主 論 文

Glycinergic transmission and postsynaptic activation of CaMKII are required for glycine receptor clustering *in vivo*

生体内において、グリシン受容体のクラスタリングには

グリシン作動性伝達と後シナプスにおける **CaMKII** の活動が必要である

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This thesis is based on the following work:

Glycinergic transmission and postsynaptic activation of CaMKII are required for glycine receptor clustering *in vivo*

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1. Abstract

Synaptic transmission-dependent regulation of neurotransmitter receptor accumulation at postsynaptic sites underlies the formation, maintenance and maturation of synaptic function. Previous *in vitro* studies revealed that glycine receptor (GlyR) clustering requires synaptic inputs. However, *in vivo* GlyR regulation by synaptic transmission is not fully understood. Here, we established a model system using developing zebrafish, in which GlyRs are expressed in Mauthner cells (M-cells), a pair of giant, reticulospinal, hindbrain neurons, thereby enabling analysis of GlyR clusters over time in identifiable cells. Bath-application of a glycinergic blocker, strychnine, to developing zebrafish prevented postsynaptic GlyR cluster formation in the M-cells. After strychnine removal, the GlyR clusters appeared in the M-cells. At a later stage, glycinergic transmission blockade impaired maintenance of GlyR clusters. Interestingly, the necessity of glycinergic transmission appears to decrease with age. It was also found that pharmacological blockade of either L-type Ca^{2+} channels or calcium/calmodulin-dependent protein kinase II (CaMKII) disturbed GlyR clustering. In addition, the M-cell specific CaMKII inactivation using the Gal4-UAS system significantly impaired GlyR clustering in the M-cells. Thus, the formation and maintenance of GlyR clusters in the M-cells in the developing animals is regulated in a synaptic transmission-dependent manner and CaMKII activation at the postsynapse is essential for GlyR clustering. This is the first demonstration of synaptic transmission-dependent

modulation of synaptic GlyRs *in vivo*.

2. Introduction

2.1 General introduction

Synapses are asymmetric contacts between neurons that mediate the transmission of signals across the synaptic cleft. The synaptic transmission involves a chemical signal mediated by neurotransmitters released from the presynaptic terminal, which is then converted to an electric signal by neurotransmitter receptors on the postsynaptic cells.

To ensure reliable synaptic transmission, neurotransmitter receptors are anchored at postsynaptic sites via scaffolding proteins and cytoskeletal elements, and form clusters (Choquet and Triller, 2003; Specht and Triller, 2008) (Fig. I1). Accumulation of necessary synaptic components including neurotransmitter receptors and scaffolding elements is an essential step in the formation of functional synapses (McAllister, 2007). Furthermore, there are many kinds of neurotransmitter chemicals in brain, and matching of presynaptic neurotransmitters and their appropriate postsynaptic receptors is critical for synaptic function. It was indicated that the synaptic activity contributes to matching of postsynaptic components at synapses during synaptic development (Anderson *et al.*, 2004; Fritscy *et al.*, 2006).

Even after synapses are formed, postsynaptic receptors can still move between synaptic and extrasynaptic sites, and between the plasma membrane and the intracellular compartments

(Fig. I1). The receptors also undergo basal turnover under steady state conditions. The regulation of neurotransmitter receptor clustering at postsynaptic sites can be achieved through insertion of receptors to the plasma membrane and removal from plasma membrane via endocytosis, and/or through lateral diffusion events along the plasma membrane between synaptic and extrasynaptic sites (Cognet *et al*., 2006; Triller and Choquet, 2005; Dumoulin *et al*., 2010; Gerrow and Triller, 2010; Renner *et al.,* 2008). It has been suggested that the receptor clustering can be regulated by receptor activity.

Complex and plastic functions of the central nervous system (CNS) such as learning and memory are believed to result from activity-dependent modulation of the synaptic transmission between neurons, like long-term potentiation (LTP) and long-term depression (LTD) (Malenka and Bear, 2004). Since one way to modulate synaptic strength/transmission is to alter the number of neurotransmitter receptors at postsynaptic sites, the activity-dependent regulation of neurotransmitter receptor clustering is one of the crucial mechanisms for synaptic modulation/plasticity.

Taken together, playing a crucial role both in synaptic development and plastic modulation of synapse, activity-dependent regulation of receptor clustering is a key mechanism underlying establishment as well as maintenance of precise neural circuit.

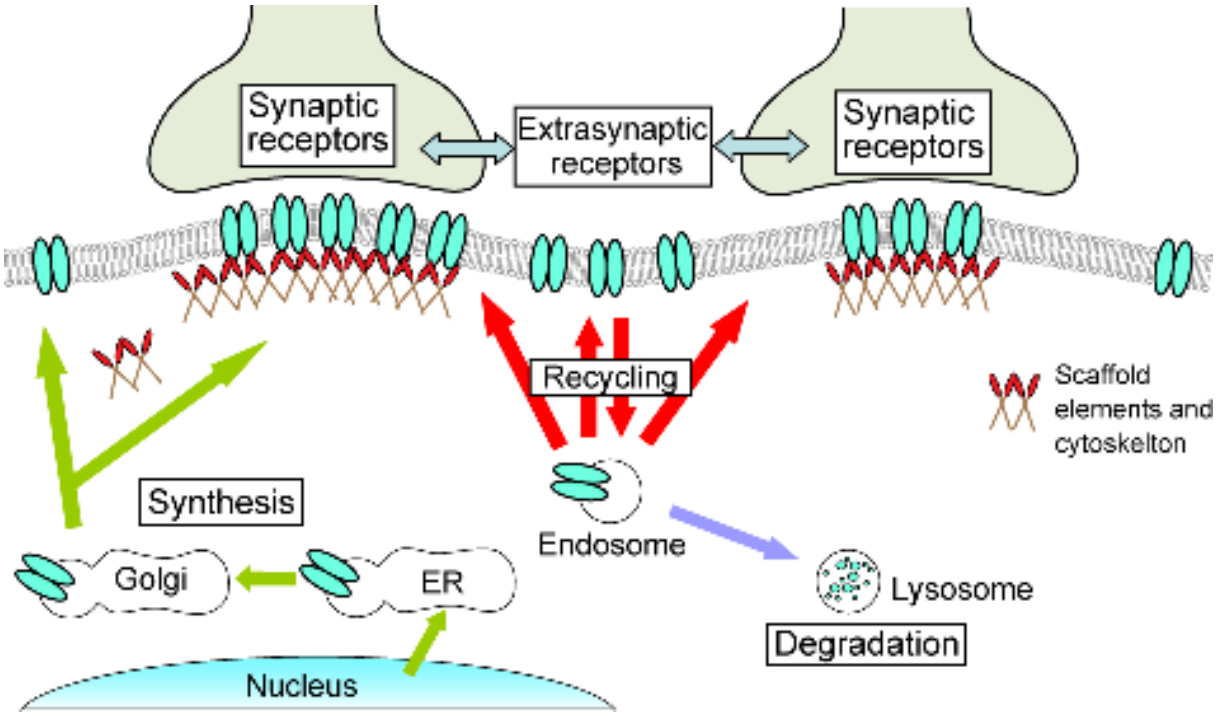


Figure. I1 Dynamic behavior of neurotransmitter receptors.
Receptors (blue) diffuse at extrasynaptic locations and are immobilized by the scaffold-cytoskelton complex (red and brown) at postsynaptic region. Receptors move between synaptic, extrasynaptic and intracellular compartments, and undergo turnover. They maintain equilibrium at steady state. The number of receptors at postsynaptic sites changes by shifting the equilibrium. (modified from Choquet and Triller, 2003)

2.2 Clustering mechanisms of neurotransmitter receptor in excitatory synapses

The regulation of postsynaptic receptors clustering has been extensively studied in excitatory glutamatergic synapses (Craig, 1998; Specht and Triller, 2008; Newpher and Ehlers, 2008). Two types of glutamate receptors playing major roles at glutamatergic synapses are α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid type receptors (AMPARs) and N-methyl-D-aspartic acid type receptors (NMDARs). The postsynaptic density (PSD) at excitatory synapses brings together the receptors, scaffold and adhesion proteins, cytoskeletal elements, and kinases and phosphatases. The glutamatergic receptors are anchored in the PSD via scaffolding proteins such as PSD-95, PSD-93, SAP97 and Shank, and cytoskeletal elements (Newpher and Ehlers, 2008; Okabe, 2007; Sheng and Hoogenraad, 2007). Thus, several types of scaffold proteins bind to receptors and each other at the excitatory synapses. In contrast, the inhibitory synapses have only single type of scaffold protein, gephyrin (see below). Moreover, the PSD at excitatory synapses is thicker than that at inhibitory synapses. Thus, PSD at excitatory synapses is much more complex than that at inhibitory synapses (Renner *et al.,* 2008).

The number of glutamate receptors at a given synapse is not fixed but changes through development. A number of electrophysiological and immunocytochemical studies showed that the number of glutamate receptors can be also altered by synaptic activity (Rao and Craig,

1997; Lissin *et al*., 1998; O'Brien *et al*., 1998; Shi *et al.*, 2001; Matsuzaki *et al*., 2004). Furthermore, it is reported from many laboratories that changes in the receptor number are achieved by insertion, endocytosis, degradation, or diffusion of the receptors (Dell'Acqua *et al*., 2006; Malenka and Bear, 2004).

Synaptic activity-dependent regulation of the number of postsynaptic receptors plays a key role in creating synaptic plasticity, which occurs in LTP and LTD (Malenka and Bear, 2004). The events are proposed to be initiated by increases in the intracellular Ca^{2+} level caused by synaptic activity, and the Ca^{2+} signalling is mediated through several Ca^{2+}-binding proteins, including calmodulin (CaM). Calcium /calmodulin-dependent protein kinase II (CaMKII) is the most representative CaM-regulated enzyme to be implicated in synaptic plasticity. It is known that CaMKII is one of the most abundant proteins in the PSD and enhances recruitment of AMPARs to postsynapses during LTP.

2.3 Clustering mechanisms of neurotransmitter receptor in inhibitory synapses

Postsynaptic clustering of neurotransmitter receptors has been studied also in inhibitory synapses. At inhibitory synapses, glycine receptors (GlyRs) or $GABA_A$ receptors ($GABA_A$Rs) are embedded in PSD, where they bind to the scaffold protein, gephyrin which also binds to

tubulin and are anchored at postsynaptic sites (Kirsch *et al*., 1993; Feng *et al*., 1998; Dumoulin *et al*., 2000; Lévi *et al*., 2004; Jacob *et al*., 2005; Meyer *et al*., 1995; Tretter *et al.*, 2008; Kirsch *et al.*, 1991). Real time observation of GlyRs and $GABA_A$Rs in cultured neurons has revealed that GlyRs and $GABA_A$Rs continuously move on the plasma membrane by lateral diffusion and stay for a longer time at gephyrin-rich synaptic sites than at extrasynaptic regions (Meier *et al*., 2001; Dahan *et al.,* 2003; Bannai *et al*., 2009).

Synaptic clustering of the receptors in inhibitory synapses also depends on activity (Choquet and Triller, 2003; Specht and Triller, 2008; Renner *et al*., 2008). For example, excitatory activation of cultured hippocampal neurons increased $GABA_A$R motility and reduced the synaptic $GABA_A$R cluster size (Naylor *et al*., 2005; Bannai *et al*., 2009).

2.4 Molecular structure and function of the glycine receptor

Glycine is one of the most important inhibitory neurotransmitters in the spinal cord and the brainstem. The GlyR is a member of the pentameric ligand-gated ion channel (LGIC) family, of which the nicotinic acetylcholine receptor cation channel (nAChR) is the prototypical member. GlyR is generally composed of two 48 kDa α-subunits and three 58 kDa β-subunits (Grudzinska *et al.,* 2005). The β-subunit is responsible for anchoring GlyRs at postsynaptic

sites via gephyrin (Meyer *et al.*, 1995; Lynch, 2004). Also in process of GlyR-transport, gephyrin bind GlyRs as an adaptor to motor proteins such as KIF and dynein (Maas *et al.,* 2006, 2009).

When glycine binds to the external domains of the receptor on the cell surface, the ion channel opens and Cl^- passively diffuse across the membrane through the pore. This Cl^- flux moves the membrane potential rapidly toward the Cl^- equilibrium potential. Depending on the value of the equilibrium potential relative to the resting potential of a cell, the Cl^- flux may cause either a depolarization or a hyperpolarization. Because the Cl^- equilibrium potential is generally close to or more negative than the resting potential, the glycinergic synapse functions as an inhibitory synapse. However, in immature neurons, because the intracellular Cl^- concentration is higher than that of extracellular, GlyR activation causes a depolarization (Ben-Ari, 2002). The Cl^- flux leading to a hyperpolarization can inhibit neuronal firing. The flux resulting in subthreshold depolarizations can also inhibit neuronal firing by shorting out excitatory responses, a phenomenon termed “shunting inhibition” (Lynch, 2004; Legendre, 2001).

