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Glycine release from astrocytes via functional reversal of GlyT1

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Abbreviation list

DA: dopamine
GPCR: G-protein coupled receptor
DA-R: dopamine receptor
GlyT1: astrocytic glycine transporter 1
PLC: phospholipase C
TRPA1: transient receptor potential ankyrin 1
TRPV4: transient receptor potential vanilloid 4
TRPC: transient receptor canonical
HEK293T: human embryonic kidney 293T cell
EDTA: ethylenediaminetetraacetic acid
PLL: poly-L-lysine
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Abstract

It was previously reported that functional glycine receptors were expressed in neonatal prefrontal cortex; however, the glycine-releasing cells were unknown. We hypothesized that astrocytes might be a major glycine source, and examined the glycine-release properties of astrocytes. We also hypothesized that dopamine (DA) might be a trigger for the astrocytic glycine release, since numerous DA terminals localize in the cortex. We combined two different methods to confirm the glycine release from astrocytes. Firstly, we analyzed the supernatant of astrocytes by amino acid analyzer after DA stimulation, and detect significant glycine peak. Furthermore, we utilized a patch clamp biosensor method to confirm the glycine release from astrocytes by using GlyRα1 and Glyβ-expressing HEK293T cells, and detected significant glycine-evoked current upon DA stimulation. Thus, we clearly demonstrated that DA induces glycine release from astrocytes. Surprisingly, DA caused a functional reversal of GlyT1, an astrocytic type of glycine transporter, causing astrocytes to release glycine. Hence, astrocytes transduce pre-synaptic DA signals to glycine signals through a reversal of GlyT1 to regulate neuronal excitability.

Introduction

Astrocytes are important in the regulation of synaptic transmission. Gliotransmitters such as ATP, D-serine and glutamate that are released from astrocytes can actively regulate neural excitation (Shibasaki et al, 2014; Shibasaki et al, 2013). Recently, we reported that a novel subtype of astrocytes enhance neuronal excitability via release of glutamate (Shibasaki et al, 2014).

Glutamate can be released via astrocytic TREK-1 and Best1 channels upon G-protein coupled receptor (GPCR) activation (Woo et al, 2012), and this mechanism may contribute to synaptic plasticity (Araque et al, 2014; Shibasaki et al, 2014) and survival of neuroblast cells (Platel et al,

2010). Thus, gliotransmitters transfer active signaling from astrocytes to neurons, and regulate many physiological functions (Araque et al, 2014). It is reported that astrocytes can control breathing through pH-dependent release of ATP (Gourine et al, 2010). Astrocyte-derived ATP in the prefrontal cortex and hippocampus also modulates depressive-like behaviors (Cao et al, 2013). Gliotransmitter release is typically evoked by neurotransmitters through GPCR activation (Araque et al, 2014; Woo et al, 2012). Furthermore, the importance of intracellular pH change for astrocytic glutamate release is also reported (Beppu et al, 2014).

Dopamine (DA) is an important neurotransmitter for a variety of brain functions, such as motor control and motor learning (Schultz, 2013). DA release and dopamine receptor (DA-R) activity have been reported in the cerebral cortex and multiple other areas (Albrecht et al, 2014). DA release might be expected to affect astrocytes, since previous reports have demonstrated a functional expression of DA-R in astrocytes (Miyazaki et al, 2004; Zanassi et al, 1999). The activation of DA-R in astrocytes might produce gliotransmitter release following an increase of intracellular Ca^{2+} levels. The release of inhibitory gliotransmitters such as GABA from astrocytes was also reported, and astrocyte-derived GABA may contribute to tonic inhibition in the cerebellum (Lee et al, 2010).

An alternative mediator of cortical inhibition can arise from glycine, another major inhibitory neurotransmitter. Similar to $GABA_A$ receptors, glycine receptors are pentameric ligand-gated ion channels that are permeable to Cl^- (Salling & Harrison, 2014). Previous studies reported the expression of glycine receptor subunits in the forebrain (Jonsson et al, 2012), and glycine evokes Cl^- currents through the activation of glycine receptors in the prefrontal cortex (Lu & Ye, 2011). Glycine receptors may exert an important role not only in the spinal cord and brain stem but also in higher brain areas, although the cells responsible for glycine release are largely undiscovered (Dutertre et al, 2012) . It is also reported that high K^+ stimulation might evoke possible serine, taurine and glycine release from C8-1A cell (an astrocytic cell line), although those were not naïve astrocytes (Hogerton & Bowser, 2013).

In the present study, we examined whether DA-R activation of cortical astrocytes induces glycine release. Both neurons and astrocytes express DA-Rs (Albrecht et al, 2014; Zanassi et al, 1999). Furthermore, the astrocytic glycine transporter GlyT1 can release glycine under a reversal mode (Roux & Supplisson, 2000; Sakata et al, 1997). GlyT1 normally takes up waste glycine in synaptic sites after glycine release, accompanying co-uptake of $2Na^+$ and Cl^- (Huang et al, 2004). When intracellular glycine concentration is increased, the GlyT1 reverses and can release glycine (Huang et al, 2004). In addition, acute increases in intracellular Na^+ concentration or acute depolarization of membrane potential reverse GlyT1 (Roux & Supplisson, 2000; Sakata et al, 1997). However, whether neurotransmitters can evoke the reverse operation of GlyT1 is unknown. Furthermore, an *in vitro* microdialysis based capillary electrophoresis study in astrocyte cell line raised a possibility of glycine

release from the astrocytes (Hogerton & Bowser, 2013). Hence, it is highly possible that astrocytes can release glycine depending on their excitation. Here, we examined whether DA induces an excitation of astrocytes via DA-R and reverses GlyT1 to release glycine from astrocytes.

