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SUBUNIT-SPECIFIC AND HOMEOSTATIC REGULATION OF GLUTAMATE RECEPTOR LOCALIZATION BY CAMKII IN *DROSOPHILA* NEUROMUSCULAR JUNCTIONS

T. MORIMOTO,[a*] M. NOBECHI,[a] A. KOMATSU,[b1] H. MIYAKAWA[a] AND A. NOSE[c,d]

[a]*Laboratory of Cellular Neurobiology, School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

[b]*Department of Physiology, School of Medicine, Tokyo Women's Medical University, 8-1 Kawatachou, Shinjukuku, Tokyo, 162-8666, Japan*

[c]*Department of Physics, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyoku, Tokyo 113-0033, Japan*

[d]*Department of Complexity Science and Engineering, Graduate School of Frontier Sciences, University of Tokyo, Kashiwanoha 5-1-5, Kashiwa, Chiba 277–8561, Japan*

Abstract—For the efficient transfer of information across neural circuits, the number of synaptic components at synapses must be appropriately regulated. Here, we found that postsynaptic calcium/calmodulin dependent protein kinase II (CaMKII) modulates the localization of glutamate receptors (GluRs) at *Drosophila* larval neuromuscular junctions (NMJs). Expression of an inhibitory peptide of CaMKII, AIa, in muscle cells enhanced the density of GluRIIA, which is a major and calcium-permeable subunit of GluR, at synapses of third instar larval NMJs. On the other hand, postsynaptic expression of a constitutively active form of CaMKII (T287D) reduced synaptic GluRIIA. These results suggest that CaMKII regulates GluRIIA at NMJs. Moreover, postsynaptic expression of T287D abolished the accumulation of the scaffolding protein discs large (DLG) at synapses, while exerting no significant effects on the presynaptic area and the localization of cell adhesion molecule fasciclin II (FasII). The amplitude of excitatory junctional potentials (EJPs) was enhanced in AIa-expressing larvae, whereas it was unaffected in T287D-expressing larvae in spite of the prominent loss of GluRIIA. The amplitude of miniature EJPs (mEJPs) was significantly reduced and quantal content was significantly increased in T287D-expressing larvae. Notably, another class of GluR containing GluRIIB was enhanced by the postsynaptic expression of T287D. These results suggest that the homeostatic mechanism in T287D larvae works to maintain the level of synaptic responses. Thus, the *Drosophila* larval NMJs have several regulatory systems to ensure efficient muscle excitability which is necessary for proper larval movement.

Key words: homeostasis, excitatory junctional potential, larvae, Gal4-UAS, calcium.

Precise regulation of the amount and the localization of synaptic components is critical for the stable and efficient transfer of information within neural circuits. Neural activity is thought to regulate the assembly and disassembly of synaptic components and is essential for synaptic development and plasticity (Lee and Sheng, 2000; Sanes and Lichtman, 1999, 2001). One of the key molecules in such regulation is calcium/calmodulin dependent protein kinase II (CaMKII). Calcium entry through postsynaptic *N*-methyl-D-aspartic acid (NMDA) receptors and subsequent activation of CaMKII trigger synaptic plasticity in many brain regions (Lisman et al., 2002). CaMKII-triggered translocation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors has been reported to be involved in synaptic potentiation (Lisman et al., 2002; Shi et al., 1999). Further, CaMKII is also an attractive candidate molecule that monitors the level of Ca^{2+} in pre- and postsynaptic specialization. Depending on the magnitude and frequency of Ca^{2+} signals, initial activation of CaMKII can be followed by autophosphorylation of a threonine residue at position 286 (Thr287 in *Drosophila*) leading to persistent kinase activity (Lisman et al., 2002; but see Lee et al., 2009, which reports transient (~1 min) CaMKII activation). Because of this unique character and its accumulation at postsynaptic sites, CaMKII can act as a frequency detector of calcium oscillations and a potential sensor for synaptic activity (Koninck and Schulman, 1998; Lisman et al., 2002). However, how CaMKII modulates synapses according to the levels of Ca^{2+} is not yet fully understood.