2.5 Activity-dependent clustering mechanisms of GlyRs

It was immunocytochemically investigated that GlyR clusters are not formed without

glycinergic transmission suggesting that GlyR-clustering is regulated by GlyR-activation (Kirsch and Betz 1998; Lévi *et al*., 1998). Furthermore, it was indicated that Ca^{2+} influx through L-type Ca^{2+} channels and CaMKII involved in GlyR clustering (Kirsch and Betz, 1998; Charrier *et al.*, 2010). The measurements of GlyR diffusion on spinal cord neurons using quantum dots (QDs) showed that GlyR displayed confined diffusion at synapses and were shown to exchange between synaptic and extrasynaptic locations within minutes (Dahan *et al.,* 2003) and diffusional GlyR mobility at synaptic and extrasynaptic regions and the cluster are modified by neural activity (Lévi *et al*., 2008). Thus, the synaptic activity-dependent GlyR clustering mechanisms are being unveiled using cultured neurons. However, it remains unclear whether formation and maintenance of GlyR clusters is regulated by synaptic activity in CNS *in vivo* and what components are involved in the GlyR clustering. The elucidation of the molecular mechanisms *in vivo* is essential to elucidate molecular basis of synaptic function leading to plastic functions of the CNS.

2.6 Overview of our research

In this study, we investigated receptor clustering *in vivo* by focusing on GlyRs in developing zebrafish Mauthner cell (M-cell), which is a single pair of identifiable reticulospinal neurons

in the hindbrain. In zebrafish, M-cell is formed at 7.5 hours post fertilization (hpf) and this single pair is maintained to adulthood (Mendelson 1986). M-cells receive numerous glycinergic inputs on their soma-dendritic membrane (Faber and Korn, 1978; Koyama *et al.,* 2011; Moly and Hatta, 2011).

We performed GlyR cluster immunolabeling and *in vivo* whole-cell recording of glycinergic miniature postsynaptic currents (mPSCs) in M-cells in the presence or absence of strychnine, a specific GlyR blocker. We found that glycinergic transmission is necessary for the formation and the maintenance of GlyR clusters. In addition, to study its subcellular mechanisms, we applied GAL4-mediated gene expression in M-cells and revealed that CaMKII activation at the postsynapse is essential for synaptic GlyR clustering in developing M-cells.

This GlyR clustering assay in zebrafish M-cells provides an excellent model for analyzing synaptic transmission-dependent receptor accumulation and its underlying mechanisms in a developing single cell.

3. Results

Glycinergic transmission blockade leads to a lasting functional deficit in the glycinergic synapse *in vivo*

To assess whether the lack of glycinergic transmission affects glycinergic synapses, we recorded mPSCs *in vivo*. Whole-cell recordings of M-cells at 3 dpf in the presence of TTX, bicuculline, DNQX, and APV revealed spontaneous inward currents at 6.8 ± 1.2 Hz (n = 7; Fig. 1A, C), which is comparable with a previous report that found mPSCs in M-cells of 7-16 Hz at 52 hpf (Legendre and Korn 1994). These mPSCs are mediated by the glycinergic synapse, because they were eliminated by application of strychnine (0.3 ± 0.2 Hz, n = 3; Fig. 1A, C). However, when zebrafish embryos were raised in the presence of strychnine from 22 hpf to 3 dpf, glycinergic mPSCs were not observed after strychnine washout for more than 10 min (0.01 ± 0.01 Hz, n = 4; Fig. 1B, C). It is unlikely that the remaining strychnine disturbed the glycinergic mPSCs, because glycinergic mPSCs were not observed in the larvae after 6-10 h of washout before recording (0.0 ± 0.0 Hz, n = 3, Fig. 1D). Thus, glycinergic transmission blockade results in a lasting functional deficit in the glycinergic synapse.

Glycinergic input is required for GlyR cluster formation during zebrafish development

It has been reported that GlyR clusters are formed at postsynaptic sites in a synaptic

transmission-dependent manner in cultured spinal neurons (Kirsch and Betz 1998; Lévi *et al*., 1998). To examine whether the loss of glycinergic transmission in strychnine-treated larvae is attributable to defects in GlyR cluster formation, we next examined the formation of GlyR clusters in developing M-cells using anti-GlyR antibody immunolabeling. GlyR immunoreactivity was detected as dots at the M-cell surface beginning at 2 dpf (Fig. 2Aa-e). The number of GlyR clusters at the surface of a single M-cell soma increased until at least 5 dpf (Fig. 2B, Table S1). The membrane surface area of the M-cell somata, which was calculated by integration of the soma circumferences, also increased over time during development (Fig. 2C, Table S1). The GlyR cluster density (cluster number/surface area) reached a plateau by 3 dpf and was maintained thereafter to adulthood (Fig. 2Aa-i, D; Table S1). Double labeling with anti-synaptophysin, a marker for presynaptic terminals, showed that GlyR clusters were apposed to synaptophysin, indicating that GlyR clusters are formed at postsynaptic sites (Fig. 2E).

To address whether blocking glycinergic transmission affects GlyR clusters, strychnine was bath-applied to embryos from 22 hpf (before GlyR clusters had formed). This strychnine treatment significantly impaired the formation of GlyR clusters on M-cells both in number ($P < 0.05$ at 2 dpf, $P < 0.01$ at 3 dpf) and in density ($P < 0.05$ at 2 dpf, $P < 0.001$ at 3 dpf, Fig. 2Aj-l, B, D; Table S1). The M-cell surface area was unaffected by strychnine treatment (Fig. 2C, Table S1). Strychnine-mediated impairment of GlyR clustering was

observed at various strychnine concentrations (control (0 μM): 0.36 ± 0.02/μm^2, n = 3; 30 μM: 0.02 ± 0.004/μm^2, n = 9; 100 μM: 0.03 ± 0.005/μm^2, n = 6; 400 μM: 0.02 ± 0.003/μm^2, n = 9; at 3 dpf; Fig. 3). These results show that glycinergic transmission is necessary for GlyR cluster formation *in vivo*. The lack of normal GlyR cluster formation in the presence of strychnine might be attributable to the withdrawal of presynaptic terminals. To test this possibility, we examined the projection of glycinergic terminals onto the M-cells using glyt2:GFP transgenic zebrafish following application of strychnine from 22 hpf to 3 dpf. GFP-positive presynaptic boutons were observed on the soma-dendritic membranes in strychnine-treated larvae (Fig. 4). Thus, blocking glycinergic input disturbed postsynaptic GlyR cluster formation without withdrawal of presynaptic terminals.

To further investigate the role of glycinergic transmission in GlyR clustering, we examined whether GlyR clusters in the M-cells are restored after removal of strychnine. Zebrafish were treated with strychnine from 22 hpf to 2 dpf, and the embryos were then raised in breeding water and examined for immunolabeling at the indicated stages. After strychnine wash out, GlyR clusters were observed on M-cells beginning at 3 dpf and increased in density over time (Fig. 5Aa-e, B; Table S2). Similarly, when embryos were treated with strychnine until 3 dpf, the formation of GlyR clusters appeared within 2 days of strychnine washout (Fig. 5Af-i, B; Table S2). The GlyR clusters were apposed to synaptophysin, confirming that they were formed at postsynaptic sites (Fig. 5C). Therefore, GlyR clustering at synaptic sites is

regulated in a synaptic transmission-dependent manner, even if the initiation of glycinergic transmission is delayed during the early developmental stages. Taken together, present labeling and electrophysiological recordings showed that glycinergic transmission is required for the *in vivo* formation of GlyR clusters, and thus, of functional glycinergic synapses.

Glycinergic transmission is required to maintain previously formed GlyR clusters

To assess whether glycinergic transmission is necessary for the maintenance of GlyR clusters at synapses, I labeled GlyR clusters after a 24-h application of strychnine at later stages when GlyR clusters had been already formed. we were surprised to find that the previously formed GlyR clusters disappeared from the surface of M-cells after a 24-h strychnine treatment in the early larval stages (2-3 dpf, Fig. 6Aa, B; 3-4 dpf, Fig. 6Ab, B; 4-5 dpf, Fig. 6Ac, B; Table S3). Similarly, GlyR clusters were markedly decreased following a 24-h application of strychnine in late larvae and juveniles (10-11 dpf, Fig. 6Ad, B; 15-16 dpf, Fig. 6Ae, B; 30-31 dpf, Fig. 6Af, B; Table S3). Interestingly, the extent of the strychnine-induced reduction in the GlyR cluster density decreased as the fish aged (95.6 ± 2.2% at 5 dpf; 82.7 ± 5.7% at 11 dpf; 73.4 ± 5.7% at 16 dpf; 51.3 ± 6.6% at 31 dpf; Fig. 6C). Thus, glycinergic transmission is required not only for the formation but also for the maintenance of GlyR clusters during larval and juvenile periods. The importance of glycinergic input in maintaining GlyR clusters seems to attenuate in an age-dependent manner.

Glycinergic transmission dynamically regulates GlyR clusters

To investigate whether glycinergic transmission could dynamically regulate GlyR clusters, we examined effect of short-term strychnine treatment on GlyR clusters. Zebrafish were treated with strychnine for 1 h at 3 dpf. Surprisingly, strychnine treatment for only 1-h almost completely eliminated the GlyR clusters at the M-cell surface (Fig. 7Ab, B; Table S4).

we then examined the recovery of GlyR clusters at the surface of M-cells following the 1 h-strychnine treatment. After strychnine washout, the GlyR clusters started to be reformed on M-cells in 10 h, and increased thereafter (Fig. 7Ab-f, B; Table S4). It took less than one day for M-cells to recover a significant level of GlyR clusters on their surface. The GlyR clusters recovered much faster than did those in the longer-term (22 hpf-3 dpf) strychnine treatment experiment (Fig. 5Af-i, B; Fig. 7B). These data suggest that the GlyR clusters are dynamically and continuously regulated by GlyR activities.

CaMKII activation in the postsynaptic cell is required for GlyR clustering

To elucidate the molecular basis underlying synaptic transmission-dependent GlyR clustering *in vivo*, we focused on Ca^{2+}-mediated events. It has been reported that pharmacological application of either nifedipine or KN-93, which inhibit the L-type Ca^{2+} channels and CaMKII, respectively, compromises the formation of GlyR clusters in cultured spinal neurons

(Kirsch and Betz 1998; Charrier *et al*., 2010). We applied nifedipine or KN-93 to zebrafish embryos from 24 hpf to 3 dpf and immunolabeled GlyRs at 3 dpf. Application of either nifedipine or KN-93 to zebrafish embryos significantly impaired GlyR clustering similar to the strychnine treatment (Fig. 8Aa, b, d, e, B; Table S5). In contrast, application of cyclosporine A, a calcineurin inhibitor, did not affect GlyR clusters (Fig. 8Ac, B; Table S5). The impairment of GlyR clustering in the presence of nifedipine or KN-93 is not caused by the elimination of presynaptic terminals, because glycinergic presynaptic boutons, which can be labeled by GFP in glyt2:GFP transgenic zebrafish, were not morphologically affected in M-cells after 2 days of the drug application (Fig. 9). Glycinergic presynaptic terminals were also unchanged following treatment with cyclosporine A. Thus, the L-type Ca^{2+} channels and CaMKII, but not calcineurin, play important roles in GlyR clustering *in vivo*.

Since pharmacological application of drugs to developing zebrafish embryos affects presynaptic and postsynaptic cell targets, we cannot simply conclude that postsynaptic CaMKII regulates GlyR clustering. Indeed, CaMKII is involved in the release of neurotransmitters at the presynaptic terminals (Nichols *et al*., 1990). To clarify this issue, we inhibited CaMKII specifically in M-cells using the Gal4-UAS system (Asakawa *et al*., 2008). we used a Gal4 driver zebrafish line, hspGFF62A, in which a modified GAL4 transcription activator is expressed in the M-cells (Fig. 10A). We generated UAS transgenic lines expressing mCherry and either autocamtide-2-related inhibitory peptide II (AIP2) or its

control peptide 2 (ACP2), using the auto-cleavage peptide sequence 2A in between the two genes. AIP2 originates in the autoinhibitory domain of CaMKII and, thus, is used to inhibit CaMKII activation, whereas ACP2 is designed as a control peptide by changing some critical amino acid residues in AIP2 that inhibits CaMKII (Ishida *et al*., 1998; Khoo *et al*., 2006). It was confirmed that mCherry was expressed in M-cells in transgenic larvae that carried both hspGFF62A and UAS:mCherry-2A-ACP2 and in those that carried hspGFF62A and UAS:mCherry-2A-AIP2 (Fig. 10B). In ACP2-expressing M-cells, GlyR clusters were normally formed at the surface of the M-cells (Fig. 10Ca, D; Table S5). In contrast, GlyR cluster formation was significantly impaired in AIP2-expressing M-cells ($P < 0.001$; Fig. 10Cb, D; Table S5). These results demonstrate that CaMKII activation in postsynaptic cells is essential for the GlyR clustering *in vivo*.