Materials and Methods

Animals

C57BL/6J mice of both sexes were utilized. For the astrocyte culture, we used postnatal day 0 (P0) mice. For the slice patch clamp experiments, we used P2 mice. All animal care procedures and experimental protocols were performed in accordance with the guidelines of the National Institute of Health, the National Institute for Physiological Sciences, and Gunma University.

Culture of dissociated astrocytes or HEK293 cells

Cortical astrocytes were prepared from male and female P0 mice as previously described (Shibasaki et al, 2014; Shibasaki et al, 2007; Shibasaki et al, 2015b). The cultures were maintained in Eagle's Minimum Essential Media (MEMα, Wako, Osaka, Japan) supplemented with 10% (v/v) fetal bovine serum, 0.6% (w/v) glucose, and 1 μg/ml penicillin/streptomycin for 7 to 10 days in a humidified CO_2 incubator at 37 ºC. HEK293T cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum and 1 μg/ml penicillin/streptomycin. Glyα1/β expression plasmids were transfected by Lipofectamine 2000 (Invitrogen, CA).

Reverse transcription PCR

DA-R expression was examined by reverse transcription PCR (RT-PCR). Total RNA was prepared from the cultured astrocytes using TRIzol reagent (Invitrogen). Total RNA (1 μg) was converted to cDNA using SuperScriptIII RNaseH (-) Reverse Transcriptase (Invitrogen). *DA-Rs* were PCR-amplified from cDNA with reported PCR primer sets (Zhu et al, 2007).

Fluorescent measurements and electrophysiology

Fura-2 fluorescence was measured by Fura-2-AM (Molecular Probes, Carlsbad, CA, USA) in a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH7.4. The 340:380 nm ratio was recorded as previously described (Shibasaki et al, 2010). The standard bath solution for the patch-clamp experiments was the same as that used for fluorescence measurements. The reversal potential was measured by using voltage ramps (-100 to +100 mV in 5-s intervals). Pipette solutions for whole-cell recordings of the biosensor experiments contained 140 mM KCl, 5 mM EGTA, and 10 mM HEPES, pH 7.4 (adjusted with KOH). Whole-cell recordings were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pCLAMP software, Axon Instruments, Foster City, CA, USA).

For the slice patch-clamp recordings, male or female P2 mice were deeply anaesthetized on ice and then decapitated. The brain was quickly removed and transferred to an ice-cold medium saturated with 95% O_2 and 5% CO_2. The medium contained (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose. Coronal brain slices (400 μm thickness) were prepared in the medium with a tissue slicer (VT-1200S, Leica, Germany) and placed in a humidified interface-type holding chamber for at least 1 h before recordings. A single slice was then transferred to the recording chamber and submerged with a nylon net stretched out on a U-shaped piece of flattened platinum wire beneath a continuously perfusing medium (flow rate: ~2 ml min^{-1}) that had been saturated with 95% O_2 and 5% CO_2. The composition of the medium used for recordings was the same as that used for slice preparations. Pipette solutions for acute slice recordings contained 120mM K-gluconate, 20mM KCl, 0.5mM EGTA, 2mM Mg-ATP, 2mM K_2-GTP, and 10mM HEPES, pH7.4 as previously described (Shibasaki et al, 2015a; Shibasaki et al, 2007). Whole-cell recording data were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pCLAMP9.2 software, Axon Instruments, Foster City, CA). All patch-clamp experiments were performed at room temperature. Data were statistically analyzed by using the unpaired *t* test, ANOVA, or Duncan's multiple range test.

Analysis of glycine release in cultured astrocytes

Phosphate-buffered saline with or without DA was applied to cultured astrocytes for 10 minutes, and then the conditioned media were collected. After filtration, the amino acid composition of the media was determined with an automatic amino acid analyzer (Hitachi, Ibaraki, Japan).

Data analysis

Analysis for electrophysiological data was performed using Clampfit 10.1 (Molecular Devices, Sunnyvale, CA, USA), Microsoft Excel 2013 (Microsoft, Redmond, WA, USA), Prism 5.01 (GraphPad Software Inc.,Ja Jolla, CA, USA). All data were given as means ± SEM; statistical significance was tested using student t-test, Mann-Whitney test and Dunnett's test. A value of $P < 0.05$ was considered to be statistically significant.

Results

We examined the functional expression of DA-Rs by a Ca^{2+}-imaging method using Fura-2. We prepared cultured astrocytes from neonatal cerebral cortex for these Ca^{2+}-imaging experiments. The application of DA (30 μM) induced an acute increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) under physiological 2mM Ca^{2+} conditions (Fig. 1A, control). Although the extracellular Ca^{2+}-free condition, the application of DA (30 μM) induced Ca^{2+}-elevation (supplementary figure 1). In terms of GPCR properties of DA-Rs, DA activated both Ca^{2+} influx through the plasma membrane and Ca^{2+} release through the endoplasmic reticulum. To further confirm DA-R-mediated activity in astrocytes, we applied SCH23390 (10 μM) and sulpiride (10 μM), selective D1-like (D1 and D5) and D2-like

DA-R (D2 and D3) blockers (respectively) to cultured astrocytes. Since the expressions of both D1-like and D2-like receptors in cortical astrocytes were hypothesized (Requardt et al, 2010), we used the combinational application of D1- and D2-like blockers. The combined application of SCH23390 and sulpiride (shown as DA-R BL or DA-R blockers in all figures) significantly reduced DA responses compared with control groups (Figs. 1A and 1B), while washout of DA-R blockers led to a recovery of normal DA responses (Figs. 1A and 1B). These results indicate that DA-R activation caused an increase in $[Ca^{2+}]_i$. A reduction of DA responses was also observed by the application of U73122 (5 μM), a phospholipase C (PLC) blocker, whereas application of NFPS (5 μM), a GlyT1 blocker, had no effect (Figs. 1A and 1B). To examine DA-R types involved, we performed RT-PCR experiments using cultured astrocytes from neonatal cerebral cortex. *D1* and *D5* DA-Rs expression was detected by RT-PCR (Fig. 2), although *D2-4* DA-Rs were not detected. Furthermore, we performed the protein detection of D1R an D5R by immunocytochemistry and immunoblotting in the cultured astrocytes. We detected the D1R protein (supplementary figure 2) consistent with our mRNA detection (Fig. 2), although the D5R antibody cannot be used for both immunocytochemistry and immunoblotting (supplementary figure 2). These results indicate that astrocytic PLC activation following (most likely) D5 DA-R activation is involved in the induction of $[Ca^{2+}]_i$ increase (Figs. 1 and 2).

