D-Amino acid oxidase controls motoneuron degeneration through D-serine

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Edited by Solomon H. Snyder, The Johns Hopkins University School of Medicine, Baltimore, MD, and approved December 2, 2011 (received for review September 6, 2011)

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder involving an extensive loss of motoneurons. Aberrant excitability of motoneurons has been implicated in the pathogenesis of selective motoneuronal death in ALS. D-Serine, an endogenous coagonist of *N*-methyl-D-aspartate receptors, exacerbates motoneuronal death and is increased both in patients with sporadic/familial ALS and in a G93A-SOD1 mouse model of ALS (mSOD1 mouse). More recently, a unique mutation in the D-amino acid oxidase (DAO) gene, encoding a D-serine degrading enzyme, was reported to be associated with classical familial ALS. However, whether DAO affects the motoneuronal phenotype and D-serine increase in ALS remains uncertain. Here, we show that genetic inactivation of DAO in mice reduces the number and size of lower motoneurons with axonal degeneration, and that suppressed DAO activity in reactive astrocytes in the reticulospinal tract, one of the major inputs to the lower motoneurons, predominantly contributes to the D-serine increase in the mSOD1 mouse. The DAO inactivity resulted from expressional down-regulation, which was reversed by inhibitors of a glutamate receptor and MEK, but not by those of inflammatory stimuli. Our findings provide evidence that DAO has a pivotal role in motoneuron degeneration through D-serine regulation and that inactivity of DAO is a common feature between the mSOD1 ALS mouse model and the mutant DAO-associated familial ALS. The therapeutic benefit of reducing D-serine or controlling DAO activity in ALS should be tested in future studies.

excitotoxicity | motor neuron disease | neurodegeneration | enzyme histochemistry | 2D-HPLC

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by selective loss of motoneurons in the spinal cord and brain leading to fatal paralysis. Approximately 90% of all cases are sporadic, and the remaining cases are inherited. Of inherited cases, 20% are associated with mutations in superoxide dismutase 1 (*SOD1*), and 10% involves 43-kDa transactivation response DNA-binding protein (*TDP-43*) and fused in sarcoma/translocated in liposarcoma (*FUS/TLS*). Despite extensive studies of previously identified ALS-causing genes, the mechanism underlying the selective motoneuronal loss in ALS remains uncertain. Given that the mechanism is at least, in part, common between sporadic and familial ALS, identification of the common pathology is a clue to conquering ALS. Among numerous etiological hypotheses, motoneuronal vulnerability to excitotoxicity is one of the most intensely investigated targets for the treatment of ALS because it is observed in both sporadic and familial ALS with SOD1 mutations (1, 2). For motoneurons, glutamate is the main excitatory transmitter, and excessive motoneuron excitability by glutamate through ionotropic glutamate receptors has been demonstrated.

The *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) is a subtype of the ionotropic glutamate receptors and exhibits relatively higher permeability to the calcium ion (Ca^{2+}) than non-NMDARs, which links it to a variety of physiological and pathological processes. Unlike non-NMDARs, glutamate does not activate the NMDARs unless a coagonist binding site is occupied. D-Serine, an unusual D-amino acid found in mammalian brain, is a physiological ligand of the coagonist site of the NMDARs (3, 4); hence, it is pivotal in determining excitability of glutamatergic neurons. D-Serine is endogenously converted from L-serine by serine racemase (SRR) (5) and exists at a high level in the forebrain, where it has a critical role in long-term potentiation (6) and is required for memory formation (6). D-Serine is also involved in NMDAR-mediated neurotoxicity, a process that plays a pathophysiological role in stroke and neurodegenerative diseases (7–9). We previously reported that D-serine is increased in the spinal cord in both patients with sporadic/familial ALS and in a G93A-SOD1 mouse model of ALS (mSOD1 mouse) (10). D-Serine is progressively increased with expressional elevation of SRR caused by glial activation (10). Intriguingly, a point mutation that diminishes enzyme activity in D-amino acid oxidase (DAO), a D-serine degrading enzyme, is associated with familial ALS (11). However, whether DAO is related to D-serine increase in ALS, or DAO inactivity is relevant to motoneuronal degeneration in vivo, remains uncertain. In this study, we found that DAO activity is strikingly suppressed in the reticulospinal tract of mSOD1 mice, which plays a central role in D-serine increase in mSOD1 mice, and that loss of DAO activity results in motoneuron degeneration.

