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Title:
Spinal cord-specific deletion of the glutamate transporter GLT1 causes motor neuron death in mice

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Conflicts of interest: none

Abstract:
Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative disorder characterized by the selective loss of motor neurons. The precise mechanisms that cause the selective death of motor neurons remain unclear, but a growing body of evidence suggests that glutamate-mediated excitotoxicity has been considered to play an important role in the mechanisms of motor neuron degeneration in ALS. Reductions in glutamate transporter GLT1 have been reported in animal models of ALS and the motor cortex and spinal cord of ALS patients. However, it remains unknown whether the reduction in GLT1 has a primary role in the induction of motor neuron degeneration in
ALS. Here, we generated conditional knockout mice that lacked GLT1 specifically in the spinal cord by crossing floxed-GLT1 mice and Hoxb8-Cre mice. Hoxb8-Cre/GLT1^{flox/flox} mice showed motor deficits and motor neuron loss. Thus, loss of the glial glutamate transporter GLT1 is sufficient to cause motor neuron death in mice.

**Keywords:**
- glutamate transporter, GLT1, motor neuron, amyotrophic lateral sclerosis, excitotoxicity

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that is characterized by the selective loss of motor neurons [1,2]. ALS can be classified into two major types: familial ALS (FALS), which accounts for approximately 10% of cases, and sporadic ALS (SALS), which comprises the remaining 90%. Among the several potential pathogenic mechanisms of ALS, glutamate-mediated excitotoxicity has been considered to play an important role in the mechanisms of motor neuron death [1-4]. The link between excitotoxicity and ALS was supported by early studies indicating that ALS patients exhibited large increases in the glutamate levels in cerebrospinal fluid [5,6], a finding that has since been confirmed in 40% of SALS patients [7]. Furthermore, ALS patients show reduced glutamate uptake in the motor cortex and spinal cord [8], which results from a reduction of the astrocytic glutamate transporter GLT1 (also known as EAAT2) [9]. Together with the finding that the loss of GLT1 was observed in ALS rodent models with copper/zinc superoxide dismutase (SOD1) [10] or TAR DNA-binding protein 43 (TDP-43) mutations [11], it seems most likely that deficiencies in the expression and function of the glial glutamate transporter GLT1 may result in elevated levels of extracellular glutamate, which could lead to neuronal death through excitotoxicity in both SALS and FALS. However, GLT1 impairment could be a consequence of motor neuron loss, because the expression of GLT1 is critically dependent on the presence of neurons [12]. Furthermore, previous studies showed that the chronic in vivo administration of glutamate transport inhibitors elevated extracellular glutamate but did not result in motor neuron death, gliosis or motor deficits [13,14].
Here we tested whether a primary deficit in GLT1 was sufficient to induce spinal motor neuron death and motor deficits in vivo. Because GLT1 null mice die prematurely with seizures [15], we generated GLT1 conditional knockout mice that lacked GLT1 specifically in the spinal cord.

2. Materials and methods

2.1. Mice

All animal procedures were conducted according to the animal experiment plan approved by the Tokyo Medical and Dental University Animal Care and Use Committee. Three to five mice were housed per cage on 12-hour light/dark cycle at 23-25 °C. Food and water were available ad libitum. Floxed-GLT1 [16-18] mice, Hoxb8-Cre transgenic [19] mice, and ROSA<sup>tdTomato</sup> reporter [20] mice were previously described. Male mice were used except for GLT1 immunohistochemistry. Age and number of mice used for all experiments are described in the figure legends.

2.2. Behavioral tests

Behavioral analyses were conducted as described previously [18]. For the hanging wire test, the wire netting was turned over and held for 60 sec after placing mice on it. The time until the hindlimbs disengaged from the wire netting was recorded as the latency to fall. For the hindlimb reflex score, mice were suspended from their tails for 14 sec and we evaluated the hindlimb reflex score using an established definition (Fig. 2D). 0, normal; 1, failure to stretch their hindlimbs; 2, hindlimb clasping; 3, hindlimb paralysis.

