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PRESYNAPTIC FACILITATION OF GLYCINERGIC mIPSC IS REDUCED IN MICE LACKING α3 GLYCINE RECEPTOR SUBUNITS

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Abstract—Glycinergic neurons provide an important mechanism to control excitation of motoneurons in the brainstem and a reduction or loss of glycinergic inhibition can be deleterious by leading to hyperexcitation such as in hyperekplexia or neurodegeneration and neuronal death as in amyotrophic lateral sclerosis (ALS). Second messenger systems that change cyclic AMP and lead to phosphorylation of the α3 subunit of the glycine receptor (GlyR α3) have been shown to be potent modulators of synaptic inhibition in the spinal cord and brain stem. In this study we analyzed the role of GlyR α3 in synaptic inhibition to the hypoglossal nucleus using *Glra3* (the gene encoding the glycine receptor α3 subunit) knockout mice. We observed that baseline glycinergic synaptic transmission to nucleus of hypoglossal motoneurons is rather normal in *Glra3* knockout mice. Interestingly, we found that the modulation of synaptic transmission by cAMP-mediated pathways appeared to be reduced in *Glra3* knockout mice. In the second postnatal week the forskolin-induced increase of miniature inhibitory postsynaptic potential (mIPSC) frequency was significantly larger in control as compared to *Glra3* knockout mice suggesting that presynaptic glycine release in the hypoglossal nucleus is partially depending on GlyR α3. © 2016 Published by Elsevier Ltd. on behalf of IBRO.

Key words: forskolin, glycine receptors, hypoglossal motoneurons, whole-cell recordings.

INTRODUCTION

Hypoglossal motoneurons, which innervate the tongue, are particularly vulnerable and therefore often affected in the course of amyotrophic lateral sclerosis (ALS) (von Lewinski et al., 2008). Although the pathophysiology of ALS includes neuronal and glial aspects, the vulnerability of hypoglossal motoneurons appears to be related to their low level of calcium buffers (von Lewinski and Keller, 2005; von Lewinski et al., 2008; Grosskreutz et al., 2010; Fuchs et al., 2013). However, impairment of glial glutamate transporter (Van Den Bosch et al., 2006; Valori et al., 2014), and the imbalance of excitatory and inhibitory innervation has been reported to be involved the damage of motoneurons in ALS (Kono et al., 2007; Martin and Chang, 2012; Ramirez-Jarquin et al., 2014).

The inhibitory innervation of hypoglossal motoneurons is mediated by the two major inhibitory neurotransmitters, GABA and glycine, and their receptors (Latal et al., 2010; Rahman et al., 2013). Glycine receptors (GlyR) are pentameric ligand-gated Cl^- channels made up of α1, α2, or α3 with or without β subunits (Betz and Laube, 2006). Their subunit composition in the hypoglossal nucleus undergoes changes during development with a postnatal increase of the α1 subunit and reduction of the expression of the α2 and α3 subunit during the first 3 postnatal weeks (Liu and Wong-Riley, 2013). But unlike described in the spinal cord (Becker et al., 1992) and hypoglossal nucleus (Singer et al., 1998) for mRNA, protein levels of α2 in the hypoglossal nucleus does not drop to zero (Liu and Wong-Riley, 2013) and robust expression of α3 is still observed after 4 months of life (Manzke et al., 2010).

Glycine receptors containing the α3 subunit (GlyR α3) have been attracting broad attention, because they are targets of functional modulation by cAMP and protein kinase A-dependent phosphorylation (Harvey et al., 2004). This modulation appears to be extremely relevant for modulation of pain (Harvey et al., 2004) and also for the regulation of breathing (Manzke et al., 2010). Upon phosphorylation, GlyR α3-mediated chloride-currents are inhibited (Harvey et al., 2004) providing a measure to modulate synaptic transmission by various metabotropic receptors.

For a long time it has been known that glycinergic transmission to the hypoglossal nucleus is modulated by metabotropic serotonin (Umemiya and Berger, 1995) and glutamate (Hulsmann et al., 2000) receptors. Since *Glra3* (the gene encoding the glycine receptor α3 subunit) knockout mice are now available, we aimed to analyze the role of GlyR α3 in the glycinergic transmission to the hypoglossal nucleus.