Results

DAO is highly expressed in mammalian CNS, liver, and kidney and catalyzes the oxidative deamination of D-amino acids. ddY/DAO^- mice, found in outbred ddY mice (12), lack DAO activity because of a natural point mutation (G181R) (13) and show abnormal locomotor behavior related to enhanced NMDAR function (14). Because hypofunction of the NMDAR and genetic association of DAO have been implicated in schizophrenia, most studies using ddY/DAO^- mice have focused on a therapeutic approach to schizophrenia through D-serine increase. No phenotypic analysis of spinal motoneurons in the mice, however, has yet been conducted. To study whether inactivation of DAO affects the motoneuronal phenotype in vivo, ddY/DAO^- mice were backcrossed with C57BL/6J and maintained as homozygotes ($^{B6}DAO^{-/-}$ mice). The $^{B6}DAO^{-/-}$ mice developed an abnormal limb reflex characterized by retraction of hindlimbs

Author contributions: J.S. designed research; J.S., Y.M., M.S., and K.H. performed research; M.M., R.K., and K.H. contributed new reagents/analytic tools; J.S., R.K., M.M., K.H., and S.A. analyzed data; and J.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1114639109/-/DCSupplemental.

toward the trunk when they were lifted up by their tails, whereas C57BL/6J mice showed normal extension of the hindlimbs (Fig. 1*A*). Motoneurons of the lumbar ventral horn in ^{B6}DAO$^{-/-}$ mice exhibited morphology of degeneration (Fig. 1*B*), including the reduction in number by 24% (Fig. 1*C* and Fig. S1 *A–C*) and in size of their somata by 15% on average (Fig. 1*D* and Fig. S1*D*) compared with age-matched C57BL/6J mice. In aged ^{B6}DAO$^{-/-}$ mice, ubiquitin-positive aggregations were observed in the ventral neurons (Fig. 1 *E* and *F*), and the ubiquitin-immunoreactive smear was increased in the immunoblotting of spinal cords (Fig. 1*G*), but no expressional or localization changes of NMDARs were detected except for localization shift of NR2A (Fig. S1 *E* and *F*). Furthermore, inactivation of DAO triggers axonal degeneration with muscle atrophy in aged mice (Fig. 1 *H* and *I* and Fig. S1*G*). These findings demonstrate that DAO activity influences the motoneuronal phenotype.

To elucidate the pathophysiological significance of DAO in ALS, we visualized DAO activity in cryostat sections by modifying an enzyme histochemical (EHC) technique for vibratome sections (15). EHC is based on oxidation of diaminobenzidine with peroxidase by using D-proline as a substrate of DAO. Intense enzymatic reactivity was observed in the cerebellum, and relatively weak reactivity was in the pons, medulla oblongata, and spinal cord, whereas there was no signal at all in the forebrain region (Fig. S2 *A* and *B*). The specificity of the EHC was verified by using L-proline as a substrate or tissue sections from ^{B6}DAO$^{-/-}$ mice (Fig. S2*C*). In the spinal cord, DAO was mainly distributed in the anterior columns, where motor axons descend from upper neurons, and in lamina VIII and IX of ventral spinal gray matter (Fig. 2*A*). This distribution pattern suggested a correlation between DAO activity and the motor tract. Because D-serine is essential for the neurotoxicity through NMDARs (7), DAO has been thought to associate with diseases that involve NMDAR malfunction. A R199W mutation in DAO found in patients with familial ALS resulted in dramatic deactivation of DAO (Fig. S3 *A* and *B*) and motoneuronal death in vitro (11). In mSOD1 mice (16), the most studied ALS animal model, DAO activity was significantly decreased compared with that in age-matched wild-type mice (control mice) especially in the ventral part of lumbar spinal cords (Fig. 2*A*). Quantification of DAO activity in the whole tissue of the lumbar spinal cords from mSOD1, ^{B6}DAO$^{+/-}$, and ^{B6}DAO$^{-/-}$ mice showed that the DAO activity in mSOD1 mice was suppressed to 57.9% of that in control mice and was almost identical to that in ^{B6}DAO$^{+/-}$ mice [$F_{(3, 8)} = 19.42$, $P = 0.0005$] (Fig. 2*B*). This suppressed activity of DAO resulted from marked reduction of DAO protein expression in Western blotting of spinal cords of mSOD1 mice at the end stage, although significant reduction was not observed at the onset (Fig. 2*C* and Fig. S2 *D* and *E*).

