechanism of the neuropsychiatric effects of oseltamivir remains unclear. Many studies reported that changes in neurotransmission and abnormal behaviors are closely related. We investigated the changes in dopamine and serotonin metabolism after systemic administration of oseltamivir in the medial prefrontal cortex (mPFC) of rats by using microdialysis. After systemic administration of oseltamivir (25 mg/kg or 100 mg/kg; intraperitoneally (i.p.)), extracellular dopamine in the mPFC was significantly increased as compared to the control values; 3,4-dihydroxyphenylacetic acid and homovanillic acid, the metabolites of dopamine, had also increased significantly. Serotonin was unchanged after the administration of oseltamivir. These findings suggest that oseltamivir increased dopamine release in the mPFC; further, they suggest that the increase in dopamine during oseltamivir treatment may have caused abnormal behaviors in young patients. In cases where oseltamivir is prescribed to children, close observation is required.

Influenza virus infection is a significant public health concern. Vaccination and antiviral drugs are effective in providing protection against the influenza virus. Oseltamivir (Tamiflu®) and zanamivir are neuraminidase inhibitors that prevent influenza virus replication [1,9]. Oseltamivir is the prodrug of Ro 64-0802, which is a known influenza virus neuraminidase inhibitor, and oseltamivir is effective when administered orally [5]. Many clinical studies have reported the efficacy of oseltamivir as treatment for influenza [2,12]. Moreover, since oseltamivir is expected to be effective for the clinical management of the highly pathogenic H5N1 influenza A viruses, several countries are stockpiling oseltamivir [6,7]. Oseltamivir is generally well tolerated; the most common adverse effects observed in clinical studies were nausea and vomiting [5,12]. However, abnormal behaviors such as jumping or falling from balconies by adolescents during oseltamivir treatment were reported from Japan, following which the Japanese authorities advised against prescribing oseltamivir to adolescents aged between 10 and 19 years. The U.S. Food and Drug Administration (FDA) also require that patients be closely monitored for signs of abnormal behavior throughout the treatment period. The pharmacological mechanism of the neuropsychiatric effects of oseltamivir remains unclear. However, the abnormal behaviors observed during oseltamivir administration resemble those induced by psychoactive drugs [3,8] such as amphetamines that influence monoamine metabolism in the brain. However, no change in monoamine metabolism in the brain during oseltamivir administration has been revealed thus far. Therefore, in the present study, we evaluated the effect of oseltamivir on dopamine (DA) and serotonin (5-HT) metabolism in the medial prefrontal cortex (mPFC) of rat by using microdialysis.

Male Wistar rats (Clea Japan Inc., Japan) weighing 200–250 g were used in this study. The rats were housed at a constant room temperature (23 ± 1 °C) and maintained on rat chow and tap water ad libitum. All animal procedures were approved by the Animal Investigation Committee of Jichi Medical University and were in strict accordance with the National Institute for Health (NIH) Guide for the Care and Use of Laboratory Animals. In vivo microdialysis was performed as previously described [16,17]. In brief,