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Abbreviations: AC, adenylate cyclase; aCSF, artificial cerebrospinal fluid; ALS, amyotrophic lateral sclerosis; EGFP, Green fluorescent protein; GlyR α3, α3 subunit of the glycine receptor; GlyT1, glycine transporter 1; GlyT2, glycine transporter 2; KCC2, K+–CL− co-transporter 2; mIPSC, miniature inhibitory postsynaptic potential; NKCC1, Na+–K+–2Cl− co-transporter 1.

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EXPERIMENTAL PROCEDURES

Ethics statement

Breeding and handling of animals and experiments were performed in compliance with the guidelines for the welfare of experimental animals issued by the European Communities Council Directive (86/609/EEC) and the laws of the Federal Government of Germany. In accordance with the German Protection of Animals Act (Tierschutzgesetz; TierSchG §4 Abs. 3), all procedures were approved by the Animal Welfare Office of University Medical Center Göttingen, Germany (file number T11/27).

Animals

GlyRα3-deficient mice (Harvey et al., 2004) were obtained from a colony that was crossbred with a transgenic line (GlyT2–EGFP) that expresses the Green fluorescent protein (EGFP) under the control of the glycine transporter 2 (GlyT2) (Zeilhofer et al., 2005). Homozygous GlyRα3-deficient mice that express EGFP are fertile. They were maintained as homozygous breeding pairs (*Glra3* −/−) and served as experimental animals. In all experiments, GlyT2–EGFP (*Glra3* +/+) mice served as controls.

Preparation of brain slices

Electrophysiological experiments were performed on medullary slice preparations from the male and female mice at different ages (P3–P21). Mice were rapidly killed under deep diethyl ether anesthesia by decapitation, brains were quickly removed from the skull, and the brainstems were mechanically separated as described before (Latal et al., 2010). The isolated brainstem was placed in carbogen-saturated (95% O_2, 5% CO_2) ice-cold artificial cerebrospinal fluid (aCSF) that contained 118 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 30 mM D-glucose. The osmolarity was 310 mOsm/L and pH was adjusted to 7.4 using 1 M NaOH. Transverse slices (300 μm) were cut with a vibratome (VT1200S, Leica, Bensheim, Germany) in a rostro-caudal-direction using various steps until the lower brainstem was reached and the hypoglossal nucleus became visible. Slices were stored in aCSF at room temperature for at least 30 min before starting an experiment. Slices were subsequently transferred to a recording chamber and kept submerged by a grid of nylon fibers for mechanical stabilization. The chamber was mounted on a Zeiss Axioscope microscope (Zeiss, Germany) and continuously perfused with aCSF (room temperature; 20–23 °C) at a flow rate of 4–7 ml/min.

Electrophysiological recordings

Whole-cell recordings were obtained with EPC9 patch-clamp amplifier (Heka) using voltage-clamp mode. Recording electrodes were pulled from borosilicate glass capillaries (Biomedical Instruments, Zöllnitz, Germany) on a horizontal pipette-puller (Zeitz, Munich, Germany). Electrodes had resistances ranging from 4 to 6 M when filled with intracellular solution containing (in mM): 110 CsCl, 30 TEA-Cl, 1 $CaCl_2$, 2 $MgCl_2$, 4 Na_2ATP, 10 HEPES, 10 EGTA (pH adjusted to 7.2 with KOH). 5-Lidocaine-N-ethyl bromide (QX-314, 5 mM) was added to the electrode solution to block fast voltage–dependent sodium currents. Hypoglossal neurons visually identified under an upright microscope (Axioscope FS1, Zeiss) with Dodt gradient contrast optics (Luigs & Neumann) and voltage-clamped at a holding potential of −70 mV. The equilibrium potential of chloride was about 0 mV. Therefore, we observed IPSCs as inward currents at the holding potential of −70 mV. Currents were filtered at 2 kHz with a four-pole Bessel filter. Further noise reduction was achieved by electrically filtered the signals with a hum-bug-noise eliminator (Quest Scientific, North Vancouver, Canada). Signals were digitized at 10 kHz using an EPC9 amplifier interface and TIDA software (HEKA) and stored on hard disk for offline analysis.