Next, we examined whether application of DA to cultured astrocytes induces glycine release. We utilized an amino acid analyzer to examine the contents of conditioned media from cultured astrocytes. In the control group (no DA application), we observed a very low amount of glycine (Figs. 3A and 3B). Compared to the control group, DA application evoked glycine release from astrocytes in a dose-dependent manner (Figs. 3A and 3B). Notably, 30 μM of DA stimulation, which is considered as a physiological concentration (Caravaggio et al, 2014), was enough to induce the glycine release from cultured astrocytes (Figs. 3A and 3B).

To further confirm DA-induced glycine release, we utilized a bio-sensor method (Mandadi et al, 2009; Shibasaki et al, 2014). In this system, ionotropic receptors expressed in HEK293 cells detect molecules released from adjacent astrocytes that have been stimulated by DA (Fig. 4A). We used GlyRα1 and GlyRβ-expressing HEK cells as our bio-sensor, and we applied 30 μM DA (Fig. 4A). During the application at a holding potential of -60 mV, GlyRα1/β currents were recorded only from whole-cell patch-clamped HEK293 cells that were in close proximity to the astrocytes (located within 10 μm; Fig. 4B; n = 6). Under this whole-cell patch-clamp mode, we applied ramped pulses from -100 mV to +100 mV (Fig. 4B) at 5-second intervals. Exposure to DA evoked an outwardly rectifying current-voltage relationship (Fig. 4B, red arrowhead and red trace labeled 2 in the graph on the right), although the basal current-voltage relationship exhibited a linear pattern (Fig. 4B, black arrowhead and black trace labeled 1 in the graph on the right). In the end of experiments, we moved the biosensor cells far away from the astrocytes (to avoid the possible effects of glycine release from

astrocytes), and the expression of functional GlyRα1/β in HEK293 cells was confirmed by applying 100 μM glycine (Fig. 4B, green arrowhead and green trace labeled 3 in the graph on the right). To avoid the possibilities that naïve HEK293 cells can response to glycine or dopamine, or GlyRα1/β-expressing HEK293 cells can response to dopamine, we performed the control experiments in naïve HEK293 cells or GlyRα1/β-expressing HEK293 cells. Then, We confirmed that DA-induced glycine release was observed in only GlyRα1/β-biosensors on the astrocytes (Fig. 4 and supplementary figure 3). These results strongly indicate that we succeeded to real-time monitoring of glycine release by DA stimulation from the astrocytes. Next, we examined the effects of DA-R blockers on glycine release (Fig. 4C). First, we pre-applied the DA-R blockers. Exposure to 30 μM DA with DA-R blockers did not evoke significant outwardly rectified current-voltage relationship (Fig. 4C, blue arrowhead and blue trace labeled 2 in the graph on the right), although the basal current-voltage relationship exhibited a linear pattern (Fig. 4C, black arrowhead and black trace labeled 1 in the graph on the right). Next, we washed out the DA-R blocker. After washout of the DA-R blocker, we again applied the 30 μM DA. In this case, the 2nd DA-induced current was significantly larger than the 1st DA-induced current (Fig. 4C, red arrowhead and red trace labeled 3 in the graph on the right). Furthermore, we quantified the current density of DA-induced GlyRα1/β current between naïve DA (DA alone) condition and DA with DA-R blockers (Fig. 4D). DA-R blockers significantly suppressed the DA-induced GlyRα1/β current (Fig. 4D). These results strongly indicate that the DA induces glycine release through DA-Rs activation in astrocytes. These results also clearly indicate that GlyRα1/β bio-sensors detect glycine under our experimental conditions. To our knowledge, this is the first real-time monitoring of glycine release from astrocytes via DA-R activation. Consistent with our results by the amino acid analyzer (Fig. 3), the 30 μM of DA stimulation, which is considered as a physiological concentration, was enough to detect GlyRα1/β evoked current (Fig. 4), suggesting that physiological concentration of DA can induce glycine release from astrocytes.

Next, we examined the glycine release mechanisms produced by astrocytic DA-R activation. We applied different inhibitors to identify intracellular signaling pathways, and quantified the DA-induced glycine release via an amino acid analyzer method. In contrast to control conditions, a DA-R blocker (10 μM) or U73122 (5 μM) significantly reduced glycine release from astrocytes (Fig. 5), although neither SQ22536 (10 μM, an inhibitor of adenylate cyclase) nor forskolin (10 μM, an activator of adenylate cyclase) had any effects (Fig. 5). These results are consistent with the idea that DA induces astrocytic D5 receptor (D5-R) activation, resulting in an activation of PLC, and eventually glycine release from astrocytes (Fig. 5). In this case, how does D5-R and PLC activation lead to glycine release from astrocytes? One strong candidate is GlyT1, the astrocytic glycine transporter, since GlyT1 can operate in a reverse mode to release glycine (Huang et al, 2004). Hence, we applied GlyT1 (astrocytic glycine transporter) or GlyT2 (neuronal glycine transporter) inhibitors to identify the release pathways, and quantified DA-induced glycine release by an amino acid

analyzer method. An application of NFPS (5 μM, a GlyT1 inhibitor shown as GLYT1 BL in all figures) completely blocked the DA-induced glycine release (Fig. 6), whereas no effect was detected following application of Org25543 (10 μM, a GlyT2 inhibitor shown as GLYT2 BL in Fig. 6). Thus, DA activates D5-R and PLC pathways, causing a functional reversal of GlyT1 to release glycine from astrocytes (Figs. 4 , 5 and 6).