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the rats were anesthetized with sodium pentobarbital (50 mg/kg; intraperitoneally (i.p.)) and placed in a stereotaxic frame (David Kopf Instruments; nose bar positioned 3.5 mm below inter-aural zero). The skull was exposed during surgery, and a burr hole was drilled to accommodate the plantation. Straight-type cellulose dialysis tubing (length, 1.0 mm; internal diameter, 0.16 mm; molecular weight cutoff, 50,000; Eicom Co. Ltd., Kyoto, Japan) was steadily and carefully inserted into the right mPFC (AP, 3.2 mm; ML, 0.6 mm; DV, −5.2 mm from the Bregma) after reference to a brain atlas and fixed with acrylic dental cement and three skull screws. Two days after the surgery, the probe was connected to a microinfusion pump and perfused with Ringer's solution (NaCl, 147 mM; KCl, 4 mM; $CaCl_2$, 1.9 mM) at a flow rate of 2 μl/min. The rats were allowed to move freely during this period. The outflow from the mPFC was automatically collected every 30 min for an additional 240 min and directly injected into a high-performance liquid chromatography (HPLC) unit using an automatic injector (Eicom AS-100, Eicom, Kyoto, Japan). A reverse-phase column (Eicompack CA-5ODS, 150 mm × 2.1 mm, Eicom, Japan) was used for DA and 5-HT separation and another reverse-phase column (Eicompack SC-5ODS, 150 mm × 3.0 mm, Eicom, Japan) was used for 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) separation; the results were immediately analyzed. The DA, DOPAC, HVA, 5-HT, and 5-HIAA concentrations in the samples were determined by an electrochemical detector (Eicom ECD-300, Eicom, Kyoto, Japan). The column and detector were kept in a temperature controller at 25 °C. The composition of the mobile phase used for the DA and 5-HT analyses were 0.1 M phosphate buffer (pH 6.0), 18% methanol, 0.6 g/l sodium 1-octane-sulfonate, and 50 mg/l Na_2EDTA; the flow rate was set at 0.23 ml/min. The mobile phase used in the DOPAC, HVA, and 5-HIAA analyses was 0.1 M acetate–citrate buffer (pH 3.5), 17% methanol, 0.19 g/l sodium 1-octane-sulfonate, and 5 mg/l Na_2EDTA; the flow rate was set at 0.5 ml/min. After a 3-h stabilization period, three consecutive dialysate samples were collected and the baseline DA levels were measured. Capsules containing 75 mg oseltamivir (Tamiflu®) were purchased from Chugai Pharmaceutical Co. (Japan). The capsule ingredients were dissolved in distilled deionized water. Either 25 mg/kg or 100 mg/kg of the drug was administered i.p. to the rats. Following the microdialysis experiments, the brains were removed and frozen at −80 °C. In each case, the position of the dialysis probe was macroscopically verified on 150-μm-thick serial coronal slices. When a bloody region was found around the probe site, the data were excluded. The average of 3 fractions prior to drug administration was defined as 100% (control), and the subsequent perfusate levels were expressed as percentages of the control. Statistical analyses were conducted by two-way ANOVA and one-way ANOVA followed by Dunnett's test. The Windows-compatible software Dr. SPSS2 (SPSS Japan Inc., Tokyo, Japan), was used.

The mean basal extracellular DA, 5-HT, DOPAC, HVA, and 5-HIAA values per 60 μl dialysate for each group were as follows: DA = 0.60 ± 0.08 pg, 5-HT = 3.26 ± 0.85 pg, DOPAC = 86.37 ± 19.05 pg, HVA = 194.10 ± 35.90 pg, and 5-HIAA = 1867.42 ± 289.80 pg.

When 25 and 100 mg/kg of oseltamivir were administered i.p., the DA level increased to 156% and 223% of the pre-administration level, respectively and was also significantly greater than those in the vehicle-administered rats (Fig. 1a). Ataxia was observed following systemic administration of 100 mg/kg of oseltamivir. When 25 and 100 mg/kg oseltamivir was administered i.p., the DOPAC level increased to 147% and 169% of the pre-administration level, respectively (Fig. 1b). The HVA concentration increased to 127% and 146% of the pre-administration level after 25 and 100 mg/kg oseltamivir was administered i.p. (c).