Miniature inhibitory postsynaptic potentials (mIPSCs) were recorded in aCSF containing 20 μM 6-cyano-7-nitro quinoxaline-2,3-dione (CNQX), 100 μM DL-2-amino-5-phosphonopentanoate (AP5, Alexis) and 0.5 μM tetrodotoxin (TTX). 20 μM bicuculline was added subsequently to the external solution to block GABAergic mIPSCs. Forskolin (10 μM) was applied to activate Adenylate cyclase. All drugs were obtained from Sigma (St. Louis, MO, USA) or Tocris Bioscience (Ellisville, MO, USA).

Statistical analysis

Values are expressed as mean values ± SEM. Statistical significance was tested using SigmaPlot 12.5 software (Erkrath, Germany). Statistical tests are given in the results section, a $P < 0.05$ was considered significant. Normality Test (Shapiro-Wilk) was performed to test normal distribution prior to comparison. Dunn's Method was used for pairwise multiple comparison procedures.

RESULTS

mIPSC-amplitude in GlyR α3-deficient hypoglossal motoneurons is increased after the second postnatal week

To analyze the role of GlyR α3 in glycinergic synaptic transmission to hypoglossal motoneurons, we recorded miniature glycinergic IPSCs (mIPSCs). In the control (*Glra3* +/+) mice, the mIPSC amplitude was 44.69 ± 7.88 pA at P3–7 ($n = 9$ slices), 49.27 ± 4.24 pA at P8–14 ($n = 17$ slices), and 52.95 ± 7.54 pA at P15–21 ($n = 21$ slices). There was no significant difference between the age group (ANOVA, n.s.). The frequency of glycinergic mIPSCs was 0.72 ± 0.23 Hz ($n = 9$ slices) at P3–7, 1.49 ± 0.27 Hz ($n = 17$ slices) at P8–14, and 1.38 ± 0.16 Hz ($n = 21$ slices) at P15–21 (Fig. 1). Miniature IPSC frequency at P15–21 mice was larger as compared to P3–7 mice (ANOVA on Ranks, $p = 0.019$; Dunn's Method ($p < 0.05$); Fig. 1).

In the GlyR α3-deficient (*Glra3* −/−) mice, an averaged mIPSC amplitude of 34.88 ± 4.48 pA ($n = 9$

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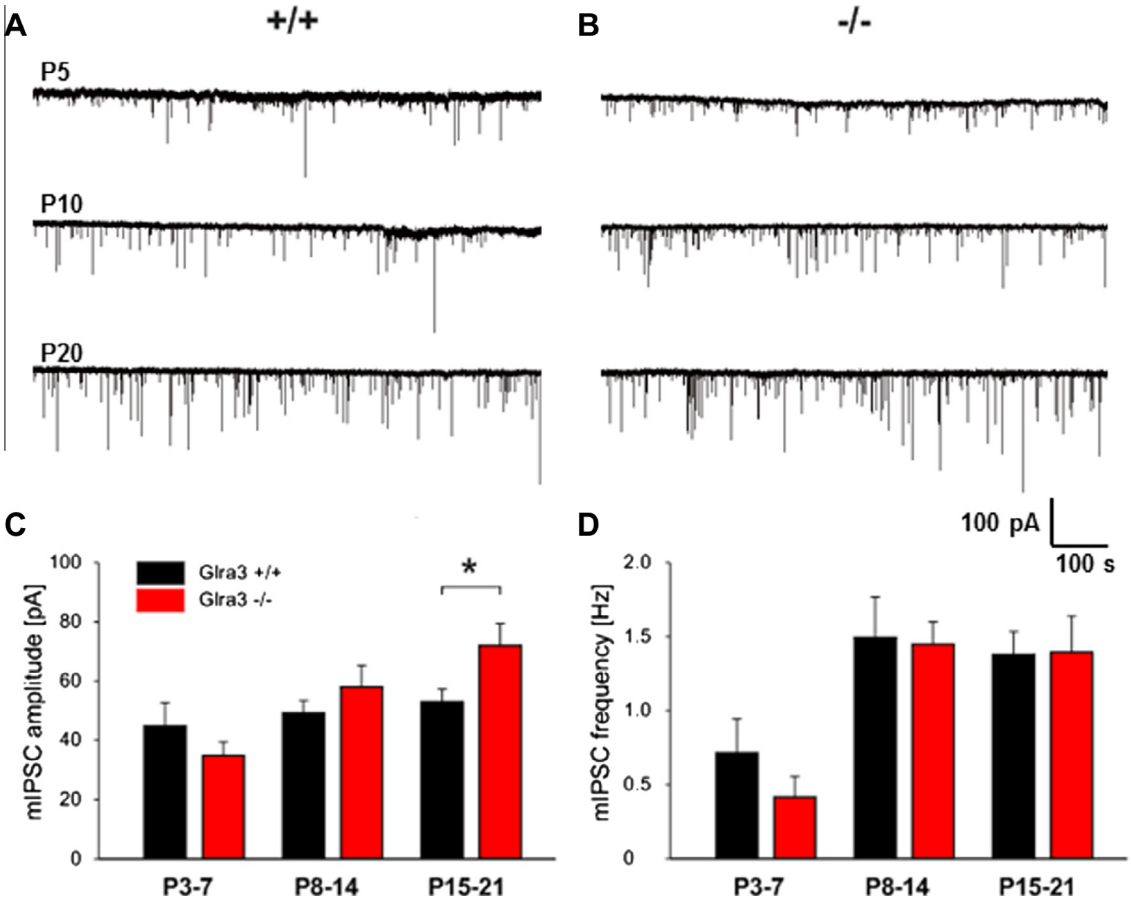

