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Responses of Several $GABA_A$ Receptors to Facial Nerve Injury in Rat

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ABSTRACT

Facial motoneurons express an ion channel-type gamma-aminobutyric acid receptor ($GABA_AR$) that is constructed from five subunits, including α, β, and γ subunits. The transection of rat facial nerve was reported to lead to a significant downregulation of the $GABA_AR\alpha1$ subunit. In this study, we investigated a transition of $GABA_AR\beta2,3$ and $GABA_AR\beta1$ subunits in axotomized (i.e., injured) rat facial nucleus. Immunoblotting indicated that the levels of $GABA_AR\alpha1$ significantly decreased in axotomized facial nucleus from 3 days to 5 weeks post-injury. However, the levels of $GABA_AR\beta2,3$ transiently decreased during the period from 5 to 14 days post-insult, but they recovered to the control level at 3–5 weeks post-insult. The profile of $GABA_AR\beta1$ in a time course experiment resembled that of $GABA_AR\alpha1$. These results suggest that the individual subunits of $GABA_AR$ in injured motoneurons are not regulated as a group; rather, they are separately regulated by an unknown mechanism.

INTRODUCTION

The *nervus facial is* the VII cranial nerve that governs the contraction of muscles for facial expressions [1]. The facial motoneurons exist in the facial nucleus of the brainstem, and they extend their axons to the target muscles across the skull. Facial motoneuron activity is essentially dependent on the commands of superior motoneurons in the cerebral cortex [2,3]. The actions of facial motoneurons are modified by inhibitory interneurons satelliting around motoneurons in the nucleus [4,5]. Facial motoneurons express inhibitory gamma-aminobutyric acid (GABA) receptors as well as excitatory Glu receptors [6]. There are two classes of GABA receptors (Rs). $GABA_ARs$ are ligand-gated ion channels (ionotropic receptors), and $GABA_BRs$ are G protein-coupled metabotropic-type receptors [7,8]. $GABA_AR$ is comprised of five subunits, including α, β, and γ subunits. The most well-known form is a complex of α1, β2, and γ2 subunits in the central nervous system [9], and there are other combination forms including αβγ and αβδ complexes. In addition to these predominant subunits, minor subunit components have been observed in the brain, including α2-4, β1, γ1, δ and ε [10].

Thus far, major subunits of $GABA_AR$ have been recognized in rat facial nucleus, and the subunits were found to be decreased in axotomized facial motoneurons. Vassias et al. [11] reported that the mRNA levels of $GABA_AR$ subunits (α1, β2, and γ2) and the immunoreactivity of antibodies against the α1/γ2 subunits were downregulated in axotomized rat facial motoneurons. We also reported that the levels of

GABA$_A$Rα1 protein declined significantly in injured rat facial nucleus [12]. It is not known whether all of the receptor subunits would be similarly influenced in axotomized facial motoneurons. In this study, we analyzed GABA$_A$R subunits α1, β2,3 and β1 at the protein level in injured rat facial nucleus. Our findings indicate that the protein levels of GABA$_A$Rα1, GABA$_A$Rβ2,3, and GABA$_A$Rβ1 are not commonly regulated, and they showed different profiles during the 5 weeks post-insult.

MATERIALS AND METHODS

Reagents and antibodies

Anti-GABA$_A$ receptor α1 chain (GABA$_A$Rα1) antibody (AB5592), anti-GABA$_A$Rβ2,3 chain antibody (MAB341), and anti-GABA$_A$Rβ1 chain antibody (AB9680) were purchased from Millipore (Temecula, CA, USA). Anti-actin antibody (sc-1615) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). As secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (sc-2055), HRP-conjugated anti-rabbit IgG (sc-2374) and HRP-conjugated anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology.

Animals and operation

Eight-weeks-old male Wistar rats were obtained from Clea Japan (Tokyo) and kept on a 12-hr daylight cycle with food and water provided ad libitum. The animal experiments were carried out in accordance with the guidelines laid down by the U.S. National Institutes of Health (NIH) regarding the care and use of animals, and were approved by the ethics committee of Soka University (approval no. 19014).