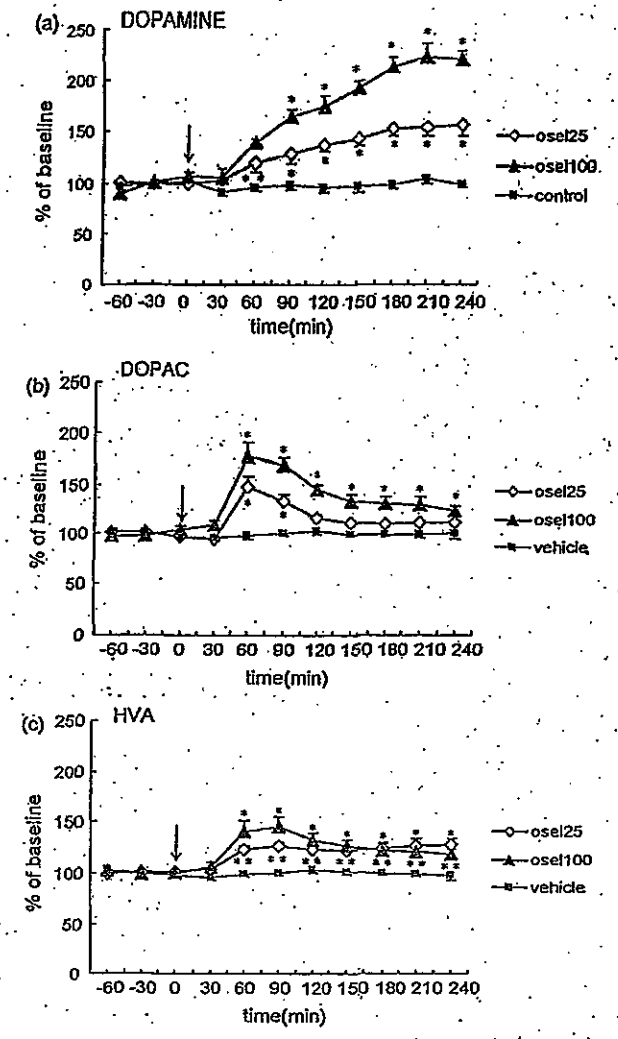

Fig. 1. Effects of oseltamivir on the extracellular DA concentration in the rat mPFC. (a) Effects of oseltamivir on the extracellular 5-HT concentration in the rat mPFC. (b) Drug injection is indicated by the arrow. Data are represented as the mean ± S.E.M. of the values obtained in five rats. $^{*}p < 0.01$, $^{**}p < 0.05$ compared with the corresponding value for vehicle (one-way ANOVA followed with Dunnett's test).

5-HT levels were unchanged after 25 and 100 mg/kg oseltamivir was administered i.p. (Fig. 2a). The 5-HIAA level showed a mild increase after 25 mg/kg oseltamivir administration i.p.; the increase was not significant (Fig. 2b). When 100 mg/kg oseltamivir was administered i.p., the 5-HIAA level showed a significant increase to 126% of the pre-administration level (b).

The present study showed that oseltamivir increased DA levels in dialysates of the rat mPFC in a dose-dependent manner. This is the first demonstration of the effects of oseltamivir on the DA system by using in vivo dialysis. Since the increase in DA levels in animal brains is closely related with abnormal behaviors [13], the abnormal behaviors of patients while being administered oseltamivir may be explained by the present findings.

The increase in extracellular DA levels is assumed to be a consequence of the DA-releasing or DA-reuptake-inhibiting effect of oseltamivir. If oseltamivir can inhibit DA reuptake, reuptake of DA in synaptic clefts should be inhibited and the oxidative deamination of DA by monoamine oxidase within presynaptic sites should be decreased, resulting in a decrease in DOPAC and HVA concentra-

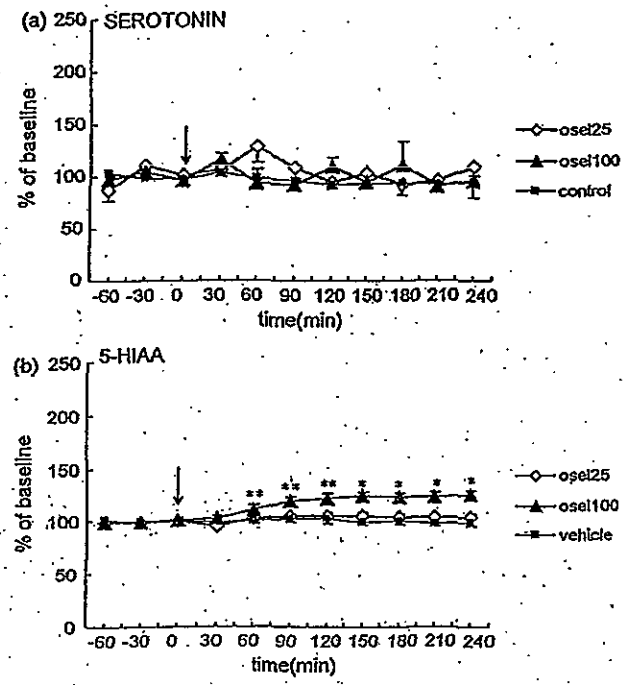

Fig. 2. Effects of oseltamivir on the extracellular DOPAC concentration in the rat mPFC. (a) Effects of oseltamivir on the extracellular HVA concentration in the rat mPFC. (b) Effects of oseltamivir on the extracellular 5-HIAA concentration in the rat mPFC. (c) Drug injection is indicated by the arrow. Data are represented as the mean ± S.E.M. of the values obtained in five rats. $^{*}p < 0.01$, $^{**}p < 0.05$ compared with the corresponding value for vehicle (one-way ANOVA followed with Dunnett's test).

tions. However, in the present study, the levels of DOPAC, HVA, DA metabolites, and DA in the rat mPFC were increased by oseltamivir administration. Therefore, oseltamivir may cause the release of DA from presynaptic sites.

