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Changes in the spontaneous calcium oscillations for the development of the preconditioning-induced ischemic tolerance in neuron/astrocyte co-culture

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Running title: calcium oscillations and ischemic tolerance

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Abstract

Spontaneous Ca^{2+} oscillations are believed to contribute to the regulation of gene expression. Here we investigated whether and how the dynamics of Ca^{2+} oscillations changed after sublethal preconditioning (PC) for PC-induced ischemic tolerance in neuron/astrocyte co-cultures. The frequency of spontaneous Ca^{2+} oscillations significantly decreased between 4 and 8 h after the end of PC in both neurons and astrocytes. Treatment with 2-APB, an inhibitor of IP3 receptors, decreased the oscillatory frequency, induced ischemic tolerance and a down-regulation of glutamate transporter GLT-1 contributing to the increase in the extracellular glutamate during ischemia. The expression of GLT-1 is known to be up-regulated by PACAP. Treatment with PACAP38 increased the oscillatory frequency, and antagonized both the PC-induced down-regulation of GLT-1 and ischemic tolerance. These results suggested that the PC suppressed the spontaneous Ca^{2+} oscillations regulating the gene expressions of various proteins, especially of astrocytic GLT-1, for the development of the PC-induced ischemic tolerance.

Key words: spontaneous Ca²⁺ oscillations, ischemic tolerance, GLT-1, PACAP, oxygen and glucose deprivation, neuron/astrocyte co-cultures.

Abbreviations

PC	Preconditioning
CM	Conditioned medium from astrocytes cultured for more than 2 weeks
GFAP	Glial fibrillary acidic protein
OGD	Oxygen-glucose deprivation
NADH	Nicotinamide adenine dinucleotide
2-APB	2-Aminoethyl diphenylborinate
IP3	Inositol 1, 4, 5-trisphosphate
PACAP	Pituitary Adenylate Cyclase Activating Polypeptide

INTRODUCTION

Spontaneous oscillations of the intracellular concentration of calcium (Ca^{2+} oscillations) are a signaling system in cells. The oscillations play functional roles in neuronal migration and development, axonal and dendritic growth, and the formation of the neuronal network [1-5]. In addition, recent studies have reported that frequency is more important than amplitude, and the frequency of Ca^{2+} oscillations regulates the efficiency of the gene expression of specific proteins [6-8]. Previous studies have revealed that the oscillatory frequency is regulated by neurotransmitters, growth factors, and cytokines [9-11], suggesting that the intracellular Ca^{2+} oscillations are influenced by the external environment, and have the role of adapting cells to the external environment.

Ischemic tolerance is the phenomenon whereby a brief sub-lethal ischemia (preconditioning, PC) provides protection against the neuronal degeneration caused by a subsequent prolonged lethal ischemia [12-15]. Previous studies have demonstrated that the ischemic tolerance is induced about 24 h after the PC *in vitro* [14, 15] or after about 3 d *in vivo* [13], and

the PC is believed to induce ischemic tolerance via the regulation of various genes and proteins important for neuronal protection [16-19]. The regulation of intracellular free Ca^{2+} has been intensively studied as a key factor in the induction of ischemic neuronal cell death or in the development of PC-induced ischemic tolerance. In addition, a number of studies have been performed on the dynamics of intracellular Ca^{2+} oscillations during either lethal ischemia or PC, but have reported contradicting results; some studies reported that the changes contribute to neurodegeneration [20-21], others that they contribute to neuroprotection [22-24]. And, the relationship between the changes in the dynamics of intracellular Ca^{2+} oscillations during the period between PC and lethal ischemia and the development of ischemic tolerance has not been studied in detail. There is a possibility that the changes in Ca^{2+} oscillation-regulated gene expression during the period between PC and lethal ischemia contributes to the development of ischemic tolerance.

Here we provide experimental evidence that PC-induced changes in spontaneous Ca^{2+} oscillations in neurons and/or astrocytes are crucially involved in the development of PC-induced neuronal ischemic tolerance

by transiently down-regulating the expression of the astrocytic glutamate transporter GLT-1 in neuron/astrocyte co-cultures.

MATERIALS AND METHODS

Cell Culture

Culture methods were described previously in detail [25-27]. In brief, neurons/astrocytes were prepared from 17-day-old embryonic rat cortices, digested with 0.02 % papain (Boehringer, Germany) and mechanically dissociated, and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% Ham's F12, and 0.24% penicillin/streptomycin (culture medium). Cells were plated at a uniform density of 3.0×10^4 cells/cm² onto poly-L-lysine (100 µg/ml) and Laminin (20 µg/ml)-coated plastic dishes and maintained in a 5% CO₂ incubator at 37 °C. The cultures were fed a filtered (0.22 µm; Millipore, Bedford, TX) conditioned culture medium (CM) twice a week. After 13-15 days, the neurons in these cultures sit on the top of a confluent monolayer of astrocytes. The experiments were performed using these cultures.

To obtain the CM [28], cells from the 17-day old embryonic rat cortices were plated onto 2% polyethyleneimine-coated 6-well dishes.

The cultures were then fed a cooled culture medium and incubated for an additional day. The cultures became astrocyte-enriched with few neurons after more than 2 weeks. The CM for neuron/astrocyte co-cultures was conditioned by such cultures.

Intracellular Ca²⁺ measurements and identification of astrocytes

Intracellular Ca²⁺ was measured in both neurons and astrocytes after loading the cells with the Ca²⁺-sensitive fluorescent dye Fluo4-AM. Cell loading was performed using 5 μM of Fluo4-AM in conditioned medium supplemented with 0.03% Pluronic F-127 for 50-60 min at 37 °C. Intracellular Ca²⁺ measurements were performed in a balanced salt solution (142 mM NaCl, 0.8 mM MgSO₄, 5.4 mM KCl, 1.0 mM NaH₂PO₄, 4 mM NaHCO₃, 1.8 mM CaCl₂, 10 mM HEPES, 0.01 mM glycine, and 20 mM glucose). The fluorescence was excited at 480 nm and detected at > 510 nm with a fluorescence microscope (Olympus, IX70). Images were acquired with integration times of 400 ms at intervals of 4 s and analyzed off-line (HAMAMATSU, Aquacosmos). The change in intracellular Ca²⁺ was estimated from the ratio of the fluorescence (F/F₀). Ca²⁺ spikes were

defined as rapid elevations of $\Delta F/F_0 \geq 0.1$ or 0.2 . Although this threshold produced false positives in some cells in which noise base-line values were scored as transients, as determined by visual inspection, but in most cases this criterion represented Ca^{2+} spike correctly.

To distinguish them from neurons in the analysis of calcium oscillations, astrocytes were identified by immunostaining with antibodies against glial fibrillary acidic protein (GFAP, Sigma, 1:5000) after intracellular Ca^{2+} measurements. For labeling, a 1:200 dilution of Alexa Fluor 532 goat anti-mouse IgG (Molecular Probes) was used. The fluorescence was excited at 530 nm and detected at > 580 nm with a fluorescence microscope. We distinguished neurons and astrocytes by the images of Fluo-4 and GFAP in the same regions in the co-cultures (Fig. 1A2 and A3).

Oxygen-Glucose deprivation

Cortical cultures were subjected to oxygen-glucose deprivation (OGD) injury using a protocol described previously [26, 29, 30]. In brief, cultures were placed in an anaerobic chamber containing the

deoxygenation reagent and glucose-free balanced salt solution (BSS: 116 mM NaCl, 0.8 mM MgSO₄, 5.4 mM KCl, 1.0 mM NaH₂PO₄, 26.2 mM NaHCO₃, 1.8 mM CaCl₂, and 0.01 mM glycine) was added. Near anoxic conditions were achieved using an Anaero-Pack System (Mitsubishi Gas Chemical, Tokyo, Japan). To terminate OGD, cultures were carefully washed with glucose (20 mM) containing BSS, and then incubated again in culture medium at 37 °C in 95% air-5% CO₂ (reperfusion). Cultures with sham treatment not deprived of oxygen and glucose were placed in BSS containing 20 mM glucose.