The right facial nerves of adult rats were transected at the stylomastoid foramen under diethylether anesthesia and the ipsilateral nucleus was used as the axotomised facial nucleus as described [13]. As the controls, left facial nerves were left without treatment. The rats were reared for 1, 3, 5, 7, or 14 days, or for 3, 4, or 5 weeks, and then decapitated under anesthesia. The whole brains were removed, frozen on dry ice, and stored at −80°C until the facial nuclei were cut out.

Immunoblotting

The ipsilateral and contralateral facial nuclei were carefully cut from the frozen brainstem. The cut facial nuclei were sonicated with nonreducing sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate and 5% glycerol) and centrifuged at 100,000 g for 30 min. The supernatant of each tissue homogenate was recovered as tissue extract. The amounts of protein in the tissue extract were determined by the method of Lowry et al. [14].

The resultant tissue extract was prepared to contain 5% 2-mercaptoethanol and then used for immunoblotting. Twenty-microgram protein amounts were subjected to immunoblotting for GABA$_A$Rα1 (1:1000), GABA$_A$Rβ2,3 (1:1000), GABA$_A$Rβ1 (1:1000), and actin (1:2000). The staining methods were as described [13].

Statistical analysis

The densities of protein bands (GABA$_A$Rα1, GABA$_A$Rβ2,3 and GABA$_A$Rβ1) in the immunoblotting were measured by densitometry using ImageJ software (NIH, Bethesda, MD). These densities are expressed as the mean±SD of three separate experiments. Differences between the ipsilateral and contralateral nuclei were assessed via Student's *t*-test. In all cases, p-values <0.05 were considered significant (*p<0.05, **p<0.01).

RESULTS

Response of GABA$_A$Rα1 to motoneuron injury

The response of the ion channel-type GABA$_A$R to facial motoneuron injury was evaluated. We first examined changes in GABA$_A$Rα1 at 1–14 days post-insult. The immunoblotting results indicated that the GABA$_A$Rα1 levels in the injured nuclei decreased at 3–14 days post-insult (Figure 1A). We quantitatively estimated that the levels of GABA$_A$Rα1 in the axotomized facial nuclei decreased at 1, 3, 5, 7, and 14 days post-insult to 103.8±4.1%, 74.9±4.3%, 34.6±10.8%, 18.0±3.6%, and 16.5±3.4%, respectively (Figure 1B).

We further examined the levels of GABA$_A$Rα1 from 3 to 5 weeks post-insult. As shown in the graph in Figure 1C, the GABA$_A$Rα1 levels in the injured nuclei remained low during this period. The quantification showed that the levels of GABA$_A$Rα1 in the injured nuclei were 13.8±6.1%, 15.9±7.4%, and 22.5±2.8%, at 3, 4, and 5 weeks post-insult, respectively (Figure 1D). We thus found that the levels of GABA$_A$Rα1 in axotomized rat facial nuclei were decreased at 3 days post-insult and that low levels remained for 5 weeks post-insult.

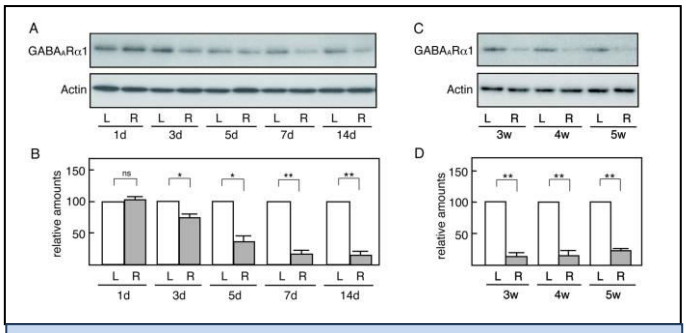

Figure 1: The response of GABA$_A$Rα1 to the motoneuron insult.

