

Regulation of AMPA Receptor Trafficking by *O*-Glycosylation

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Abstract The present study investigated the role of *O*-linked β -*N*-acetylglucosamine (*O*-GlcNAc) glycosylation (*O*-GlcNAcylation) in AMPA receptor trafficking. Alloxan, an inhibitor of *O*-GlcNAc transferase, potentiated responses of AMPA receptors composed of the GluR1 subunit expressed in *Xenopus* oocytes. No potentiating effect of alloxan was obtained with mutant GluR1 (S831A) receptor lacking CaMKII phosphorylation site. Alloxan facilitated basal synaptic transmission to approximately 120% of basal levels and enhanced Schaffer collateral-CA1 long-term potentiation (LTP) in rat hippocampal slices, especially in the late phase of the LTP. Alloxan stimulated translocation of the GluR1 and GluR2 subunit from the cytosol towards the plasma membrane in rat hippocampal slices with the LTP, although it had no effect on subcellular distribution of the NR1 subunit. Taken together, the results of the present study show that alloxan regulates AMPA receptor trafficking by inhibiting *O*-GlcNAcylation, to modulate hippocampal synaptic transmission and synaptic plasticity.

Keywords *O*-Glycosylation · Alloxan · AMPA receptor · Long-term potentiation · Trafficking

Introduction

O-linked β -*N*-acetylglucosamine (*O*-GlcNAc) glycosylation (*O*-GlcNAcylation) is engaged in the posttranslational modification of proteins as well as phosphorylation. *O*-GlcNAcylation means that *N*-acetylglucosamine is transferred to hydroxyl groups of the serine/threonine residues in proteins by *O*-GlcNAc transferase. Interestingly, *O*-GlcNAcylation occurs at the same conserved sites as protein phosphorylation, and two independent modifications due to *O*-GlcNAcylation and phosphorylation at the common sites, with each being mutually exclusive, may cause functional switches of proteins [1]. Accumulating studies have pointed to the diverse roles of the *O*-GlcNAc modification, ranging from nutrient sensing to the regulation of proteosomal degradation and gene silencing [2, 3]. Moreover, perturbations in intracellular *O*-GlcNAc levels could be a factor for cancers, Alzheimer disease, and diabetes mellitus [2, 4].

α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, that are enriched in the central nervous systems as heterotetramers composed of the GluR1 through GluR4 subunit, mediate excitatory synaptic transmission and are the major governor of synaptic plasticity relevant to learning and memory, such as long-term potentiation (LTP) and long-term depression (LTD). Much attention has focused upon activity-dependent AMPA receptor trafficking in synaptic plasticity [5, 6]. The PDZ domain-containing proteins such as protein interacting with C-kinase-1 (PICK1), glutamate receptor-interacting protein (GRIP), and AMPA receptor binding protein (ABP), are a mediator for AMPA receptor trafficking, that is regulated through protein phosphorylation [7, 8]. Then, we hypothesized that *O*-GlcNAcylation, occurring at the site same as phosphorylation, might affect AMPA receptor trafficking.

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To address this hypothesis, we recorded currents through AMPA receptors composed of the wild-type or mutant GluR1 subunit expressed in the *Xenopus* oocytes, monitored Schaffer collaterals-CA1 synaptic transmission and LTP in rat hippocampal slices, and assayed subcellular distribution of AMPA receptors in the CA1 region of rat hippocampal slices. We show here that alloxan, an *O*-GlcNAcylation inhibitor, facilitates hippocampal synaptic transmission and enhances Schaffer collateral-CA1 LTP, possibly resulting from an increase in the presence of AMPA receptors in the plasma membrane.

Materials and Methods

Animal Care

All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In Vitro Transcription and Translation in *Xenopus* oocytes

mRNAs coding for the rat AMPA receptor GluR1 subunit was synthesized by in vitro transcription. For the mutant GluR1 subunit mRNA lacking Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) phosphorylation site, Ser⁸³¹ was replaced by Ala [mGluR1(S831A)]. *Xenopus* oocytes were manually separated from the ovary, and incubated overnight in Barth's solution (in mM, 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.82 MgSO₄, 0.33 Ca(NO₂)₂, 0.41 CaCl₂, and 7.5 Tris, pH 7.6) after collagenase (1 mg/ml) treatment. Oocytes were injected with mRNAs for the GluR1 subunit or the mGluR1(S831A) subunit, and incubated at 18°C.