4. Discussion

In this study, we have presented the first *in vivo* evidence that the formation and maintenance of GlyR clusters in zebrafish M-cells require glycinergic synaptic transmission and that L-type Ca^{2+} channels and the postsynaptic CaMKII are involved in the process. We found three developmental features of GlyR clustering in M-cells. First, GlyR clusters appear by 2 dpf and subsequently increase in number. This is consistent with previous physiological findings that the frequency of mPSCs in zebrafish M-cells increases from 26 to 52 hpf (Ali *et al*. 2000). Second, blockade of glycinergic transmission impairs the formation of GlyR clusters, but termination of the blockade enables initiation of the cluster formation. Thus, GlyRs accumulate at synaptic sites in a synaptic transmission-dependent manner, even if the initiation of glycinergic transmission is delayed during the early developmental stages. Third, glycinergic transmission is necessary for the maintenance of GlyR clusters, at least until 30 dpf, and the importance of glycinergic input for the maintenance appears to decrease in an age-dependent manner. In addition, short term-strychnine treatment (1 h) experiments have suggested that continuous glycinergic transmission is crucial for the maintenance of GlyR clusters. Thus, present findings show that GlyR clusters are dynamically regulated in a synaptic transmission-dependent manner during development.

4.1 General discussion

Because clustering of neurotransmitter receptors at postsynaptic sites is crucial for formation and development of proper synaptic function, the clustering mechanisms must be ultimately investigated in developing animals to test the relevance to these phenomena. However, mechanisms for activity-dependent clustering in CNS have been studied mainly by using *in vitro* experimental systems including cultured slices of brain and spinal cord, and little has been studied *in vivo,* especially in glycinergic synapses.

In vivo mechanisms of activity-dependent neurotransmitter clustering in vertebrate were often investigated using neuromuscular junction (NMJ) because of its accessibility (Akaaboune *et al.,* 1999). The NMJ is a cholinergic synapse that develops between a motor neuron and a muscle fiber, and has been the primary model system for studying regulation of the receptor composition of synapses. Further, LTP was sometimes examined in CNS *in vivo* but there are few morphological studies.

A change in the number of neurotransmitter receptors at synapses is detected by electrophysiological techniques and/or imaging using immunostaining or fluorescent fusion protein to receptors. Pharmacological imaging studies about activity-dependent clustering *in vitro* have been performed since 1990s. In these studies, many receptors exhibit activity-dependent modulation of clustering, including GlyRs on spinal cord neurons,

$GABA_A$Rs on hippocampal neurons, AMPARs and NMDARs on hippocamal neurons (Rao and Craig, 1997; Kirsch and Betz 1998; Lévi *et al*., 1998; Lissin *et al*., 1998; O'Brien *et al*., 1998; Bannai *et al*., 2009; Lévi *et al*., 2008). However, the effect of the inhibitors on receptor clustering is not consistent across studies.

It is known that there are mainly two kinds of regulation mechanism of GlyR clustering; glycinergic transmission stabilizes GlyR clusters at the synaptic sites (Kirsch and Betz 1998; Lévi *et al*., 1998). In contrast, GlyR clustering depends on the NMDAR activity (Lévi *et al*., 2008).

In the present study, we established a model system in M-cells in developing zebrafish to examine processes and modulation of GlyR clustering *in vivo*. M-cells are easily identified morphologically as a pair of giant reticulospinal neurons in the hindbrain. GlyRs are expressed in M-cells which receive numerous glycinergic inputs as major inhibitory inputs which shape the M-cell activity (Faber and Korn, 1978; Koyama *et al*., 2011; Moly and Hatta, 2011). Furthermore, glycinergic LTP was found electrophysiologically in adult goldfish M-cells (Oda *et al*., 1995, 1998). This is the only example of the glycinergic LTP *in vivo*. Furthermore, zebrafish embryo and larvae are transparent and suitable for imaging. Genetics approaches are well established and plenty of transgenic lines help to image and manipulate a specific group of neurons. Thus, GlyRs on M-cell in zebrafish gives a valuable *in vivo* model to analyze phenomena and underlying mechanisms of receptor clustering during development.

In addition, output of M-cell is closely correlated with escape initiation behavior and the neural circuits for triggering fast escape including M-cells have been well known (Korn and Faber, 2005; Kohashi and Oda, 2008; Koyama *et al.,* 2011). Hence, comprehensive analyses at many levels ranging from molecules to behavior are possible in this system, through which elucidation of important aspects of plastic functions of CNS would be expected.

4.2 The rates and mechanisms of receptor clustering modulation

Previous immunocytochemical studies suggested that it took several hours to days to modulate clustering of neurotransmitter receptors including GlyRs in an activity-dependent manner (Rao and Craig, 1997; O'Brien *et al*., 1998; Kirsch and Betz, 1998; Lévi *et al*., 1998; Rasmussen *et al*., 2002). More recently, however, it has been reported that the modulation were seen within 1 h in cultured neurons (Lévi *et al*., 2008; Bannai *et al.,* 2009). In agreement with the report, we found in the present study that application of strychnine eliminated GlyR clusters within 1 h *in vivo*, which is a very fast response. What is the cellular mechanism underlying the rapid elimination of postsynaptic GlyRs *in vivo* by strychnine treatment? There are at least two possibilities to explain the disappearance of GlyR clusters after strychnine treatment. One possibility is that glycinergic transmission blockade accelerates GlyR lateral

diffusion by unanchoring them from the postsynaptic structure, which is similar to the regulation of glutamate receptors (Ehlers *et al*., 2007). Indeed, GlyR mobility is regulated by lateral diffusion in cultured neurons within seconds to minutes (Dahan *et al*. 2003). Another possibility is that strychnine promotes degradation of surface GlyRs. It has been reported, however, that the half-life of plasma membrane GlyRs is about 14 h in cultured neurons and that is not affected by strychnine-induced blockade of glycinergic transmission (Rasmussen *et al*., 2002). The reported half-life of the GlyR protein is much longer than 1 h, within which the GlyR clusters were eliminated by strychnine, suggesting that degradation of cell surface GlyR is not primarily responsible for the rapid elimination of GlyR clusters. We therefore suggest that blockade of glycinergic transmission facilitates the lateral diffusion of GlyR that results in the rapid disappearance of GlyR clusters.

We also found that after strychnine washout at 3 dpf, the GlyR re-clustering occurred in less time in the 1 h-strychnine treatment experiment than in the experiments with the longer (22 hpf-3 dpf) strychnine treatment (Fig. 7B). The difference in recovery time suggests that long-term strychnine treatment affects GlyR regulation more profoundly than dose the acute blockade of glycinergic transmission. The changes caused by the long-term strychnine treatment may involve expression and/or intracellular transport of GlyR, in addition to the presumed facilitation of GlyR lateral diffusion. Indeed, it was shown in cultured neurons that strychnine treatment suppresses intracellular transport of newly synthesized gephyrin, which

binds to GlyR during intracellular transport and at synaptic sites (Maas *et al*, 2009). Further studies are needed to clarify whether the GlyR supply is affected in the M-cells after long-term treatment with strychnine.

4.3 Changes of clustering mechanisms during development *in vivo*

Age-related deficit in LTP of glutamatergic synaptic transmission was observed, although it remains controversial (Lynch, 2004; Rosenzweig and Barnes, 2003; Kumar, 2011). In the present study, the synaptic transmission-dependent maintenance of GlyR clusters observed in larvae and juveniles decreased as the fish grew up. In agreement with this observation, it has been reported that glycinergic transmission blockade does not affect GlyR clusters in adult goldfish M-cells (Seitanidou *et al*., 1992). Thus, the maintenance of GlyR clusters appears to be age-dependent. The age-dependency may be partly attributable to a reduction of intracellular Cl^- concentration in developing neurons. It has been shown that glycinergic transmission causes hyperpolarization in mature neurons whereas it can induce depolarization in immature and young neurons, in which the intracellular Cl^- concentration is high (Tapia and Aguayo, 1998). The intracellular Cl^- concentration decreases due to the late onset of the K^+-Cl^--coupled co-transporter (KCC2) expression, which exports cytosolic Cl^- (Tapia and

Aguayo, 1998; Ben-Ari, 2002; Reynolds *et al*., 2008; Zhang *et al*., 2010). Although developmental shift in the actions of inhibitory neurotransmitter has been observed in nearly all brain structures in rodents and organisms examined, there is considerable variation in developmental time course of KCC2 expression among brain structures (Ben-Ari, 2002). KCC2 expression increases during zebrafish development (Reynolds *et al*., 2008), but it remains to be elucidated whether or when the Cl^- reversal potential changes in the M-cells.

4.4 Mechanisms for the synaptic activity-dependent clustering

How does glycinergic transmission regulate GlyR clustering? Strychnine directly blocks glycinergic inputs to the M-cells. At the same time, it might induce excessive excitation of M-cells and connected neurons by disinhibition, since glycinergic inputs can inhibit action potentials through their shunting effects (Takahashi *et al*., 2002). Therefore, strychnine-induced disappearance of GlyR clusters can be caused directly by the elimination of glycinergic inputs or, alternatively, indirectly by the induction of excessive excitation. However, it is shown here that blockade of voltage-gated L-type Ca^{2+} channels inhibited GlyR clustering *in vivo*. This result is difficult to explain using the model of elevated excitation in which the Ca^{2+} channels should be activated. In contrast, possible glycinergic

transmission-induced postsynaptic depolarization appears to account for the result.

The present *in vivo* data suggest that L-type Ca^{2+} channels and CaMKII are essential for GlyR clustering. In addition, we found that targeted expression of CaMKII inhibitor peptides in the M-cells disturbed GlyR clusters at the surface of the cells. This is the first demonstration that CaMKII activation in the postsynaptic cells is required for GlyR clustering. In adult goldfish M-cells, CaMKII is abundant at postsynaptic sites in the soma and lateral dendrites (Pereda *et al*., 1998; Flores *et al*., 2010). CaMKII is also associated with the L-type Ca^{2+} channels has in cardiac muscles (Hudmon *et al*., 2005). In addition, glycinergic currents can be enhanced by intracellular application of activated CaMKII in cultured spinal neurons (Wang and Randić, 1996). Thus, CaMKII may be a crucial mediator that links Ca^{2+} and GlyR clustering. The present research provides a model where glycinergic transmission causes the local depolarization of postsynaptic membranes that activates L-type Ca^{2+} channels and then increases Ca^{2+} permeability. The intracellular Ca^{2+} activates CaMKII, thereby facilitating the GlyR clustering at postsynaptic sites (Fig. 11).

If this is the case, how does CaMKII mediate GlyR clustering? Numerous studies have found a critical role for CaMKII in the activity-dependent recruitment of AMPARs at glutamatergic synapses during plastic changes in synaptic transmission. (Asrican *et al*., 2007; Correia *et al*., 2008; Hayashi *et al*., 2000; Lee *et al*., 2009; Lisman *et al*., 2002; Rongo and Kaplan, 1999; Sanhueza *et al*., 2007). Although it has been suggested that CaMKII activation

enhances the exocytosis of AMPARs, a recent study showed that CaMKII leads to trapping of diffusing AMPARs; CaMKII directly phosphorylates the AMPAR auxiliary subunit stargazin and the its binding to PSD-95 immobilized AMPARs at postsynaptic sites in an activity-dependent manner (Opazo *et al*., 2010). Regarding glycinergic synapses, it has been shown that gephyrin has several serine residues that can be phosphorylated by CaMKII *in vitro* (Charrier *et al*., 2010). Phosphorylation of several specific gephyrin serine residues promotes interaction with peptidyl-prolyl isomerase Pin1, a molecular chaperone, and causes a conformational change in gephyrin that enhances gephyrin binding to GlyRs (Zita *et al*., 2007). However, these serine residues of gephyrin are different from the actual phosphorylation targets identified by an *in vitro* CaMKII reaction (Charrier *et al*., 2010). Although the gephyrin phosphorylation site that is critical for GlyR clustering *in vivo* is still unknown, CaMKII or CaMKII-dependent protein kinase likely promotes gephyrin phosphorylation, which accelerates the GlyR clusters formation at the postsynaptic sites. Protein kinase C (PKC) may also be involved in the process; a recent study has demonstrated that PKC-induced phosphorylation of the GlyRβ subunit regulates GlyRβ-gephyrin interaction (Specht *et al*., 2011).

Two independent groups reported that GlyR activation is required for stabilization of synaptic receptor in cultured neurons (Kirsch and Betz, 1998; Lévi *et al*., 1998). However, they showed inconsistent results about gephyrin clusters. Kirsch and Betz indicated that

gephyrin clusters were eliminated as well as the receptors (Kirsch and Betz, 1998), whereas Lévi and colleagues showed that gephyrin distribution did not change by strychnine treatment (Lévi *et al*., 1998). The former would indicate that an activity-dependent process controls clustering of gephyrin either directly or indirectly which in turn controls GlyR clutering, while the latter would indicate that it controls GlyR clustering independently of gephyrin clustering. This is a very important issue and need to be addressed it in future studies in zebrafish M-cells.

4.5 Remaining Questions and Conclusions

Studies of activity-dependent modulation of GlyR clustering raise some important questions. Can synaptic receptor clustering be also modulated by more subtle long-term changes in synaptic activity, rather than presumed drastic changes caused by the pharmacological manipulations employed in this study? Does activity control GlyR clustering at the level of an individual synapse? To answer these questions is very crucial for determining which activity of neuron or GlyR directly regulates GlyR clustering and clarifying a role of the activity-dependent clustering during development in more details.