A: Changes in the GABA$_A$Rα1 level over the 1–14 days after the insult. Sets of control (left: L) and injured (right: R) facial nuclei recovered at 1, 3, 5, 7 and 14 days after transection were immunoblotted for GABA$_A$Rα1 and actin. A representative result is shown. B: Quantification of GABA$_A$Rα1 levels. The intensities of the GABA$_A$Rα1 bands in panel (A) were determined by a densitometer, and the value for the axotomized facial nucleus (R) is expressed relative to that for the control nucleus (L) (defined as 100%). The data are mean±SD from three independent experiments (ns: not significant; *p<0.05; **p<0.01). C: Changes in GABA$_A$Rα1 levels over the 3–5 weeks post-insult. Sets of contralateral (left: L) and ipsilateral (right: R) facial nuclei recovered at 3, 4 and 5 weeks after axotomy were immunoblotted for GABA$_A$Rα1 and actin. A representative result is shown. D: Quantification of the GABA$_A$Rα1 levels. The intensities of the bands in panel (C) were determined by a densitometer, and the value for the axotomized facial nucleus (R) is expressed relative to that for the control nucleus (L) (defined as 100%). The data are mean±SD from three independent experiments (**p<0.01).

Response of GABA$_A$Rβ2,3 to motoneuron injury

We next investigated the levels of GABA$_A$Rβ2,3 in axotomized rat facial nuclei over time. The levels in the injured sites appeared to decrease at 5–14 days post-insult (Figure 2A). The quantified results indicated that the levels of GABA$_A$Rβ2,3 in the axotomized facial nuclei decreased to 103.7±5.7%, 78.4±13.8%, 45.6±12.7%, 40.3±4.9%, and 51.9±4.8%, at 1, 3, 5, 7, and 14 days post-insult, respectively (Figure 2B), but the levels then seemed to recover during the 3–5 weeks post-insult (Figure 2C). The values in the injured nuclei were 91.9±5.8%, 105.1±5.4%, and 100.2±4.6%, at 3, 4, and 5 weeks post-insult, respectively (Figure 2D). Interestingly, the levels of GABA$_A$Rβ2,3 in the axotomized facial nuclei were transiently reduced at 5–14 days post-insult but recovered at 3–5 weeks post-insult.

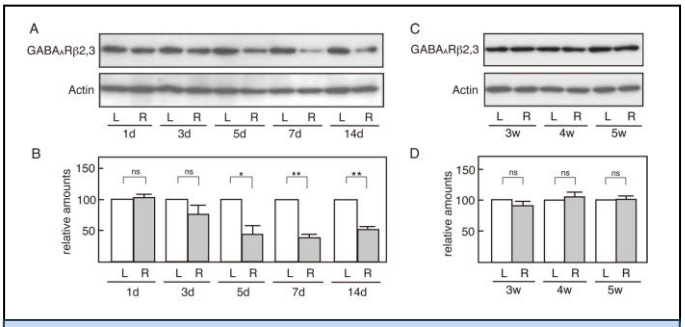

Figure 2: The response of GABA$_A$Rβ2,3 to the motoneuron insult.

A: Changes in GABA$_A$Rβ2,3 levels over the 1–14 days after insult. The samples used in Fig. 1A were immunoblotted for GABA$_A$Rβ2,3 and actin. A typical result is shown. B: Quantification of GABA$_A$Rβ2,3 levels. The intensities of the GABA$_A$β2,3 bands in panel (A) were determined by a densitometer and statistically analyzed in the same manner as that in Fig. 1B. The data are mean±SD from three independent experiments (ns: not significant; *p<0.05; **p<0.01). C: Changes in GABA$_A$Rβ2,3 levels over the 3-5 weeks after insult. The samples used in Fig. 1C were immunoblotted for GABA$_A$Rβ2,3 and actin. A typical result is shown. D: Quantification of GABA$_A$Rβ2,3 levels. The intensities of the GABA$_A$β2,3 bands in panel (C) were determined by a densitometer and statistically analyzed in the same way as that in Figure 1D. The data are mean±SD from three independent experiments (ns: not significant).