Using FITC-conjugated tyramide, which reacts sensitively with peroxidase and enhances the sensitivity of EHC (Fig. S2 *A* and *B*), we performed double staining of DAO activity and glial fibrillary acidic protein (GFAP; an astrocytic marker), Iba1 (a microglial marker), or nonphosphorylated neurofilament H (npNFH, a marker for motoneuron) and found that DAO activity was located in a portion of quiescent astrocytes (Fig. 2 *D–F* and Fig. S2 *F* and *G*), but not in microglia (Fig. 2*G*) or somata/axons of motoneurons (Fig. 2 *H* and *I*). Pyramidal neurons such as Purkinje cells in the cerebellum and large neurons in the brain-stem reticular formation did not exhibit any activity of DAO (Fig. S2 *H* and *I*). Of note is that in mSOD1 mice, DAO activity was severely suppressed in reactive astrocytes (Fig. 2 *D* and *E, Right*). To further observe the DAO activity in the upper motor tract in mSOD1 mice, sagittal sections were stained with EHC enhanced with FITC-tyramide. DAO activity was strikingly decreased in reactive astrocytes in brainstem reticular formation of mSOD1 mice compared with control mice (Fig. 2 *J* and *K*), whereas the activity in the cerebellum, pons, and the dorsal part of the medulla oblongata (vestibular nuclei) remained unchanged (Fig. 2*J*). Together with the finding in Fig. 2*A*, in mSOD1 mice, DAO suppression was restricted in the reticulospinal tract, which mainly regulates motoneuronal excitability