Measurement of extracellular glutamate

Extracellular glutamate was measured using an enzymatic assay [31, 32]. In the presence of glutamate and β -nicotinamide adenine dinucleotide (NAD⁺), L-glutamic dehydrogenase (GDH) produces α -ketoglutarate and NADH, a product that fluoresces when excited at 360 nm. In the presence of α -ketoglutarate and L-Alanine, glutamate pyruvate transaminase (GPT) produces L-glutamate. Therefore, GDH (50 U/mL), NAD⁺ (2 mM), GPT (4 U/mL), and L-Alanine (2 mM) were

added to the external solution, and the fluorescence was excited at 360 nm and detected at > 510 nm with a fluorescence microscope. Images were acquired with integration times of 5 s at intervals of 20 s. The extracellular glutamate was detected as an increase in NADH fluorescence.

Western blot analysis

Cell lysates were diluted 3:1 in sample buffer (187.5 mM Tris-HCl containing 6% SDS, and 15% glycerol, 15% 2-mercaptoethanol) and denatured at 100 °C for 3 min. Proteins were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Nonspecific binding sites were blocked with 5% nonfat milk for 60 min, and then the membrane was incubated overnight at 4 °C with the following antibodies: polyclonal antibody to GLT-1 (1:10000), and monoclonal antibody to actin (1:2000) (Sigma), and then horseradish peroxidase-labeled anti-guinea pig (1:2000) (Cell Signaling Technology) or anti- mouse IgG antibody for actin (1:2000) (Cell Signaling Technology), respectively. The immunoreactive

bands were detected with an enhanced chemiluminescence kit (NEN Life Science Products, Boston, MA). Quantification of the levels was performed by densitometric analysis using Scion Image Beta 4.02 (Scion Corporation).

Survival rate of neurons

Neuronal death was analyzed following observation of the nuclear morphology by using the fluorescent DNA-binding dyes, Hoechst 33342 (H33342) and propidium iodide (PI). Cells were incubated with these dyes for 15 minutes at 37 °C. Individual nuclei were observed using a fluorescence microscope (Olympus, IX70) and subsequently analyzed. PI was used to identify nonviable cells. More specifically, an average of 450-500 neurons from fields chosen at random were analyzed in each experiment. The survival rate of neurons — meaning the percentage of viable neurons remaining — was determined by placing images of nuclear staining on phase-contrast images, and calculated as $(\text{viable neurons}/\text{total neurons before drug treatment}) \times 100$, since some neurons came off the dishes at the time of inspection. At least 4 independent experiments ($n \geq$

4) were conducted.

Chemicals

Forskolin, 2-Aminoethyl diphenylborinate, Pluronic F-127, NAD⁺, GDH, L-Alanine, GPT, Hoechst 33342 and propidium iodide were obtained from Sigma. Pituitary Adenylate Cyclase Activating Polypeptide 6-38 (Human) and Pituitary Adenylate Cyclase Activating Polypeptide 38 (Human) were obtained from Peptide Institute (Osaka, Japan). Fluo-4 AM was purchased from Molecular Probes. All other compounds were obtained from Wako Chemical (Tokyo, Japan).

Statistics

The data are expressed as the mean±S.D. Comparisons were made using the one-way analysis of variance (ANOVA) followed by a paired t-Test. A *P* value of less than 0.01 or 0.05 was considered statistically significant.

RESULTS

We investigated whether oscillations in the intracellular concentration of calcium (Ca^{2+} oscillations) in neurons and/or astrocytes have any functional role in the development of preconditioning-induced neuronal ischemic tolerance in mixed cultures of neurons and astrocytes from fetal rat brain (embryonic day 17). Our recent studies have demonstrated that sublethal oxygen-glucose deprivation (OGD) for 60 min induces neuronal ischemic tolerance against subsequent lethal OGD for 90 min in neuron/astrocyte co-cultures [26, 27]. Neuronal ischemic tolerance was observed when the time between the PC and lethal OGD was 20-24 h, but not when the interval was 6 h or 48 h [26]. Therefore, we first investigated whether the dynamics of Ca^{2+} oscillations changed during reperfusion 20 h after the end of PC.

Neurons and astrocytes were distinguished by GFAP staining after intracellular Ca^{2+} measurements (Figs. 1A2 and 1A3; see Materials and Methods). Spontaneous Ca^{2+} oscillations were observed in both neurons and astrocytes in our co-cultures (Fig. 1B1 and 1C1). About 50% of the cultured neurons, and about 15% of cultured astrocytes located close to or

under the aggregates of neurons, showed Ca^{2+} oscillations. At 4 h after the end of sublethal OGD (PC), the frequency of the spontaneous Ca^{2+} oscillations significantly decreased as compared with cells without PC (control) in both neurons and astrocytes (Fig. 1B2, 1B3, 1C2, and 1C3). The frequency of the oscillations then gradually increased, and had almost returned to the control level at 20 h after the PC in neurons and astrocytes (Fig. 1B3 and 1C3). The question then arises as to whether the transient decrease in the frequency of Ca^{2+} oscillations after the PC insult has anything to do with the development of the PC-induced ischemic tolerance of neurons.

We therefore investigated whether the forced suppression of spontaneous Ca^{2+} oscillations without the PC treatment before the onset of lethal OGD induced neuronal ischemic tolerance. Previous studies have suggested that the inositol 1, 4, 5-trisphosphate (IP3) receptors are involved in the generation of Ca^{2+} oscillations [8, 33], and the treatment of cells with 2-APB, an inhibitor of IP3 receptors, suppresses the oscillations [33]. The other chemicals that directly modulate the dynamics of intracellular Ca^{2+} oscillations, such as Ca^{2+} chelators and Ca^{2+} ionophore,

would affect the basal level in intracellular Ca^{2+} concentration rather than the frequency of Ca^{2+} oscillations. We thus used 2-APB to suppress the spontaneous Ca^{2+} oscillations in our cultured cells. Treatment of cultures with 2-APB (100 μM) didn't induce marked change in the base-line of the concentrations of intracellular free Ca^{2+} , but significantly suppressed the spontaneous Ca^{2+} oscillations in both neurons and astrocytes in the cultures without PC (Fig. 2A and 2B). In addition, to clarify whether the 2-APB-induced suppression of Ca^{2+} oscillations contributed to the ischemic tolerance of neurons, the co-cultures were incubated in conditioned culture medium (CM) containing 2-APB (100 μM) for 12 h before the onset of lethal OGD and then in CM without 2-APB for 8 h instead of being subjected to the PC treatment. Treatment with 2-APB significantly reduced neuronal cell death induced by lethal OGD (Fig. 2C). The survival rate of neurons with 2-APB treatment was almost the same as that with the prior PC insult.

We next investigated whether the inhibition of the PC-induced suppression of spontaneous Ca^{2+} oscillations reversed the PC-induced ischemic tolerance. A previous study [10] has suggested that treatment

with forskolin, an activator of adenylate cyclase, increases the frequency of spontaneous Ca^{2+} oscillations. Although the frequency of spontaneous Ca^{2+} oscillations significantly decreased at 4 h after the end of the PC, treatment of cultures with forskolin (50 μM) at 4 h after the end of the PC significantly increased the frequency oscillations in both neurons and astrocytes (Fig. 3A and 3B). In addition, we incubated the cultures in CM containing forskolin (50 μM) for 12 h after the end of PC, and then in CM without the drug for 8 h after that. Treatment with forskolin significantly reversed the PC-induced ischemic tolerance of neurons (Fig. 3C). These results suggested that the increase in the oscillatory frequency before the onset of lethal OGD decreased the neuronal resistance to lethal OGD.

Our recent study [29] has demonstrated that the rise in the extracellular concentration of glutamate during OGD was mainly caused by a reversal in the operation of the astrocytic glutamate transporter GLT-1 in neuron/astrocyte co-cultures. In addition, the PC-induced transient decrease in the expression of astrocytic GLT-1 contributes both to the suppression of the rise in the extracellular concentration of glutamate

during ischemia, and to the development of PC-induced ischemic tolerance. We therefore investigated whether the suppression of the spontaneous Ca^{2+} oscillations by 2-APB decreased the expression of astrocytic GLT-1 and the rise in the extracellular concentration of glutamate during ischemia. Figure 4A shows the time-course of the change in the extracellular concentration of glutamate during OGD in cultures treated with or without 2-APB (100 μM) for 12 h before the onset of OGD and then in CM without 2-APB for 8 h. OGD resulted in a marked rise in the concentration, but the elevation during OGD was significantly suppressed in co-cultures treated with 2-APB before the onset of lethal OGD (Fig. 4A and 4B). In addition, Western blotting showed that treatment with 2-APB for 12 h and then in CM without 2-APB for 8 h significantly reduced the expression level of GLT-1 (Fig. 4C and 4D), but did not reduce that of GLAST (Fig. 4E). These results suggested that the suppression of the spontaneous Ca^{2+} oscillations by 2-APB induced a down-regulation of GLT-1 expression contributing to PC-induced neuronal ischemic tolerance.