2.3. Histology

Histological analysis was performed as previously described [16-18]. Mice deeply anesthetized with pentobarbital (100 mg/kg, i.p.) were fixed by perfusion with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). After further fixation of brains and spinal cords with 4% PFA overnight, tissues were transferred to 30% sucrose/PBS for cryoprotection and embedded in OCT compound (Sakura, Tokyo, Japan). Cryosections were prepared at a 20 µm thickness for spinal cords, and a 50 µm
thickness for sagittal sections and mounted on MAS-coated slides (Matunami, Osaka, Japan) (Leica CM3050s). Cryosections were washed, permeabilized and blocked with 0.3% Triton X-100, 1% BSA and 10% normal goat serum in PBS, and incubated with primary antibodies overnight at 4 °C. For ChAT immunofluorescence, sections were incubated in 10mM citrate buffer (pH 6.0) at 100 °C for 20 min. After air-cooling, sections were permeabilized and blocked with 0.3% Triton X-100, 1% BSA and 10% normal horse serum in PBS. The following antibodies were used: polyclonal anti-GLT1 (1:5,000, a gift from M. Watanabe, Hokkaido University) [21], polyclonal anti-ChAT (1:200, MAB144P, Merck Millipore), polyclonal anti-glial fibrillary acidic protein (GFAP, 1:1,000, Z0334, Dako), polyclonal anti-CD68 (1:100, MCA1957, AbD Serotec), and polyclonal anti-MAP2 (1:200, MAB3418, Millipore) antibodies. Sections were washed, incubated with secondary antibodies conjugated with Alexa Fluor 488, 594, or 633, and DAPI as nuclear marker (Life Technologies) for 2 hours, and mounted with Fluoromount (Diagnostic BioSystems). For ChAT immunofluorescence, sections were incubated with biotinylated donkey anti-goat IgG (1:200, Vector Lab) for 2 h, washed and incubated with streptavidin conjugated with Alexa Fluor 488 (1:200, Invitrogen) for 2 h. For GLT1 immunohistochemistry, sections were incubated with biotinylated goat anti-rabbit IgG (1:1,000, Vector Lab.) for 1 h, immersed in 1% H$_2$O$_2$/PBS, and incubated with AB complex (1:500, Vector Lab) for 1 h, and then visualized with a DAB substrate kit (Vector Lab). Images were acquired using an LSM710 laser scanning confocal microscope (Carl Zeiss), a BX-700 fluorescent microscopy (KEYENCE) for GLT1 immunohistochemistry, or an Olympus SZX16 stereoscopic microscope (Olympus).

2.4. Cell counts

Cell counts were conducted as described previously [18]. The number of ChAT positive cells in the bilateral ventral horns of L3-L5 in 6-9 serial sections was counted using Image J software with the cell counter plug-in (NIH, Bethesda, MD) under the conditions blind to genotype.

2.5. Statistical analysis
Results are presented as the mean ± SEM. Student’s t-test was used to compare differences between any two groups. For longitudinal observations, repeated-measure ANOVA with post hoc unpaired two-tailed t-test was performed. To analyze the significant differences in survival rate, Kaplan-Meier and logrank test were used. All statistical analyses were performed using Statcel3 software. A p-value of less than 0.05 was considered statistically significant.

3. Results
3.1. Generation of spinal cord-specific GLT1 knockout mice

Because GLT1 knockout animals die prematurely with seizures [15], we have generated GLT1 conditional mutant mice that lacked GLT1 specifically in the spinal cord to examine the effects of GLT1 loss on spinal cord motor neuron survival. We crossed mice carrying a loxP-flanked GLT1 allele (GLT1^{flox/flox}, denoted as control) [16-18] with Hoxb8-Cre mice [20] to obtain spinal cord-specific GLT1 knockout mice (Hoxb8-Cre/GLT1^{flox/flox} mice, Fig. 1A). The Hoxb8-Cre line expresses Cre recombinase in the spinal cord caudal to cervical level C5 but express very little Cre in the brain [19]. To assess Cre expression, we crossed the Hoxb8-Cre mice to the ROSA^{tdTomato} reporter mice. Consistent with previous report [19], the strong tdTomato signal was observed caudal to cervical spinal cord of Hoxb8-Cre/ROSA^{tdTomato} mice (Fig. 1B). Thus, using this Cre mouse line enabled us to delete GLT1 in the spinal cord.

