Note

Caffeine Increases Hippocampal Sharp Waves *in Vitro*

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Caffeine promotes memory consolidation. Memory consolidation is thought to depend at least in part on hippocampal sharp waves (SWs). In the present study, we investigated the effect of bath-application of caffeine in spontaneously occurring SWs in mouse acute hippocampal slices. Caffeine induced an about 100% increase in the event frequency of SWs at concentrations of 60 and 200 μM. The effect of caffeine was reversible after washout of caffeine and was mimicked by an adenosine A_1 receptor antagonist, but not by an A_{2A} receptor antagonist. Caffeine increased SWs even in dentate-CA3 mini-slices without the CA2 regions, in which adenosine A_1 receptors are abundantly expressed in the hippocampus. Thus, caffeine facilitates SWs by inhibiting adenosine A_1 receptors in the hippocampal CA3 region or the dentate gyrus.

Key words ripple; adenosine; memory; hippocampus; slice; learning

The hippocampus plays roles in memory formation and consolidation.[1)] Recent literatures have demonstrated that sharp wave/ripple complexes (SWs), a form of transient high-frequency oscillations, contribute to memory consolidation.[2)] SWs are believed to arise from the hippocampal CA3 region and propagate to the hippocampal CA1 region and thereafter to various brain regions.[3,4)] During a SW period of 40–100 ms, previously activated neurons are serially reactivated in behaviorally relevant orders, a phenomenon known as memory replay.[5,6)] Disrupting SWs after learning resulted in memory impairment.[7,8)] Thus, SWs are likely to contribute to the memory consolidation process and are often regarded as a cognitive biomarker for memory.[9)]

Caffeine, a non-selective adenosine receptor antagonist, promotes some forms of memory consolidation. In rodents, intraperitoneal administration of caffeine immediately after training in passive avoidance tasks and in the Morris water maze task enhances memory test performance. In another case, honeybees rewarded with caffeine were nearly three times as likely to remember a learned floral scent as were honeybees rewarded with sucrose.[10)] In humans, caffeine intake immediately after learning improves discrimination performance 24 h after study.[11)] The memory-enhancing effect was rarely observed when caffeine was administered before training, 30 min after training, or before test.[12,13)] Thus, the caffeine effect appears specific for the memory consolidation process.

Based on these observations, in the present work, we hypothesized that caffeine affects hippocampal SWs and thereby potentiates memory consolidation. Because endogenous adenosine is tonically released in *in vitro* hippocampal slice preparations even without apparent external stimulation and thereby regulates the excitability of hippocampal synaptic transmission,[14)] it is possible that caffeine alters the occurrence of SWs in hippocampal slices. To test this possibility, we bath-applied caffeine to mouse hippocampal slices that spontaneously generate SWs.[15)]

METHODS

Animal Ethics Animal experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo (approval number: P24–8) and according to the University of Tokyo guidelines for the care and use of laboratory animals. These experimental protocols were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71 of 2006), the Standards for Breeding and Housing of and Pain Alleviation for Experimental Animals (Ministry of the Environment, Notice No. 88 of 2006) and the Guidelines on the Method of Animal Disposal (Prime Minister's Office, Notice No. 40 of 1995). All animals were housed under a 12-h dark–light cycle (light from 07:00 to 19:00) at 22±1°C with *ad libitum* food and water.

Drugs Caffeine was dissolved at 60 mM in deionized distilled water (ddw) and stocked at 4°C. N^6-Cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and SCH58261were dissolved at 10 μM in 10% dimethyl sulfoxide (DMSO)/90% ddw and were stocked at 4°C. Immediately before use, they were diluted to the final concentrations with artificial cerebrospinal fluid (aCSF) containing (in mM): 126 NaCl, 3.5 KCl, 1.24 NaH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose.

Hippocampal Slice Preparations Experiments were performed using brain slices prepared acutely from 21-to-36-d-old C57BL/6J mice (SLC, Shizuoka, Japan). Mice were briefly anaesthetized with isoflurane, decapitated, and removed of the brain. The brain was horizontally sliced (400 μm thick) at an angle of 12.7° to the fronto-occipital axis using a vibratome in ice-cold oxygenated modified aCSF containing (in mM): 27 $NaHCO_3$, 1.5 NaH_2PO_4, 2.5 KCl, 0.5 Ascorbic acid, 1 $CaCl_2$, 7 $MgSO_4$, and 222.1 sucrose. Slices were transferred in oxygenated aCSF at 35°C in submerged chamber and were allowed to recover for at least 90 min.