The present study showed that DA increased to 156% and 223% of the pre-administration level following the intraperitoneal administration of 25 and 100 mg/kg oseltamivir, respectively. In rats, ataxia was observed within 10 min after 100 mg/kg oseltamivir was administered, and the ataxia continued for a few minutes. However, compared with the marked increase in DA levels (over 10-fold of the pre-administration level) induced by psychostimulants including phencyclidine and methamphetamine [13], the action of oseltamivir as a DA releaser is less potent. It is reported that oseltamivir does not readily cross the blood–brain barrier (BBB) [6]. Recently, Morimoto et al. [10] reported that P-glycoprotein affects the accumulation of oseltamivir in the brain. The rats used in our experiment were normal; however, if the experimental animals are infected with the influenza virus or have high fever due to infection or insufficient P-glycoprotein activity, it is possible that the BBB is impaired and oseltamivir may reach the central nervous system more easily [14]. In this case, the extracellular DA levels may markedly increase.

Because 5-HT as well as DA neurotransmission in the central nervous system influences animal behavior [4,15], we also measured the levels of 5-HT and 5-HIAA, a metabolite of 5-HT, in the rat mPFC after oseltamivir injection. Although the 5-HIAA levels were significantly increased (126% of the pre-administration level), the 5-HT levels were unchanged after oseltamivir injection. Currently, it is not clear whether 5-HT neurotransmission is closely associated with the abnormal behaviors observed during oseltamivir treatment.

Recently, Izumi et al. [6] reported that oseltamivir and oseltamivir carboxylate, the active metabolite of oseltamivir, affect the central nervous system in vitro, and that the metabolite is more active than oseltamivir. Therefore, further studies are required to investigate the effects of oseltamivir carboxylate on the DA system.

The abnormal behaviors of many patients during oseltamivir treatment may be due to influenza-associated encephalopathy [11]. However, patients showing abnormal behaviors have been observed only in Japan where 70–80% of all oseltamivir consumed and not in Western countries. This fact suggests that not all abnormal behaviors were due to influenza-associated encephalopathy and that some of the abnormal behaviors may result from the use of oseltamivir. The present study suggests that the mechanism underlying the abnormal behaviors observed during oseltamivir use may, to some extent, be due to the transmission of overactive DA.

In conclusion, systemic administration of oseltamivir increased the concentrations of DA and its metabolites in the rat mPFC in a dose-dependent manner. These findings indicate that oseltamivir treatment is related to the manifestation of abnormal behaviors.

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Oseltamivir Enhances Hippocampal Network Synchronization

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Abstract. Oseltamivir, a widely used anti-influenza drug, inhibits virus neuraminidase. A mammalian homologue of this enzyme is expressed in the brain, yet the effect of oseltamivir on central neurons is largely unknown. Patch-clamp recordings ex vivo revealed that oseltamivir enhanced spike synchronization between hippocampal CA3 pyramidal cells. Time-lapse multineuron calcium imaging revealed that oseltamivir and its active metabolite evoked synchronized population bursts that recruited virtually all neurons in the network. This unique, so-far-unknown, event was attenuated by muscarinic receptor antagonist. Thus, oseltamivir is a useful tool for investigating a new aspect of neural circuit operation.

Supplementary Fig. and movie: available only at http://dx.doi.org/10.1254/jphs.SC0070467

Keywords: oseltamivir, functional multineuron calcium imaging, network excitability

Sialic acid is a component of glycoproteins in the cell membrane and regulates various biological functions by inhibiting cellular adhesion. In the central nervous system, sialic acid is mainly present as a chain of neural cellular adhesion molecules, which is abundant in the paleocortical dentate gyrus-CA3 region (1, 2). Neuraminidase (exo-α-sialidase) is a key regulator of the length of sialic acid chains, and the malfunction of this enzyme is associated with epileptic conditions (2). Indeed, sialylation in rat CA3 pyramidal neurons changes the action potential threshold by modulating the properties of voltage-sensitive sodium channels, resulting in an alteration in the excitability of hippocampal networks (3).

