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*Short Communication*

# 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

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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-$\alpha$-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-$\mu$m-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, $\phi$25 mm; Millipore, Bedford, MA, USA) (6). Cultures were fed with 1 ml of 50% minimal essential medium, 25%

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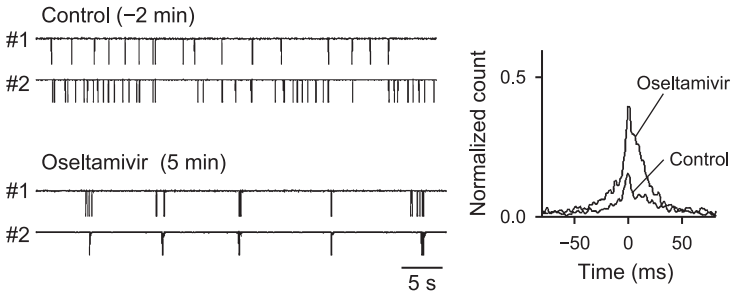


**Fig. 1.** Oseltamivir synchronizes action potentials between neuron pairs in hippocampal CA3 networks. Dual loose-patch-clamp recordings were performed with glass pipettes filled with ACSF to record extracellular single-unit activity from adjacent hippocampal CA3 pyramidal cell pairs. Recordings were 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 $\mu$M 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 $\mu$M 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 $\mu$M 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 $\mu$M 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 $\mu$M (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 $\mu$M for oseltamivir and 0.7 $\mu$M 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

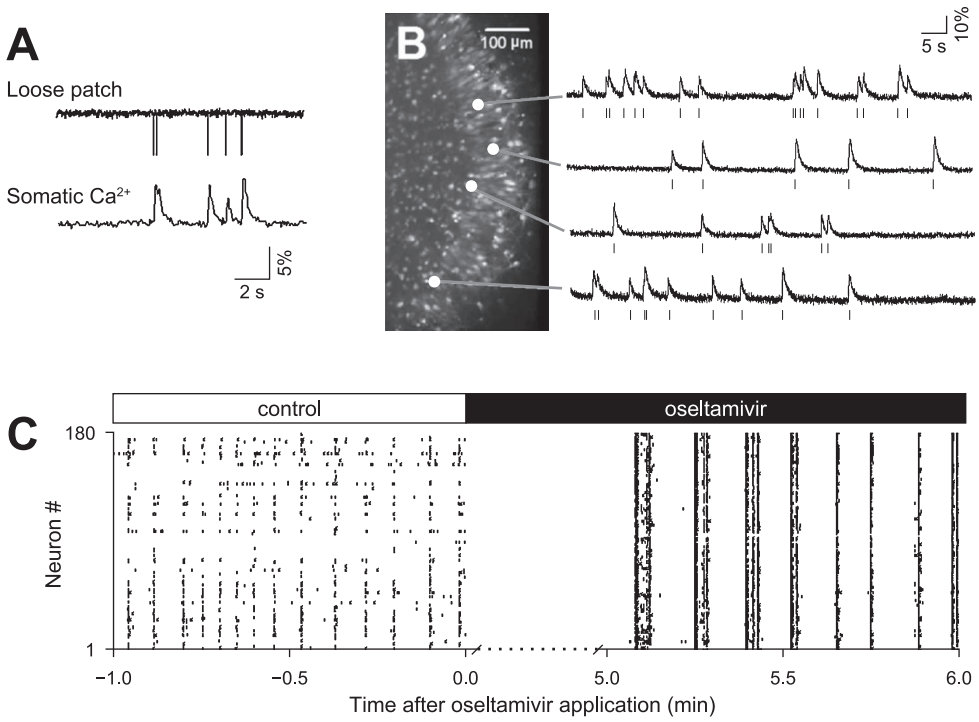


**Fig. 2.** Oseltamivir induces globally synchronized "population burst" activity. A: Simultaneous loose-patch-clamp recordings and time-lapse imaging of somatic $Ca^{2+}$ signals from a CA3 pyramidal neuron loaded with Oregon green 488 BAPTA-1 reveal that action potentials are faithfully reflected as $Ca^{2+}$ 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 $\mu$M 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.