Hippocampal Dentate-CA3 Mini-Slice Preparations

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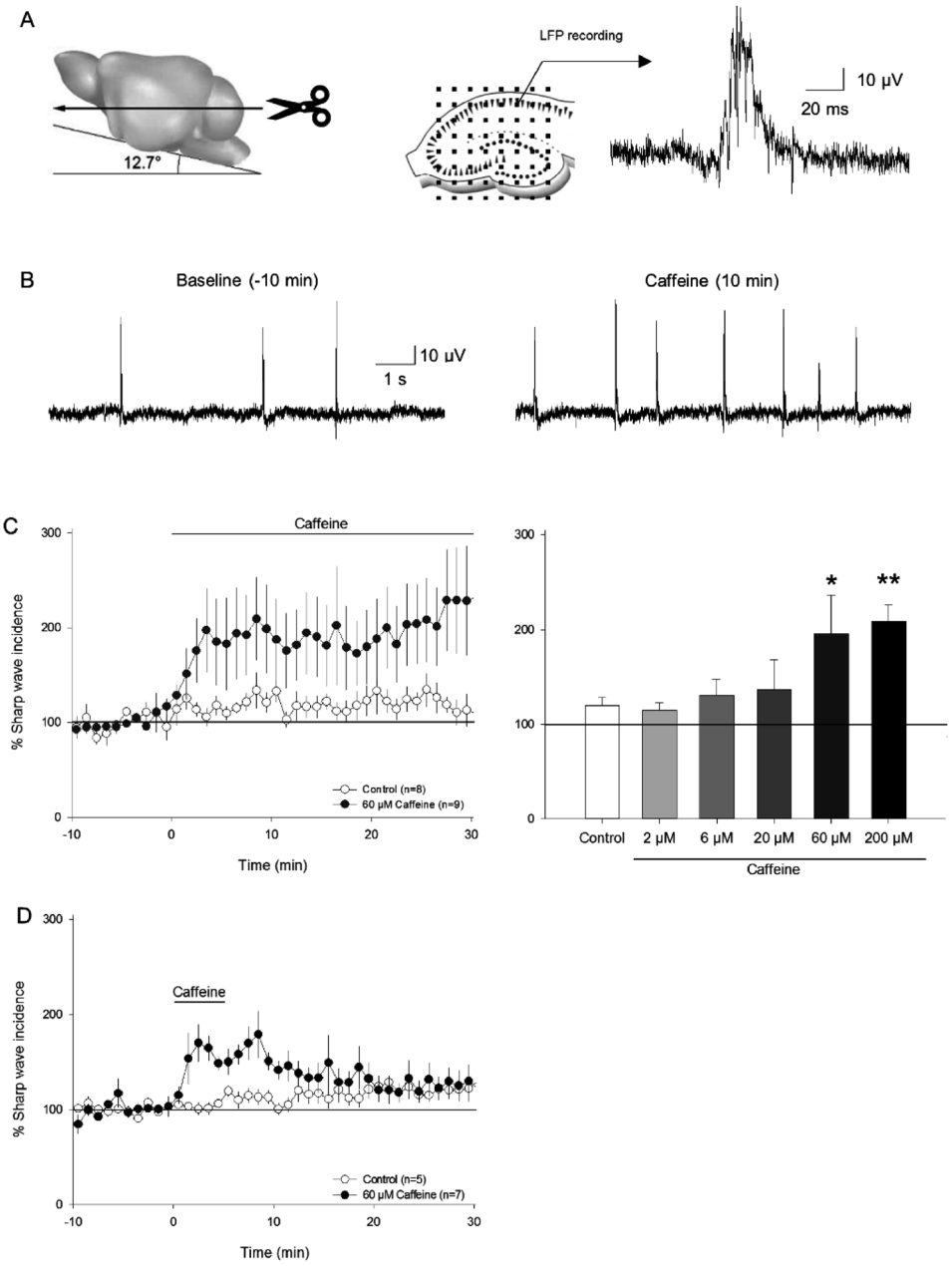