Response of GABA$_A$Rβ1 to motoneuron injury

To examine whether another type of β subunit also temporarily decreases and later returns to control levels, we analyzed the levels of GABA$_A$Rβ1 in axotomized rat facial nuclei. The levels of GABA$_A$Rβ1 in the injured nuclei decreased after 3 days post-insult (Figure 3A). The quantification indicated that the levels of GABA$_A$Rβ1 in the axotomized facial nuclei decreased to 98.9±7.0%, 38.9±7.8%, 29.0±9.4%, 35.2±6.1%, and

23.9±9.8%, at 1, 3, 5, 7, and 14 days post-insult, respectively (Figure 3B). The declined levels of GABA$_A$Rβ1 were maintained during the 3–5 weeks post-insult (Figure 3C). The quantified values in the injured nuclei were 23.2±6.0%, 16.5±5.4%, and 20.6±7.6%, at 3, 4, and 5 weeks post-insult, respectively (Figure 3D).

Thus, the GABA$_A$Rα1 and GABA$_A$Rβ1 levels in lesioned rat facial nuclei decrease from 3 days to 5 weeks post-injury, but the GABA$_A$Rβ2,3 levels recovered at 3–5 weeks post-insult after the transient reduction at 5–14 days post-insult.

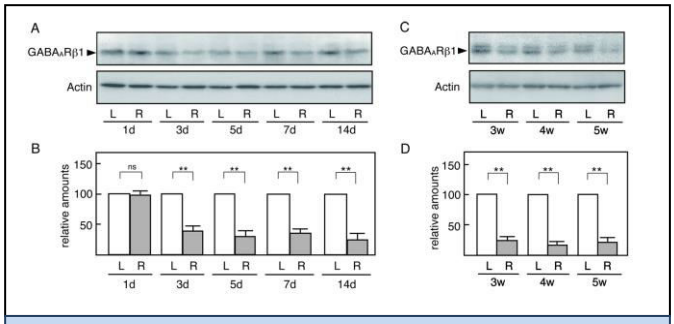

Figure 3: The response of GABA$_A$Rβ1 to the motoneuron insult.

A: Changes in GABA$_A$Rβ1 levels over the 1–14 days after insult. The samples used in Fig. 1A were immunoblotted for GABA$_A$Rβ1 and actin. A representative result is shown. B: Quantification of GABA$_A$Rβ1 levels. The intensities of the GABA$_A$β1 bands in panel (A) were determined by a densitometer and analyzed in the same manner as in Figure 1B. The data are mean±SD from three independent experiments (ns: not significant; **p<0.01). C: Changes in GABA$_A$Rβ1 levels over the 3–5 weeks after insult. The samples used in Fig. 1C were analyzed for GABA$_A$Rβ1 and actin. A representative result is shown. D: Quantification of GABA$_A$Rβ1 levels. The intensities of the GABA$_A$Rβ1 bands in panel (C) were determined by a densitometer and analyzed in the same manner as in Figure 1D. The data are mean±SD from three independent experiments (**p<0.01).

DISCUSSION

We focused on ion channel-type GABAR (GABA$_A$R) in facial motoneurons, and we examined its response to a motor nerve injury. GABA$_A$R is comprised of five subunits including α, β, γ. In the central nervous system, the most common GABA$_A$R is constructed from three subunits (α1, β2 and γ2) at the ratio of 2:2:1 [10]. In addition to these subunits, minor subunits including α4, α5, α6, β1 and γ1 are also known. Thus, the apparent variety of GABA$_A$ receptors constructed of different subunits [15,16] let us to speculate that there is functional diversity in inhibitory transmission in the mammalian nervous system. To date, we have detected the α1 subunit of GABA$_A$R in the rat facial nucleus, and we reported that its levels were significantly decreased in axotomized motoneurons [12]. However, a question arose regarding the responses of other subunits of GABA$_A$R. It had not been known whether the levels of other subunits change in injured motoneurons. To address this question, we analyzed the levels of GABA$_A$Rβ2,3 and GABA$_A$Rβ1 proteins in addition to that of GABA$_A$Rα1.