Oseltamivir was designed according to the X-ray crystal structure of sialic acid analogues bound to the active site of neuraminidase. This drug is an ethyl-ester prodrug; its active metabolite inhibits virus-type neuraminidase and thereby prevents influenza virus from emerging from infected cells. The structure of viral neuraminidase, however, is very similar to that of HsNEU2, one of four human sialidases (4). HsNEU2 contains exactly the same active site residues, thus being a possible target of oseltamivir. Consistent with this, a recent study has demonstrated that oseltamivir and its active metabolite facilitate the presynaptic function of CA1 excitatory synapses in rat hippocampal slices (5). Here we use electrophysiological recording and functional multineuron calcium imaging (fMCI) techniques to examine the effect of oseltamivir on the excitability of CA3 networks.

Hippocampal slice cultures were prepared from postnatal day 7 Wistar/ST rats (SLC, Shizuoka). Rat pups were decapitated, and the brains were cut into horizontal 300-μm-thick slices using a DTK-1500 microslicer (Dosaka, Kyoto) in aerated, ice-cold Gey's balanced salt solution (Invitrogen, Gaithersburg, MD, USA) supplemented with 25 mM glucose. Entorhino-hippocampal stumps were cultivated for 7 – 14 days on Omnipore membrane filters (JHWP02500, φ25 mm; Millipore, Bedford, MA, USA) (6). Cultures were fed with 1 ml of 50% minimal essential medium, 25%

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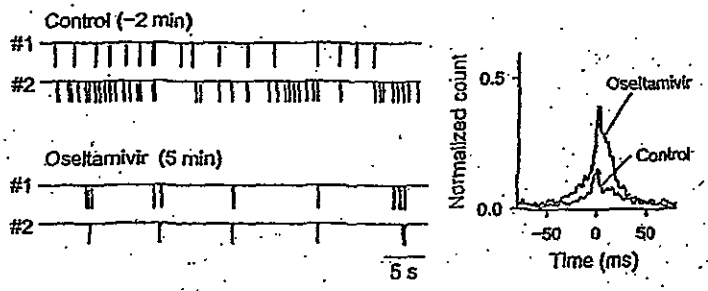

Fig. 1. Oseltamivir synchronizes action potentials between neuron pairs in hippocampal CA3 networks. Dual loose-patch-clamp recordings were performed with glass pipettes filled with ACSF to record extracellular single-unit activity from adjacent hippocampal CA3 pyramidal cell pairs. Recordings were carried out using Axopatch 700B amplifiers (Molecular Devices, Union City, CA, USA), and signals were low-pass filtered at 1 kHz, digitized at 10 kHz, and analyzed with pCLAMP 8.0 software (Molecular Devices). Bursts were defined as any series of spikes with an interval of less than 1 s. Left panels: representative traces of simultaneous loose-patch recordings from two CA3 pyramidal cells 2 min before (top) and 5 min after bath application of 100 μM oseltamivir (bottom). Right panel: crosscorrelogram of spike counts in the same neurons. Oseltamivir increased the degree of spike synchronization. Similar results were obtained in all 4 cases tested.

Hanks' balanced salt solution (Invitrogen), and 25% horse serum (Cell Culture Laboratory, Cleveland, OH, USA) in a humidified incubator at 37°C in 5% CO_2. The medium was changed every 3.5 days.

Experiments were performed in artificial cerebrospinal fluid (ACSF) consisting of 127 mM NaCl, 26 mM $NaHCO_3$, 3.3 mM KCl, 1.24 mM KH_2PO_4, 1.0 mM $MgSO_4$, 1.0 mM $CaCl_2$, and 10 mM glucose, bubbled with 95% O_2 and 5% CO_2 (7). Slices were pre-incubated in ACSF at room temperature for more than 30 min and transferred to a 36°C recording chamber perfused with ACSF at a rate of 1.5 to 2 ml/min. Oseltamivir was chemically synthesized (8–10). *N*-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (NADNA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Both inhibitors were dissolved in ASCF and bath-applied.