Immunohistochemistry of GLT1 showed that the amount of GLT1 protein in the spinal cord caudal to cervical level C5 in Hoxb8-Cre/GLT1^{flox/flox} mice was much lower than age-matched GLT1^{flox/flox} mice but that GLT1 expression in other brain regions was normal (Fig.1C). There was no compensatory upregulation of other subtypes of glutamate transporters, GLAST and EAAC1, in Hoxb8-Cre/GLT1^{flox/flox} mice [18].

3.2. Hoxb8-Cre/GLT1^{flox/flox} mice develop motor dysfunction

In contrast to GLT1 null mice, Hoxb8-Cre/GLT1^{flox/flox} mice did not show lethal seizures and survived to adulthood (Fig. 2A). Starting at 4 months of age, Hoxb8-Cre/GLT1^{flox/flox} mice had slightly lower body weight than did the controls and showed both grip strength reduction and hindlimb reflex impairment (Fig. 2B, C, E).
These motor deficits stabilized after 6 months of age and Hoxb8-Cre/GLT1\textsuperscript{flox/flox} mice exhibited no overt paralysis until at least 22 months of age (data not shown). These results indicate that GLT1 deletion in spinal cord causes mild motor deficits in mice.

3.3. Hoxb8-Cre/GLT1\textsuperscript{flox/flox} mice show motor neuron loss and gliosis

We next examined whether GLT1 deletion caused motor neuron loss and gliosis. Immunohistochemical analysis using an anti-ChAT antibody showed a loss of spinal motor neurons in the lumbar ventral horn of Hoxb8-Cre/GLT1\textsuperscript{flox/flox} mice at 5 months of age (symptomatic stage) (Fig. 3A, B). In contrast, there was no difference in the number of ChAT-positive cells between control and Hoxb8-Cre/GLT1\textsuperscript{flox/flox} mice at 2 months of age (presymptomatic stage) (Fig. 3A, B). In addition, immunoreactivity for GFAP, a marker of astrocyte, and CD68, a marker of activated microglia, was increased in the ventral horn of the lumbar spinal cord of Hoxb8-Cre/GLT1\textsuperscript{flox/flox} mice at 5 months of age (Fig. 3C). Taken together, these results indicate that GLT1 deletion in spinal cord is sufficient to induce motor neuron loss and gliosis in vivo.

4. Discussion

Although several lines of evidence suggest the possibility that glutamate transport dysfunction may play an important role in the mechanisms of motor neuron degeneration in ALS [5-9], a few reports do not support glutamate-transport deficits as a primary cause of neuronal death in ALS. Spinal infusion of glutamate transporter inhibitors in rats failed to cause motor neuron death or motor deficits [13,14]. Furthermore, adeno-associated virus-based GLT1 overexpression failed to prevent loss of phrenic nerve motor neurons or rescue respiratory function in SOD1(G93A) mice, a widely studied ALS model [22]. Thus, currently the evidence for glutamate transporter dysfunction as a primary cause of motor neuron death is inconclusive. In this study, we found that conditional knockout mice that lacked GLT1 specifically in the spinal cord developed motor dysfunction and spinal motor neuron loss. These data demonstrate that dysfunction of GLT1 is sufficient to cause motor neuron death in vivo.