Although we confirmed the DA-induced glycine release from cultured astrocytes, so far we had huge gap about *in vivo* situation of DA-induced glycine release. To further investigate the glycine release from astrocytes, we prepared acute slices of cerebral cortex from postnatal day 2 (P2) mice, and performed whole-cell patch-clamp recordings in layer V pyramidal neurons. Glycine application (100 μM) to the slices significantly depolarized the resting membrane potentials (Supplementary Fig. 4). In the cerebral cortex of P2 mice, E_{Cl} values are around -30 mV in contrast to approximately -60 mV in adult mice (Sebe et al, 2010). Thus, glycine induced depolarization of the neuronal membrane potentials at P2 (Supplementary Fig. 4), instead of hyperpolarization in the adult (Betz & Laube, 2006). DA application (30 μM) to the slices also depolarized the resting membrane potentials similar to glycine (Supplementary Fig. 1). We co-applied DA with NFPS (5 μM) to confirm whether the inhibition of GlyT1 in astrocytes abolishes the depolarization of membrane potentials. The blockade of GlyT1 by NFPS significantly reduced the depolarization by DA (Supplementary Fig. 4). These results strongly indicate that DA induces glycine release from astrocytes via GlyT1 in *ex vivo*, and that glycine depolarizes membrane potentials (Supplementary Fig. 4). Thus, we identified that astrocytes are an important glycine source that excite neurons in the developing cortex. These results imply that astrocytes may exhibit a transducer function linking presynaptic DA signals to postsynaptic glycine signals.

Discussion

We demonstrated glycine release from cultured astrocytes by using an amino acid analyzer and bio-sensor method (Figs. 1-4). Further, an involvement of GlyT1 in the glycine release was confirmed by application of DA with NFPS both in culture and in acute brain slices (Fig. 6). Thus, astrocytes may well act as a glycine source (Figs. 1-6) to activate post-synaptic glycine receptors in neonatal cortex (Fig. 4). Astrocytes express D5-R and appear to transduce DA signals through Gq-PLC pathway activation, at least in part (Fig. 2). Astrocytes express various types of Gq-coupled GPCRs (Araque et al, 2014); therefore other GPCRs might also participate in the observed glycine release in addition to D5-Rs. In our analysis, extracellular Ca^{2+} free conditions did not inhibit the glycine release from astrocytes (supplementary figure 5). These results indicate that Ca^{2+} influx is not required for the release after Gq-PLC pathway activation. It is reported that the glycine transport by GlyT1 is required for the elevation of intracellular Ca^{2+} levels and cytoskeletal remodeling in Muller glia (Gadea et al, 2002). Hence, the mode change of GlyT1 might be also required the intracellular Ca^{2+} elevation and cytoskeletal remodeling for astrocytic glycine release. Astrocytic glycine release was also confirmed

in acute slices of neonatal cortex (Supplementary Fig. 4). Recently, it was reported that tonic activation of glycine receptors is modulated via the normal operation of GlyT1 (glycine uptake) in neonatal rat hippocampus (Sipila et al, 2014), although it is unclear how the tonic glycine signal originates. In light of our results (Figs. 1-6), it is possible that the reverse operation of GlyT1 can significantly increase extracellular glycine concentrations. In fact, once DA elicited glycine release from astrocytes, the depolarization of pyramidal neurons continued for a long time after elimination of DA from the extracellular space (Supplementary Fig. 4). These results imply that the reversal mode of GlyT1 is a candidate for tonic glycine signaling. After GlyT1 reversal is reset via down-regulation of the PLC pathway in astrocytes, GlyT1 might recover to a normal mode to restore proper extracellular glycine concentrations. Another recent study has also reported the tonic activation of glycine receptors and its modulation under the normal operation of GlyT1 in the adult prefrontal cortex (Salling & Harrison, 2014). Thus, it is interesting to speculate that astrocytic glycine release (Figs. 3 and 4) occurs, and acts as the tonic glycine source in the adult cortex. Glycine receptors can also be activated by taurine and β-alanine (Mori et al, 2002). However, DA-induced taurine and β-alanine release is unlikely in the present study, since the release of these substances was not detected in parallel with glycine release from astrocytes in our analysis based on the amino acid analyzer (Fig. 3A). Thus, DA-induced glycine regulation is a likely mechanism for the activation of post-synaptic glycine receptors in the neonatal cortex. Our electron-microscopic analysis has suggested that GlyT1 is localized at synaptic endfeets of astrocytes (manuscript under preparation), which is suitable for releasing glycine to post-synaptic sites. What is the physiological role of astrocytic glycine release in neonatal pyramidal neurons? It was reported that neuronal firing is required for axonal growth and arborization, whereas an inhibition of firing reduces axonal growth significantly (Mizuno et al, 2007). Hence, DA-induced astrocytic glycine release might contribute to the axonal growth and arborization.