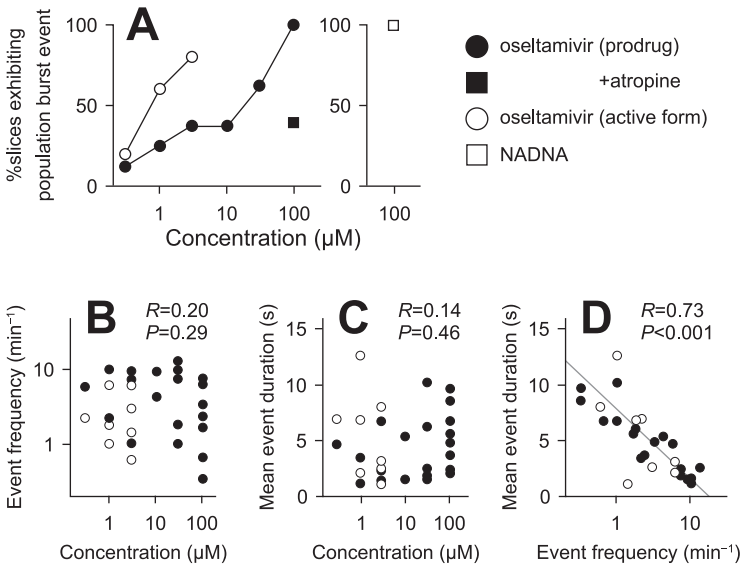


**Fig. 3.** Characterization of oseltamivir-induced population bursts. A: Concentration-dependent emergence of population bursts. The ordinate indicates the percentage of slices that showed population bursts in response to oseltamivir (closed circles), its hydrolyzed active metabolite (open circles), and NADNA (open square). Atropine was co-applied with 100 $\mu$M 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 $\mu$M atropine, a muscarinic receptor antagonist, 3 out of 5 slices failed to generate population bursts in response to 100 $\mu$M 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.

Cholinergically induced gamma oscillations are accompanied by rhythmic activity of the inhibitory network, which shunts excitatory synaptic inputs and sharpens the window in which pyramidal neurons can fire action potentials, leading to a tighter synchrony among pyramidal neurons (12). Thus, oseltamivir-induced population bursts may be shaped by the phasic activity of inhibitory interneurons, rather than pyramidal cells. Our preliminary data indicate that even at a high concentration of 1 mM, oseltamivir does not induce either depolarization or hyperpolarization of membrane potential of hippocampal excitatory neurons in primary dispersed cultures ($n = 8$, data not shown). Thus, our data imply two, but not mutually exclusive, possibilities concerning the action site of oseltamivir: i) inhibitory interneurons are an action target of oseltamivir, and ii) the effect of oseltamivir requires network activity flows, rather than single neurons. Given that two structurally unrelated neuraminidase inhibitors, that is, oseltamivir and NADNA, exerted the sane effect on network activity and that sialic acid, a neuraminidase substrate, regulates neurite adhesion between hippocampal neurons (1), we speculate that oseltamivir modulates sialylation-mediated neurite connectivity and enhances network synchronicity through interneurons.

Animal experiments with rodents demonstrate that orally (30 – 300 mg/kg) or intravenously (8 $\mu$mol/h per kg) administered oseltamivir accumulates in the brain via the blood-brain barrier, the brain-to-plasma concentration ratio ranging from 0.1 – 0.7 (roughly equal to 0.1 – 5 $\mu$M in the brain) (13, 14). Safety examinations of Tamiflu$^{TM}$ (oseltamivir), conducted by Roche, show that in 7 – 14-day-old rats, the brain concentration reaches more than 500 times greater than that in adult animals (see basic product information of Tamiflu$^{TM}$), suggesting a higher risk of a side-effect in younger brains. Interestingly, a minor allele with single nucleotide polymorphism in HsNEU2, which shows a strong binding affinity to oseltamivir, is frequently observed in Asians (9.29%), but not in Europeans and African Americans (15). This Asian population may be highly susceptible to oseltamivir and thus affected by neuropsychiatric disorders. Because our current data are not linked to behavioral alternations in human and animals, investigations in vivo will be necessary to examine whether oseltamivir-induced population bursts are related to some psychologic behaviors frequently seen in influenza-infected children.

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