Although Hoxb8-Cre/GLT1\textsuperscript{flox/flox} mice showed motor neuron death, motor dysfunction and the loss of motor neurons in Hoxb8-Cre/GLT1\textsuperscript{flox/flox} mice were very mild, which suggested that Hoxb8-Cre/GLT1\textsuperscript{flox/flox} mice could not faithfully
recapitulate the rapidly progressive clinical phenotype observed in ALS patients. In a recent paper, we demonstrated that a concurrent reduction in the GLAST (another glial glutamate transporter) expression levels from Hoxb8-Cre/GLT1<sup>flox/flox</sup> mice accelerated motor deficits and resulted in earlier motor neuron loss and a reduction in survival [18], thus recapitulating the clinical phenotypes of ALS as accurately as SOD1 and TDP-43 mutants [10,11]. Since GLAST knockout mice did not show grip strength reduction, hindlimb reflex impairment, and spinal motor neuron loss [23], these results suggest that loss of GLAST is a contributor to, but not the cause of, spinal motor neuron loss.

Taken together, our studies suggest that glial glutamate transporter GLT1 plays an important role in the survival of spinal motor neurons and loss of GLT1 in spinal cord directly contributes to spinal motor neuron death and motor deficits.

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Figure Legends

Figure 1. Generation of spinal cord-specific GLT1 knockout mice
(A) Schematic diagram showing the generation of spinal cord-specific GLT1 knockout (Hoxb8-Cre/GLT1<sup>flox/flox</sup>) mice.
(B) Characterization of the brain-sparing Hoxb8-Cre line. Red fluorescence is observed in the spinal cord caudal to cervical level C5 in Hoxb8-Cre mice harboring the reporter R26tdTomato (ROSA<sup>tdTomato</sup>) at postnatal day 0.
(C) GLT1 immunohistochemistry on brain and spinal cord from control (GLT1<sup>flox/flox</sup>) and Hoxb8-Cre/GLT1<sup>flox/flox</sup> mice at 4 weeks of age.
Scale bars, (A and C) 2 mm.

Figure 2. Mild motor deficits in spinal cord-specific GLT1 knockout mice.
(A) Percent survival of control (n = 14) and Hoxb8-Cre/GLT1<sup>flox/flox</sup> (n = 17) mice calculated using the Kaplan-Meier method (p = 0.36, logrank test). n. s. = not significant.
(B) Body weight changes, (C) hanging wire test, and (E) hindlimb reflex score of control (n = 12) and Hoxb8-Cre/GLT1<sup>flox/flox</sup> animals (n = 9). *p < 0.05, **p < 0.01, ***p < 0.001 (post hoc unpaired two-tailed t-test at corresponding time-point after two-way repeated measures ANOVA).
(D) The scoring criteria for the hindlimb reflex score. Mice were suspended by the tail approximately 30 cm above a tabletop for 14 sec. The posture adopted was scored according to the following criteria: score of 0, normal; score of 1, failure to stretch hindlimbs; score of 2, hindlimb clasping; score of 3, hindlimb paralysis.

Figure 3. Motor neuronal loss and gliosis in spinal cord-specific GLT1 knockout mice.
(A) ChAT immunofluorescence of the lumbar ventral horn from control and Hoxb8-Cre/GLT1<sup>flox/flox</sup> mice at 2 and 5 months of age.
(B) Quantification of ChAT-positive motor neurons from 2 and 5 months of age of control (P2M, n = 5; P5M, n = 3) and Hoxb8-Cre/GLT1<sup>flox/flox</sup> (P2M, n = 5; P5M, n = 4) mice. **p < 0.01, n.s. = not significant (unpaired two-tailed t-test).
(C) GFAP and CD68 immunofluorescence in the lumbar ventral horn from control and
Hoxb8-Cre/GLT1$^{\text{flox/flox}}$ mice at 5 months of age. Mice were triple labeled with GFAP (red), CD68 (cyan) and MAP2 (green) antibodies. Scale bars, (A and C) 50 µm.
Highlights:
GLT1 deletion in spinal cord causes motor deficits.
GLT1 deletion in spinal cord induces spinal motor neuron loss.
GLT1 deletion in spinal cord induces gliosis in the ventral horn.