We next investigated possible factors that regulate the dynamics of

spontaneous Ca^{2+} oscillations after the sublethal PC. Previous studies [34, 35] have suggested that pituitary adenylate cyclase activating polypeptide (PACAP), increases GLT-1 protein levels via the activation of PAC1 receptors in rat glial cultures. Our recent study [29] has demonstrated a possibility that PC insult suppresses the release of the neuron-derived factors up-regulating GLT-1 and that the down-regulation of GLT-1 contributes to the development of PC-induced ischemic tolerance. These findings have led to a hypothesis that PACAP would contribute to the regulation of astrocytic GLT-1 and the development of ischemic tolerance via the changes in the dynamics of Ca^{2+} oscillations regulating the expression of various genes. In addition, it has been suggested that both neurons and astrocytes express PAC1 receptors [36]. Therefore, there is a possibility that PACAP contributes to the regulation of spontaneous Ca^{2+} oscillations in both astrocytes and neurons. We next investigated whether PACAP was involved in the PC-induced changes in the dynamics of spontaneous Ca^{2+} oscillations and in the PC-induced neuronal ischemic tolerance. Treatment of co-cultures with PACAP6-38 (1 μM), an inhibitor of PAC1 receptors [34], significantly suppressed the

spontaneous Ca^{2+} oscillations in both neurons and astrocytes in the co-cultures without PC (Fig. 5A and 5B). Incubation of the cultures in conditioned medium (CM) containing PACAP6-38 (1 μM) for 12 h before the onset of lethal OGD and then in CM without PACAP6-38 for 8 h resulted in a significant reduction in the neuronal cell death induced by lethal OGD (Fig. 5C), and the expression level of GLT-1 (Fig. 5D and 5E). In contrast, although the frequency of spontaneous Ca^{2+} oscillations significantly decreased at 4 h after the end of the PC, treatment of cultures with PACAP38 (100 nM) at 4 h after the sublethal PC insult significantly increased the frequency of spontaneous Ca^{2+} oscillations in the neurons and astrocytes (Fig. 6A and 6B). Incubation of the cultures in CM containing PACAP38 (100 nM) for 12 h after the end of PC and then in CM without PACAP38 for 8 h resulted in a significant decrease in the survival rate of neurons exposed to lethal OGD; that is, in the suppression of the PC-induced neuronal ischemic tolerance (Fig. 6C). In addition, the PC significantly lowered GLT-1 protein levels, but treatment with PACAP38 antagonized the PC-induced down-regulation of GLT-1 expression (Fig. 6D and 6E).

Above findings suggested that treatment with PACAP38 antagonized PC-induced ischemic tolerance via PACAP-induced increase in Ca^{2+} oscillatory frequency. However, there remains a possibility that the PACAP-induced increase in Ca^{2+} oscillatory frequency does not directly contribute to the antagonization of PC-induced ischemic tolerance, since PACAP is known to be a factor activating a variety of intracellular signal transduction pathways. We finally investigated whether the attenuation of PC-induced ischemic tolerance by treatment with PACAP38 was recovered by co-treatment with 2-APB. Co-treatment of co-cultures with 2-APB (100 μM) significantly suppressed the PACAP38 (100 nM)-induced increase in Ca^{2+} oscillatory frequency in both neurons and astrocytes (Fig. 7A and 7B). Incubation of the cultures in CM containing both PACAP38 (100 nM) and 2-APB (100 μM) for 12 h after the end of PC and then in CM without them for 8 h significantly reversed the PACAP38-induced reduction in the survival rate of neurons (Fig. 7C). These results supported our inference that the PACAP38-induced significant attenuation of the PC-induced ischemic tolerance was mainly caused by the PACAP38-induced increase in Ca^{2+} oscillatory frequency.

DISCUSSION

This study showed that the frequency of spontaneous oscillations in the intracellular concentration of calcium (Ca^{2+} oscillations) significantly decreased 4-8 h after the end of sublethal preconditioning (PC), and the decrease contributed to the development of ischemic tolerance in neurons 20-24 h after the PC. Previous studies have reported that the rise in the intracellular concentration of calcium was caused by a rise in the extracellular concentration of glutamate during PC, leading to the activation of calcium-dependent enzymes for the development of ischemic tolerance [22-24]. The present results, however, suggested that the changes in the dynamics of Ca^{2+} oscillations after the PC insult were crucial to the development of neuronal ischemic tolerance. In addition, here we provide experimental evidence suggesting that the changes in spontaneous Ca^{2+} oscillations in neurons and/or glia are involved in regulating the expression of some proteins, especially of astrocytic GLT-1 for the survival of neurons. Our recent study [29] and the other previous studies [37, 38] have reported that the decrease in the expression or the inhibition of astrocytic GLT-1 contributes to the suppression of the rise in

the extracellular concentration of glutamate during ischemia, and to the protection to the ischemia-induced neuronal cell death. The present study suggested that the transient down-regulation of GLT-1 caused by the suppression of spontaneous Ca^{2+} oscillations after PC was one of the mechanisms for PC-induced ischemic tolerance.

The present study demonstrated that the frequency of spontaneous Ca^{2+} oscillations was suppressed at 4 and 8 h after the PC insult in both neurons and astrocytes, and the suppression contributed to the development of ischemic tolerance obtained 20 h after the PC. We also investigated the spontaneous calcium oscillations during the period 12 - 20 h after the PC. The frequency at 14 h after the PC almost recovered to the control level, but that at 18 h significantly increased (data not shown). However, the inhibition of the PC-induced increase in frequency at 18 h by 2-APB did not attenuate the PC-induced neuronal ischemic tolerance obtained at 20-24 h after the PC (data not shown). Although PC-induced ischemic tolerance is maintained for several hours *in vitro* and a few days *in vivo*, it disappears after that [13-15]. A previous study has demonstrated that the PC-induced tolerance in our co-cultures disappears

at 48 h after the end of PC [26], and the PC-induced reduction in the level of astrocytic GLT-1 also recovers 48 h after the PC, suggesting a close relationship between the PC-induced change in GLT-1 expression and that in ischemic tolerance [29]. Therefore, the PC-induced rebound-like increase in the oscillatory frequency at 18 h after the PC may contribute to the recovery process, antagonizing the PC-induced temporal reduction in the expression of astrocytic GLT-1. In fact, the level of astrocytic GLT-1 had almost returned to that before the PC at 48 h after the insult [29]. In addition, the PC-induced ischemic tolerance was also attenuated significantly in association with the recovery of GLT-1 expression. The ischemic tolerance was observed at 20 h after the PC, when the PC-induced reduction in the frequency of Ca^{2+} oscillations was already reversed in both neurons and astrocytes (Fig. 1B3 and C3). There may be a considerable time delay linking the changes in the oscillatory frequency with those in the expression of proteins such as astrocytic GLT-1. This is probably why neuronal ischemic tolerance was still observed at 20 h after the PC.

The present study suggested that the PC-induced down-regulation of

astrocytic GLT-1 and the resultant suppression in the rise in the concentration of extracellular glutamate during lethal ischemia were considered as the primary cause responsible for the development of PC-induced neuronal ischemic tolerance. However, there is a possibility that a sublethal PC would decrease the glutamate sensitivity in neurons, contributing to the PC-induced ischemic tolerance. Our previous study, however, has revealed that neurons rather become vulnerable to glutamate-mediated toxicity by the prior PC insult, probably caused by the PC-induced down-regulation of astrocytic GLT-1 leading to the reduction in the clearance capability of extracellular glutamate, suggesting that PC does not decrease the neuronal glutamate sensitivity at least in our co-cultures [29]. Taken together, our results suggested that the PC-induced down-regulation of astrocytic GLT-1 and the resultant suppression in the rise in the concentration of extracellular glutamate during lethal ischemia was critical to the development of PC-induced ischemic tolerance for neurons. However, under normal conditions, astrocytic GLT-1 plays a crucial role in the protection of neurons from glutamate-mediated toxicity. Therefore, it seems pathophysiologically

meaningful for neurons that the PC-induced down-regulation of astrocytic GLT-1 expression is a transient phenomenon [29].