Fig. 1. Comparison of glycinergic mIPSC in *Glra3* +/+ and *Glra* 3 −/− mice. (A, B): glycinergic mIPSCs recorded from hypoglossal motoneurons at different age groups in control mice (*Glra3* +/+, A) and GlyR α3-deficient (*Glra3* −/−) mice (B) at different ages as indicated. Bar; horizontal axis 100 ms, vertical axis 100 pA. (C) Basic statistics of the development of glycinergic mIPSC-amplitude sorted by postnatal week. (D) Basic statistics of the development of glycinergic mIPSC-frequency. Amplitude sorted by postnatal week. Significance ($p < 0.05$) is only shown for comparisons between genotypes (see text for details).

slices) was measured in P3–7 slices. At P8–14 the mIPSC amplitude measured 57.96 ± 7.34 pA ($n = 18$ slices), and 71.98 ± 7.54 pA ($p = 10$ slices) in the P18–21 group. In older mice (P15–21) the amplitude was larger as compared to the P3–7 group (ANOVA on Ranks; $p < 0.05$, Dunn's method). Miniature IPSC frequency of *Glra3* −/− mice was 0.42 ± 0.14 Hz ($n = 9$ slices) at P3–7 and increased to 1.45 ± 0.15 Hz (P8–14; $n = 18$ slices) and 1.39 ± 0.25 Hz (P15–21; $n = 10$ slices). In the older age groups (P8-14 and P15-21) the frequency was significantly larger as compared to the P3–7 group (ANOVA on Ranks, $p < 0.001$; Dunn's Method ($p < 0.05$)).

The comparison of glycinergic mIPSCs between *Glra3* +/+ and *Glra3* −/− mice revealed a larger mIPSC-amplitude in *Glra3* −/− mice at P15–21 (*t*-test; two-tailed, $p = 0.0261$) but no significant difference in mIPSC frequency.

Modulation of mIPSC by forskolin

Since GlyR α3 are a target for modulation by cAMP and protein kinase A (PKA)-dependent phosphorylation, we tested the effect of forskolin, an adenylate cyclase (AC) activator on glycinergic mIPSCs. To evaluate of role of glycine receptors containing α3 subunits in modulation of glycine synaptic transmission, we compare the effect of forskolin in slices from *Glra3* +/+ and *Glra3* −/− mice.