As described above, it is reported that serine, taurine and glycine release was observed in C8-1A cell (an astrocytic cell line) by high K^+ stimulation (Hogerton & Bowser, 2013). In this case, the astrocyte cell line released 3 types of amino acids including glycine different from our results (Fig. 3A). Hence, astrocytes might have several abilities to release glycine by using many different pathways. We have reported that astrocytes can be functionally divided into several groups, and found that the unique function and localization of novel and specific subtypes of astrocytes might form a localized core that triggers neural excitation in response to the release of one type of neurotransmitter (Shibasaki et al, 2014; Shibasaki et al, 2013). From our studies, DA-responsive astrocytes might well form one such subtype. In our previous study, we revealed that TRPV4 activation triggers ATP and glutamate release from astrocytes (Shibasaki et al, 2014). It is also reported that astrocytic TRPA1 reduced GABA transport by inhibition of GAT-3, a GABA transporter, and elevated extracellular GABA (Shigetomi et al, 2012). In our study, we have never identified the down-stream molecules of Gq-PLC pathway (Fig. 5). According to the specific properties of TRPV4 and TRPA1 for the gliotransmitter release (Shibasaki et al, 2014; Shigetomi et al, 2012), TRP channels are good

candidate molecule for the down-stream. Furthermore, several TRPC channels are functionally expressed in the astrocytes and have important physiological functions (Munakata et al, 2013; Shirakawa et al, 2010). Especially, the some of TRPC channels can be activated in down-stream of Gq-PLC signaling. Therefore, we examined the possibility of the involvement of TRP channels in DA-induced glycine release (Cheng et al, 2013). Unfortunately, application of ruthenium red (10 μM), a broad TRP channel and non-selective cation channel inhibitor, did not inhibit the glycine release from astrocytes (Supplementary Fig. 6). These results indicate that TRP channels do not act as the down-stream target of Gq-PLC signaling. It is previously reported that Bergman glial GlyT1 can be reverse and release the glycine depending on the cell state, which high intracellular Na^+ concentration, depolarization of membrane potential and high intracellular glycine (Huang et al, 2004). Our ruthenium red experiments (Supplementary Fig. 6) suggest that the reverse of GlyT1 by high intracellular Na^+ concentration (Huang et al, 2004) is unlikely mechanism in our results (Figs. 3 and 4). We also recorded the changes of astrocytic membrane potentials by DA application, and it was changed within ~5 mV range. These results suggest that the reported reverse of GlyT1 by depolarization (around 20 mV) of membrane potential (Huang et al, 2004) is also unlikely mechanism in our results (Figs. 3 and 4). Is it possible that DA-R activation causes high glycine production, and this induces the reverse of GlyT1 as reported (Huang et al, 2004)? Notably, it is reported that DA reduced NAD(P)H and activated glycolysis and oxidative phosphorylation (Requardt et al, 2010) in astrocytes. This report indicates that DA might increase intracellular glycine concentration through activation of glycolysis and oxidative phosphorylation, since glycine production is linked to the intracellular metabolic map. Hence, it is highly possible that DA-Rs activation firstly activate intracellular metabolic states such as glycolysis and oxidative phosphorylation (Requardt et al, 2010), and then intracellular glycine concentration is elevated as hypothesized in Fig. 7. Finally the increased glycine reverses the GlyT1 (Huang et al, 2004).

The most important finding of our data is cortical DA release can modify the neuronal excitability through the glycine release from astrocytes (Figs. 4-7 and supplementary Fig. 4). Although DA release have been reported in the cerebral cortex (Albrecht et al, 2014) as described above, the physiological meaning had been unclear. Our data suggests that the cortical DA signals are transduced by the astrocytes, and the transduced astrocytic glycine signals might influence many GlyRs in the cortex. Further detail analysis is required to identify the physiological meaning of the astrocytic glycine release by the GlyT1 reverse.

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ARRIVE guidelines have been followed:
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=> if No, skip complete sentence
=> if Yes, insert "All experiments were conducted in compliance with the ARRIVE guidelines."

Conflicts of interest: none
=> if 'none', insert "The authors have no conflict of interest to declare."
=> otherwise insert info unless it is already included

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Figure legends

Figure 1

Astrocytes respond to DA.

A: The average traces from cells (n=105-136) exposed to DA (30μM) in Ca^{2+} imaging experiments are shown (blue trace). Together with DA, 10 μM DA-R blockers including D1-like and D2-like blockers (DA-R BL, dark orange trace), 5 μM U73122 (a PLC blocker, green trace), or 5 μM GlyT1 blocker (GLYT1 BL) were applied. **B:** Quantification of DA-induced 1st $[Ca^{2+}]_i$ response in the presence of blockers compared with control responses. * $P < 0.05$, Dunnett's test (vs. control).

Figure 2

Astrocytes expresses D1-R and D5-R.

RT-PCR of *DA-Rs* in cultured astrocytes. The product size of *D1-R* is 501 bp, and that of *D5-R* is 422 bp.

Figure 3

Astrocytes release glycine by DA application.

A: A representative trace of the analysis of amino acids present in the conditioned media from the cultured astrocytes exposed to DA (30 μM or 300 μM). Red arrowheads indicate glycine. **B:** Quantification of glycine release [in basal media (control) or conditioned media from cells exposed to DA] from the cultured astrocytes. * $P < 0.05$, Dunnett's test (vs control).

Figure 4

Activation of DA-Rs causes acute glycine release from cultured astrocytes.