Two-Electrode Voltage-Clamp Recording

Two to seven days after injection with the wGluR1 or mGluR1(S831A) subunit mRNA oocytes were transferred to a recording chamber continuously superfused with standard frog Ringer's solution (in mM: 115 NaCl, 2 KCl, 1.8 CaCl₂, and 5 HEPES, pH 7.0) at 22°C, and kainate-evoked whole-cell membrane currents were recorded using two-electrode voltage-clamp techniques with a GeneClamp-500 amplifier (Axon Instruments, Inc., Foster City, CA, USA).

Field Excitatory Postsynaptic Potential (fEPSP) Recording

Rat hippocampal slices (400 μm) (male Wistar rat, 6 w) were prepared and incubated in standard artificial

cerebrospinal fluid (ACSF) (in mM: 117 NaCl, 3.6 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.5 CaCl₂, 25 NaHCO₃, 11.5 glucose) oxygenated with 95% O₂ and 5% CO₂ at room temperature for 1 h. Then, fEPSPs were recorded from the CA1 region by electrically stimulating the Schaffer collateral (0.03 Hz, 0.1 ms in duration) in ACSF oxygenated with 95% O₂ and 5% CO₂ at 34°C in the presence and absence of alloxan (10 mM). Schaffer collateral-CA1 LTP was induced by applying high frequency stimulation (HFS) (100 Hz for 1 s, one train).

Analysis for Subcellular Distribution of AMPA Receptor Subunits in Fractions Using a Sucrose Density Gradient Centrifugation

The CA1 region of rat hippocampal slices, obtained before and after recording Schaffer collateral-CA1 LTP in the presence and absence of alloxan (10 mM), was resected, and sonicated in 320 mM sucrose/Tris buffer (pH 7.4) containing 2 mM EDTA and 1% protease inhibitor cocktail at 4°C, followed by centrifugation at 800×g for 10 min at 4°C. The supernatants were layered on the top of sucrose density gradient comprising 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, and 5% of 0.3 ml sucrose solution with 10 mM Tris-HCl (pH 7.4) in a SW 55Ti Beckman rotor tube, centrifuged at 100,000×g for 1 h at 4°C, and 12-fraction samples were collected (300 μl for each fraction). Subsequently, Western blotting was carried out in each fraction using an anti-GluR1 subunit antibody (Upstate Biotechnology, Lake Placid, NY, USA), an anti-GluR2 subunit antibody (Chemicon, Temecula, CA, USA), and an anti-pan cadherin (Sigma, St. Louis, MO, USA) antibody, and immunoreactive signals were quantitatively analyzed using an NIH image program.

Analysis for Subcellular Distribution of Glutamate Receptor Subunits in the Cytosolic and Plasma Membrane Component

The CA1 region was isolated from rat hippocampal slices (400 μm) (male Wistar rat, 6 w) with and without Schaffer collateral-CA1 LTP in the presence and absence of alloxan (10 mM), and homogenized in an ice-cold mitochondrial buffer [210 mM mannitol, 70 mM sucrose and 1 mM EDTA, 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5 containing 1% protease inhibitor cocktail (Nacalai, Kyoto, Japan)] followed by centrifugation at 3,000 rpm for 5 min at 4°C. The supernatants were centrifuged at 11,000 rpm for 15 min at 4°C and further, the collected supernatants were ultracentrifuged at 100,000×g for 60 min at 4°C to separate the cytosolic and plasma membrane fraction. Protein concentrations for each fraction were determined using a BCA

protein assay kit (Pierce, Rockford, IL, USA). Plasma membrane fraction proteins were resuspended in the mitochondrial buffer containing 1% sodium dodecyl sulfate (SDS). Proteins for each fraction were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes. After blocking with TTBS (150 mM NaCl, 0.1% Tween20, and 20 mM Tris, pH7.5) containing 5% bovine serum albumin (BSA), blotting membranes were reacted with antibodies against the GluR1 subunit (Upstate Biotechnology), the GluR2 subunit (Chemicon), and the NR1 subunit (Chemicon), followed by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (FUJIFILM, Tokyo, Japan). Signal density was measured with an Image Gauge software (FUJIFILM).

Statistical Analysis

Statistical analysis was carried out using unpaired *t*-test, Fisher's Protected Least Significant Difference (PLSD) test, and one-way analysis of variance (ANOVA).