Fig. 1. Caffeine Reversibly Increases the Frequency of SWs *in Vitro*

(A) Schematic illustrations for hippocampal slices that spontaneously generate SWs. Left: the mouse brain was obliquely sliced. Right: a slice was placed on a 64-channel electrode array, and LFPs were recorded from one electrode on the CA1 pyramidal cell layer. (B) Representative LFP traces 10 min before (left) and 10 min after (right) the onset of bath application of 200 μM caffeine. (C) Summarized data of the caffeine effect on SWs. Left, the time course of the SW frequency after application of 60 μM caffeine. In control, slices were continuously perfused with normal aCSF. Right: the mean percentages of the SW frequencies during 5–30 min to the baseline was plotted against the concentrations of caffeine. $*p<0.05$, $**p<0.01$ *versus* baseline; paired *t*-test. Error bars represent S.E.M. of 5–9 slices. (D) Caffeine (60 μM) was applied for 5 min and washed out. The SW-increasing effect was reversible after washout of caffeine. Error bars represent S.E.M. of 5–7 slices.

Dentate-CA3 slices were prepared in a similar way to hippocampal slices, but the CA1 and CA2 regions in hippocampal slices were carefully removed under a stereoscopic microscope using a scalpel in ice-cold oxygenated modified aCSF immediately after slicing.

Electrophysiological Recording *in Vitro* Slices were mounted on 8×8 planar multi-electrode arrays (electrode size, 50×50 μm; inter-polar distance, 300 μm; Alpha MED Scientific MED-P530A) and were perfused at 1.8 mL/min with 95% O_2/5% CO_2-bubbled aCSF at 35°C. Spontaneous local field potentials (LFPs) were recorded at 10 kHz using the Alpha MED Scientific MED64 system.

Data Processing and Analysis Data were processed using custom-made routines in MATLAB (The MathWorks, Natick, MA, U.S.A.). SWs were detected at a threshold at 5×standard deviation (S.D.) above the baseline noise in 2-to-30-Hz band-pass-filtered traces and were visually inspected by eyes. We report data as mean±S.D. unless otherwise specified.

RESULTS AND DISCUSSION

Although standard hippocampal slice preparations do not reliably emit spontaneous SWs,[16,17] our previous studies demonstrated that obliquely sliced hippocampi reproducibly generate spontaneous SWs,[15,18] presumably because they preserve more intact intrinsic neuronal circuits critical for the initiation