The present results demonstrated that the suppression of Ca^{2+} oscillations by treatment with either 2-APB (Fig. 4) or PACAP6-38 (Fig. 6) decreased the protein level of GLT-1. Then the question arises as to how Ca^{2+} oscillations participate regulating the expression of GLT-1. One possibility is the participation of a transcription factor, NF- κ B. NF- κ B is known to be one of the factors regulating the expression of GLT-1, and its activation leads to the up-regulation of GLT-1 expression [39, 40]. Dolmetsch et al. [7] reported that a decrease in Ca^{2+} oscillatory frequency reduced the gene expression induced by the activation of NF- κ B, suggesting that the decrease in oscillatory frequency results in the expression of astrocytic GLT-1 being down-regulated.

Our previous study has reported that the neuronal nitric oxide synthase (nNOS) derived NO produced during PC contributes to PC-induced down-regulation of GLT-1 and ischemic tolerance [41]. In the present study, there is no direct evidence that links NO production during PC and the suppression of Ca^{2+} oscillations after PC. However,

some previous studies have reported the relationship between the regulation of Ca^{2+} oscillations or IP₃ receptors and NO production. For example, the study in intact rat megakaryocytes reported that NO-cGMP inhibited IP₃-induced Ca^{2+} release [42]. In addition, the study in neuronal nuclei of newborn piglets concluded that hypoxia resulted in increased functions for IP₃ receptors by NO, and the modification of IP₃ receptors may contribute to programmed cell death [43]. Thus, NO is involved in both physiological and pathological events, and is believed to play different roles in both lethal and sub-lethal events. Our present report demonstrated a possibility that NO produced during PC might affect the dynamics of Ca^{2+} oscillations after PC for PC-induced ischemic tolerance.

The protective role of astrocytic GLT-1 during ischemia is controversial. The operation of GLT-1 depends on the extracellular environment. Under the normal condition, astrocytic GLT-1 removes extracellular glutamate, and contributes to the protection against neurotoxic damage by glutamate. However under ischemic condition, astrocytic GLT-1 releases glutamate to extracellular space via the

reverse-operation of GLT-1, and contributes to the induction of neurotoxic damage by glutamate. The studies about ischemia have been performed using *in vitro* [37, 44, 45] or *in vivo* [38, 46, 47] models, being different from the technique to induce ischemia, such as global or focal, and brain region, and these studies have reported inconsistent results [46, 47]. The changes in the extracellular environment during ischemia strongly depend on such ischemic models, and affect the function of glutamate transporters during ischemia. This point needs to be further investigated.

Previous studies have reported that several genes and thus proteins are involved in PC-induced neuronal ischemic tolerance. For some of the proteins, levels decrease [16, 17, 29], but for others, levels increase following the PC insult [18, 19]. For example, the expression of Bcl-2, one of the anti-apoptosis proteins, increases on the activation of cyclic AMP-responsive element binding protein (CREB) after PC, and possibly contributes to the development of ischemic tolerance [18]. The present results couldn't explain all of the PC-induced changes in the expression of proteins. However, here we show that the PC-induced suppression of Ca^{2+} oscillations in neurons and/or astrocytes leading to a reduction in the

expression of astrocytic GLT-1 was crucial to the development of PC-induced ischemic tolerance in neurons. Further study will be needed to clarify the precise mechanisms involved.

Previous studies have reported that PACAP induces neuroprotective effects through the phosphorylation of extracellular signal-regulated kinase (ERK); that is, mitogen-activated protein (MAP) kinase [48, 49, 50]. PACAP is also involved in the inhibition of Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38, leading to anti-apoptotic effects under ischemic conditions [50]. The present study, however, demonstrated that in PACAP38-treated co-cultures, the PC-induced ischemic tolerance was significantly attenuated. In contrast, inhibition of the activity of PACAP receptors induced neuroprotective effects against OGD. Unlike previous reports, the present study indicated that neither the PACAP-induced activation of neuroprotective kinases such as a MAP kinase nor the PACAP-induced inhibition of apoptosis-inducing kinases such as SAPK had a primary role in the PC-induced increase in the ischemic tolerance of neurons.

Previous studies have reported that neuronal and astrocytic Ca^{2+}

oscillations are regulated via extracellular messengers [9, 11]. In fact, in our co-cultures, neurons and astrocytes showed synchronized Ca^{2+} oscillations in most of the experiments. It remains unclear whether the PC-induced changes in neuronal Ca^{2+} oscillations cause the changes in astrocytic oscillations or vice versa. We thus tried to clarify this issue using either PACAP, a neuron-derived peptide, which regulates the expression of astrocytic GLT-1, or an inhibitor of PACAP receptors. However, both PACAP38 and PACAP6-38 affected the calcium oscillations in neurons and in astrocytes. Not only neurons but also astrocytes have PACAP receptors [36]. In addition, the expression of PACAP has been confirmed in both neurons [51, 52] and cultured astrocytes although PACAP is expressed in astrocytes only from aged P0 rats [53]. Our previous paper, however, reported that the decrease in the release of PACAP from neurons is possibly involved in the PC-induced down-regulation of astrocytic GLT-1 expression [29]. A number of studies have also reported that the expression of astrocytic GLT-1 needs the existence of neurons [34, 54, 55]. Therefore, these results supported that the Ca^{2+} oscillation-regulated controlled release of PACAP from

neurons rather than from astrocytes would play an essential role in the regulation of the expression of astrocytic GLT-1. More studies are needed to test this possibility.

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Figure legends

Fig.1.

Changes in the spontaneous calcium oscillations in neurons and astrocytes after the end of the preconditioning (PC) insult. Figures A1, A2, and A3 show a representative photomicrograph, a fluorescent image using Fluo4, and the fluorescent labeling of GFAP in the same region of a co-culture, respectively. B1-B3: Representative spontaneous calcium oscillations observed in a neuron in the cultures without PC (control, Cont.) (B1), at 4 h after the PC (B2), and a bar graph showing the average frequency of calcium oscillations of neurons for 15 min (B3). C1-C3: Representative spontaneous calcium oscillations in an astrocyte in the non-PC cultures (control, Cont.) (C1), at 4 h after the PC (C2), and a bar graph showing the average frequency of calcium oscillations of astrocytes for 15 min (C3). Data are expressed as the mean +SD (n = 4, different cultures). * $P < 0.05$.

Fig.2.

Treatment with 2-APB suppressed the spontaneous calcium oscillations, and induced neuronal ischemic tolerance. A: Representative spontaneous calcium oscillations in a neuron with (A2) or without (A1) 2-APB (100 μ M) treatment in the cultures without PC, and in an astrocyte with (A4) or without (A3) 2-APB treatment in the cultures without PC. B: Average frequency of calcium oscillations in neurons (B1) or astrocytes (B2) in the control (Cont.) and on treatment with 2-APB for 10 min. C: Statistical comparison of the survival rate of neurons following lethal OGD. Data are expressed as the mean \pm SD (n = 4 ~ 5, different cultures). * P < 0.05

Fig.3.

Treatment with forskolin enhanced the spontaneous calcium oscillations and reversed the PC-induced neuronal ischemic tolerance. A: Representative spontaneous calcium oscillations in a neuron with (A2) or without (A1) forskolin (50 μ M) treatment at 4 h after the end of PC, and in an astrocyte with (A4) or without (A3) forskolin treatment at 4 h after the end of PC. B: Average frequency of the calcium oscillations in neurons

(B1) or astrocytes (B2) in the cultures at 4 h after the end of the PC (PC 4h), and in the cultures treated with forskolin for 10 min at 4 h after the end of the PC,. C: Statistical comparison of the survival rate of neurons following lethal OGD. Data are expressed as the mean +SD (n = 4, different cultures). * $P < 0.05$.

Fig.4.

