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A glycine transporter 2-Cre knock-in mouse line for glycinergic neuron-specific gene manipulation

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ABSTRACT

Glycine is an inhibitory neurotransmitter in the brainstem and spinal cord. Glycine transporter 2 (GLYT2) is responsible for the uptake of extracellular glycine. GLYT2 is specifically expressed in glycinergic neurons and thus has been used as a marker of glycinergic neurons. Here, we generated GLYT2 promotor-driven Cre recombinase (Cre)-expressing mice (GLYT2-Cre knock-in mice) to develop a tool for manipulating gene expression in glycinergic neurons. Cre activity was examined by crossing the GLYT2-Cre knock-in mice with a Cre reporter mouse line, R26R, which express β-galactosidase (β-gal) in a Cre-dependent manner. X-gal staining of GLYT2-Cre/R26R double transgenic mouse brains and spinal cords revealed that the Cre activity was primarily distributed in the brainstem, cerebellum, and spinal cord. These areas are rich in glycinergic neurons. Furthermore, we performed immunohistochemistry for β-gal combined with *in situ* hybridization for GLYT2 in the GLYT2-Cre/R26R double transgenic mouse brains to determine whether Cre activity is specifically localized to glycinergic neurons. The β-gal protein and GLYT2 mRNAs were colocalized in the cerebellar Golgi cells, dorsal cochlear nucleus, gigantocellular reticular nucleus, spinal trigeminal nucleus, nucleus of the trapezoid body, and lateral lemniscus. More than 98% of the GLYT2 mRNA-expressing cells in these brain regions also expressed β-gal, whereas 90–98% of the β-gal-positive cells expressed the GLYT2 mRNAs. Thus, Cre activity is specifically localized to glycinergic neurons with high fidelity in the GLYT2-Cre knock-in mice. The GLYT2-Cre knock-in mouse line will be a useful tool for studying glycinergic neurons and neurotransmission.

1. Introduction

Glycine is a major inhibitory neurotransmitter in the brainstem and spinal cord, and glycinergic neurotransmission participates in a variety of functions, such as sensory processing, motor rhythm generation, and control of the respiratory network (Legendre, 2001; Ezure et al., 2003; Yevenes and Zeilhofer, 2011). Glycine is released from presynaptic terminals in glycinergic neurons through a vesicular mechanism (Wojcik et al., 2006; Saito et al., 2010) and acts on postsynaptic glycine receptors (GlyRs) (Laube et al., 2002). GlyR activation leads to an influx of chloride to hyperpolarize and subsequently inhibit postsynaptic neurons (Aragón and López-Corcuera, 2003). Extracellular glycine is transported to the intracellular space by two types of glycine transporters (GLYTs), GLYT1 and GLYT2, which are encoded by two separate genes (Ebihara et al., 2004; Betz et al., 2006). Both GLYTs belong to a large family of Na^+/Cl^--dependent transporter proteins (Nelson, 1998). GLYT1 and GLYT2 have different functions at glycinergic synapses. GLYT1 eliminates glycine from the synaptic cleft, whereas GLYT2 is essential for glycine uptake into the presynaptic terminal and the subsequent refilling of synaptic vesicles with glycine. Deficiencies of GLYT1 and GLYT2 cause the opposite effects on glycinergic neurotransmission. GLYT1 deficiency causes an increased extracellular glycine, which leads to a sustained activation of GlyRs. This effect generates symptoms found in human glycine encephalopathy. On the other hand, GLYT2 deficiency causes the decreased inhibitory postsynaptic currents via GlyRs and induces human hyperekplexia phenotype *in vivo* (Gomeza et al., 2003a, 2003b; Eulenburg et al., 2005; Rees et al., 2006; Carta et al., 2012). GLYT1 is predominantly expressed in glial cells of the brain, whereas GLYT2 is specifically expressed in glycinergic neurons and is a reliable marker of glycinergic neurons (Poyatos et al., 1997).

Genetically modified mice have become a powerful tool in the analysis of neural networks. The Cre/loxP system, in which the Cre

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Abbreviations:

4V	fourth ventricle
7	facial nucleus
7n	facial nerve
8n	vestibulocochlear nerve
12	hypoglossal nucleus
Aq	aqueduct
BAC	bacterial artificial chromosome
β-gal	β-galactosidase
Cre	Cre recombinase
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
DC	dorsal cochlear nucleus
DIG	digoxigenin
DTg	dorsal tegmental nucleus
EGFP	enhanced green fluorescent protein
GC	granule cell layer
Gi	gigantocellular reticular nucleus
GiA	gigantocellular reticular nucleus, alpha part
GLYT	glycine transporter
GLYT1	glycine transporter 1
GLYT2	glycine transporter 2
GM	gray matter
Gr	granular layer
IC	inferior colliculus
icp	inferior cerebellar peduncle
Int	interposed cerebellar nucleus
IO	inferior olive
Lat	lateral cerebellar nucleus
MdD	medullary reticular nucleus, dorsal part
MdV	medullary reticular nucleus, ventral part
Mo	molecular layer
MVe	medial vestibular nucleus
neo	neomycin-resistant gene expression cassette
Pa4	paratrochlear nucleus
PBS	phosphate-buffered saline
PC	Purkinje cell layer
Pn	pontine nuclei
PnC	pontine reticular nucleus, caudal part
PnO	pontine reticular nucleus, oral part
Pr	prepositus nucleus
Pr5	principal sensory trigeminal nucleus
py	pyramidal tract
RMg	raphe magnus nucleus
SC	superior colliculus
SO	superior olive
SP5	spinal trigeminal nucleus
TBS	Tris-buffered saline
TBST	TBS containing 0.1% Tween-20
Tz	nucleus of the trapezoid body
VC	ventral cochlear nucleus
VLL	ventral nucleus of the lateral lemniscus
WM	white matter