A: Cartoons representing the bio-sensor system. HEK293 cells expressing GlyRα1/β (the bio-sensor) are placed in close proximity to the astrocytes, then DA (30 μM) is applied to the astrocytes. At the end of each experiment, the reliability of the bio-sensors is confirmed by directly applying glycine

(100 μM) to a location that is distant to the astrocytes. **B:** Representative trace showing that application of DA evokes whole-cell current responses (left panel) with outward rectification (red trace labeled "2" in the right panel) in HEK293 cells transfected with GlyRα1/β cDNA. Application of glycine (100 μM) was used to confirm GlyRα1/β -mediated responses (left panel) with linear rectification (green traces labeled "3" in the right panel). Black traces labeled "1" in the right panel represent control currents. Ramp pulses (from -100 mV to +100 mV for 500 msec) were applied at 5-second intervals. The holding potential was -60 mV. Traces are representative of a typical experiment (n=6). **C:** Representative trace showing that application of DA-R blockers (10 μM) inhibits whole-cell current responses (left panel) with linear rectification (right panel) in HEK293 cells transfected with GlyRα1/β cDNA. Black traces labeled "1" in the right panel represent control currents. The traces are representative of a typical experiment (n=6). **D:** Quantification of the peak current density of the GlyRα1/β current. * $P < 0.01$, *t* test (vs. DA alone).

Figure 5

Glycine release is induced by D5-R and PLC pathways in astrocytes.

Quantification of glycine release in conditioned media from cells (n=6 dishes) exposed to DA alone (none, the blue bar graph) or DA with various inhibitors (10 μM DA-R blockers, 5 μM U73122, 10 μM SQ22536, or 10 μM forskolin) from the cultured astrocytes. * $P < 0.05$, Dunnett's test (vs none).

Figure 6

GlyT1 reversal is critical determinant of DA-induced glycine release from astrocytes.

Quantification of glycine release in conditioned media from cells (n=6 dishes) exposed to DA alone (none, blue bar graph) or DA with glycine transporter inhibitors [5 μM NFPS (a GlyT1 blocker, GLYT1BL) or 10 μM Org25543 (a GlyT2 blocker, GLYT2BL) from the cultured astrocytes. * $P < 0.05$, Dunnett's test (vs none).

Figure 7

Summary of DA-induced glycine release from astrocytes.

Schematic representation of glycine release from astrocytes through the GlyT1 reversal. DA activates D5R, and causes PLC activation. Following the PLC activation, GlyT1 reversal is activated, and then astrocytes release glycine.

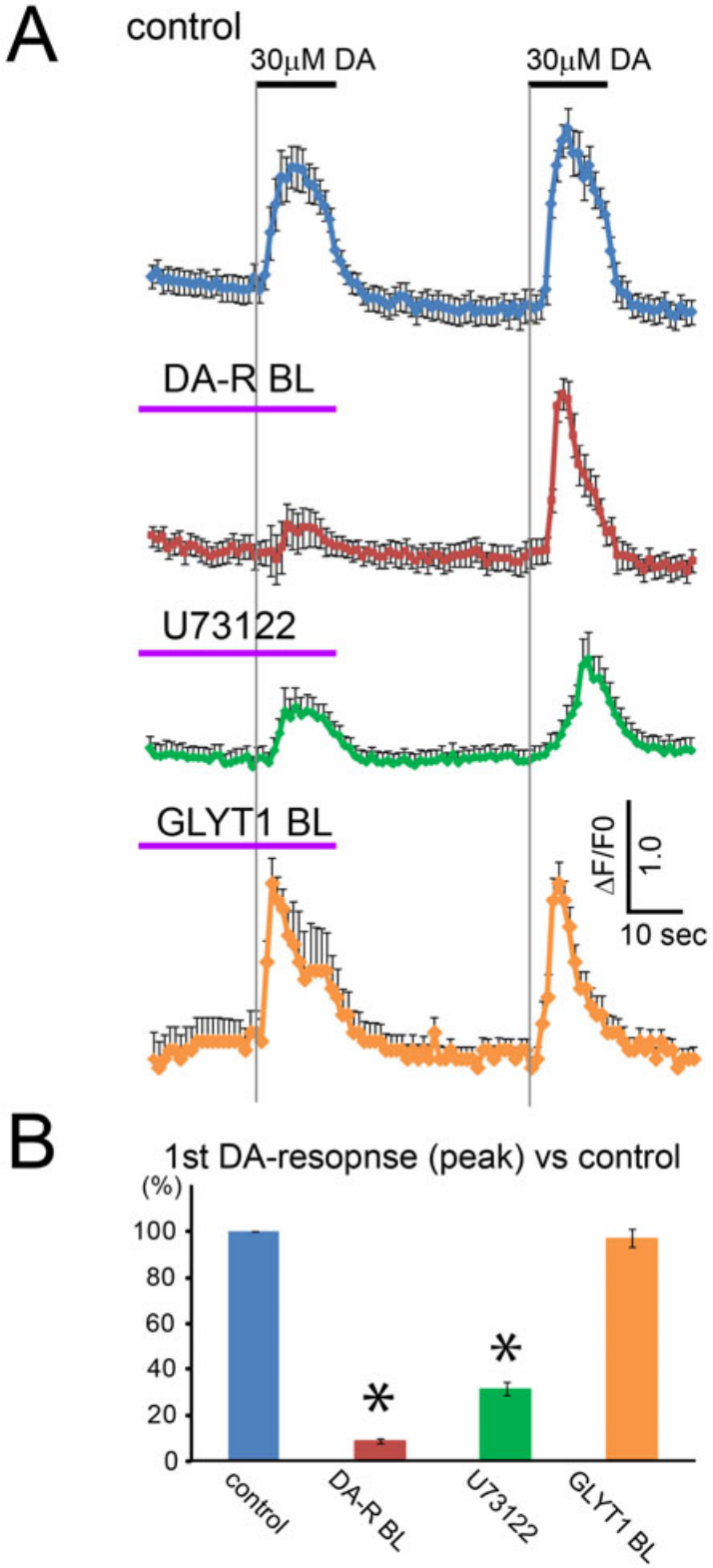


Fig. 1 Shibasaki et al.