Treatment with 2-APB reduced the rise in the extracellular concentration of glutamate during OGD and the expression of GLT-1. A: Time course of the change in the NADH fluorescence ratio during OGD in control cultures (triangle), and in the cultures incubated in CM for 8 h following the treatment with 2-APB (100 μ M) for 12 h before the onset of OGD (square), respectively. Control (Cont.): cultures treated with 0.1 % DMSO, a vehicle of 2-APB. OGD begins at 0 min. Vertical bars indicate either + or - SD. B: Statistical comparison of the relative fluorescence intensity 80 min after the onset of OGD. Data are expressed as the mean +SD (n = 4-7 different cultures). * $P < 0.05$. C: GLT-1, GLAST and actin protein expression levels analyzed by Western blotting in the control

cultures or the cultures incubated in CM for 8 h following the treatment with 2-APB for 12 h. D and E: Statistical comparison of the expression of GLT-1 and GLAST normalized to that of actin. Data are expressed as the mean +SD (n = 4 different cultures). * $P < 0.05$.

Fig.5.

Treatment with PACAP 6-38 suppressed the spontaneous calcium oscillations, and induced neuronal ischemic tolerance. A: Representative spontaneous calcium oscillations in a neuron with (A2) or without (A1) PACAP 6-38 (1 μ M) treatment in the cultures without PC, and in an astrocyte with (A4) or without (A3) PACAP 6-38 treatment in the cultures without PC. B: Average frequency of calcium oscillations in neurons (B1) or astrocytes (B2) in the control cultures (Cont.) and in the cultures treated with PACAP 6-38 for 10 min. C: Statistical comparison of the survival rate of neurons following lethal OGD. D: Expression of GLT-1 and actin analyzed by Western blotting in the cultures incubated in CM for 8 h following the treatment with or without PACAP 6-38 for 12 h. E: Statistical comparison of the expression of GLT-1 normalized to the

expression of actin. Data are expressed as the mean +SD (n = 4, different cultures). * $P < 0.05$

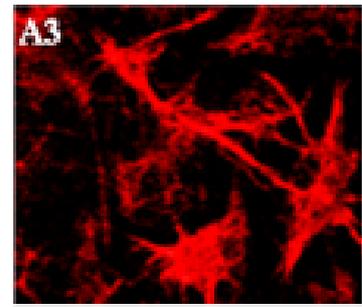
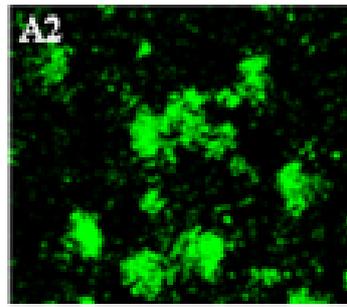
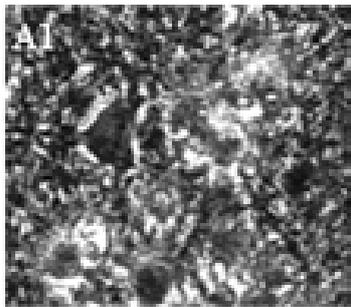
Fig.6.

Treatment with PACAP 38 enhanced the spontaneous calcium oscillations, and reversed the PC-induced neuronal ischemic tolerance. A: Representative spontaneous calcium oscillations in a neuron with (A2) or without (A1) PACAP 38 (100 nM) treatment at 4 h after the end of PC, and in an astrocyte with (A4) or without (A3) PACAP 38 treatment at 4 h after the end of PC. B: Average frequency of calcium oscillations in neurons (B1) or astrocytes (B2) in the cultures at 4 h after the end of the PC (PC 4h), and in the cultures treated with PACAP 38 for 10 min at 4 h after the end of the PC,. C: Statistical comparison of the survival rate of neurons following lethal OGD. D: Expression of GLT-1 and actin analyzed by Western blotting in the control cultures (sham-treated) and in the cultures incubated in CM for 8 h following treatment with or without PACAP38 for 12 h after the end of PC. E: Statistical comparison of the expression of GLT-1 normalized to the expression of actin. Data are

expressed as the mean +SD (n = 4, different cultures). * $P < 0.05$.

Fig.7.

Co-treatment with 2-APB antagonized both the PACAP-induced increase in the spontaneous calcium oscillatory frequency and the PACAP-induced attenuation of the PC-induced neuronal ischemic tolerance. A: Figures A1-A3 show representative spontaneous calcium oscillations in a neuron co-treated with PACAP 38 (100 nM) and 2-APB (100 μ M) (A3), treated with PACAP 38 only (A2), and control (A1) at 4 h after the end of PC, respectively. Figures A4-A6 show calcium oscillations in an astrocyte co-treated with PACAP 38 (100 nM) and 2-APB (100 μ M) (A6), treated with PACAP 38 only (A5), and control (A4) at 4 h after the end of PC, respectively. B: Average frequency of calcium oscillations in neurons (B1) or astrocytes (B2) in the cultures at 4 h after the end of the PC (PC 4h), and in the cultures treated with PACAP 38 or co-treated with PACAP 38 and 2-APB for 10 min at 4 h after the end of the PC. C: Statistical comparison of the survival rate of neurons following lethal OGD.

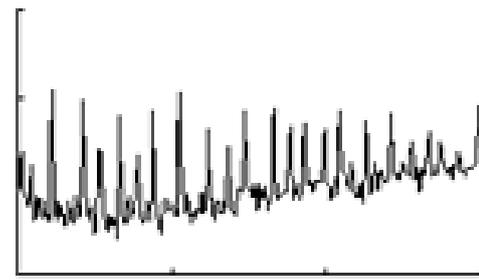
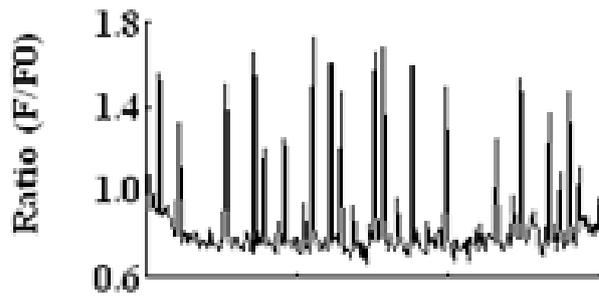


Neuron

Astrocyte

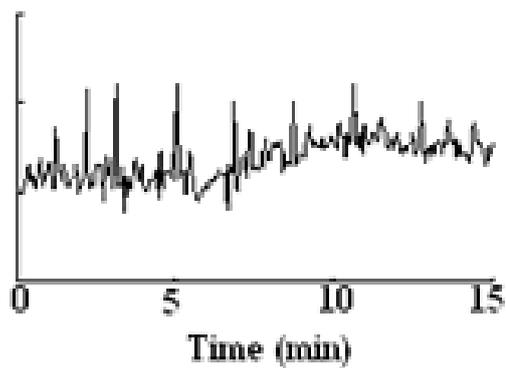
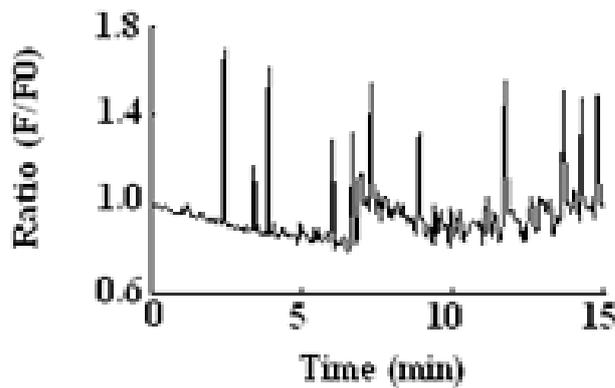
B1

C1



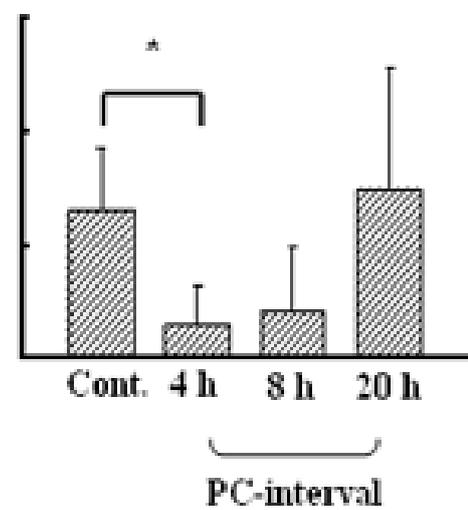
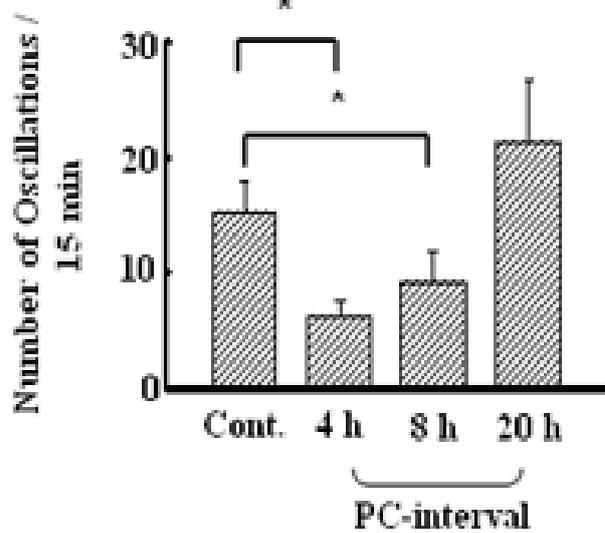
B2