For calcium imaging, slices were incubated with 0.0005% Oregon green 488 BAPTA-1AM (Invitrogen) for 1 h at 37°C and then with ACSF at room temperature for >30 min (11). They were transferred to a 36°C recording chamber. The CA3 stratum pyramidale was illuminated at 488 nm and imaged at 10–100 frames/s with a CSU22/CSUX1 Nipkow-disk confocal unit (Yokogawa Electric, Tokyo) and a cooled CCD camera (iXon DV887/DU860; Andor Technology, Belfast, UK). Spike-triggered Ca^{2+} signals were detected with custom-written software in Microsoft Visual Basic (Microsoft, Seattle, WA, USA) (11).

We recorded spike activity simultaneously from two CA3 pyramidal cells under control conditions for a total time of 13 min and then in the presence of oseltamivir for a total time of 11 min (n = 4 pairs) (Fig. 1). Neurons spontaneously fired a series of action potentials. Under control conditions, the timings of the action potentials were almost uncorrelated between the neuron pairs, but after bath application of 100 μM oseltamivir, the same pairs became to emit intermittent bursts of action potentials. On average, single bursts included 5.3 ± 0.2 spikes at 44.7 ± 3.4 Hz (mean ± S.E.M., n = 234 bursts). This burst frequency corresponds to the so-called "gamma frequency" range. Importantly, the bursts concurred between neuron pairs; spike-timing crosscorrelogram (Fig. 1 right) shows that oseltamivir increased the peak amplitude at a time difference of 0 ms, indicating that it enhanced spike synchronization between hippocampal CA3 neurons.

To address the effect of oseltamivir at the network level, we used functional multineuron calcium imaging (11). By taking advantage of the fact that action potentials evoke Ca^{2+} transients in the soma (Fig. 2A), this optical technique can reconstruct spike activity from hundreds of neurons in a network with single-cell resolution (Fig. 2B). Under control conditions, the activity of CA3 neurons was sparse in time and space, but after bath application of 100 μM oseltamivir, they showed a gradual increase in activity rates and abruptly started to show globally synchronized "population burst" activity after 3–20 min (Fig. 2C; see also supplementary Fig. 1 and movie 1; available at online version only). The same effect was produced by 100 μM NADNA, another neuraminidase inhibitor (Fig. 3A, n = 5 slices).

We sought to characterize the population burst events. The emergence of these events, that is, the percentage of slices that showed population bursts, depended on the concentration of oseltamivir in the range from 0.3–100 μM (Fig. 3A, n = 5–8 slices for each concentration). This dose-response curve shifted leftward for the active metabolite of oseltamivir (Fig. 3A). With least-square regression to a sigmoid curve, the ED_{50} value was estimated to be 10.2 μM for oseltamivir and 0.7 μM for the active form. The event frequency, that is, the number of events per min, had no relation to the oseltamivir concentration ($R = 0.20$, $P = 0.29$, Pearson's test) (Fig. 3B) nor did the event duration, that is, the mean period during which individual burst persisted, have any