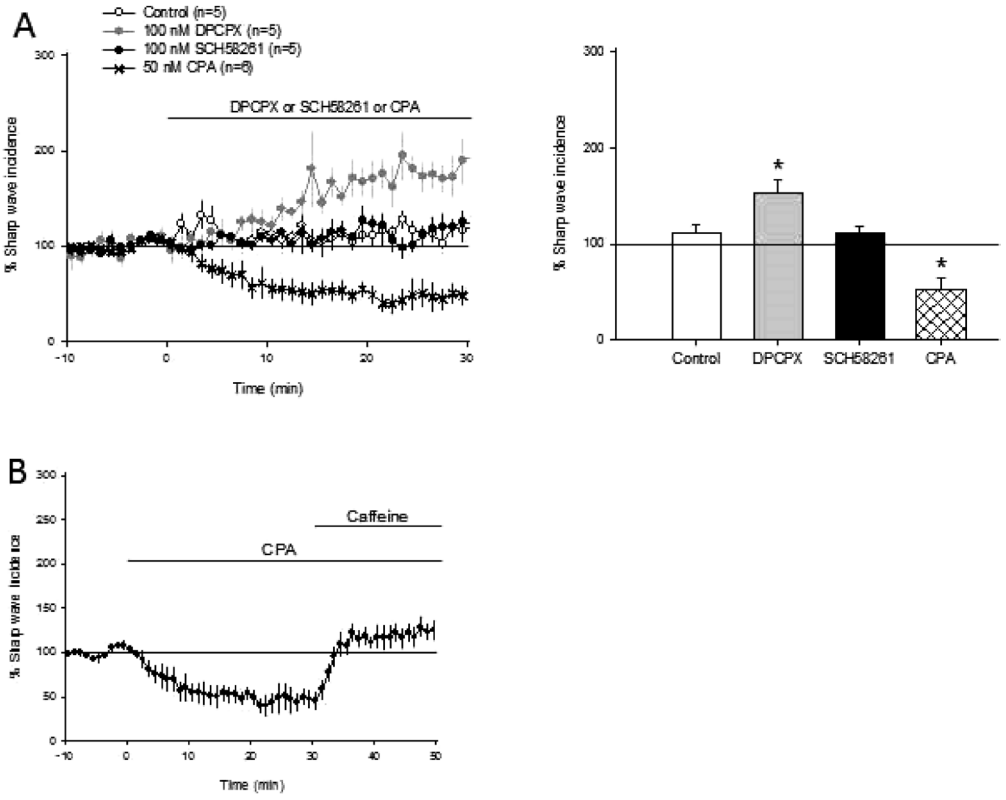

Fig. 2. Adenosine A_1 Receptor Antagonist Increases the SW Frequency

(A) Left: time course of SWs while 100 nM DPCPX, 100 nM SCH58261, 50 nM CPA were bath-applied at time 0. In control, slices were continuously perfused with 0.1% DMSO in normal aCSF. Right: the mean percentages of the SW frequencies during 5–30 min to the baseline was plotted for three experimental conditions. *$p<0.05$ *versus* baseline; paired *t*-test. Error bars represent S.E.M. of 5–6 slices. (B) Time course of SWs while 60 μM caffeine was added 30 min after bath-application of 50 nM CPA. Error bars represent S.E.M. of 6 slices.

and propagation of SWs. In the present study, we used oblique slices and examined the effect of caffeine on the spontaneously occurring SWs (Fig. 1A). The frequencies of SW events were 0.57±0.30 Hz in the CA3 region and 0.59±0.38 Hz in the CA1 region and did not differ between two regions ($p=0.70$, $t_{52}=0.38$, paired *t*-test, $n=53$ slices); however, CA3 SWs occurred earlier than CA1 SWs with the mean time lag of 9.5±8.6 ms of 11114 SW events. Therefore, SWs are likely to originate from the CA3 networks and reliably propagate to the CA1 network.

Caffeine was continuously bath-perfused to hippocampal acute slices while LFPs were recorded from the CA1 pyramidal cell layer (Figs. 1A, B). Note that the concentration of normally taken caffeine in the human brain under physiological conditions is estimated to reach tens of μM.[19–23] Within 5 min of perfusion with 60 μM caffeine, the incidence of SWs increased to approximately 200%, and the increase was stably maintained in the presence of caffeine (Fig. 1C left). We tested five different concentrations of caffeine, 2, 6, 20, 60, and 200 μM, and the mean frequencies of SWs for a period of 5–30 min during the caffeine application were calculated. The SW-increasing effect of caffeine depended on its concentrations (Fig. 1C right; 60 μM: $p=4.9\times10^{-2}$, $t_8=2.33$, paired *t*-test, $n=9$ slices from 9 mice; 200 μM: $p=8.7\times10^{-4}$, $t_6=6.13$, paired *t*-test, $n=7$ slices from 7 mice). To examine whether the effect of caffeine is reversible, we applied 60 μM caffeine for 5 min and washed out. The increased SW frequency was reduced to the baseline level within 20 min after wash-out (Fig. 1D; $n=7$ slices from 5 mice).

We next explored which subtype of receptors are required for the SW-increasing effect of caffeine. Caffeine non-selectively antagonizes adenosine receptors. In human, the K_D values of caffeine for individual receptors are reported to be 12 μM (adenosine A_1 receptor), 2.4 μM (A_{2A}), 13 μM (A_{2B}), and 80 μM (A_3).[24] Therefore, either subtype could be candidate for our caffeine effect. However, the major forms of the adenosine receptors in the brain are A_1 and A_{2A} receptors.[23] Thus, we examined the A_1 receptor-selective antagonist DPCPX ($K_D=3.9$ nM in human) and the A_{2A} receptor-selective antagonist SCH58261 ($K_D=0.6$ nM in human).[25] Likewise caffeine, 100 nM DPCPX increased the incidence of SWs (Fig. 2A; $p=1.5\times10^{-2}$, $t_4=4.10$, paired *t*-test, $n=5$ slices from 5 mice), whereas 100 nM SCH58261 did not ($p=0.22$, $t_4=1.46$, paired *t*-test, $n=5$ slices from 5 mice). Moreover, 50 nM CPA, an A_1 receptor-selective agonist, alone reduced the SW event frequency (Fig. 2A; $p=1.0\times10^{-2}$, $t_5=4.04$, paired *t*-test, $n=6$ slices from 2 mice), an effect that was reverted by addition of 60 μM caffeine (Fig. 2B; $n=6$ slices from 2 mice). These results suggest that the effect of caffeine is mediated mainly by adenosine A_1 receptor inhibition.