C2

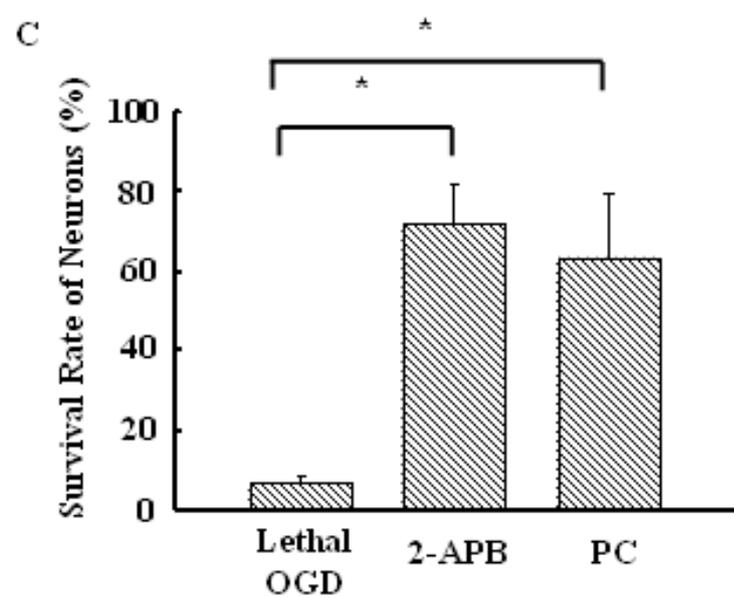
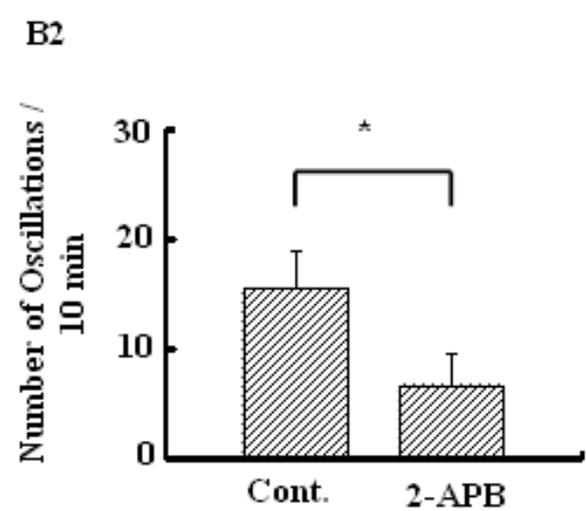
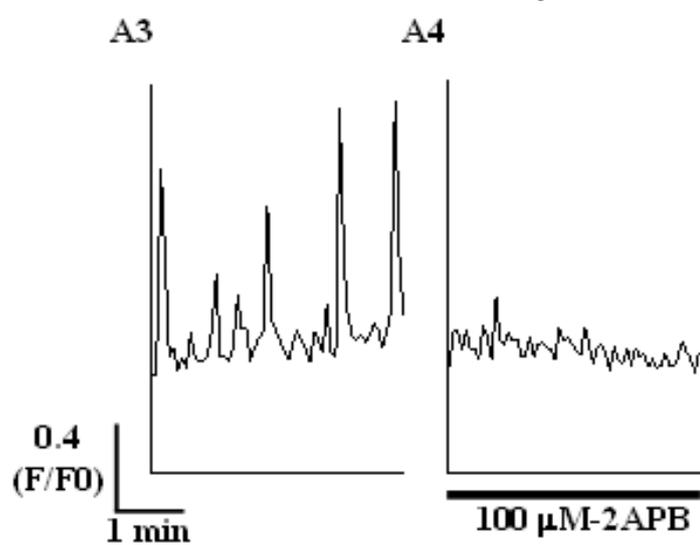
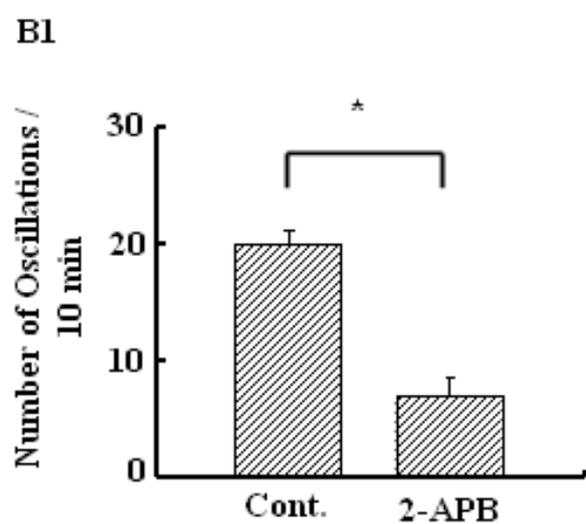
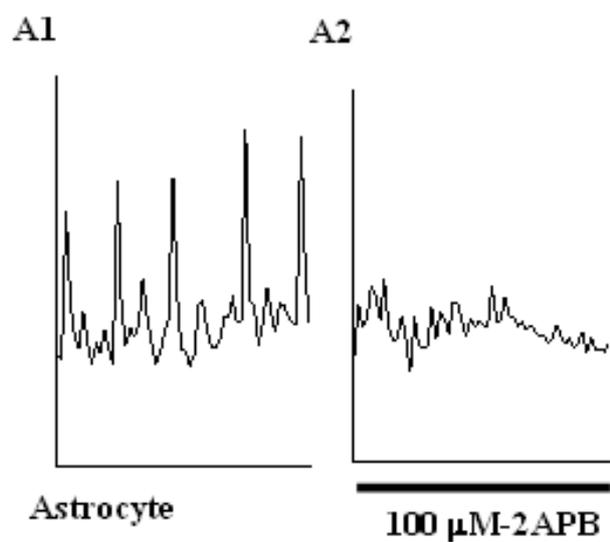