Forskolin did not change the amplitude of glycinergic mIPSC in slice from *Glra3* +/+ at P3–7 (aCSF: 49.54 ± 13.96 pA vs. forskolin 40.61 ± 3.52pA ($n = 5$ slices; paired *t*-test; n.s.)), at P8–14 (aCSF: 50.10 ± 5.31 pA vs. forskolin 47.44 ± 5.29 pA ($n = 13$ slices; paired *t*-test; n.s.) nor at P 15–21 (aCSF: 61.13 ± 7.10 pA vs. forskolin 62.39 ± 9.321 pA ($n = 6$ slices; paired *t*-test; n.s.), however, the frequency was affected. In *Glra3* +/+ mice, forskolin (10 μM) significantly increased the mIPSC-frequency in the age groups P8–14 (aCSF 1.14 ± 0.25 Hz vs. forskolin 1.88 ± 0.38 Hz; $p = 0.00135$, paired *t*-test, two-tailed) and P15–21 (aCSF 1.12 ± 0.19 Hz vs. forskolin 1.978 ± 0.21 Hz; $p = 0.012$, paired *t*-test, two-tailed), while there was no change at P3–7 (aCSF 0.63 ± 0.21 Hz vs. forskolin 0.79 ± 0.36 Hz; Wilcoxon Signed Rank Test; n.s.).

In *Glra3* −/− mice we measured mIPSC frequencies at P3–7 (aCSF 0.46 ± 0.19 Hz vs. forskolin 0.90 ± 0.51 Hz ($n = 4$ slices; paired *t*-test; n.s.), at P8–14 (aCSF 1.46 ± 0.20 Hz vs. forskolin 1.77 ± 0.33 Hz

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(*n* = 14 slices; paired *t*-test; n.s.); but at P15–21 there was an increase of mIPSC-frequency measured in *Glra3* −/− mice (aCSF: 1.40 ± 0.24 Hz vs. forskolin 2.23 ± 0.41 Hz (*n* = 10 slices; *p* = 0.009, paired *t*-test, two-tailed). mIPSC amplitudes in *Glra3* −/− mice were (P3–7: aCSF 36.39 ± 9.66 pA vs. forskolin 45.29 ± 7.57 pA (*n* = 4 slices; paired *t*-test; n.s); P8–14: aCSF 62.65 ± 8.69 pA vs. forskolin 59.86 ± 6.45 pA (*n* = 14 slices; paired *t*-test; n.s) and at P18–21 (aCSF 71.98 ± 7.54 pA vs. forskolin 72.93 ± 8.50 pA (*n* = 10 slices; paired *t*-test; n.s)).

Reduced forskolin effects in Glra3 −/− mice

Interestingly, when comparing *Glra3* +/+ with *Glra3* −/− mice, we found that the forskolin-induced increase of mIPSC-frequency was smaller in *Glra3* −/− measurements. In the P8-14 group the forskolin-induced increase of mIPSC-frequency was 76 ± 17% of control in *Glra3* +/+ mice and diminished to 22 ± 12% in *Glra3* −/− (*p* = 0.0179; *t*-test, two-tailed). The forskolin-effect was also smaller in the P15–21 group (112 ± 43% in *Glra3* +/+ mice and 64 ± 17% in *Glra3* −/−) however this difference did not reach significant (Mann–Whitney Rank Sum Test; n.s.; Fig. 2D). No significant differences between *Glra3* +/+ and *Glra3* −/− mice were observed regarding forskolin effects on mIPSC amplitude.

DISCUSSION

Our present study suggests that the α3 subunit of the glycine receptor (GlyR α3) is a novel presynaptic element of the regulation of glycinergic inhibition in the hypoglossal nucleus. Since the effect of forskolin on the mIPSC is limited on the frequency, which depends on release of vesicle in the ready releasable pool and how efficient vesicles can fuse after priming we have to assume that in hypoglossal nucleus presynaptic GlyR α3 are involved in the presynaptic mechanism that mediates inhibition at glycinergic terminals

Presynaptic GlyR α3 can provide a negative feedback regulation of glycine release

Glycinergic neurons in the brainstem express high levels of α3 subunits (Manzke et al., 2010) and it is known that some of these neurons project to the hypoglossal nucleus (Li et al., 1997). The concept that presynaptic α3 receptors are contribution to the regulation of release is novel, but back up by the fact that presynaptic glycine receptors exist and that they might be involved in the pathophysiology of hyperekplexia (Xiong et al., 2014). In the hypoglossal nucleus, activation of presynaptic GlyR α3, e.g.by glycine that escapes the clearance by the astroglial glycine transporter 1 (GlyT1; (Gomeza et al., 2003a)) could therefore cause presynaptic inhibition and provide a negative feedback regulation of glycine release in those presynaptic terminals that do not release GABA as a co-transmitter (Lim et al., 2000; Rahman et al., 2013, 2015).