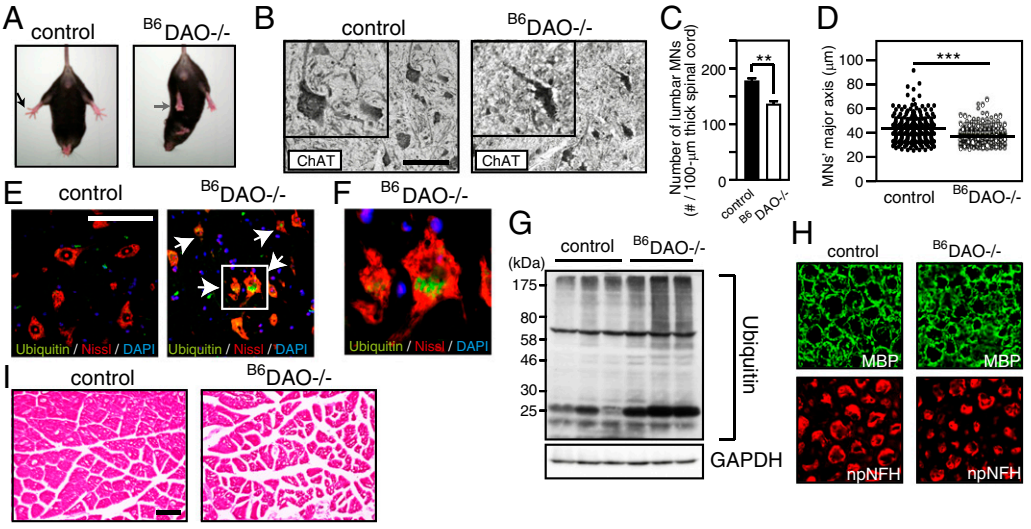

Fig. 1. DAO inactivation triggers motoneuron degeneration. (*A*) Abnormal limb reflex in ^{B6}DAO$^{-/-}$ mice (8 mo). (*B*) Motoneurons in spinal cords (L2) of ^{B6}DAO$^{-/-}$ and wild-type control (control) mice (8 mo) were immunolabeled with a choline acetyltransferase (ChAT) antibody. *Insets* are enlarged ChAT-positive neurons. (*C*) Number of motoneurons (MN) in lumbar spinal cord of ^{B6}DAO$^{-/-}$ ($n = 4$) and control ($n = 5$), normalized to thickness of coronally sliced sections. $^{**}P = 0.0028$ (Student's *t* test). (*D*) The longest diameter of MN's soma (major axis) was measured (^{B6}DAO$^{-/-}$, $n = 275$; control, $n = 272$). $^{***}P < 0.0001$ (Student's *t* test). (*E* and *F*) Motoneurons in the spinal cords of ^{B6}DAO$^{-/-}$ and control mice (15 mo) were costained with fluorescent analysis of Nissl (red), ubiquitin (green), and DAPI (blue). Arrows indicate ubiquitin aggregation-positive cells. Squared region was enlarged in *F*. (*G*) Western blotting of spinal cord lysates from ^{B6}DAO$^{-/-}$ and control (15 mo) was performed by using antibodies to ubiquitin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (*H*) Motor fibers in tibial nerve from ^{B6}DAO$^{-/-}$ and control (15 mo) were visualized with immunofluorescent analysis of myelin basic protein (MBP) and npNFH. (*I*) Gastrocnemius from ^{B6}DAO$^{-/-}$ and control (15 mo) was stained with H&E. Data are plotted as mean ± SEM (*C*). (Scale bars: 100 μm.)

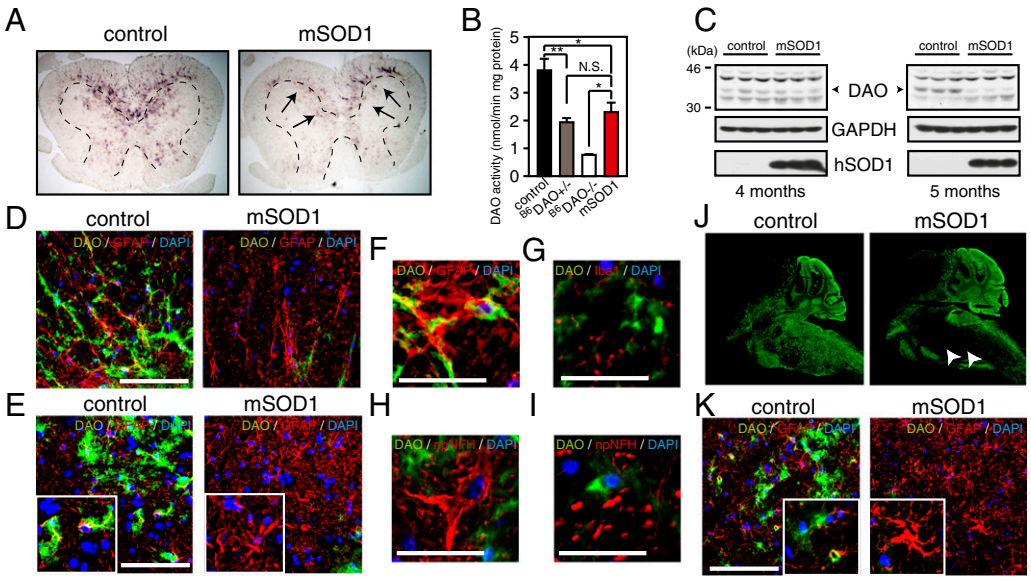

Fig. 2. DAO activity is suppressed in the reticulospinal tract. (*A*) DAO enzyme histochemistry of lumbar spinal cord sections of mSOD1 and control mice (5 mo). The dashed line indicates a border between the gray and white matter. DAO activity was suppressed in ventral gray and white matter (arrows). (*B*) DAO activity in spinal cords of control, ^{B6}DAO$^{+/-}$, ^{B6}DAO$^{-/-}$, and mSOD1 mice (5 mo) was assayed ($n = 3$ each). $P = 0.0005$ (one-way ANOVA), $^{*}P < 0.05$, $^{**}P < 0.01$, N.S., not significant (followed by Tukey's multiple comparison test). Data are plotted as mean ± SEM. (*C*) Immunoblot of spinal cords of mSOD1 and control mice ($n = 3$ each) at 4 mo (onset) and 5 mo (end stage) was performed with antibodies to DAO, GAPDH, and human SOD1. Arrowheads are DAO with a size of 38 kDa. (*D–K*) Coronal sections of lumbar spinal cord (*D, F, G,* and *I*, ventral white matter; *E* and *H*, ventral gray matter, lamina IX) or sagittal ones of hindbrain (*J*) and brainstem reticular formation (*K*) of control and mSOD1 mice (5 mo) were costained with DAO enzyme histochemistry (green), immunofluorescent analysis of GFAP/Iba1/npNFH (red), and DAPI (blue). Squared areas in (*E* and *K*) are high magnification images. (*J*) Shown are joint images. Arrowheads indicate diminished DAO activity in brainstem reticular formation. (Scale bars: *D, E,* and *K,* 100 μm; *F–I,* 25 μm.)

in rodents (17). In contrast to the brainstem and spinal cord, DAO activities in the tissues rich in DAO, the cerebellum and kidney, were not altered in mSOD1 mice (Fig. S2*J*), suggesting that the DAO inactivation in the reticulospinal tract was not caused simply by expression of G93A-SOD1, but was affected by ALS pathophysiology.