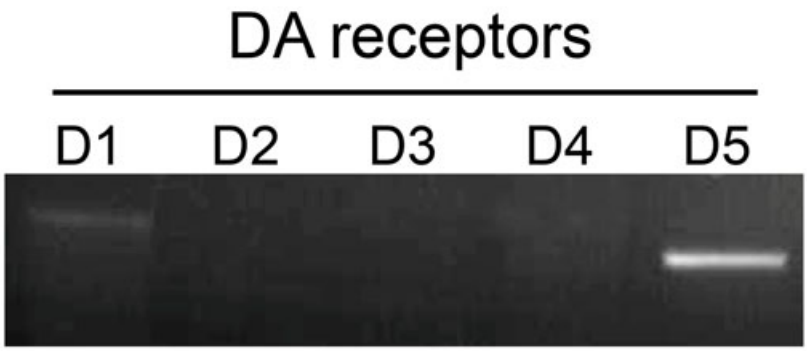


Fig 2 Shibasaki et al.

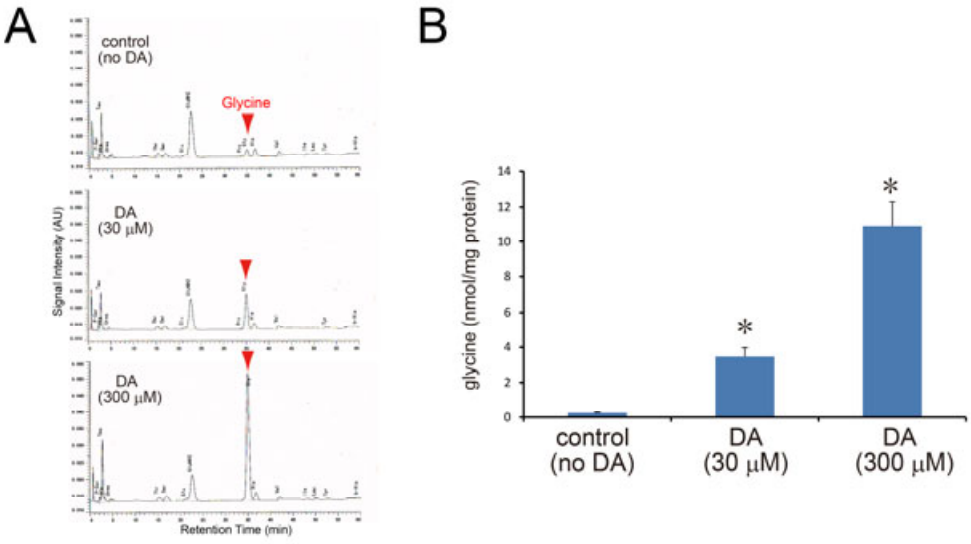
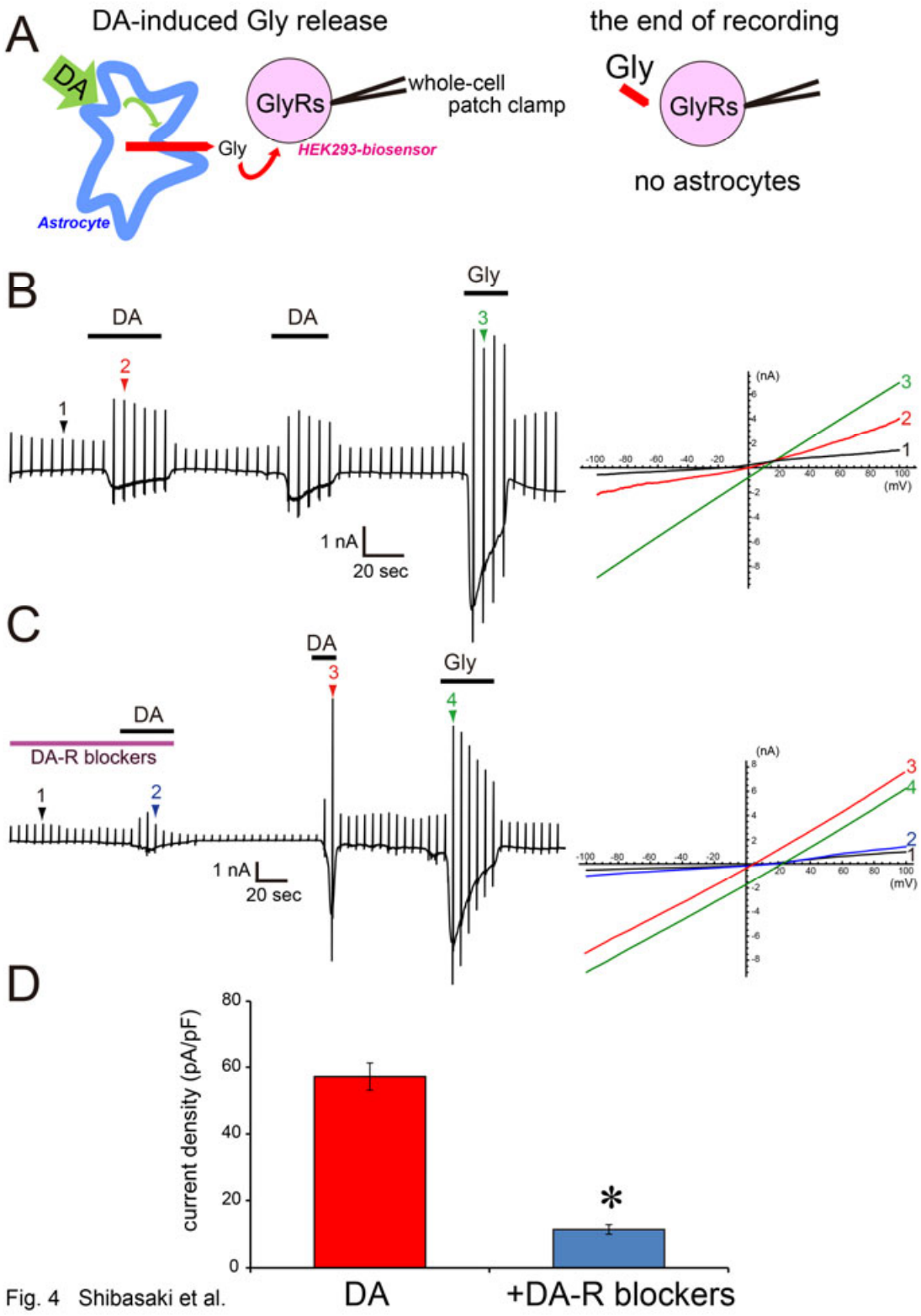


Fig. 3 Shibasaki et al.