B3

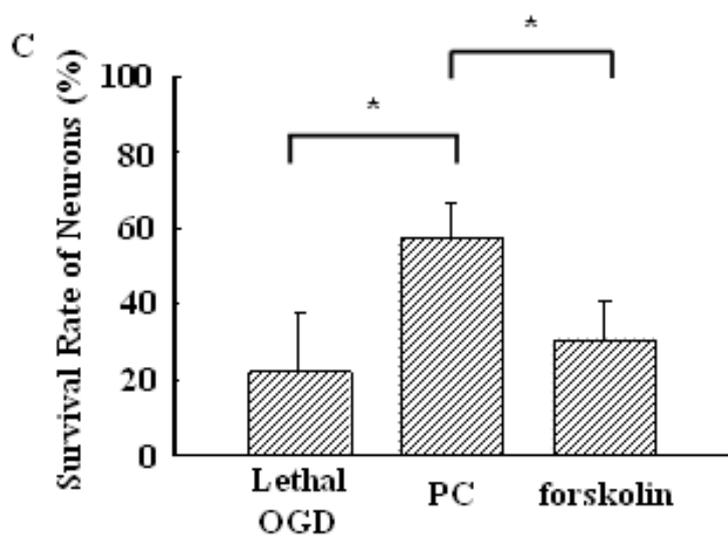
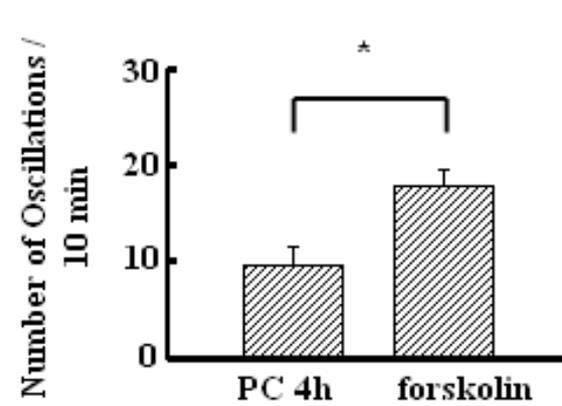
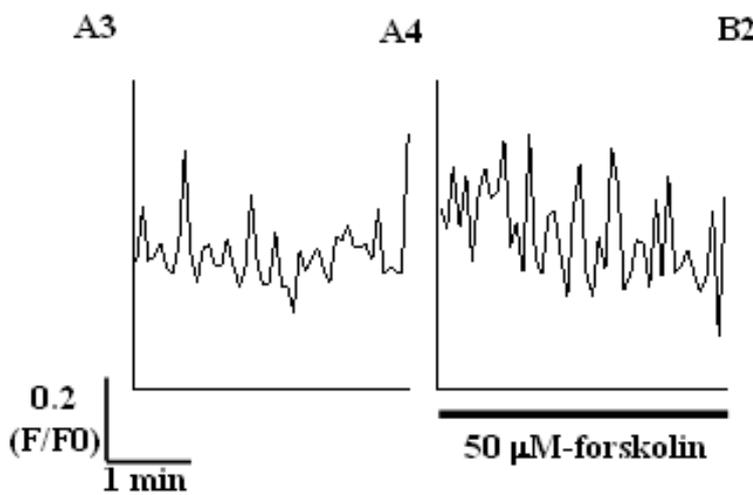
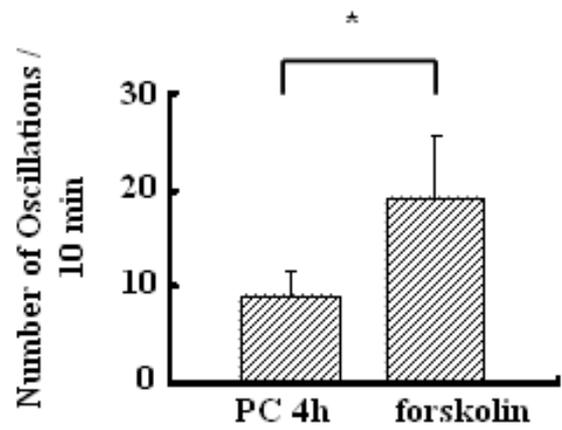
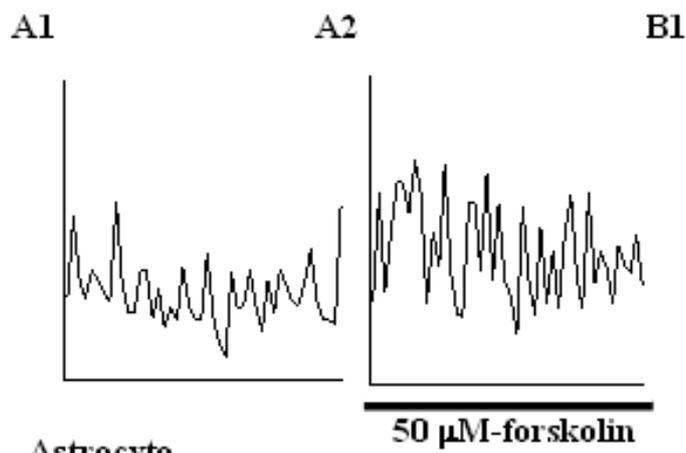
C3

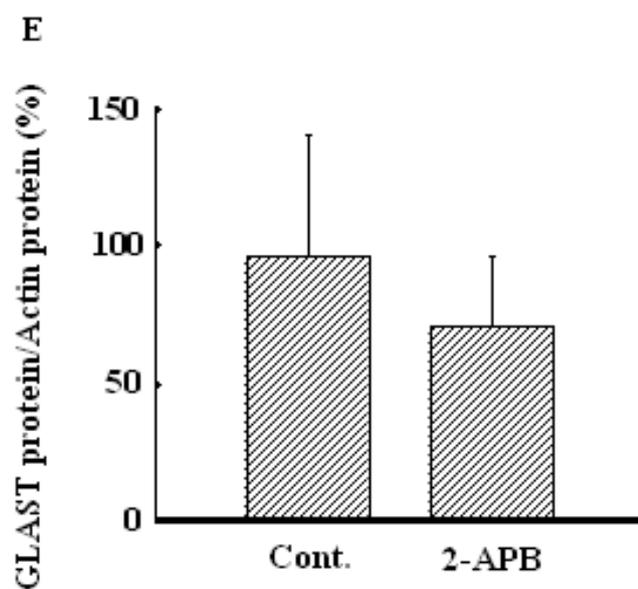
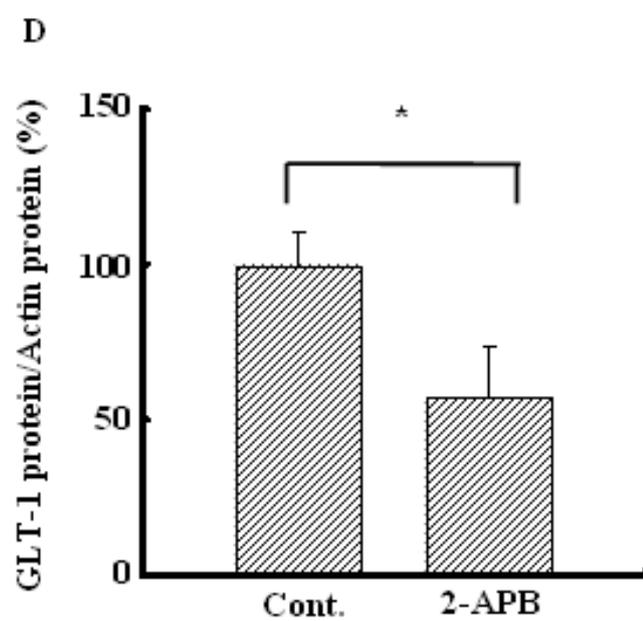
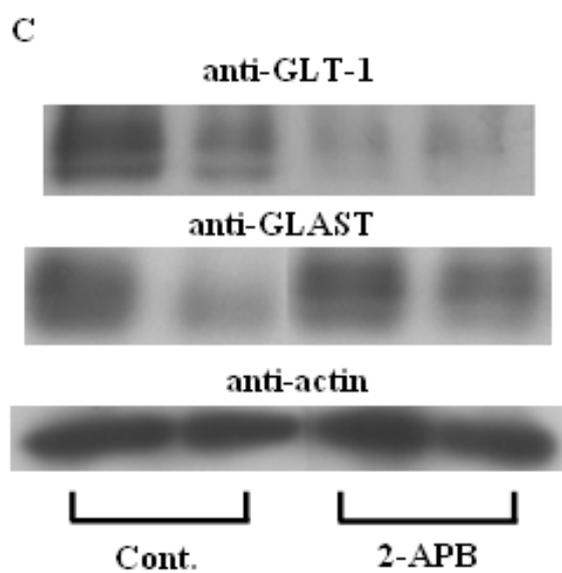
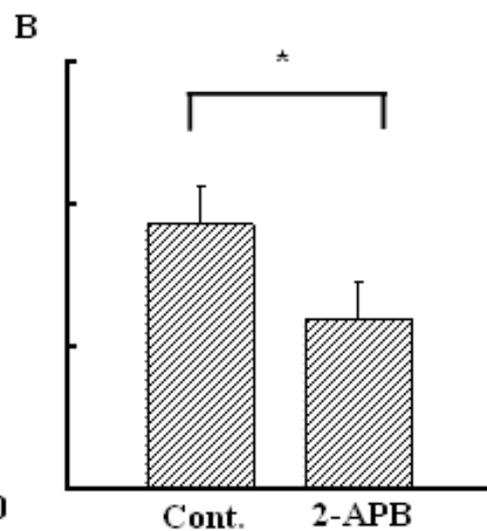
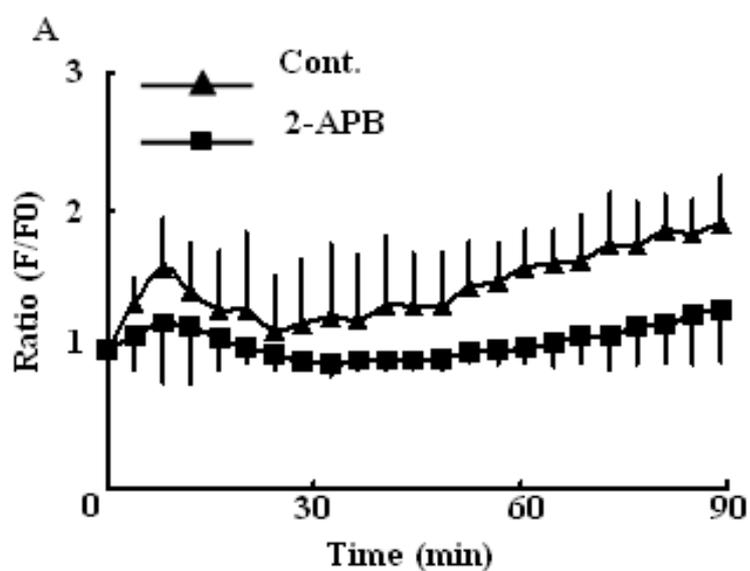