Our GlyR clustering assay in zebrafish M-cells provides an excellent model for

analyzing synaptic transmission-dependent receptor accumulation and its underlying mechanisms in a developing single cell. In this study, we also succeeded in inducing the expression of certain genes in M-cells using the Gal4-UAS system. Zebrafish embryos and larvae are transparent and are thus suitable for visualizing fluorescence-tagged receptor behavior *in vivo*, which will be useful in future studies.

Figure 1

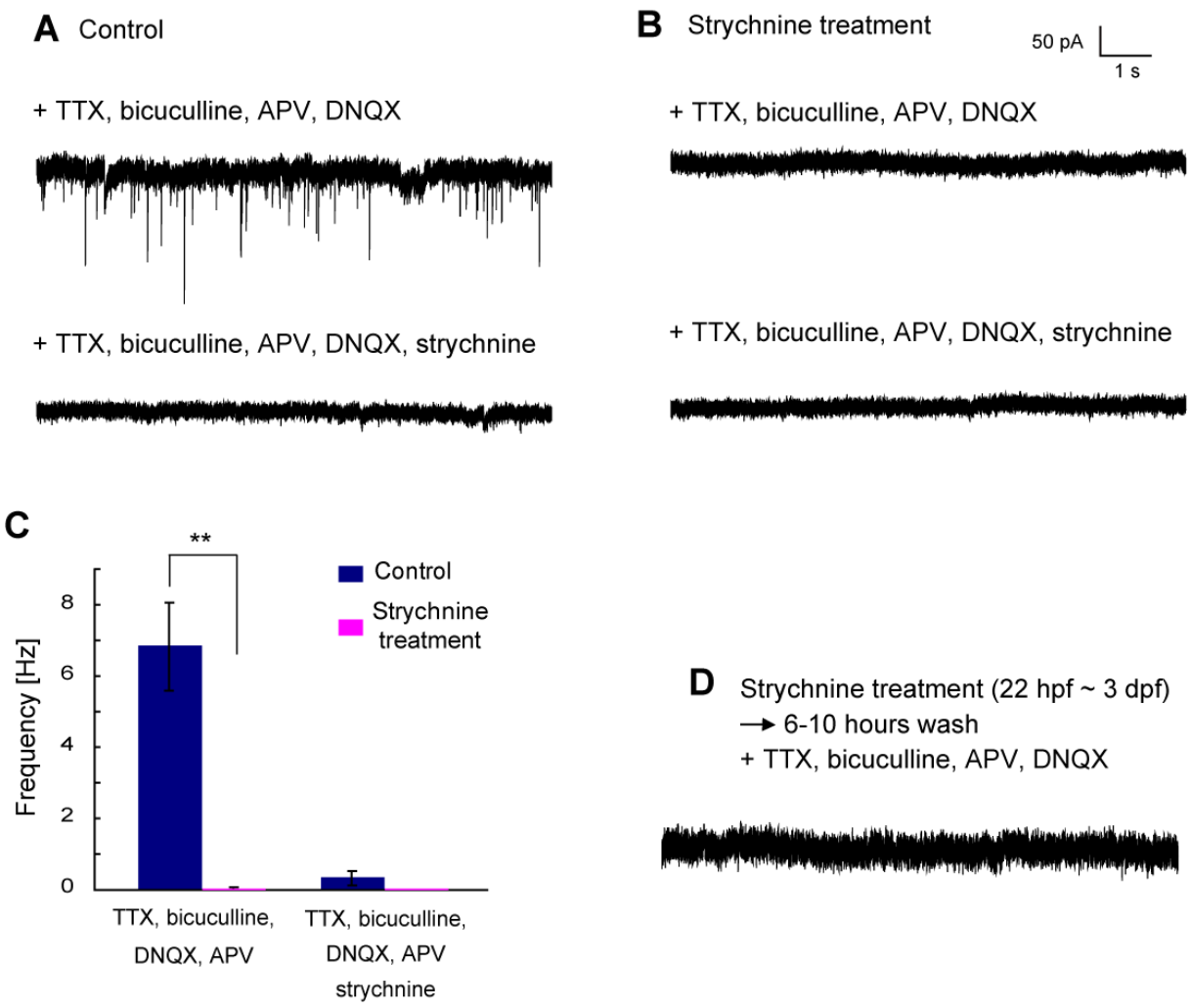

A Control
+ TTX, bicuculline, APV, DNQX
+ TTX, bicuculline, APV, DNQX, strychnine
B Strychnine treatment
50 pA
1 s
+ TTX, bicuculline, APV, DNQX
+ TTX, bicuculline, APV, DNQX, strychnine
C
**
Control
Strychnine treatment
Frequency [Hz]
0
2
4
6
8
TTX, bicuculline, DNQX, APV
TTX, bicuculline, DNQX, APV strychnine
D
Strychnine treatment (22 hpf ~ 3 dpf)
→ 6-10 hours wash
+ TTX, bicuculline, APV, DNQX

Figure 1. Blockade of glycinergic transmission leads to a lasting functional deficit in glycinergic synapses *in vivo.*

Miniature PSCs (mPSCs) were recorded from M-cells in 3-dpf larvae. (**A**) In the presence of 1-2 μM TTX, 10 μM bicuculline, 50 μM APV, and 50 μM DNQX, frequent mPSCs were recorded in control larvae (top; 6.8 ± 1.2 Hz, n = 7). These mPSCs were blocked by application of 5 μM strychnine in the recording condition (bottom; 0.3 ± 0.2 Hz, n = 3). Note that mPSCs in (A) represent strychnine-sensitive glycinergic currents. (**B**) In larvae raised in the presence of 800 μM strychnine, no mPSCs were recorded in M-cells at 3 dpf (top; 0.01 ± 0.01 Hz, n = 4) using the same recording protocol as in (A)**.** Application of strychnine in the recording condition had no effect on mPSCs (bottom; 0.0 ± 0.0 Hz, n = 3). (**C**) Histograms showing the frequencies of mPSCs in the M-cells. Blue bars represent recordings from larvae raised in control conditions. Pink bars represent recording from larvae raised in strychnine-containing water. (***P* < 0.01, Mann-Whitney test). (**D**) Disappearance of the glycinergic mPSCs by GlyR blockade is likely not a result of remaining strychnine after washout. Zebrafish larvae were raised in the presence of strychnine from 22 hpf to 3 dpf, and then the strychnine was removed from the breeding water. After 6-10 h of strychnine washout, miniature PSCs (mPSCs) were recorded from the M-cells. Glycinergic mPSCs in the M-cells remained absent (0.0 ± 0.0 Hz, n = 3), which eliminates the possibility that the glycinergic mPSCs were affected by remaining strychnine in the larvae after wash out (Fig. 1B).

Figure 2

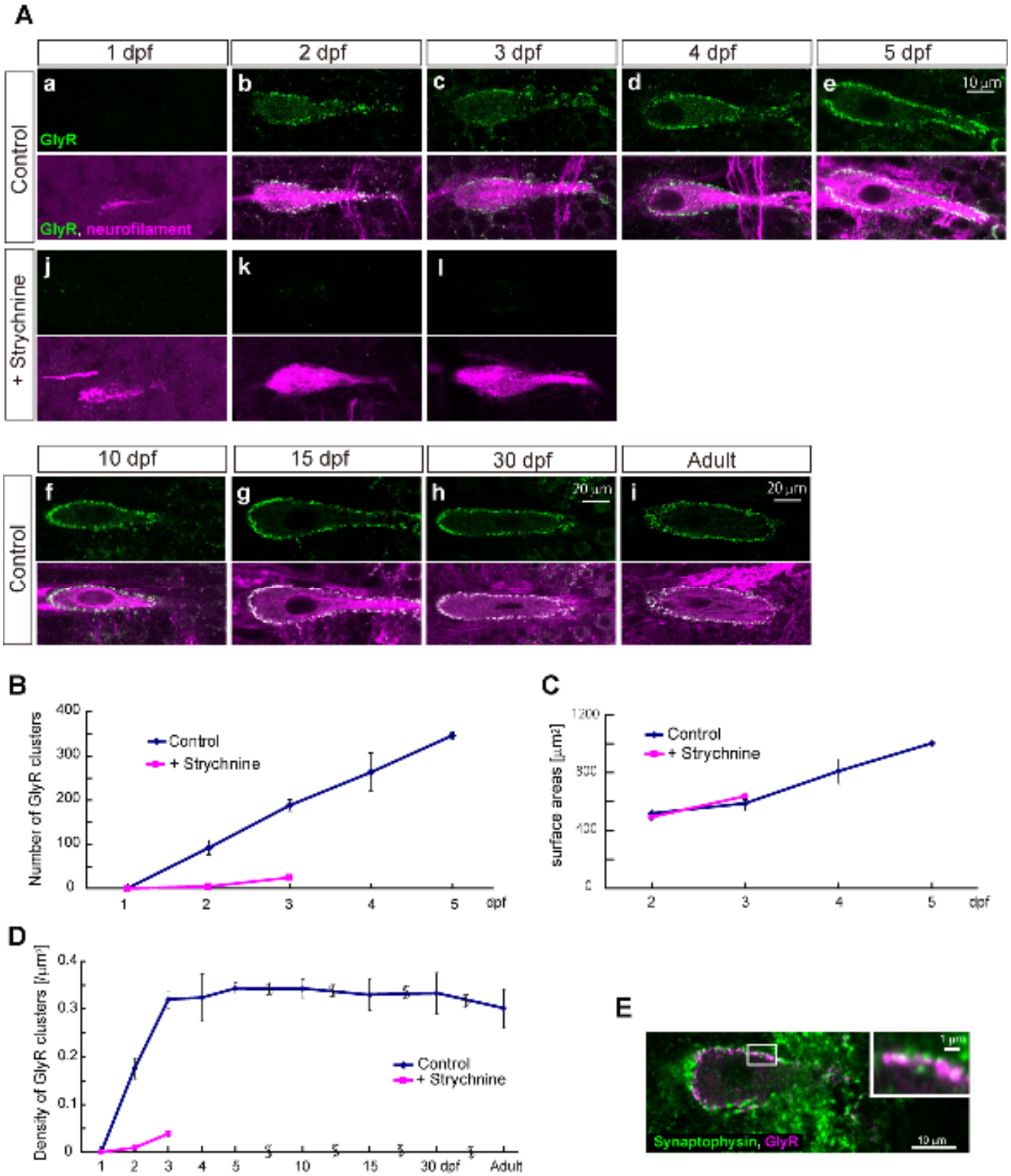

A
1 dpf
2 dpf
3 dpf
4 dpf
5 dpf
Control
a
b
c
d
e
10 µm
GlyR
GlyR, neurofilament
+ Strychnine
j
k
l
10 dpf
15 dpf
30 dpf
Adult
Control
f
g
h
20 µm
i
20 µm
B
Number of GlyR clusters
400
300
200
100
0
Control
+ Strychnine
1
2
3
4
5
dpf
C
surface areas [µm²]
1200
800
400
0
Control
+ Strychnine
2
3
4
5
dpf
D
Density of GlyR clusters [/µm²]
0.4
0.3
0.2
0.1
0
Control
+ Strychnine
1
2
3
4
5
10
15
30 dpf
Adult
E
1 µm
Synaptophysin, GlyR
10 µm

Figure 2. Formation of GlyR clusters in M-cells is blocked by strychnine treatment.

(**A**) Distribution of GlyRs in M-cells during normal development (a-h) and in adult fish (i). In each image, the upper panel is an immunostained horizontal section of the M-cell soma and lateral dendrite with anti-GlyR (mAb4a, green), and the lower panel is a double labeled with anti-GlyR (green) and anti-neurofilament (3A10, magenta). GlyR immunoreactivities were observed as dots at the M-cell surface. Blockade of glycinergic transmission was examined by treating larvae with 800 μM strychnine starting at 22 hpf (j-l). In strychnine-treated larvae, GlyR clusters were not observed at the M-cells' surface. (**B**) The total number of GlyR clusters in a single M-cell soma in control and strychnine-treated larvae. (**C**) The surface area of the M-cell soma increased until 5 dpf in controls and in strychnine-treated larvae at least until 3 dpf. (**D**) GlyR cluster density in controls reached a plateau at 3 dpf and was maintained thereafter until adulthood. GlyR cluster density did not increase in strychnine-treated larvae. (Control: n = 3~6; +Strychnine: n = 3~5 at each time point in B-D). (**E**) Double labeling of M-cells using anti-synaptophysin (green) and anti-GlyR (magenta) at 5 dpf. Apposition of GlyRs with synaptophysin, a marker for presynaptic terminals, reveals that GlyR clusters were formed at postsynaptic sites. Inset: magnified view of the boxed area (white rectangle).