Does the suppression of DAO activity affect D-serine levels? The net amount of D-serine is controlled in balance with synthesis and degradation. To evaluate each contribution, we measured the amount of D-/L-serine with a 2D-HPLC system, a highly selective and sensitive method (Fig. S4 and *Materials and Methods*) (18, 19). The D-serine level in the spinal cord is negatively correlated with the activity of DAO (Fig. 3*A*), suggesting that DAO crucially determines the D-serine level in this region. In the spinal cords of mSOD1 mice, D-serine was progressively increased (Fig. 3*B*) (10), whereas no significant alteration of D-serine was detected in those of control mice (Fig. 3*B*) or in the cortices between mSOD1 and control mice (Fig. S4 *F–H*). Although the L-serine level was also increased (Fig. 3*C*) consistent with a previous report (20), the D-/L-serine ratio was still higher in mSOD1 mice than in control mice (Fig. 3*D*). The elevated D-/L-ratio implies three possibilities: D-serine synthesis was increased, D-serine degradation was decreased, or both. To evaluate the contribution of synthesis and degradation to the D-serine increase, we generated mSOD1 mice lacking DAO activity (^{B6}DAO$^{-/-}$/mSOD1 mice). D-Serine content in the lumbar spinal cords of ^{B6}DAO$^{-/-}$/mSOD1 mice showed only a 12.9% increase compared with that in ^{B6}DAO$^{-/-}$ mice (Fig. 3*E*). If D-serine synthesis through L-serine increase and up-regulation of SRR (Fig. S5) (10) exceeded its degradation by DAO in mSOD1 mice, the level of D-serine in ^{B6}DAO$^{-/-}$/mSOD1 mice should have been distinctly higher than in ^{B6}DAO$^{-/-}$ mice. Therefore, this result demonstrates that DAO inactivation is a dominant contributing factor for D-serine increase in mSOD1 mice.

DAO catalyzes oxidative deamination of neutral and basic D-amino acids. Among D-amino acids, free D-serine and D-alanine are good intrinsic substrates of DAO in mammalian tissues. To study whether the inactivation of DAO affects D-amino acids other than D-serine, we measured D-serine and D-alanine as well as D-aspartate (18, 19, 21), as a control D-amino acid that is not metabolized by DAO. Genetic inactivation of DAO markedly increased the D-alanine level, whereas D-aspartate was not affected at all (Fig. S6*A*). In contrast to D-serine, the levels of D-alanine and D-aspartate in the spinal cord of mSOD1 mice did not differ significantly from control (Fig. S6 *A–C*). We speculate that the D-alanine level was not increased in mSOD1 mice because it is a better substrate of DAO than D-serine (22): D-serine was not fully degraded in heterozygotes (^{B6}DAO$^{+/-}$) with half the DAO activity of control mice (^{B6}DAO$^{+/+}$) (Fig. 2*B* and Fig. S6*A*), whereas D-alanine in ^{B6}DAO$^{+/-}$ mice was kept as low as that in ^{B6}DAO$^{+/+}$ mice (Fig. S6*A*). Therefore, DAO inactivation increased solely D-serine because DAO activity was half, but not fully, inactivated in mSOD1 mice (Fig. 2*B*).