Results

Alloxan Potentiates GluR1 AMPA Receptor Responses by Blocking *O*-GlcNAcylation at the CaMKII Phosphorylation Site

Our initial attempt was to see the effect of alloxan, an inhibitor of *O*-GlcNAc transferase [9], on responses of homomeric wild-type GluR1 (wGluR1) AMPA receptors expressed in *Xenopus* oocytes. Bath-application of kainate (30 μ M), an agonist of non-*N*-methyl-D-aspartate (NMDA) receptors, generated inward whole-cell membrane currents at a holding potential of -60 mV (Fig. 1a, b). Alloxan (1 mM) induced a gradual-developing potentiation of the currents, reaching approximately 200% of original amplitude 80 min after treatment (Fig. 1a, b), although alloxan by itself produced no current (data not shown). Higher concentrations of alloxan (5 and 10 mM) induced a transient depression of wGluR1 AMPA receptor currents at 10 min after treatment and in turn, sustained huge increase in the currents, reaching about 500% of original amplitude at 5 mM and 350% at 10 mM 80 min after treatment (Fig. 1a). This suggests that alloxan modulates GluR1 AMPA receptor responses.

O-GlcNAcylation is shown to occur at the site same as phosphorylation [1]. We, therefore, examined the effect of alloxan on AMPA receptors composed of the

mGluR1(S831A) subunit lacking CaMKII phosphorylation site [10]. For mGluR1(S831A) AMPA receptors, no potentiation of the currents was obtained with alloxan (1 mM), with significant difference as compared with the wGluR1 AMPA receptor currents ($P < 0.0001$, Fisher's PLSD test) (Fig. 1b). This confirms that alloxan potentiates

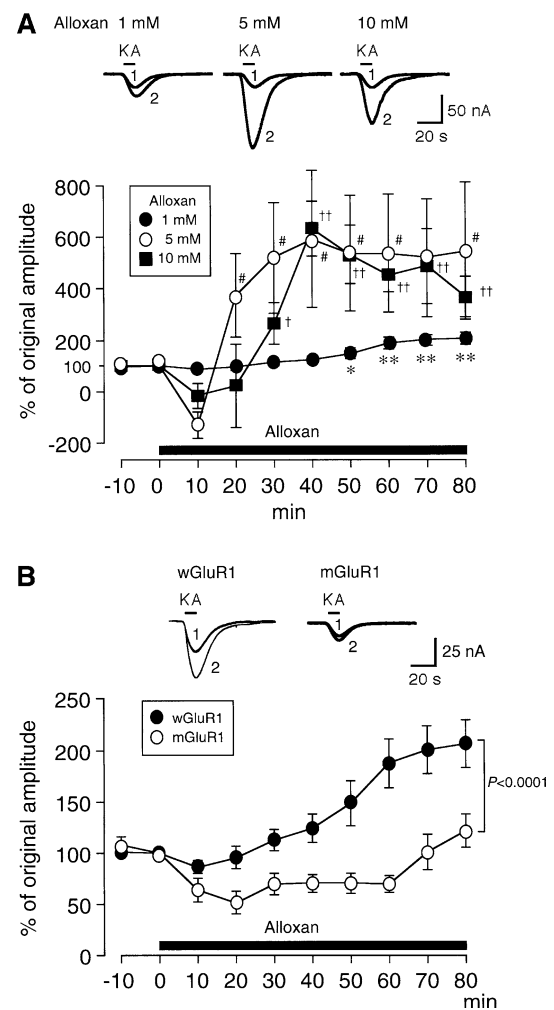


Fig. 1 Effect of alloxan on GluR1 AMPA receptor responses. wGluR1 and mGluR1(S831A) AMPA receptors (mGluR1) are expressed in *Xenopus* oocytes, and kainate (KA) (30 μ M)-evoked whole-cell membrane currents were monitored at a holding potential of -60 mV in the presence and absence of alloxan. **a** Oocytes were treated with alloxan at concentrations as indicated. The illustrated currents recorded 0 min (1) and 80 min (2) are superimposed. In the graph, each point represents the mean (\pm SEM) percentage of original amplitude (0 min) ($n = 8$ for 1 mM, 5 for 5 mM, and 5 for 10 mM). $*. \#. \dagger P < 0.05$, $**. \#\#. \dagger\dagger P < 0.01$ as compared with original amplitude (0 min), unpaired *t*-test. **b** Oocytes expressing the wGluR1 and mGluR1 subunit were treated with alloxan (1 mM). The illustrated currents recorded 0 min (1) and 80 min (2) are superimposed. In the graph, each point represents the mean (\pm SEM) percentage of original amplitude (0 min) ($n = 8$ for wGluR1 and 6 for mGluR1). P value, Fisher's PLSD test

GluR1 AMPA receptor responses by blocking *O*-GlcNAcylation at the CaMKII phosphorylation site.