Figure 3

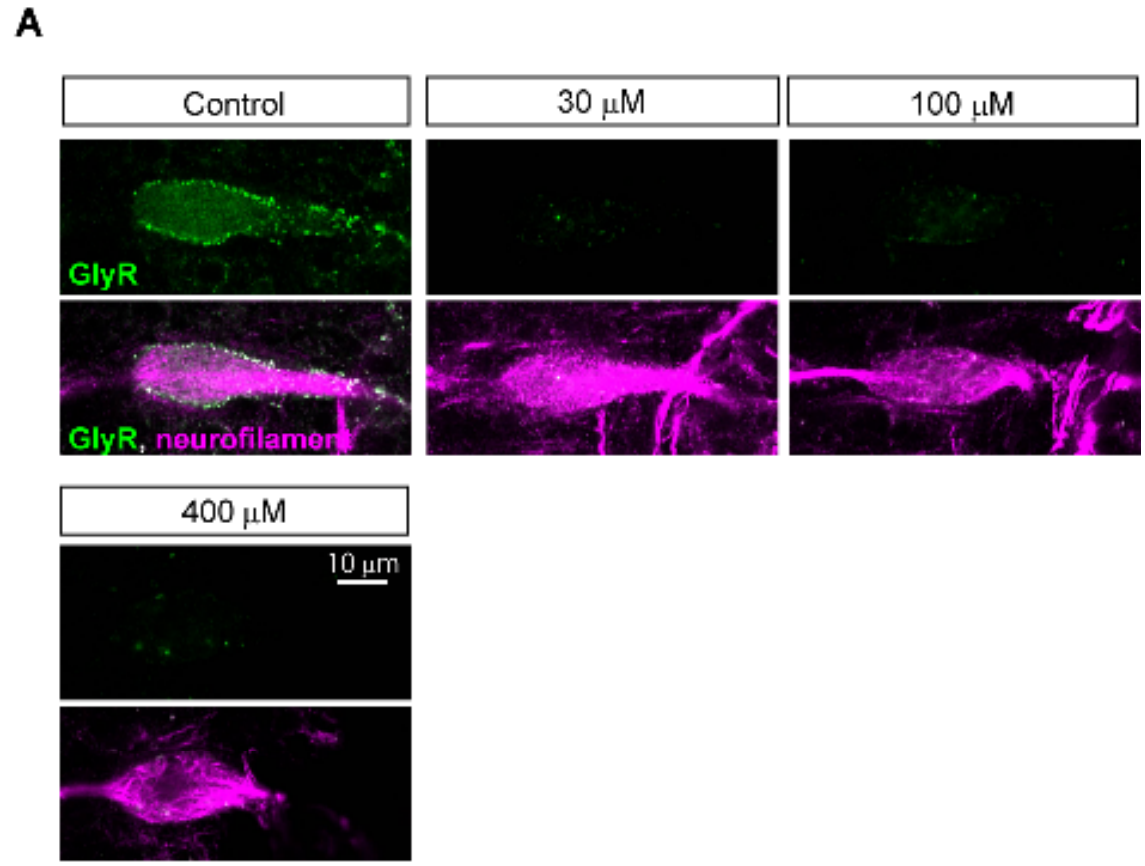

A
Control
30 μM
100 μM
GlyR
GlyR, neurofilament
400 μM
10 μm
B
GlyR clusters density [/μm²]
0.4
0.3
0.2
0.1
0
**
**
**
0 μM
30 μM
100 μM
400 μM

Figure 3. Strychnine treatment with various concentrations also impaired GlyR clustering.

(**A**) Distribution of GlyRs in the M-cells after exposure to strychnine at 0 (control), 30, 100, and 400 μM from 22 hpf to 3 dpf. In each image, the upper panel represents M-cell immunostaining using anti-GlyR (mAb4a, green), and the lower panel shows a double labeling with anti-GlyR (green) and anti-neurofilament (3A10, magenta). (**B**) GlyR cluster density on the M-cell soma in the strychnine-treated larvae at 3 dpf (**$P <$ 0.01). Application of different strychnine concentrations (30-800 μM) each disturbed GlyR clustering to a similar extent.

Figure 4

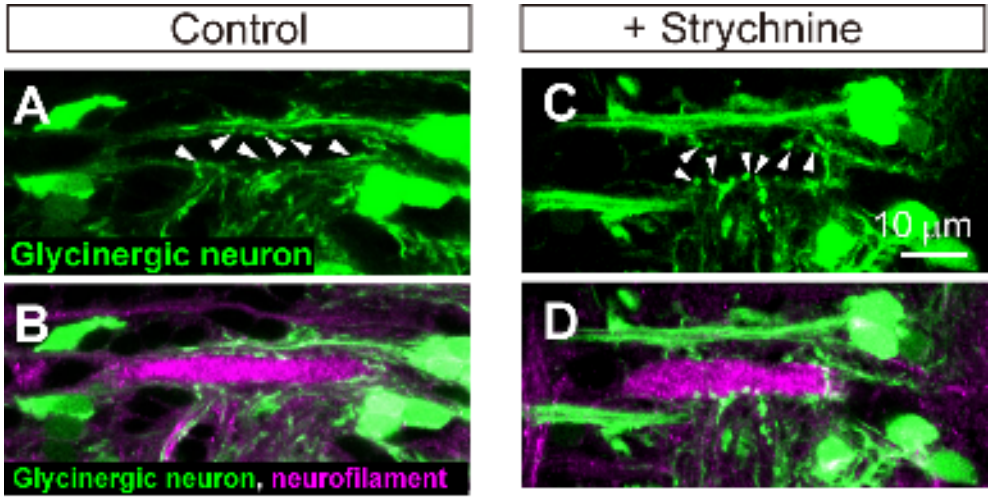

Control
+ Strychnine
A
Glycinergic neuron
B
Glycinergic neuron, neurofilament
C
10 μm
D

Figure 4. Glycinergic nerve terminals project on M-cell in larvae exposed to strychnine.

(**A**) Presynaptic boutons (arrowheads) of glycinergic neurons faced toward an M-cell at 3 dpf in glyt2:GFP transgenic larvae. (**B**) Double labeling of glycinergic neurons (green) and an M-cell soma (magenta) in glyt2:GFP larvae. (**C**) Presynaptic boutons (arrowheads) of glycinergic neurons on M-cell in glyt2:GFP transgenic larvae following 800 μM strychnine treatment from 22 hpf to 3 dpf. (**D**) Double labeling of glycinergic neurons and an M-cell soma in a glyt2:GFP larvae after strychnine treatment. Glycinergic presynaptic terminals contact on the M-cell after exposure to strychnine for 3 days.

Figure 5

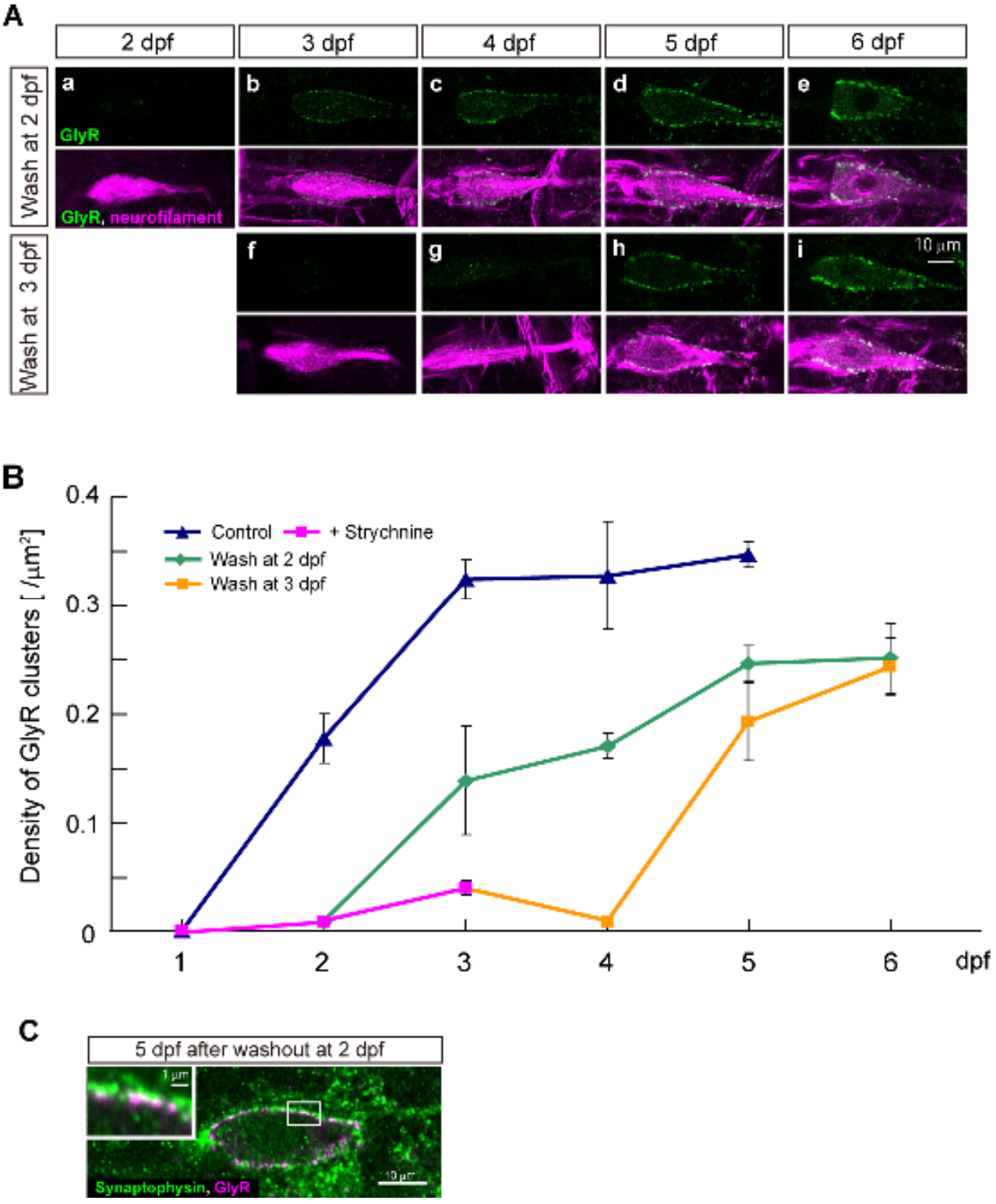

A
2 dpf
3 dpf
4 dpf
5 dpf
6 dpf
Wash at 2 dpf
Wash at 3 dpf
a
b
c
d
e
f
g
h
i
GlyR
GlyR, neurofilament
10 µm
B
Density of GlyR clusters [/µm²]
0.4
0.3
0.2
0.1
0
Control
+ Strychnine
Wash at 2 dpf
Wash at 3 dpf
1
2
3
4
5
6
dpf
C
5 dpf after washout at 2 dpf
1 µm
10 µm
Synaptophysin, GlyR

Figure 5. GlyR clusters form on the M-cell surface following strychnine removal.

(**A**) (**a-e**) Zebrafish embryos were raised in the presence of strychnine (800 μM) from 22 hpf to 2 dpf and then kept in the absence of strychnine after it was washed out at 2 dpf. GlyR clusters on the M-cells were immunolabeled at the indicated stages. Note that GlyR clusters were missing at 2 dpf but were formed on the cell surface after the removal of strychnine. (**f-i**) Strychnine was bath-applied to embryos from 22 hpf to 3 dpf and the larvae were raised without strychnine after 3 dpf. In each image, the upper panel represents M-cell immunolabeling with anti-GlyR (green) and the lower panel represents M-cell co-labeling with anti-GlyR (green) and anti-neurofilament (magenta). (**B**) This graph represents the recovery of GlyR cluster density. Control: n = 3~4; strychnine treatment: n = 3~6; wash out at 2 dpf: n = 3~6; wash out at 3 dpf: n = 3~5. The control and strychnine treatment graph contains the same data as in Fig. 2D. (**C**) Immunolabeling of an M-cell with anti-synaptophysin (green) and anti-GlyR (magenta) in zebrafish larvae (5 dpf) treated with strychnine from 22 hpf to 2 dpf. GlyRs were apposed with the presynaptic terminals. GlyR clusters that appeared after strychnine removal were formed at postsynaptic sites. Inset, magnified view of the boxed area (white rectangle).