In agreement with the concept that GlyR α3 can serve as a mediator of presynaptic feedback inhibition, we have shown earlier that metabotropic glutamate receptor activation (mGluR; group II and III) can lead to reduction of mIPSC-frequency (Hulsmann et al., 2000). Since both types of mGluR couple to $G_{i/o}$-proteins that are lowering cAMP via inhibition of the AC their activation will reduce phosphorylation of presynaptic GlyR α3 leading to presynaptic inhibition (Fig.3). Interestingly, Umemiya and Berger (1995) were able to show that serotonin causes a blockade of glycinergic mIPSC via a calcium-dependent presynaptic mechanism. Since they identified the $5HT_{1B}$ receptor to mediate this type of presynaptic inhibition (Umemiya and Berger, 1995) and $5HT_{1B}$-receptor is coupled to $G_{i/o}$-proteins (Maroteaux et al., 1992) it appears that, whenever cAMP-levels in the presynaptic terminal are lowered, the conductance of alpha3 receptors is increased, leading to inhibition of glycine release. In contrast, when forskolin is applied, GlyR α3 is phosphorylated and facilitation of glycine release will be the consequence.

As explained above, we suggest that GlyR α3 have to be discussed as a novel presynaptic modulator of glycinergic inhibition. However, we must assume that developmental changes, which require further investigation, have contributed to the apparent lack of effects in younger age. The developmental decreasing of Na+–K+–2Cl− co-transporter 1 (NKCC1) and increasing of K+–CL− co-transporter 2 (KCC2) (Liu and Wong-Riley, 2012), and presynaptic morphological maturation at inhibitory synapses are candidates that might mask GlyR α3 effects.

Interestingly, a reduction of mIPSC frequency was found in alanine-serine-cysteine-1 (Asc-1) (Safory et al., 2015) and GlyT1 knockout mice (Gomeza et al., 2003a). However, since this is beyond the scope of the current paper, we suggested that there is the possibility that dysregulation of presynaptic mechanisms controlled by GlyR α3 may underlie the reduction of mIPSC frequency observed in these mice models. Nevertheless, we could recently show high levels of *slc7a10* mRNA in brainstem astrocytes (Schnell et al., 2015).

Alternative mechanisms of forskolin-mediated modulation of presynaptic inhibition

The AC activator forskolin has been shown to increase the amplitude of glycinergic mIPSCs in other CNS regions before, such as the spinal cord, by increasing presynaptic release probability (Choi et al., 2009). Additionally, there is evidence that extracellular glycine and also the neuronal GlyT2; (Gomeza et al., 2003b) are involved in the cellular response of the presynaptic terminal (Nakamura and Jang, 2010). Of course, one cannot exclude that a GlyT2-mediated mechanism or even other mechanisms, which are independent of glycine, are involved in our model system. As possible additional pathways we have to consider the direct modulation or phosphorylation of proteins of the presynaptic release machinery (Trudeau et al., 1996) or phosphorylation of other presynaptic ionotropic receptors, ion channel channels (El Manira et al., 1997; Engelman and MacDermott, 2004) or chloride transporters (NKCC1 or KCC2; (Kaila