Alloxan Facilitates Basal Hippocampal Synaptic Transmission and Enhances Schaffer Collateral-CA1 LTP

If alloxan potentiates AMPA receptor responses, then the drug should facilitate hippocampal synaptic transmission. To address this point, we monitored fEPSPs in the CA1 region of rat hippocampal slices. Alloxan (10 mM) facilitates hippocampal synaptic transmission to approximately 120% of basal levels (Fig. 2a). For slices untreated with alloxan, HFS (100 Hz for 1 s, one train) to the Schaffer collateral increased fEPSP slope to nearly 150% of basal levels, being evident 120 min after HFS (control Schaffer collateral-CA1 LTP) (Fig. 2b). Alloxan (10 mM) significantly enhanced the LTP (over 200% of basal levels), especially in the late phase ($P < 0.01$ as compared control LTP, Fisher's PLSD test) (Fig. 2b).

Alloxan Stimulates Shift of AMPA Receptors Towards the Plasma Membrane in Hippocampal Slices with LTP

To see subcellular distribution of AMPA receptors, proteins from the CA1 region of rat hippocampal slices were separated into 12 fractions according to a sucrose density gradient centrifugation. Of 12 fractions, fractions of numbers 8 to 12 (No. 8–12) were reactive to an antibody against cadherin, a marker of the plasma membrane [11], confirming that the corresponding fractions contain plasma membrane components. Alloxan (10 mM) enhanced immunoreactive bands for the GluR1 subunit in fractions No. 8–12 from slices without Schaffer collateral-CA1 LTP as compared with control (non-treatment with alloxan) (Fig. 3a), and a more marked enhancement was found with slices with the LTP (Fig. 3b). A similar effect was obtained with the GluR2 subunit (Fig. 3c, d). It is indicated from these results that alloxan promotes shift of the GluR1 and GluR2 subunit from the cytosol towards the plasma membrane, particularly during LTP.

To obtain further evidence for this, we separated proteins from the CA1 region of rat hippocampal slices into the cytosolic and plasma membrane component. Unlike the results in fractions using a sucrose density gradient centrifugation, 2-h treatment with alloxan (10 mM) had no effect on subcellular distribution of the GluR1 subunit in rat hippocampal slices with and without Schaffer collateral-CA1 LTP (Fig. 4a, b). In addition, subcellular distribution of the NMDA receptor subunit NR1 was not also affected by alloxan (10 mM) in slices with and without the LTP (Fig. 4a, b). Treatment with alloxan (10 mM), on the