Figure 6

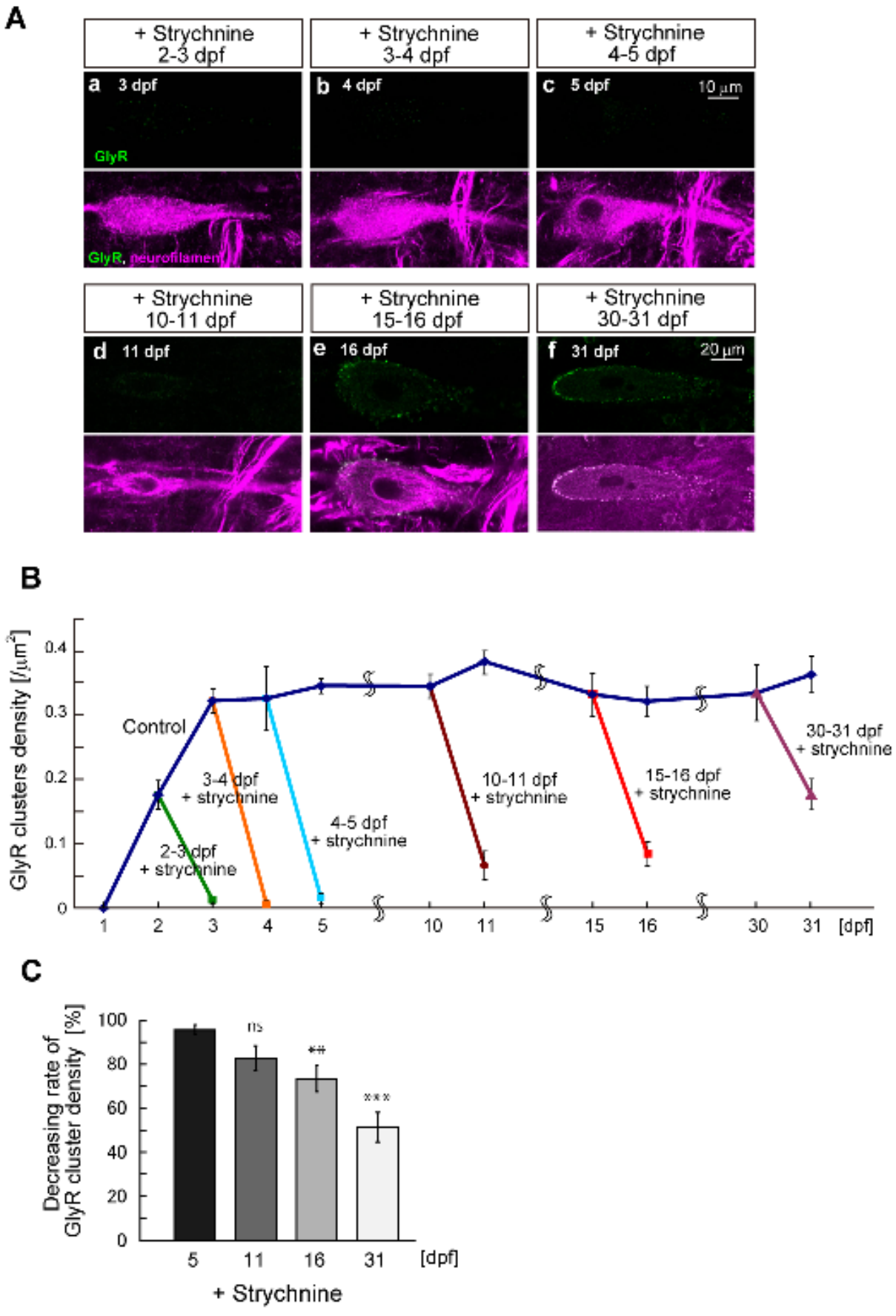
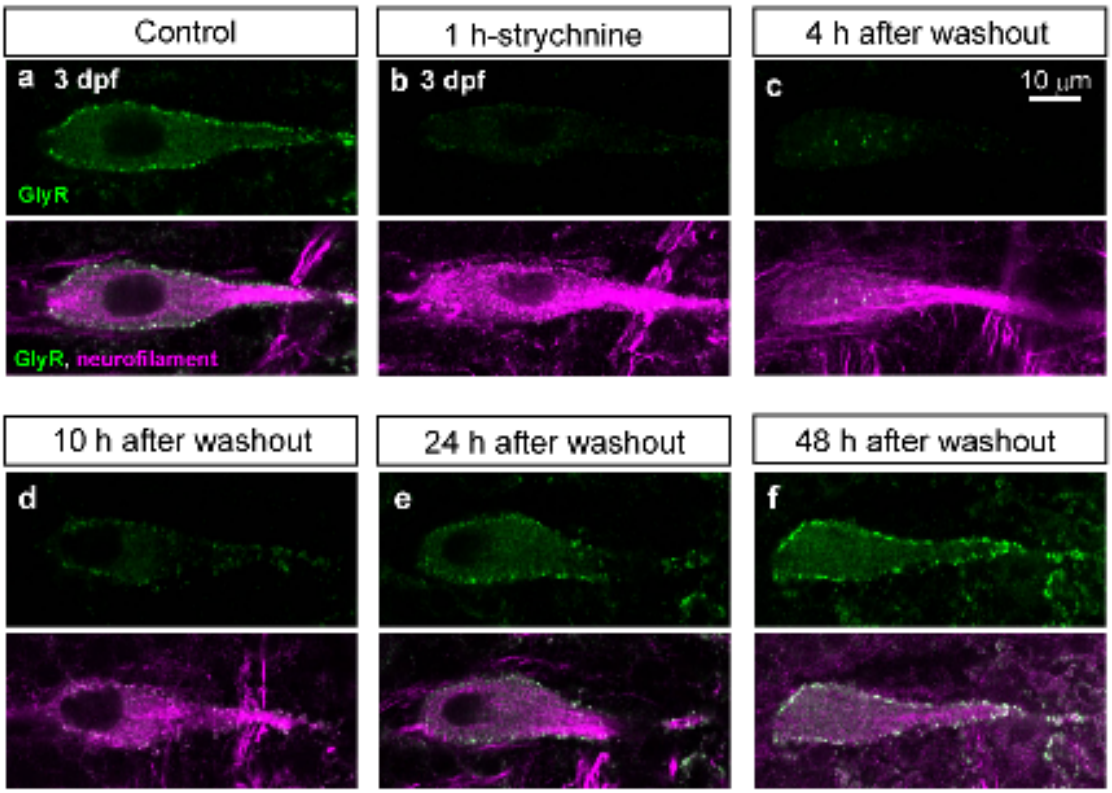

A
+ Strychnine 2-3 dpf
+ Strychnine 3-4 dpf
+ Strychnine 4-5 dpf
a 3 dpf
b 4 dpf
c 5 dpf
10 µm
GlyR
GlyR, neurofilament
+ Strychnine 10-11 dpf
+ Strychnine 15-16 dpf
+ Strychnine 30-31 dpf
d 11 dpf
e 16 dpf
f 31 dpf
20 µm
B
GlyR clusters density [/µm²]
0.4
0.3
0.2
0.1
0
Control
3-4 dpf + strychnine
2-3 dpf + strychnine
4-5 dpf + strychnine
10-11 dpf + strychnine
15-16 dpf + strychnine
30-31 dpf + strychnine
1 2 3 4 5 10 11 15 16 30 31 [dpf]
C
Decreasing rate of GlyR cluster density [%]
100
80
60
40
20
0
ns
**
***
5 11 16 31 [dpf]
+ Strychnine

Figure 6. Glycinergic transmission is also necessary for GlyR cluster maintenance.

(**A**) GlyRs (green) were immunolabeled in the M-cells (magenta) following a 24-h application of strychnine (20-30 μM) at the indicated stages. GlyR clusters disappeared from the M-cell surface after a 24-h strychnine treatment in larvae (a-e) and juveniles (f). (**B**) A graph representing surface GlyR cluster elimination after a 24-h strychnine treatment. The effect of strychnine was less remarkable in juveniles than in larvae. Control: n = 3~6; after a 24-h strychnine treatment: n = 6~19. The control plots are the same data as in Fig. 2D. (**C**) Decreasing rate of the surface GlyR cluster density after strychnine treatment for 24 h. The decreasing rate was obtained by dividing the reduction in the density by the control density at each stage. Note that the rate of the surface GlyR cluster decrease declined as the fish aged (***P* < 0.01, *** *P* < 0.001 vs. 5 dpf).

Figure 7

A


Control
1 h-strychnine
4 h after washout
a 3 dpf
b 3 dpf
c
10 µm
GlyR
GlyR, neurofilament
10 h after washout
24 h after washout
48 h after washout
d
e
f

B

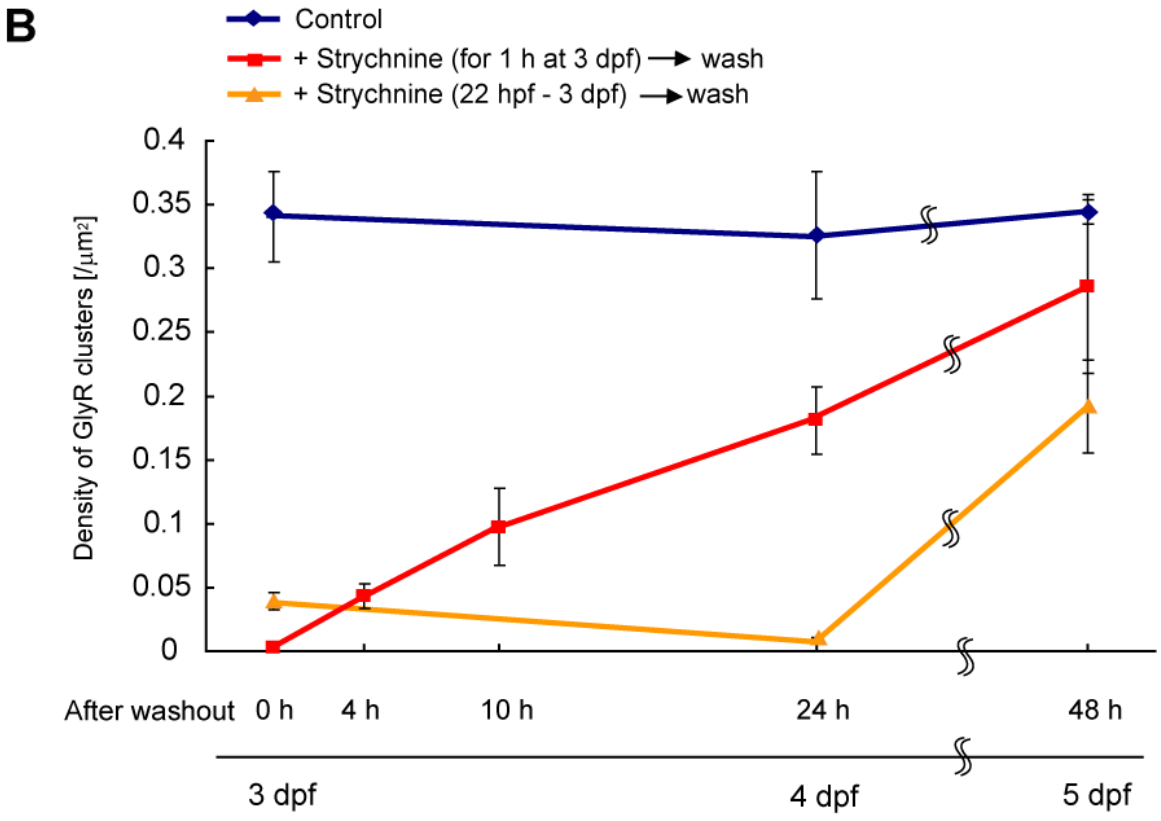

Control
+ Strychnine (for 1 h at 3 dpf) → wash
+ Strychnine (22 hpf - 3 dpf) → wash
Density of GlyR clusters [/µm²]
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
After washout 0 h
4 h
10 h
24 h
48 h
3 dpf
4 dpf
5 dpf

Figure 7. Glycinergic transmission dynamically regulates GlyR.

(**A**) Strychnine (800 μM) was bath-applied to larvae for 1 h at 3 dpf and washed out. The larvae were then raised in breeding water and examined for immunolabeling at the indicated stages. GlyR (green) was immunolabeled in the M-cells (magenta). Strychnine treatment for 1 h eliminated GlyR clusters and the clusters were observed within 1 day after strychnine washout. (**B**) GlyR cluster densities were reduced following a 1-h strychnine treatment and increased after washout. The graph of + Strychnine (22 hpf- 3 dpf) and washout are the same data as in Fig. 5B.