Adenosine A_1 receptors are highly expressed in the CA2 region,[26] and caffeine is more likely to induce synaptic plasticity in the CA2 region, compared to CA1 or CA3 regions.[27] Because bidirectional projections exist between the CA2 and CA3 regions,[28] it is possible that caffeine acts on adenosine A_1 receptors in the CA2 region and thereby increases hippocampal SWs. To address this possibility, we surgically removed the CA2 and CA1 regions from hippocampal slices

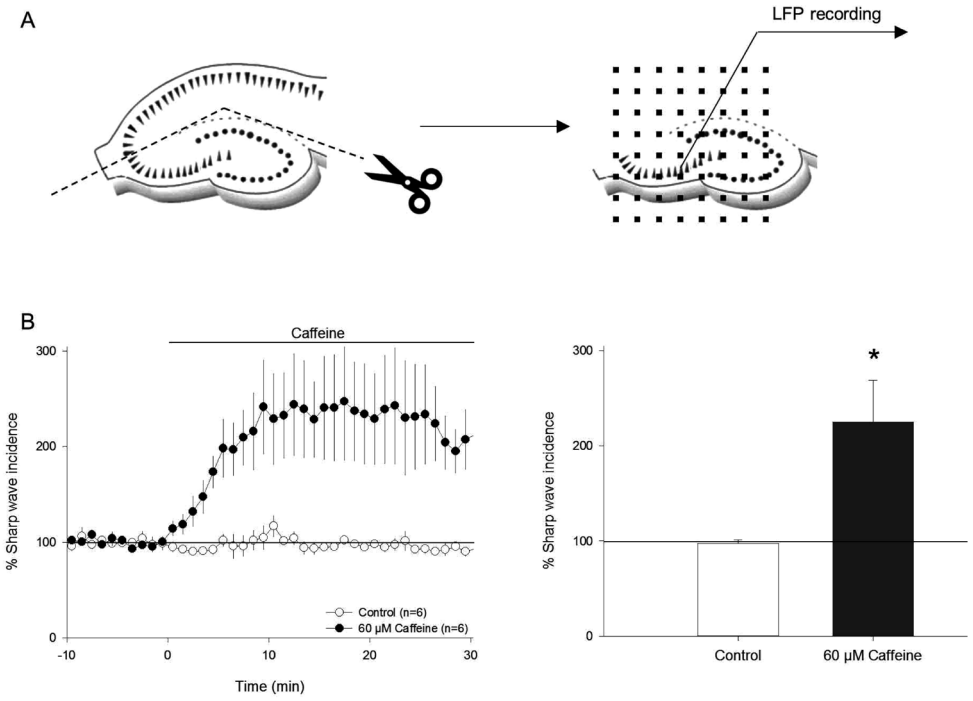

Fig. 3. Caffeine Increases the SW Frequency in Isolated Dentate-CA3 Mini-Slices

(A) The CA1 and CA2 regions were removed from a hippocampal slice (left), and LFPs were recorded from the CA3 pyramidal cell layer. (B) Caffeine increases the SW frequency in dentate-CA3 mini-slices. Left, the time course of the SW frequency after application of 60 μM caffeine. In control experiments, slices were continuously perfused with normal aCSF. Right: the mean percentages of the SW frequencies during 5–30 min to the baseline was plotted for both conditions. *$p<0.05$ *versus* baseline; paired *t*-test. Error bars represent S.E.M. of 5 slices.

using a scalpel and prepared dentate-CA3 mini-slices (Fig. 3A). When 60 μM caffeine was bath-applied to the mini-slices, SWs still increased in frequency (Fig. 3B; $p=3.6\times10^{-2}$, $t_4=2.835$, paired *t*-test, $n=5$ slices from 3 mice). The CA2 region is dispensable for the SW-increasing effect of caffeine.

Our study showed that caffeine reversibly increases SWs in acute hippocampal slices in a dose-dependent manner. This effect was likely mediated *via* adenosine A_1 receptors inhibition in either the CA3 region or the dentate gyrus. The increased SWs may mediate the facilitatory effect of post-study caffeine on memory consolidation.

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Conflict of Interest The authors declare no conflict of interest.

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