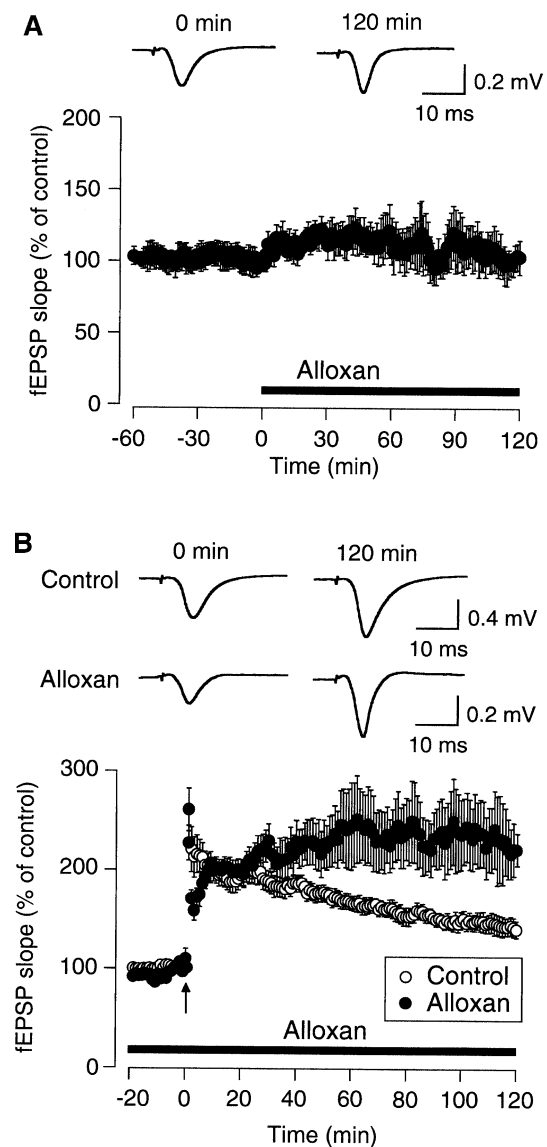
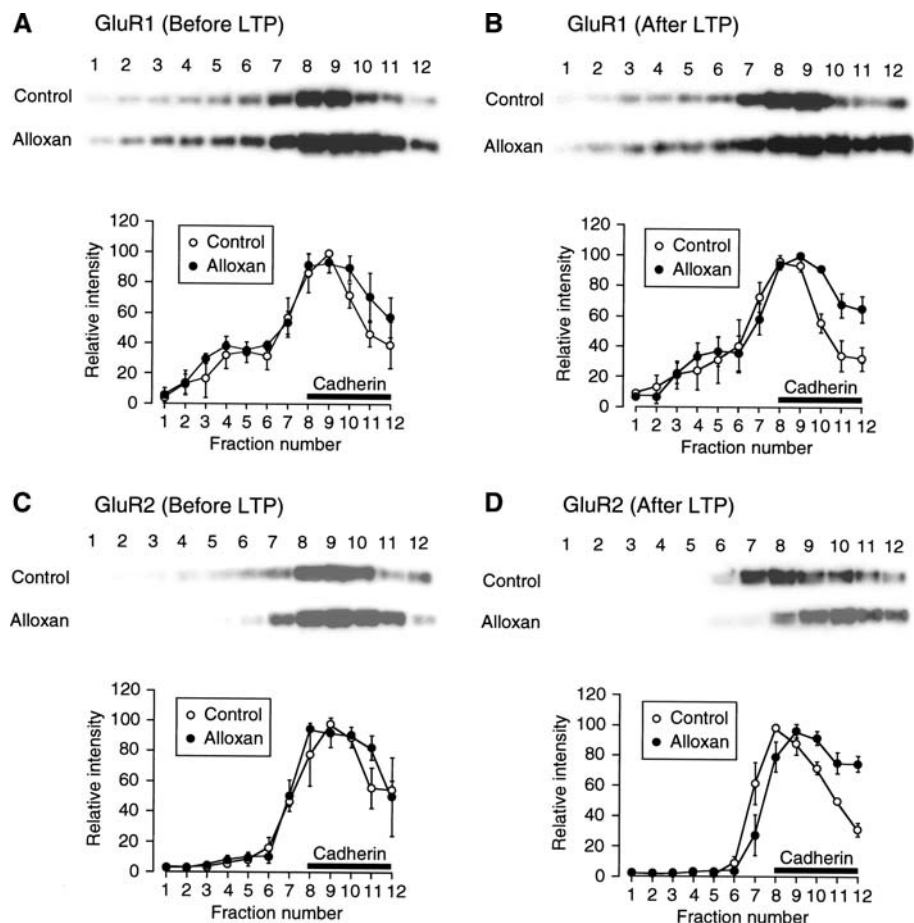


Fig. 2 Effects of alloxan on basal hippocampal synaptic transmission and Schaffer collateral-CA1 LTP. **a** fEPSPs were recorded from the CA1 region by stimulating the Schaffer collateral in rat hippocampal slices before and after treatment with alloxan (10 mM). In the graph, each point represents the mean (\pm SEM) percentage of basal fEPSP slope (0 min) ($n = 5$). **b** High frequency stimulation (100 Hz for 1 s, one train) (arrow) was applied to slices in the absence ($n = 7$) and presence of alloxan (10 mM) ($n = 13$). In the graph, each point represents the mean (\pm SEM) percentage of basal fEPSP slope (0 min)

other hand, significantly increased presence of the GluR2 subunit in the plasma membrane in slices with the LTP as compared with that for untreated slices ($P < 0.0308$, one-way ANOVA) (Fig. 3b), while it had no effect on GluR2 subunit trafficking for slices without the LTP (Fig. 3a). Alloxan, thus, appears to stimulate translocation of the GluR2 subunit from the cytosol towards the plasma membrane during LTP.