In *Drosophila*, CaMKII has been reported as a key molecule for the activity-dependent regulation of synapses (Griffith et al., 1993; Jin et al., 1998; Koh et al., 1999; Wang et al., 1994; Kazama et al., 2003, 2007; Morimoto-Tanifuji et al., 2004). Constitutive CaMKII activation at both motor neurons and postsynaptic muscles in larval neuromuscular junctions (NMJs) reduces the levels of the scaffolding protein discs large (DLG) and, consequently, a cell adhesion molecule fasciclin II (FasII), which binds to DLG (Koh et al., 1999). This study provided a model of the dynamic regulation of the assembly of synaptic components by neuronal activity and CaMKII.

The *Drosophila* larval NMJ is a glutamatergic synapse where the expression of five subunits of ionotropic gluta-

[1] Present address: Department of Judo Therapy, Faculty of Medical Engineering, Teikyo University, 1-1 Toyosatodai, Utsunomiya, Tochigi 320-8551, Japan.

*Corresponding author. Tel: +81-42-676-8963; fax: +81-42-676-8841.
E-mail address: takako@toyaku.ac.jp (T. Morimoto).

Abbreviations: AH, after hatching; ANOVA, analysis of variance; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CaMKII, calcium/calmodulin-dependent protein kinase II; DLG, discs large; EJP, excitatory junctional potential; FasII, fasciclin II; GluRs, glutamate receptors; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HRP, horse radish peroxidase; mEJP, miniature excitatory junctional potential; NMDA, *N*-methyl-D-aspartic acid; NMJ, neuromuscular junction; UAS, upstream activating sequence.

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mate receptors (GluRs) have been identified and characterized. The NMJ contains two subclasses of ionotropic GluR: those that contain GluRIIA receptors and those that contain GluRIIB receptors (Featherstone et al., 2005; Marrus et al., 2004; Qin et al., 2005). It has been shown that calcium ions flow through GluRs (Chang et al., 1994). GluRIIA, but not GluRIIB, has a calcium-permeable sequence (Petersen et al., 1997) and further, synaptic currents are smaller without GluRIIA (DiAntonio et al., 1999; Pawlu et al., 2004), suggesting either selective or the higher calcium ion flow through GluRIIA than GluRIIB. However, how the composition and the amount of GluRs are regulated is not fully understood.

We have previously reported that postsynaptic activation of CaMKII enhances the localization of GluRIIA in larvae just after hatching (AH), while reducing it in larvae 8 h AH (Kazama et al., 2003; Morimoto-Tanifuji et al., 2004). In this early developmental stage, we could not detect the effect of postsynaptic inhibition of CaMKII. For further understanding of how CaMKII involves the regulation of content and composition of synaptic GluR during development, we examined GluR localization in third instar larvae expressing CaMKII modifiers. Here, we show that modification of CaMKII activity changes the content of synaptic GluRIIA. Synaptic GluRIIA was enhanced by the inhibition of CaMKII. When CaMKII was activated, a decrease in GluRIIA and increase in GluRIIB were detected. In addition, presynaptic release was enhanced as it compensates for the loss of GluRIIA. We propose that *Drosophila* larval NMJs have several regulatory systems to ensure the contraction of muscle cells for proper movement and one of these systems is induced by CaMKII.

EXPERIMENTAL PROCEDURES

Flies and preparation of larvae

Flies were reared at room temperature (25 °C) and treated in accordance with regulations outlined by Japanese law and the Animal Care and Experimentation Committee of Tokyo University of Pharmacy and Life Sciences. *24B-Gal4* is an enhancer-trap line expressing GAL4 in all embryonic and larval somatic muscles (Luo et al., 1994). The *UAS-Ala* and *UAS-CaMKII-T287D* strains were gifts from L. C. Griffith (Jin et al., 1998). We used other muscle drivers (*Mhc-Gal4* [Haghighi et al., 2003], *G14-Gal4* [Aberle et al., 2002]) as indicated in the text. We used *24B-Gal4* crossed with *yw* (24B×*yw*) as a control. All experiments were performed using wandering third instar larvae. Larvae were dissected in Ca^{2+}-free HL3 saline to prevent muscle contraction. The saline contained (in mM): 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 5 HEPES-NaOH (pH 7.2), 115 sucrose, and 5 trehalose (Stewart et al., 1994).