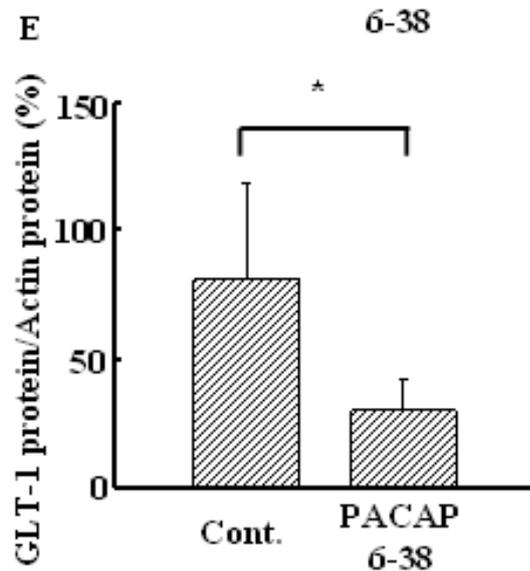
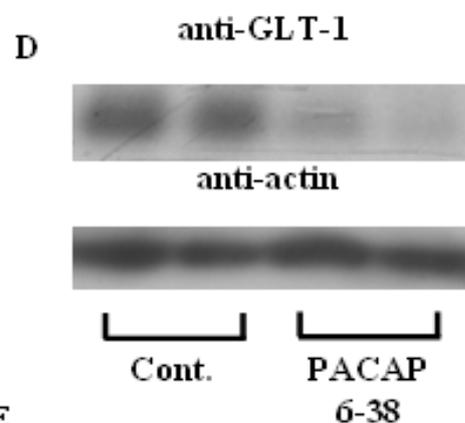
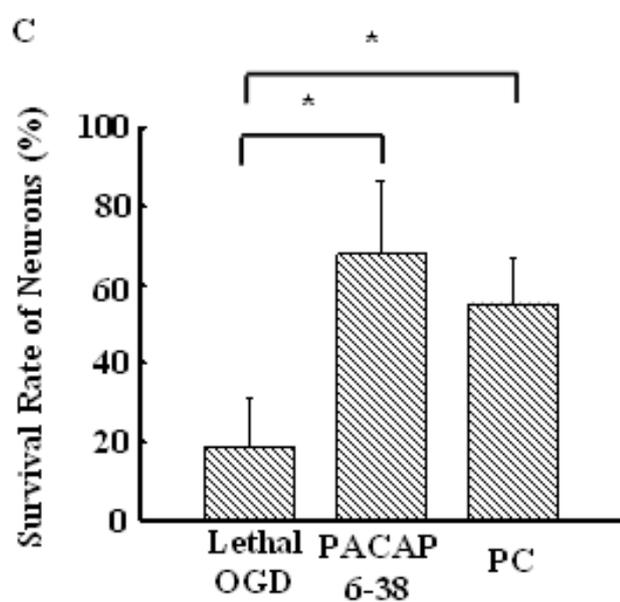
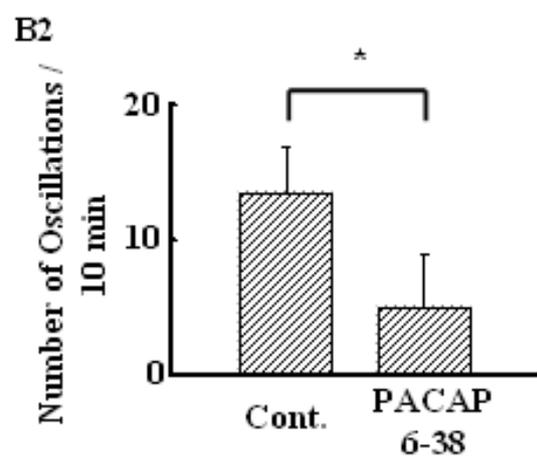
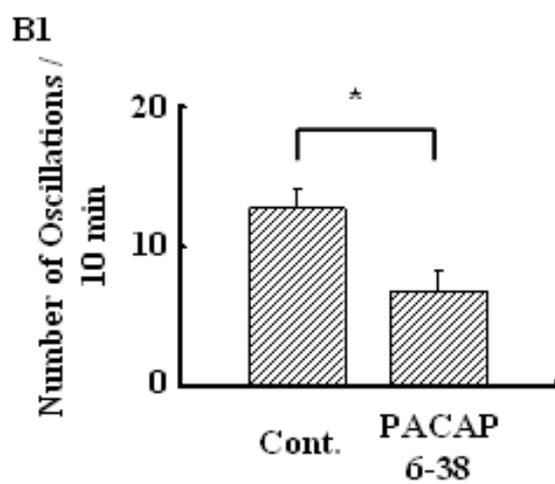
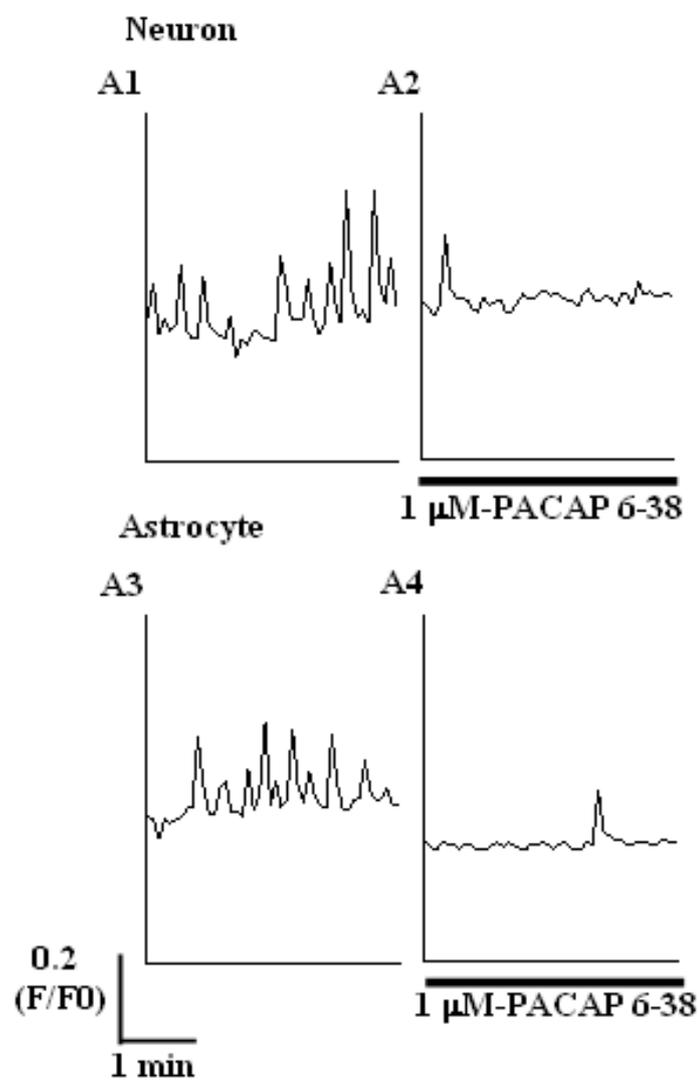
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