Fig. 3 Effect of alloxan on subcellular distribution of the GluR1 and the GluR2 subunit. Proteins from the CA1 region of rat hippocampal slices obtained before (0 min) and after Schaffer collateral-CA1 LTP (120 min) in the presence and absence of alloxan (10 mM) were subjected to a sucrose density gradient centrifugation, and then Western blotting using an anti-GluR1 subunit antibody, an anti-GluR2 subunit antibody, and an anti-pan cadherin antibody, was carried out in each fraction. The signal intensity was normalized by regarding the highest intensity in each blotting as 100. In the graphs, each value represents the mean (\pm SEM) relative intensity (3 independent experiments)



Discussion

In the present study, alloxan (1 mM), an *O*-GlcNAc transferase inhibitor, potentiated responses through homomeric wGluR1 AMPA receptors expressed in *Xenopus* oocytes. Amazingly, no potentiating effect of alloxan was found with mGluR1(S831A) AMPA receptors lacking CaMKII phosphorylation site. This explains that alloxan may potentiate GluR1 AMPA receptor responses by blocking *O*-GlcNAcylation at the CaMKII phosphorylation site, perhaps due to an enhancement in the receptor channel conductance or to an increase in the presence of the receptors in the plasma membrane. Higher concentrations of alloxan (5 and 10 mM) induced a transient depression of wGluR1 AMPA receptor currents followed by sustained huge potentiation of the currents. The mechanism underlying the transient depression is presently unknown. A plausible explanation for this is that the depression might be mediated via a pathway independent of blocking *O*-GlcNAcylation, e.g., a non-specific action of alloxan. To address this question, further experiments need to be done.

Alloxan facilitated hippocampal synaptic transmission and enhanced Schaffer collateral-CA1 LTP, especially in the late phase of the LTP. In the fractions from rat

hippocampal slices using a sucrose density gradient centrifugation, alloxan stimulated translocation of the GluR1 and GluR2 subunit from the cytosol towards the plasma membrane and the prominent effect was found in slices with Schaffer collateral-CA1 LTP. In the cytosolic and plasma membrane component from rat hippocampal slices, alloxan increased presence of the GluR2 subunit, but not the GluR1 subunit, in the plasma membrane for slices with the LTP, although it had no effect on that for slices without the LTP. Subcellular distribution of the NMDA receptor subunit NR1 was not affected by alloxan for slices both with and without the LTP. It is presently unknown why alloxan did not influence the GluR1 subunit trafficking in experiments using the cytosolic and plasma membrane component. The results presented here, however, strongly suggest that alloxan modulates hippocampal synaptic transmission and synaptic plasticity such as LTP by regulating AMPA receptor trafficking.

AMPA receptors are preferentially composed of the GluR1 subunit and the GluR2 subunit (GluR1/GluR2) in the hippocampus [12]. GluR1 subunit trafficking, therefore, would parallel GluR2 subunit trafficking. AMPA receptor delivery to synapses is stimulated through phosphorylation of the GluR1 subunit at Ser831 due to CaMKII [6].