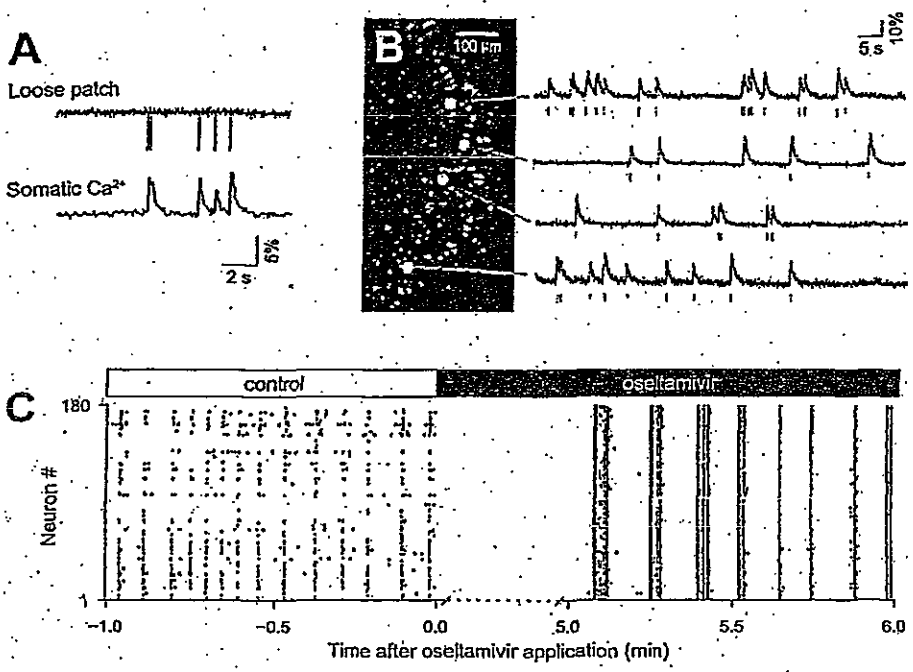

Fig. 2. Oseltamivir induces globally synchronized "population burst" activity. A: Simultaneous loose-patch-clamp recordings and time-lapse imaging of somatic Ca^{2+} signals from a CA3 pyramidal neuron loaded with Oregon green 488 BAPTA-1 reveal that action potentials are faithfully reflected as Ca^{2+} transients. B: Functional calcium imaging and reconstruction of multineuronal spike trains. Representative Ca^{2+} traces of 4 cells indicated in the confocal image of the CA3 pyramidal cell layer of a dye-loaded hippocampal slice culture. Vertical bars under each trace indicate the timings of spikes reconstructed from the raw Ca^{2+} trace. The movie was taken at 100 Hz. C: Examples of the effect of 100 μM oseltamivir on CA3 network activity. Each dot represents the onset of a single calcium transient. After 5 min of treatment with oseltamivir, spikes became synchronized in the whole network.

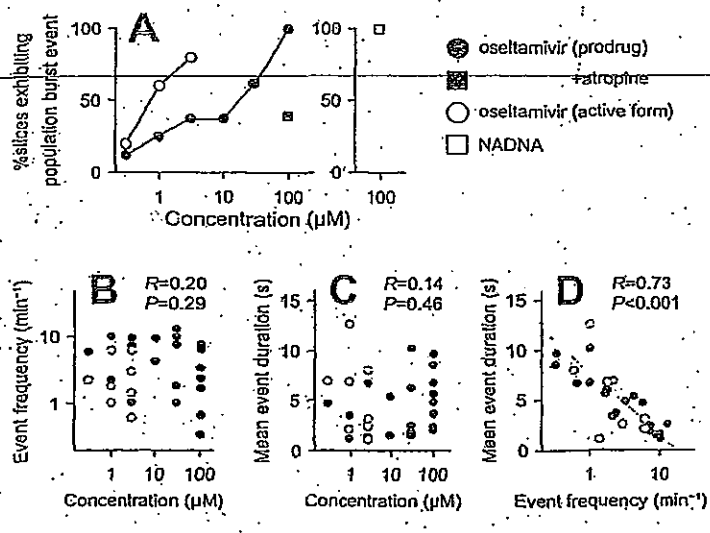

Fig. 3. Characterization of oseltamivir-induced population bursts. A: Concentration-dependent emergence of population bursts. The ordinate indicates the percentage of slices that showed population bursts in response to oseltamivir (closed circles), its hydrolyzed active metabolite (open circles), and NADNA (open square). Atropine was co-applied with 100 μM oseltamivir (closed square). n = 5–8 slices for each concentration. B and C: The frequency of population burst events per min (B) and the average duration for individual population bursts persisted (C) were plotted against the concentrations of oseltamivir and its active form. Each symbol indicates a single slice. D: Negative relationship between the frequency and the duration of population bursts. Line indicates the best linear fit with least square estimation. Significance was determined by Pearson's test.

relation to the concentration ($R = 0.14$, $P = 0.46$) (Fig. 3C). There was a negative relationship, however, between the event frequency and the event duration ($R = 0.73$, $P < 0.001$) (Fig. 3D).

As shown in the above data, oseltamivir-induced bursting had a gamma frequency rhythm. Hippocampal gamma oscillations are believed to arise from activation of muscarinic acetylcholine receptors (12). Consistent with this, we found that in the presence of 10 μM atropine, a muscarinic receptor antagonist, 3 out of 5 slices failed to generate population bursts in response to 100 μM oseltamivir, a concentration of oseltamivir that readily evoked population bursts in all 8 cases tested ($P = 0.035$, Fisher's exact test) (Fig. 3A).

We have demonstrated that oseltamivir and its active metabolite enhanced neuronal synchronization and induced population burst events of rat hippocampal CA3 networks in a concentration-dependent manner. Population bursts did not occur under normal conditions and reflected a globally synchronized state with action potentials at gamma frequency. They seemed to be mediated, at least in part, by muscarinic receptor activity.