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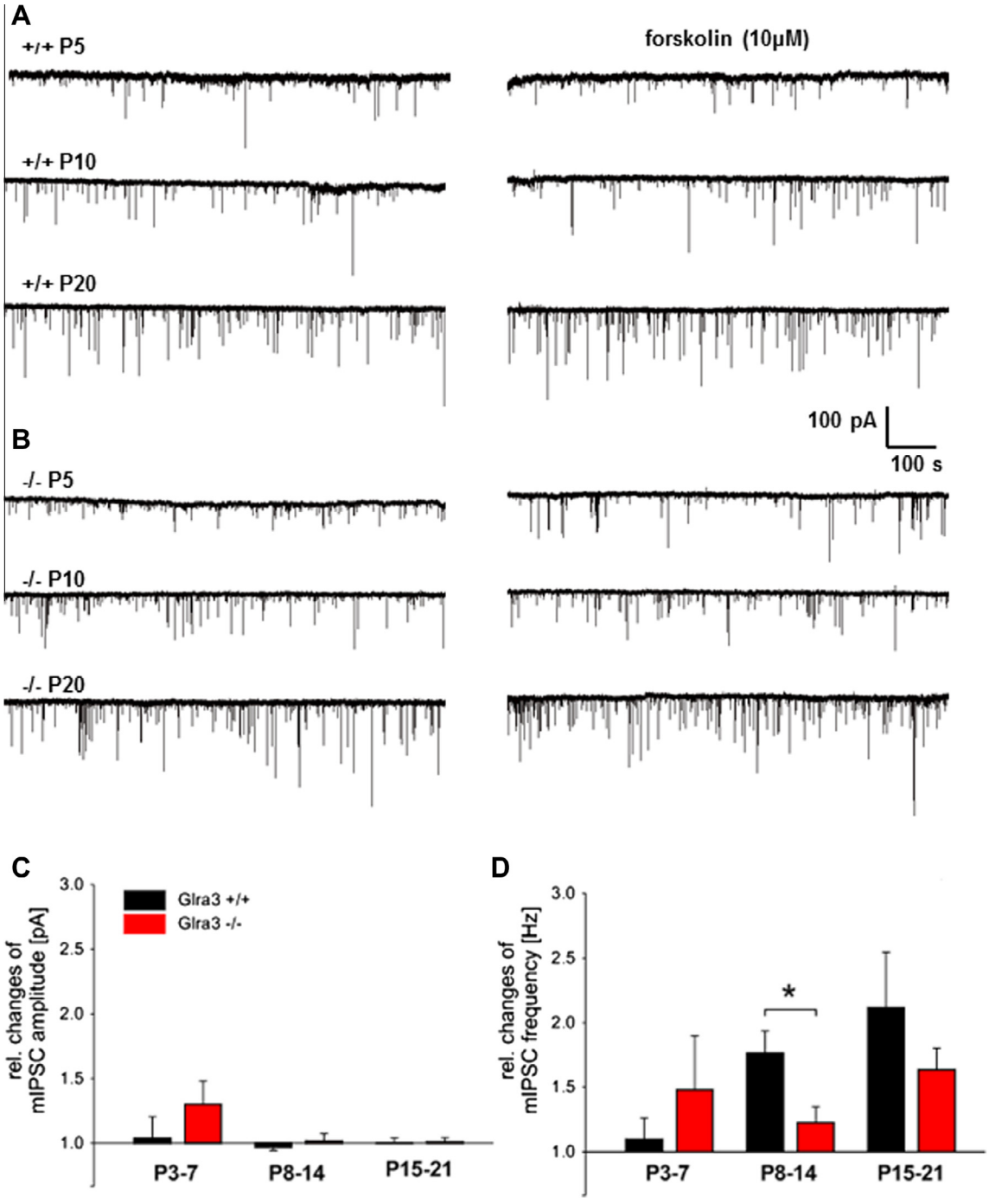

Fig. 2. Forskolin-induced increase of mIPSC-frequency is diminished in *Glra3* −/− mice. (A, B): changes of glycinergic mIPSCs induced by forskolin in P8–14 recorded from hypoglossal motoneurons of control mice (*Glra3* +/+, A) and GlyR α3-deficient (*Glra3* −/−) mice (B). Bar; horizontal axis 100 ms, vertical axis 100 pA. (C): Basic statistics of the relative increase of glycinergic mIPSC-amplitude induced by forskolin sorted by postnatal week. (D): Basic statistics of the relative change of glycinergic mIPSC-amplitude induced by forskolin sorted by postnatal week. Significance ($p < 0.05$) is only shown for comparisons between genotypes (see text for details).

et al., 2014)) and glial regulation of extracellular glutamate uptake (Hulsmann et al., 2000).