Is D-serine also increased in other neurodegenerative diseases? We examined mouse models for ALS/frontotemporal lobar degeneration (FTLD), sporadic Parkinson's disease, and familial Alzheimer's disease: A315T-TDP-43 transgenic mice (mTDP-43 mice), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, and Tg2576 mice, respectively. D-Serine was specifically increased in mSOD1 mice, but not in other principal animal models for neurodegenerative diseases (Fig. S7*A*). Although the mTDP-43 mice exhibit a motoneuronal phenotype as do the mSOD1 mice (23), DAO activity in mTDP-43 mice was almost identical to that in control mice (Fig. S7 *B* and *C*). Reactive astrocytes, also observed in mTDP-43 mice (23), did not show DAO activity, but a portion of quiescent astrocytes were supposed to retain the normal level of DAO activity in spinal cords of mTDP-43 mice (Fig. S7*D*). These

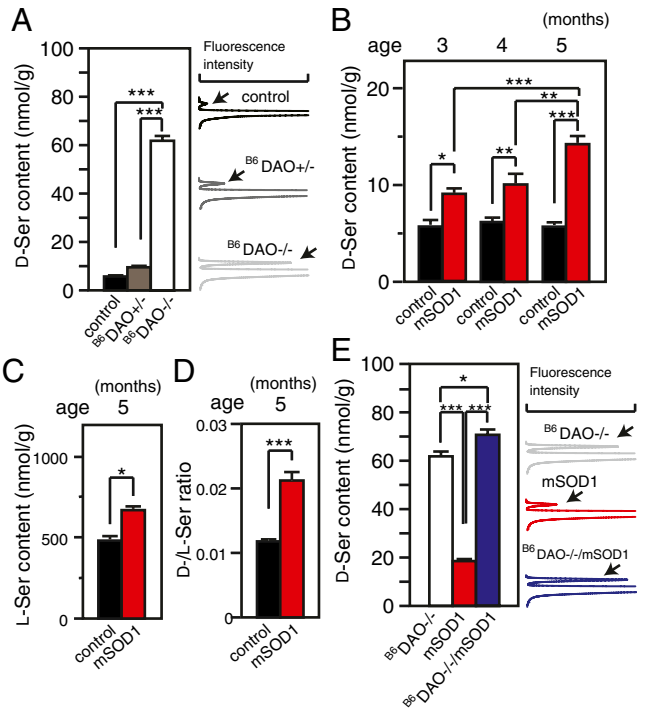

Fig. 3. DAO dominantly contributes to D-serine increase in the spinal cord. (*A* and *E*) D-Serine levels in lumbar spinal cords of control (5 mo; *A*), ^{B6}DAO$^{+/-}$ (5 mo; *A*), ^{B6}DAO$^{-/-}$ (5 mo; *A* and *E*), mSOD1 (end stage; *E*), and ^{B6}DAO$^{-/-}$/mSOD1 mice (end stage; *E*) (n = 3 each) were analyzed with 2D-HPLC. A typical chromatogram of D-serine (arrows) in each group is shown on the right. $P < 0.0001$ (one-way ANOVA). $^{*}P < 0.05$, $^{***}P < 0.001$ (followed by Tukey's multiple comparison test). (*B–D*) Amounts of D-/L-serine (*B* and *C*) and their ratio (*D*) in lumbar spinal cords of control and mSOD1 mice were quantified with 2D-HPLC at 3 mo (preonset stage around day 80, mSOD1, n = 6; control, n = 5), 4 mo (onset stage around day 120, mSOD1, n = 7; control, n = 7) and 5 mo (end stage around day 150, mSOD1, n = 7; control, n = 5) of age. $P < 0.0001$ (one-way ANOVA). Data are plotted as the mean ± SEM.

results suggested that the pathological significance of DAO was different between models of ALS and ALS/FTLD.