The results of our experiments revealed that the transition profile of GABA$_A$Rβ2,3 was unexpectedly different from those of GABA$_A$Rα1 and GABA$_A$Rβ1. The levels of GABA$_A$Rα1 and GABA$_A$Rβ1 in the axotomized nuclei were downregulated from 3 days to 5 weeks post-injury (Figure 1, 3), but those of GABA$_A$Rβ2,3 were temporarily reduced during the 5–14 days post-injury and restored after 3 weeks post-injury (Figure 2). The profiles of GABA$_A$Rα1 and GABA$_A$Rβ2,3 resembled those reported by Vassias et al. [11]. They investigated the changes of GABA$_A$R subunits in the rat axotomized facial nucleus by using in situ hybridaization and immunohistochemistry, and their results demonstrated that the levels of GABA$_A$Rα1 and γ2 mRNA were downregulated, which is essentially consistent with our results. The levels of GABA$_A$Rβ1 in injured motoneurons have not yet been determined, but our present analyses showed that the level of the β1 subunit declines for a long time, similarly to the α1 subunit. We thus can say that the subunits forming GABA$_A$R are not regulated as a group, and that each subunit is separately metabolized.

What does the downregulation of GABA$_A$R subunits mean? In a facial nerve transection model [17], an injury stimulus led to activation/inactivation of certain cellular signaling in motoneurons, and in turn some genes were transcribed or suppressed. In the case of GABA$_A$R subunits, the transcription might be reduced; this would probably be due to an inactivated signaling pathway, and at the same time the

degradation of $GABA_AR$ subunits would be enhanced, leading to a downregulation of their levels. However, little is known about the mechanisms underlying the manner in which the degradation of $GABA_AR$ subunits is triggered.

Regarding this issue, we obtained an interesting result in a previous study. We observed that the amounts of glial cell line-derived neurotrophic factor (GDNF) [18] transiently decreased in axotomized rat facial nucleus [19], suggesting that the lack of GDNF causes a functional decline of motoneurons with decreased levels of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAchT) [20]. We then investigated whether an administration of GDNF would protect against the reduction of $GABA_AR\alpha1$ in injured rat motoneurons [12]. Notably, the administration of GDNF at the cut nerve significantly blocked the reduction of $GABA_AR\alpha1$ in axotomized facial motoneurons. It is plausible that the degradation/proteolysis of $GABA_AR$ subunits in motoneurons is regulated by a mechanism linked to a function of specific neurotrophic factors. The analysis of the details remains to be performed.

What is the significance in the reduction of $GABA_AR$ subunits in the injured nucleus? An insult by cutting a nerve would cause severe damage to a motoneurons and an injured motoneuron must change its cellular metabolism from the normal mode to an emergency mode. Injured motoneurons might stop the use of energy in nerve conduction, as the motoneurons may consume the energy for their survival and repair as the highest priority. Simultaneously, injured motoneurons might enhance the degradation of neurotransmitter receptors so that the receptors ignore the input stimuli coming through the receptors. By such an urgent response, motoneurons could concentrate to activate the metabolism for their survivability and regeneration.

CONCLUSION

The transection of rat facial nerve led to the downregulation of $GABA_AR\alpha1$ and $GABA_AR\beta1$ levels in injured motoneurons from 3–5 days to 5 weeks post-insult. In contrast, the $GABA_AR\beta2,3$ levels in the ipsilateral nucleus were transiently reduced at 5–14 days post-insult and recovered after that. These results suggested that the amounts of each subunit comprising $GABA_AR$ are not regulated as a group in lesioned motoneurons.

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