Postsynaptic α3-glycine receptors

We also found a significant increase of the mIPSC amplitude in older (P15–21) *Glra3* −/− mice, although no changes of the mIPSC amplitude in earlier ages (P3–7 and P8–14). The remaining mIPSCs recorded in *Glra3*−/− mice might be mediated by α1β heteromeric channels with no compensation by other glycine receptor subunits (α2 or 4) (Handford et al., 1996; Graham et al., 2006: Rajalu et al., 2009). Moreover, developmental changes in the amplitudes of glycinergic mIPSCs in hypoglossal neurons might be due to both pre- and postsynaptic mechanisms (Gao et al., 2011).

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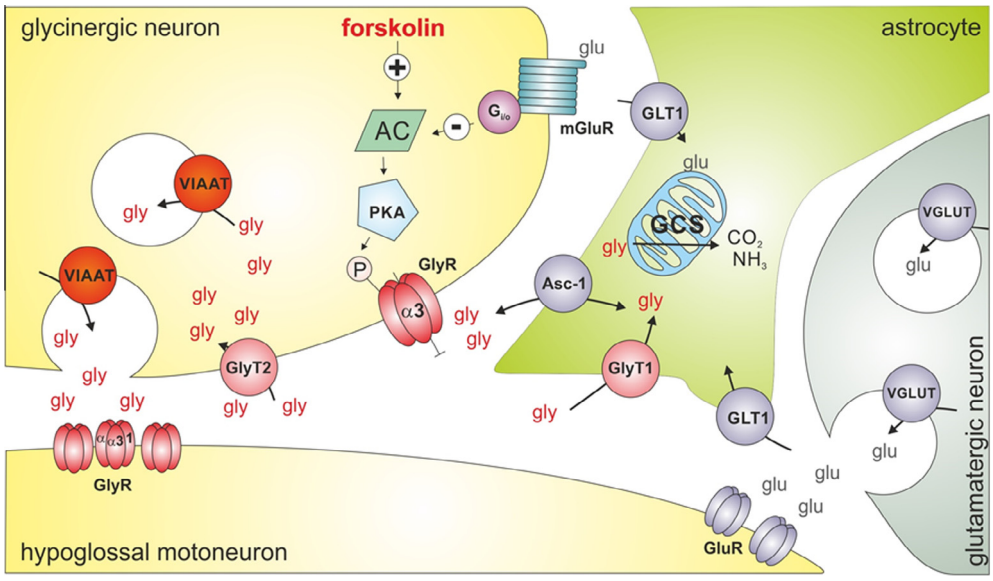

Fig. 3. Model of α3 glycine receptor subunits mediated presynaptic inhibition. The drawing demonstrated the potential role of presynaptic glycine receptors containing α3 subunit (GlyR α3) at the glycinergic synapse. $G_{i/o}$-Protein coupled mGluRs, as well as e.g., 5HT1b-receptors inhibit adenylate cyclase (AC), which reduces phosphorylation of the α3-subunit and increases the chloride flux through glycine receptors leading to presynaptic inhibition of the glycine release. In opposite, forskolin increase AC activity and this diminishes presynaptic inhibition. VIAAT = vesicular amino acid transporter; VGLUT = vesicular glutamate transporter, Asc-1 = alanine/serine/cysteine transporter 1; GlyT1 = glycine transporter 1; GlyT2 = glycine transporter 2; PKA = protein kinase A; GCS = glycine cleavage system; mGluR = metabotropic glutamate receptor; $G_{i/o}$ = G-protein, inhibitory; gly = glycine; glu = glutamate.

Thus, our results indicate that an additional postsynaptic role of the α3 subunit in development and functional compensation by α1 subunit might have a crucial role for change of amplitude in *Glra3* −/− mice.

CONCLUSION

Taken together our data identify GlyR α3 as a potential mechanism for mediation of presynaptic modulation of glycine release in the hypoglossal nucleus. Moreover, we would like to emphasize that this regulatory pathway, in future, may provide a pharmacological target for counteraction of neuronal excitation and excitotoxicity in ALS.

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