Figure 8

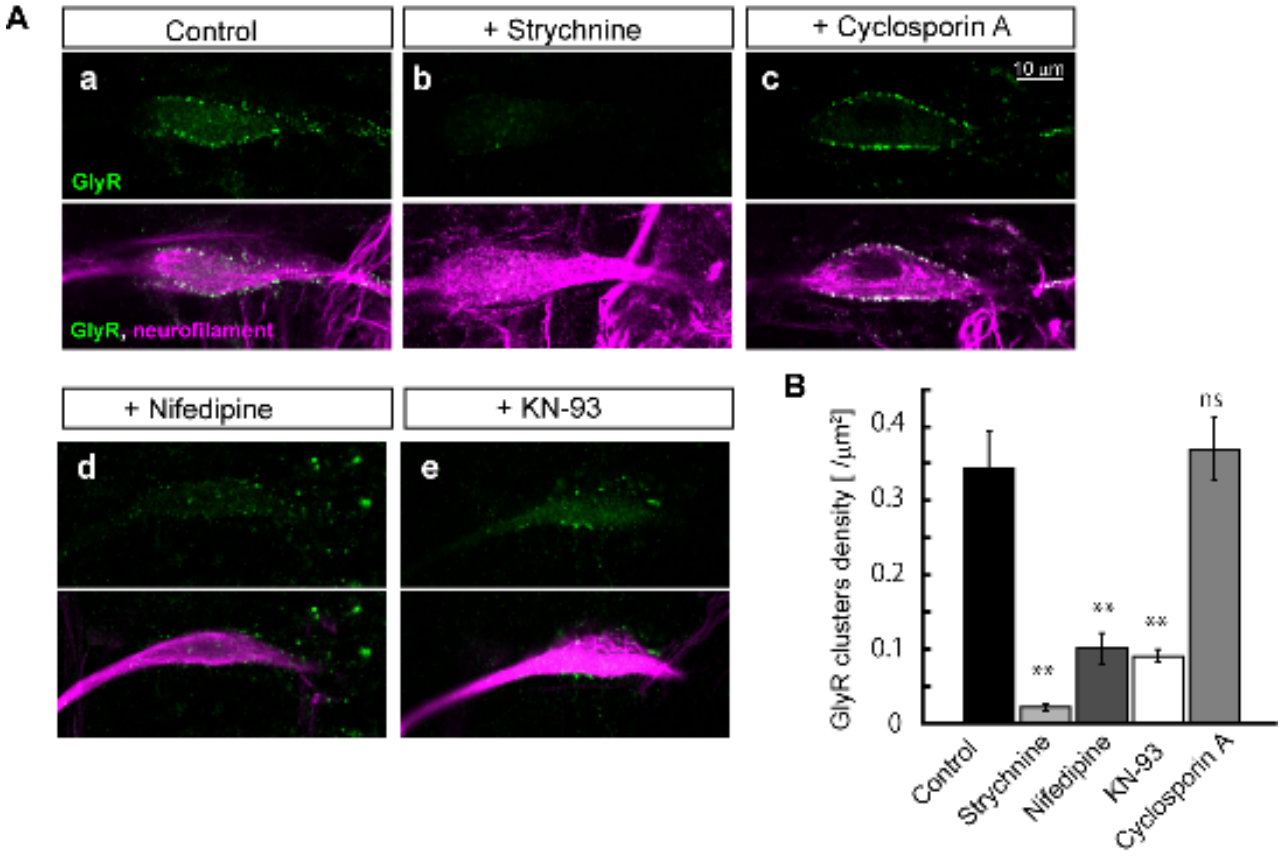


Figure 8. CaMKII and the L-type Ca^{2+} channels are required for GlyR clustering.

(**A**) GlyR clusters (green) were formed on the M-cells (magenta) at 3 dpf in controls (a) but not in 30 μM strychnine-treated larvae (b). Exposure to a calcineurin blocker (cyclosporine A) did not affect GlyR clusters (c). Treatment with an L-type Ca^{2+} channel blocker (nifedipine) or a CaMKII inhibitor (KN-93) impaired GlyR clustering at the M-cell surface (d,e). (**B**) The GlyR cluster density at 3 dpf following the drug-treatments. Control: n = 6; +strychnine: n = 5; +cyclosporine A: n = 4; +nifedipine: n = 6; +KN-93: n = 4 (**P < 0.01).

Figure 9

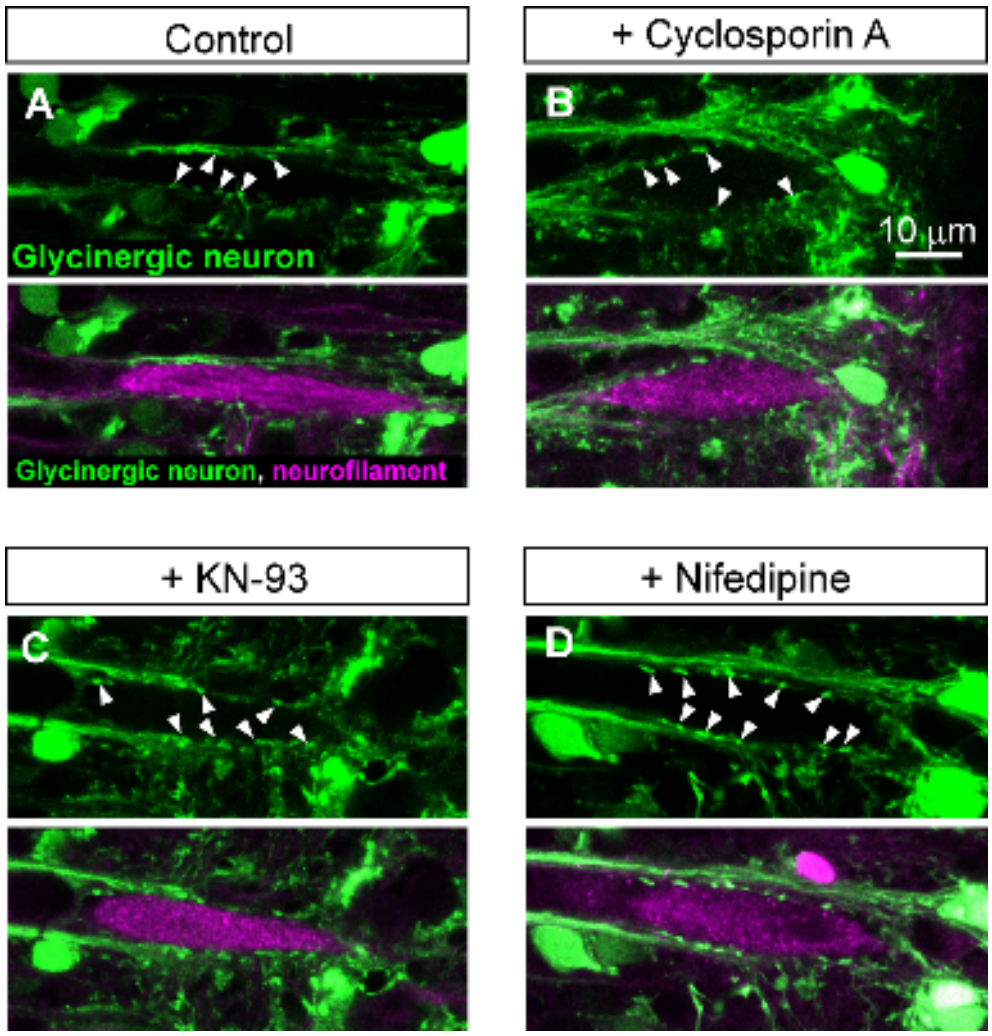


Figure 9. Glycinergic nerve terminals project on M-cell in larvae exposed to KN-93 or Nifedipine.

In each image, the upper panel is the horizontal section of the M-cell with glycinergic neurons (green) in glyt2:GFP larvae and the lower panel presents double labeling of glycinergic neurons (green) and an M-cell soma (magenta) in the same section. (**A**) Presynaptic boutons (arrowheads) of glycinergic neurons faced toward an M-cell at 3 dpf in glyt2:GFP transgenic larvae. (**C**) (**D**) (**E**) Presynaptic boutons (arrowheads) of glycinergic neurons on M-cell in glyt2:GFP transgenic larvae following cyclosporinA, KN-93 or nifedipine treatment, respectively.

Figure 10

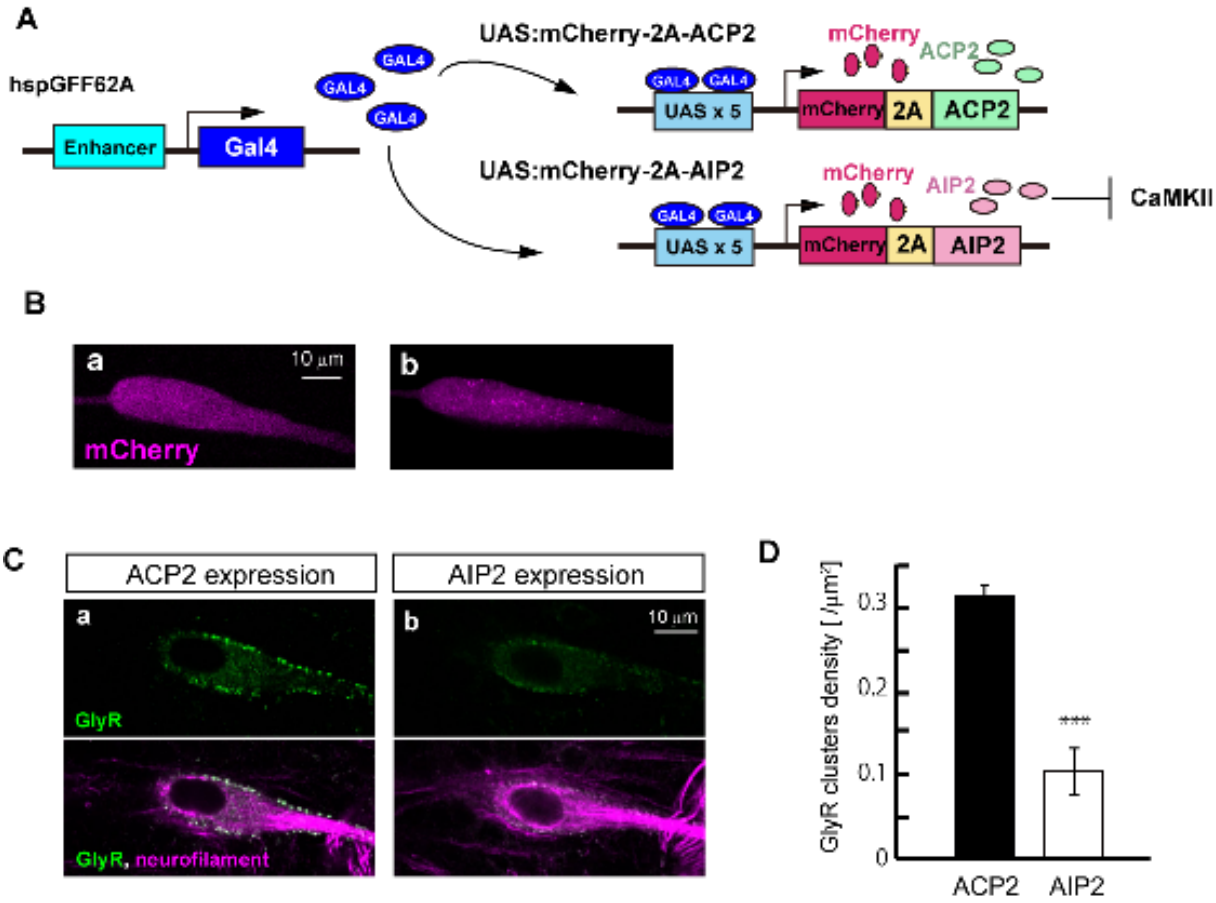


Figure 10. CaMKII is required for GlyR clustering in the M-cell.

(**A**) Schematic summary of the Gal4-UAS system. GAL4 transcriptional activators were expressed in the M-cells in hspGFF62A transgenic larvae. GAL4 drives mCherry and ACP2 expression in hspGFF62A; UAS:mCherry-2A-ACP2 double transgenic fish and AIP2 expression in the hspGFF62A; UAS:mCherry-2A-AIP2 transgenic line. AIP2 blocks CaMKII activation. The 2A is an autocleavage peptide sequence, which generates two split proteins from a single open reading frame. CaMKII in the M-cells is inhibited in hspGFF62A; UAS:mCherry-2A-AIP2 double transgenic fish. (**B**) Expression of mCherry in M-cells in transgenic larvae. mCherry is expressed in the M-cells at 3 dpf before fixation in hspGFF62A; UAS:mCherry-2A-ACP2 (a) or AIP2 (b) transgenic fish, GAL4 in an M-cell drives expression of mCherry. (**C**) In hspGFF62A; UAS:mCherry-2A-ACP2 transgenic larvae, GlyR clusters were formed on the surface of the M-cells at 3 dpf (a). GlyR clustering was impaired in the M-cells in hspGFF62A; UAS:mCherry-2A-AIP2 larvae (b). (**D**) GlyR cluster density (***$P < 0.001$).

Figure 11

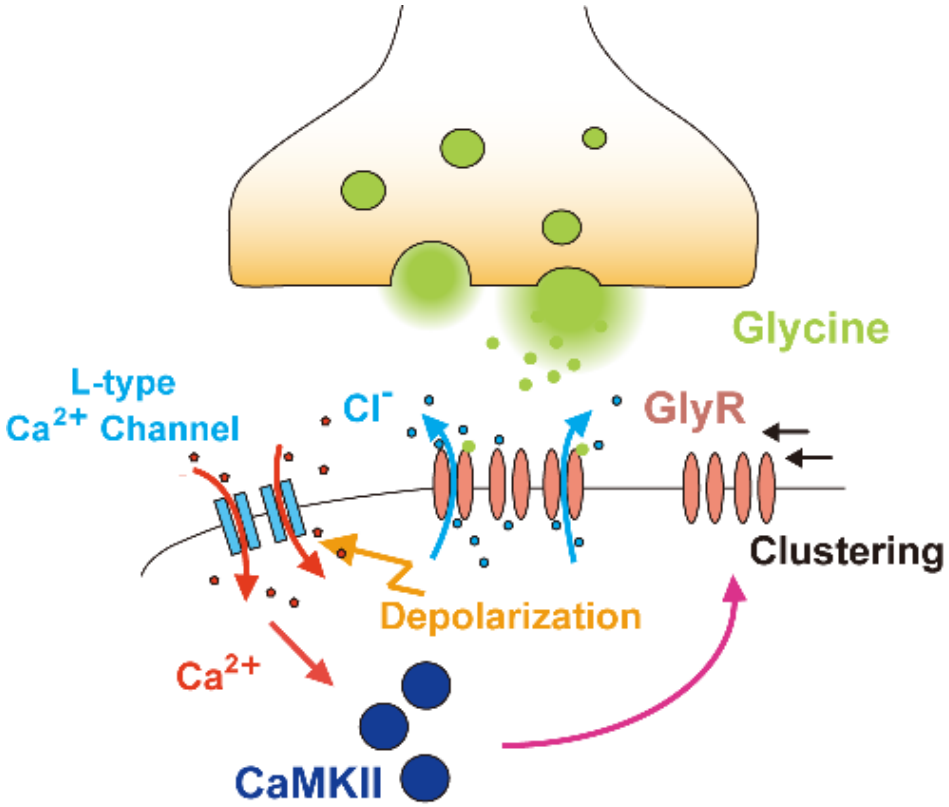


Figure 11. A model for glycinergic transmission-dependent GlyR clustering at the postsynapse.