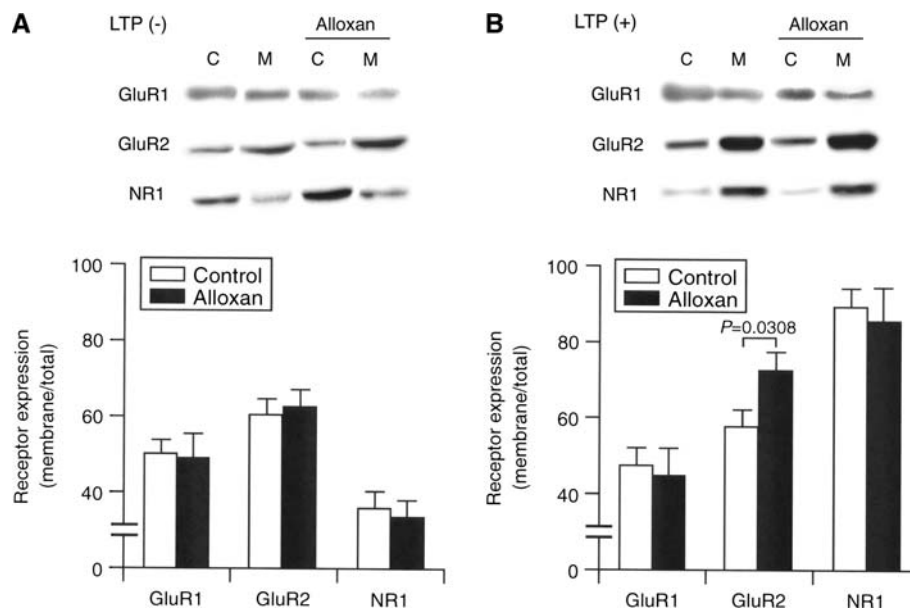


Fig. 4 Effect of alloxan on subcellular distribution of glutamate subunits. The cytosolic and plasma membrane components were separated from the CA1 region of rat hippocampal slices using an ultracentrifugation, and then Western blotting using an anti-GluR1 subunit antibody, an anti-GluR2 subunit antibody, and an anti-NR1 subunit antibody, was carried out. **a** Slices without Schaffer collateral-CA1 LTP [LTP (-)] were untreated (Control) and treated with alloxan (10 mM) for 2 h. Typical immunoreactive bands are shown. *C* cytosol; *M* plasma membrane. In the graphs, each column represents the mean (\pm SEM) ratio of signal intensity for the GluR1

subunit, the GluR2 subunit, or the NR1 subunit in the plasma membrane fraction against signal intensity for each subunit in total cells ($n = 4$ independent experiments). **b** Slices with Schaffer collateral-CA1 LTP [LTP (+)] were untreated (Control) and treated with alloxan (10 mM) for 2 h. Typical immunoreactive bands are shown. *C* cytosol; *M* plasma membrane. In the graphs, each column represents the mean (\pm SEM) ratio of signal intensity for the GluR1 subunit, the GluR2 subunit, or the NR1 subunit in the plasma membrane fraction against signal intensity for each subunit in total cells ($n = 4$ independent experiments). *P* value, one-way ANOVA

O-GlcNAcylation of the GluR1 subunit might prevent CaMKII phosphorylation of the subunit; conversely, inhibiting *O*-GlcNAcylation might promote CaMKII phosphorylation of the GluR1 subunit, leading to an increase in the presence of AMPA receptors in the plasma membrane. In support of this note, the potentiating effect of alloxan on wGluR1 AMPA receptor responses was neutralized by deleting CaMKII phosphorylation site on the GluR1 subunit in the *Xenopus* oocyte expression experiments and alloxan stimulated translocation of the GluR1 subunit from the cytosol towards the plasma membrane in the fractions from hippocampal slices using a sucrose density gradient centrifugation.

AMPA receptor trafficking is a critical factor for synaptic plasticity. The GluR1 subunit and the GluR2 subunit are implicated in activity-dependent delivery and constitutive delivery of AMPA receptors towards synapses, respectively [5, 13]. In addition, AMPA receptor delivery to postsynaptic membrane surface is found in the early phase of LTP [14, 15]. In the fractions using a sucrose density gradient centrifugation, alloxan still increased presence of the GluR1 and GluR2 subunit in the plasma membrane for hippocampal slices without Schaffer collateral-CA1 LTP. This alloxan action may account for

facilitation of basal hippocampal synaptic transmission. A definite shift of both the GluR1 and GluR2 subunit for sucrose density gradient centrifugation fractions and the GluR2 subunit alone for the cytosolic and plasma membrane component towards the plasma membrane was found in slices with the LTP. This may explain that alloxan, to inhibit *O*-GlcNAcylation, increases presence of GluR1/GluR2 AMPA receptors in the plasma membrane by stimulating delivery of the GluR1 subunit and/or the GluR2 subunit towards membrane surface during LTP.

In conclusion, the results of the present study show that alloxan potentiated GluR1 AMPA receptor responses by blocking *O*-GlcNAcylation, possibly at the CaMKII phosphorylation site on the GluR1 subunit; alloxan facilitated basal hippocampal synaptic transmission and enhanced Schaffer collateral-CA1 LTP, especially in the late phase of the LTP; and alloxan increased presence of the GluR1 subunit and/or the GluR2 subunit in the plasma membrane for hippocampal slices, prominently with the LTP. Alloxan, thus, appears to modulate hippocampal synaptic transmission and synaptic plasticity by regulating AMPA receptor trafficking. This provides new insight into *O*-GlcNAcylation as a regulatory factor for AMPA receptor trafficking.

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