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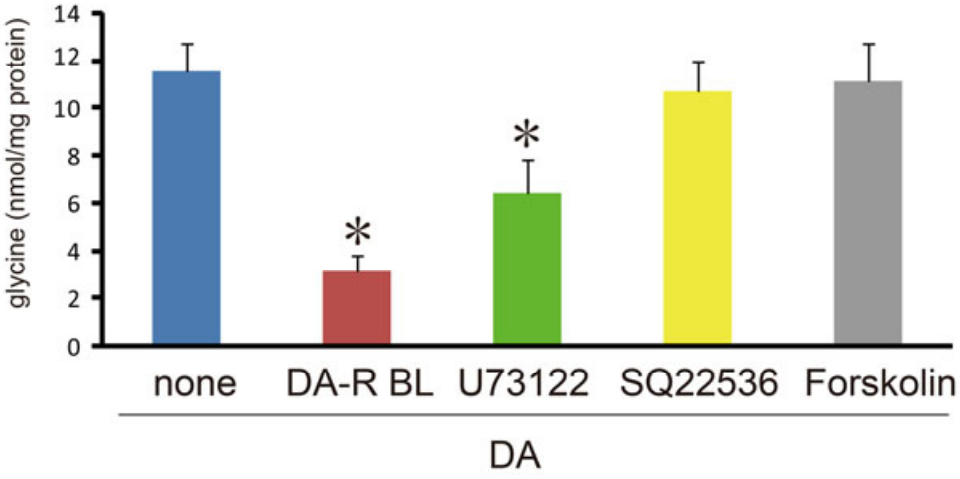

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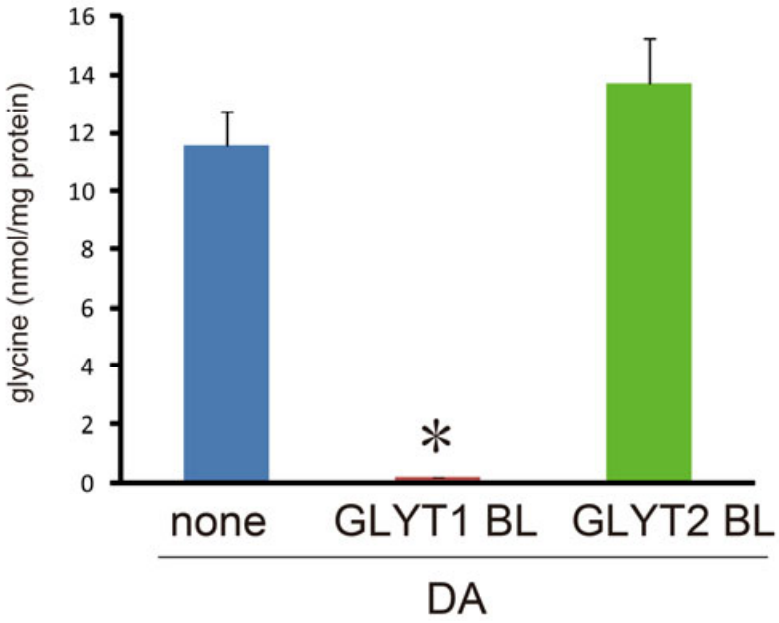

Fig. 6 Shibasaki et al.

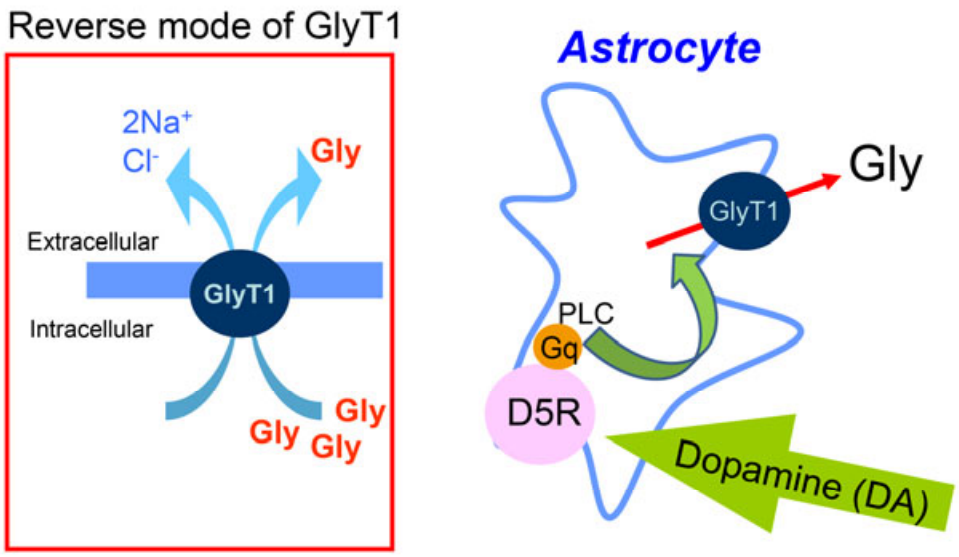

Fig. 7 Shibasaki et al.