Electrophysiology

Intracellular recordings were made from muscle 6 of A3–A4 segments, and excitatory junctional potentials (EJPs) and miniature EJPs (mEJP) were recorded. After dissection, we used HL3-containing medium at varying concentrations of extracellular Ca^{2+} (0.4 mM for mEJP recordings and 0.4, 0.75, and 1.5 mM for EJP recordings). Recordings were selected for data acquisition only when the resting membrane potential of the cell was ≤ -60 mV. The resting membrane potential was similar among different genotypes. Sharp electrodes were filled with 3 M KCl, which had a resistance of 15–25 MΩ. Recordings were performed using an M-707A amplifier (WPI, Sarasota, FL, USA). Segmental nerves were stimulated by pulling the cut end of the nerve into a suction electrode and passing a brief depolarizing pulse (0.1 ms) through it. The peak amplitudes of 10 EJPs were measured and averaged in each muscle cell. In general, recording was done from one or two muscles per animal. mEJPs were analyzed with Minianalysis software (Synaptosoft Inc., Decatur, GA, USA). Quantal content was calculated by dividing the mean amplitude of EJPs at 0.4 and 1.5 mM $CaCl_2$ by that of mEJPs in each larva recorded at 0.4 mM $CaCl_2$. Statistical analyses were done by analysis of variance (ANOVA) or Student's *t*-test.

Immunohistochemistry

The following antibodies were used at the indicated concentration: (1) goat anti-horse radish peroxidase (HRP) (Jackson, West Grove, PA, USA, 1:4000) (2) rabbit anti-GluRIIA (for Fig. 1, a generous gift from Y. Kidokoro (Gunma University, Japan), 1:1000), or mouse anti-GluRIIA for some experiments (for supplemental Fig. 1, (Hybridoma bank, University of Iowa, IL, USA), 1:100) (3) rabbit anti-GluRIIB (a generous gift from A. DiAntonio (Washington University, MI, USA), 1:2500) (4) 1D4, anti-FasII antibodies (Hybridoma bank, University of Iowa, IL, USA) (5) anti-DLG antibodies (Hybridoma bank, University of Iowa, IL, USA) (6)secondary antibodies: donkey anti-rabbit IgG Cy3 (Jackson, 1:300), donkey anti-goat IgG Alexa Fluor 594, donkey anti-mouse IgG Alexa Fluor 488 conjugated (Molecular Probes, Eugene, OR, USA, 1:300).

Staining was done as described previously (Kazama et al., 2003, 2007; Nakayama et al., 2006). Strains of interest were stained in the same dish as controls, and confocal images were acquired with an LSM 510 laser scanning microscope (Zeiss, Oberkochen, Germany) under identical conditions. Confocal images were analyzed with IPLab software (Scanalytics, Fairfax, VA, USA). Immunoreactive signals from synapses onto muscles 6 and 7 were selected and pooled for the assessment. Intensity and stained area of synaptic GluRIIA, GluRIIB, and DLG was measured and analyzed within the presynaptic area stained with either Anti-HRP or Anti-FasII antibodies. The area of presynaptic endings as well as synaptic GluRIIA, GluRIIB, FasII, and DLG were normalized by the total surface area of the two muscle fibers 6 and 7. GluRIIA clusters beneath a bouton were analyzed from randomly selected boutons. Three to five boutons were selected from one synaptic area on muscle 6 and 7. Only GluRIIA-stained boutons were analyzed. Statistical analyses were done by ANOVA or Student's *t*-test.

RESULTS

Postsynaptic CaMKII modulates localization of GluRIIA without affecting presynaptic morphology

In order to clarify the role of postsynaptic CaMKII in regulating synaptic components at synapses, we first examined the effect of postsynaptic expression of the CaMKII inhibitory peptide Ala (Griffith et al., 1993), or a constitutively active form of CaMKII, CaMKII-T287D (Wang et al., 1998), on the localization of GluRIIA at the NMJs of third instar larvae. To drive the expression of CaMKII modulators in the postsynaptic muscle cell, we used a GAL4-upstream activating sequence (UAS) expression system (Brand and Perrimon, 1993), employing the *24B-Gal4* driver, which drives strong expression in all muscle fibers from embryonic to larval stages (Luo et al., 1994). We stained the dissected preparation with antibodies specific to FasII and GluRIIA and measured the GluRIIA-immunoreactive area