recombinase (Cre) transgene activates a reporter gene by inducing recombination at loxP sites, is a widely used approach. In addition, this system facilitates cell-specific gene expression and inactivation in the mouse (Wouterlood et al., 2014). We generated a GLYT2-Cre knock-in mouse line using knock-in techniques to develop a tool for the manipulation of gene expression in glycinergic neurons and the inactivation of the endogenous GLYT2 gene. GLYT2-Cre transgenic mouse lines have been developed using bacterial artificial chromosomes (BACs), which contains more than 100 kb DNA sequences (Ishihara et al., 2010; Foster et al., 2015; Rahman et al., 2015). BAC-based transgenesis can result in ectopic expression due to the lack of all cis-regulatory elements required for proper expression and random integration into the genome (Harris et al., 2014). We generated a GLYT2-Cre knock-in mouse line expressing Cre under the control of the endogenous GLYT2 promoter using the knock-in strategy to overcome this problem. The knock-in strategy uses homologous recombination in ES cells to insert the Cre into the endogenous gene locus. The knock-in strategy generally captures endogenous gene expression patterns better than BAC transgenic strategies (Harris et al., 2014).

In the present study, we report the generation and characterization of a GLYT2-Cre knock-in mouse line. Cre activity is efficient and restricted to glycinergic neurons in the GLYT2-Cre knock-in mice. The GLYT2-Cre knock-in mouse line is very useful for studies of glycinergic neurons and neurotransmission.

2. Materials and methods

2.1. Mice

Two C57BL/6 mouse genomic BAC clones, RP23-83C16 and RP23-155A11 (BACPAC Resources, Oakland, CA, USA), containing the GLYT2 gene were purchased and used to construct the targeting vector. The knock-in vector contained an 8.1 kb fragment from the *Sal*I site (in the 5′-flanking region) to the translation initiation site (in exon 2) of the GLYT2 gene, a Cre cDNA, a polyadenylation signal (pA) from the human granulocyte-colony stimulating factor gene, a phosphoglycerate kinase (PGK) promoter-driven neomycin resistance gene (neo)-pA cassette (PGK-neo-pA cassette) flanked by two frt sites (Takeuchi et al., 2002), a 2.9 kb *Afl*II (in exon 2)-*Nco*I (in intron 2) fragment of the GLYT2 gene, and a MC1 promoter-driven diphtheria toxin A-fragment gene. A 440 bp *Kpn*I (in intron 1b of the GLYT2 gene)-*Age*I (in the Cre gene) fragment was synthesized by two-step PCR to fuse the GLYT2 gene (360 bp) to the Cre gene (80 bp) at their translation initiation sites. The Cre gene was isolated from the Cre-MC vector (Akashi et al., 2009). The PGK-neo-pA cassette and the MC1 promoter-driven diphtheria toxin A-fragment gene were isolated from the DT-NeoMC vector (Akashi et al., 2009).

The knock-in vector was linearized with *Sal*I and electroporated into the RENKA mouse C57BL/6 ES cell line (Mishina and Sakimura, 2007). G418-resistant ES clones were isolated and screened using Southern blotting to detect homologous recombination. Genomic DNAs prepared from the ES cell colonies were digested with *Eco*RV and *Eco*RI and were hybridized with the 5′ external probe (Probe A), the 3′ external probe (Probe C), and internal probe (Probe B) (see Fig. 1). These DNA probes were labeled with digoxigenin using the DIG DNA Labeling Mix, 10x conc. (Roche, Basel, Switzerland). The recombinant ES cells were microinjected into eight cell-stage embryos of the ICR mouse strain to obtain chimeric mice.

The chimeric mice were crossed with C57BL/6 mice, and mice with germline transmission of the recombined allele were then crossed with FLP66 transgenic mice (Takeuchi et al., 2002) to delete the PGK-neo-pA cassette. GLYT2-Cre knock-in mice were crossed with R26R mice to detect Cre activity (Soriano, 1999), and GLYT2-Cre/R26R double transgenic ($GLYT2^{Cre/+}$; $R26R^{lacZ/+}$) mice (Yang et al., 2003) were used for histological analyses. $GLYT2^{+/+}$; $R26R^{lacZ/+}$ or $GLYT2^{+/+}$; $R26R^{+/+}$ mice were used as control mice.

All animal procedures were conducted in accordance with the guidelines of the NIH and were reviewed and approved by the Animal Care and Experimentation Committee of Gunma University, Showa Campus (Maebashi, Japan). Every effort was made to minimize the number of animals used and their suffering.