Glycine released from presynaptic terminals binds to and activates GlyRs, which are initially present at postsynaptic sites by random distribution. This glycinergic input may cause depolarization of the postsynaptic site, where the L-type Ca^{2+} channel is activated to induce Ca^{2+} influx. The local increase of cytosolic Ca^{2+} in turn activates CaMKII at the postsynaptic sites. Activated CaMKII may eventually promote GlyR clustering. Without glycinergic input, GlyRs can freely diffuse on the plasma membrane and they do not form clusters at the postsynaptic sites.

Table S1

Number of GlyR clusters

	1 dpf	2 dpf	3 dpf	4 dpf	5 dpf
Control	0.0 ± 0.0 (n=3)	92.5 ± 16.5 (n=3)	188.3 ± 12.5 (n=3)	263.1 ± 43.3 (n=4)	346.5 ± 7.2 (n=4)
+ Strychnine	0.0 ± 0.0 (n=3)	4.3 ± 1.8 (n=3)	24.6 ± 3.7 (n=5)		

Surface areas [μm^2]

	2 dpf	3 dpf	4 dpf	5 dpf
Control	517.0 ± 31.7 (n=3)	587.8 ± 53.3 (n=3)	811.1 ± 84.6 (n=4)	1003.0 ± 22.3 (n=4)
+ Strychnine	492.2 ± 22.2 (n=3)	637.4 ± 20.3 (n=5)		

Density of GlyR clusters [/μm^2]

	2 dpf	3 dpf	4 dpf	5 dpf	10 dpf	15 dpf	30 dpf	Adult
Control	0.17 ± 0.02 (n=3)	0.32 ± 0.02 (n=3)	0.33 ± 0.05 (n=4)	0.35 ± 0.01 (n=4)	0.35 ± 0.02 (n=5)	0.33 ± 0.03 (n=6)	0.34 ± 0.04 (n=5)	0.30 ± 0.04 (n=3)
+ Strychnine	0.01 ± 0.004 (n=3)	0.04 ± 0.01 (n=5)						

Table S1. The GlyR clusters numbers and densities, and M-cell surface area in larvae shown in Fig. 2B, C, D

The numbers in parentheses indicate the number of measured cells.

Table S2

Density of GlyR clusters [/μm^2]

	2 dpf	3 dpf	4 dpf	5 dpf	6 dpf
Control	0.17 ± 0.02 (n=3)	0.32 ± 0.02 (n=3)	0.33 ± 0.05 (n=4)	0.35 ± 0.01 (n=4)	
+ Strychnine	0.01 ± 0.004 (n=3)	0.04 ± 0.01 (n=5)			
Wash at 2 dpf	0.01 ± 0.004 (n=3)	0.14 ± 0.05 (n=4)	0.17 ± 0.01 (n=3)	0.24 ± 0.02 (n=6)	0.25 ± 0.03 (n=5)
Wash at 3 dpf		0.04 ± 0.01 (n=5)	0.01 ± 0.002 (n=3)	0.19 ± 0.04 (n=5)	0.24 ± 0.03 (n=3)

Table S2. The densities of GlyR clusters following strychnine removal shown in Fig. 5B

The numbers in parentheses indicate the number of measured cells.

Table S3

Density of GlyR clusters [/μm^2]

	2 dpf	3 dpf	4 dpf	5 dpf	10 dpf	11 dpf	15 dpf	16 dpf	30 dpf	31 dpf
Control	0.17 ± 0.02 (n=3)	0.32 ± 0.02 (n=3)	0.33 ± 0.05 (n=4)	0.35 ± 0.01 (n=4)	0.35 ± 0.02 (n=5)	0.38 ± 0.02 (n=4)	0.33 ± 0.03 (n=6)	0.32 ± 0.02 (n=5)	0.34 ± 0.04 (n=5)	0.36 ± 0.03 (n=5)
2-3 dpf + strychnine		0.01 ± 0.003 (n=8)								
3-4 dpf + strychnine			0.006 ± 0.003 (n=6)							
4-5 dpf + strychnine				0.015 ± 0.008 (n=8)						
10-11 dpf + strychnine						0.07 ± 0.02 (n=10)				
15-16 dpf + strychnine								0.09 ± 0.02 (n=11)		
30-31 dpf + strychnine										0.18 ± 0.02 (n=19)

Table S3. The densities of GlyR clusters after 24 h-strychnine treatment as shown in Fig. 6B

The numbers in parentheses indicate the number of measured cells.

Table S4

	Density of GlyR clusters [/μm²]				
	After washout				
	0 h	4 h	10 h	24 h	48 h
Control	0.34 ± 0.036 (n=3)			0.33 ± 0.05 (n=4)	0.35 ± 0.01 (n=4)
+ 1 h-Strychnine at 3 dpf	0.0027 ± 0.001 (n=5)	0.04 ± 0.01 (n=7)	0.097 ± 0.03 (n=5)	0.18 ± 0.03 (n=6)	0.29 ± 0.07 (n=3)
+ Strychnine 22 hpf - 3 dpf	0.04 ± 0.01 (n=5)			0.01 ± 0.002 (n=3)	0.19 ± 0.04 (n=5)

Table S4. The densities of GlyR clusters after 1 h-strychnine treatment and washout of the strychnine shown in Fig. 7B

The numbers in parentheses indicate the number of measured cells.

Table S5

Density of GlyR clusters [/μm²]

Control	+ Strychnine	+ Nifedipine	+ KN-93	+ Cyclosporin A
0.34 ± 0.05 (n=6)	0.02 ± 0.004 (n=9)	0.11 ± 0.02 (n=6)	0.09 ± 0.01 (n=4)	0.37 ± 0.04 (n=4)

ACP2 expression	AIP2 expression
0.31 ± 0.01 (n=7)	0.10 ± 0.03 (n=8)

Table S5. The densities of GlyR clusters after blockade of L-type Ca^{2+} channel or CaMKII shown in Fig. 8B, 10D

The numbers in parentheses indicate the number of measured cells.

5. Materials and Methods

Animals

Zebrafish (*Danio rerio*) embryos, larvae, juveniles and adults were maintained at 28.5°C. Experiments were performed at room temperature (25-28°C). Wild-type and five of transgenic lines were used: glyt2:GFP (McLean *et al*., 2007), Tol-056 (expressing GFP in the M-cells; Satou *et al*., 2009; Tanimoto *et al*., 2009), hspGFF62A (Asakawa *et al*., 2008), UAS:mCherry-2A-AIP2 (this study), and UAS:mCherry-2A-ACP2 (this study). From 26 hpf, the hspGFF62A fish expresses Gal4FF (Asakawa *et al*., 2008) in the M-cells, other neurons, and cardiac muscles. For CaMKII inhibition, UAS:mCherry-2A-AIP2 was used to express Autocamtide-2 related CaMKII inhibitory peptide (AIP2, KKKLRRQEAFDAL) (Ishida *et al*., 1998). As a control, UAS:mCherry-2A-ACP2 was similarly used to drive expression of Autocamtide-2 related control peptide II (ACP2, KKKGRAQERFDCL) (Khoo *et al*., 2005). These two UAS transgenic lines were generated using a *Tol2*-mediated transgenesis method as described previously (Kawakami *et al*., 2000). All procedures were performed in compliance with the guidelines set by Nagoya University.

Pharmacological treatment

Zebrafish were bathed in a solution containing strychnine hydrochloride (Sigma-Aldrich, St Louis, MO, USA) at 20 ~ 800 μM. Zebrafish were bathed in the strychnine solution for

various lengths of time: 22 hpf – 48 hpf; 22 hpf – 72 hpf; 22 hpf – 96 hpf; 22 hpf – 120 hpf; 2 dpf – 3 dpf; 3 dpf – 4 dpf; 4 dpf – 5 dpf;10 dpf – 11 dpf; 15 dpf – 16 dpf; or 30 dpf – 31 dpf. Similarly, nifedipine (Nacalai Tesque), KN-93 (Biomol, Plymouth Meeting, PA, USA) and cyclosporine A (Biomol) were bath-applied at 100 to 200 μM, 5 μM and 42 μM, respectively, in breeding water starting at 24 hpf.

In the experiment with cyclosporine A, we used a higher concentration than those in a previous study (Ponnudurai *et al*., 2012), where cyclosporine A was shown to have clear effects on zebrafish embryos. Therefore, although we have not confirmed directly whether cyclosporine A actually inhibited calcineurin, cyclosporine A is likely to be effective in present experiments.

Immunostaining

GlyRs were immunolabeled as described previously (Hirata *et al*., 2005). Briefly, horizontal sections (20-30 μm) and whole body zebrafish embryos/larvae were fixed in 4% paraformaldehyde at room temperature for 30 min and 7 h, respectively, and then washed several times in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% Tween 20. The samples were incubated with PBS containing 2% BSA, 5% goat serum and 0.5% Triton X-100 to block nonspecific reactions, and then with the following primary antibodies: mAb4a (1:1000, anti-GlyR, Synaptic Systems, Goettingen Germany), anti-Synaptophysin1 (1:50,

Synaptic Systems) or 3A10 (1:50, anti-neurofilament, Developmental Studies Hybridoma Bank, University of Iowa). The signals were visualized with the secondary fluorescent antibodies Alexa 488- or 555-conjugated anti-mouse IgG (1:1000) and anti-rabbit IgG (1:150). The fluorescent images were captured using a confocal microscope (FV300, Olympus, Tokyo, Japan).

Quantitative analysis

To count the number of GlyR clusters on M-cell somata, 20-30 μm thick horizontal sections that included the whole M-cell soma were immunolabeled with mAb4a and 3A10. Serial optical sections were acquired at 1 μm intervals through the whole cell body. The contours of the M-cell soma on 3A10 immunoreactive images were outlined using Adobe Photoshop CS3. The surface area of each M-cell soma was calculated by integration of the soma circumferences in each optical section. GlyR clusters were defined as puncta with a diameter of 0.5-2.0 μm according to the established protocol (Triller *et al*., 1985). Statistics are represented by mean ± s.e.m. and a t-test was used except where indicated otherwise.

Electrophysiological recordings

Whole-cell recording of M-cells in *Tol-056* larvae, in which the M-cells are identifiable as GFP-positive neurons, was performed at 26-28 °C as described previously (Tanimoto *et al*.,

2009). Larvae at 3 dpf (3.0-3.6 dpf) were temporarily anesthetized and immobilized in 0.02% tricaine methanesulphonate (MS-222; Sigma-Aldrich) and 1 mM D-tubocurarine (Sigma-Aldrich) for approximately 20 min. The larvae were then rinsed and pinned on a silicone-coated dish with fine tungsten pins. The larvae were soaked in extracellular solution containing (in mM) 134 NaCl, 2.9 KCl, 1.2 $MgCl_2$, 2.1 $CaCl_2$, 10 HEPES, and 10 glucose; Tetrodotoxin (TTX, 1 μM, Wako, Osaka, Japan), was used as a voltage-gated sodium channel blocker, and the pH was adjusted to 7.8 with NaOH. In some experiments, D-2-amino-5-phosphonopentanoic acid (APV, 50 μM, Sigma-Aldrich, an NMDA receptor blocker), 6,7-dinitroquinoxaline-2,3-dione (DNQX, 50 μM, Tocris Bioscience, Bristol, UK, an AMPA receptor blocker), bicuculline (10 μM, Sigma-Aldrich, a $GABA_AR$ blocker), or strychnine hydrochloride (5 μM, Sigma-Aldrich, a GlyR blocker) was added to the extracellular solution. Miniature postsynaptic currents (mPSCs) were recorded at a holding potential of -60 mV and sampled at 100 kHz with a MultiClamp 700B amplifier controlled by Clampex 10.2 (Molecular Devices, Sunnyvale, CA, USA). Recording pipettes with a resistance of 2.3-6.5 MΩ were filled with an intracellular solution containing (in mM) 130 CsCl, 2 $MgCl_2$, 10 HEPES, 10 EGTA, and 4 Na_2ATP, at 290 mOsm, and adjusted to pH 7.2 with KOH. Series resistance was compensated by 70%-80%. Patched neurons were labeled with 0.005% Alexa Fluor 594 hydrazide (Invitrogen, Carlsbad, CA, USA) in the intracellular solution. Data were filtered at 3 kHz. Synaptic events were detected using the template

function for events more than three standard deviations above the basal noise with Clampfit 10.2 (Molecular Devices).

6. References

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