What decreased DAO protein level in mSOD1 mice? The mRNA level of DAO was suppressed even at the preonset stage of mSOD1 mice and continued to decrease over the course of the disease (Fig. 4*A*). Because DAO activity was suppressed in reactive astrocytes (Fig. 2 *D, E,* and *K*), we speculated that some extracellular soluble factor, such as proinflammatory cytokines or glutamate (24), influenced DAO expression in mSOD1 mice. Incubation of primary cultured glia with spinal cord lysates of the end-staged mSOD1 mouse reduced mRNA expression of DAO in glia by 34% compared with that of the control mouse (Fig. 4*B*). Among specific inhibitors for principal intracellular signaling pathways [PD98059: for MAPK/extracellular signal-regulated kinase (MEK) 1, SP600125: for c-Jun N-terminal kinase, SB203580: for p38, wortmannin: for phosphatidylinositol-3 kinase, AG490: for Janus kinase 2, caffeic acid phenethyl ester (CAPE): for NF-κB; ref. 25], only PD98059 recovered the down-regulation of DAO caused by the mSOD1 lysate (Fig. 4*B*). Indeed, the mSOD1-lysate treatment activated extracellular signal-regulated kinase (ERK) 1/2 and cAMP responsive element binding protein (CREB) for a longer duration than did the control lysate treatment (Fig. 4*C*). In contrast, CAPE rather decreased DAO expression (Fig. 4*B*), and proinflammatory stimuli such as TNF-α or lipopolysaccharide elevated it without affecting ERK activation (Fig. S8 *A* and *B*), suggesting proinflammatory factors potentially counteract repression of DAO caused by ERK activation. However, ERK-mediated down-regulation of DAO caused by mSOD1 lysate was far more potent than TNF-α–induced up-regulation (Fig. S8*C*). In support of these findings, astrocytes with phosphorylated ERK1/2 were increased and did not show DAO activity in the spinal ventral horn of the mSOD1 mouse (Fig. 4 *D* and *E*). Furthermore, incubation of primary cultured glia with PD98059 lowered the D-/L-serine ratio in the cultured medium (Fig. 4*F*), suggesting that the MEK/ERK signal is pivotal in DAO down-regulation.

In agreement with these results and the fact that stimulation of NMDAR activates the MAPK pathway, glutamate reduced DAO expression in primary cultured glia (Fig. S8*D*). MK-801, a selective noncompetitive NMDAR antagonist, suppressed the decrease of DAO expression in primary cultured glia treated with the mSOD1 lysate, whereas 6,7-dinitroquinoxaline-2,3-dione (DNQX) (a non-NMDAR antagonist) or a peptide antagonist (WP9QY) for TNF receptor did not affect it (Fig. 4*G*). The glial expressions of NMDARs were observed mainly in fibrous astrocytes in ventral white matter of control mice, whereas NMDARs were expressed also in reactive astrocytes in mSOD1 mice (Fig. S8 *E–H*), supporting our view that glutamatergic action mediated by the NMDAR-MEK/ERK pathway seemed to be responsible for astrocytic DAO down-regulation in mSOD1 mice.

Discussion

Our data highlight the pathologic relevance of D-serine increase derived from reduced DAO activity in ALS. Using inbred mice lacking DAO activity, we provide evidence that loss of DAO activity triggers motoneuron degeneration. Moreover, using biochemical assays and sensitive quantitative methods to detect DAO activity and D-amino acids in the tissues, we have shown that (*i*) DAO activity is drastically suppressed in reactive astrocytes of the reticulospinal tract of mSOD1 mice, (*ii*) decreased degradation due to DAO inactivation contributes dominantly to the increase of D-serine, and (*iii*) the reduction of DAO activity is caused by progressive repression of DAO gene expression that mediates the NMDAR/ERK pathway.

In this study, we show that D-serine homeostasis is disrupted in the reticulospinal tract in mSOD1 mice (Fig. S9 *A* and *B*). The reticulospinal tract is a major descending motor pathway in mammals and is assumed to be responsible for coordinated gross movements primarily of proximal muscles, whereas the corticospinal tract mediates fine movements, particularly of the hand (26). The corticospinal tract is especially well developed in primates (26, 27), and its sclerosis observed in the lateral columns of the spinal cord is a major characteristic of ALS. In nonprimate mammals including rodents, however, there are no direct cortico-motoneuronal connections. The reticulospinal tract, located in the anterior columns, primarily relays cortical input to spinal motoneurons (17, 28, 29). Therefore, D-serine degradation by DAO in the reticulospinal tract is speculated to have a physiological significance in controlling motoneuronal excitability in rodents. Although, in humans, it is unknown whether DAO also exists in the reticulospinal tract or the other descending motor pathways, considering the R199W mutation in DAO is associated with familial ALS, D-serine homeostasis by DAO might also be physiologically important in the excitability of motoneurons in humans.

Because of the hindbrain-shifted distribution of DAO activity, physiological D-serine level in the spinal cord is strictly maintained at ≈1/50th of that in the forebrain (30). In the mSOD1 mice, reduced DAO activity in the reactive astrocytes caused by repressions of its mRNA and protein expression contributes dominantly to the progressive D-serine increase and raises D-serine level by nearly three times at the end stage. Previously, we showed that proinflammatory factors also contribute to D-serine increase through SRR in activated microglia (10). Although such

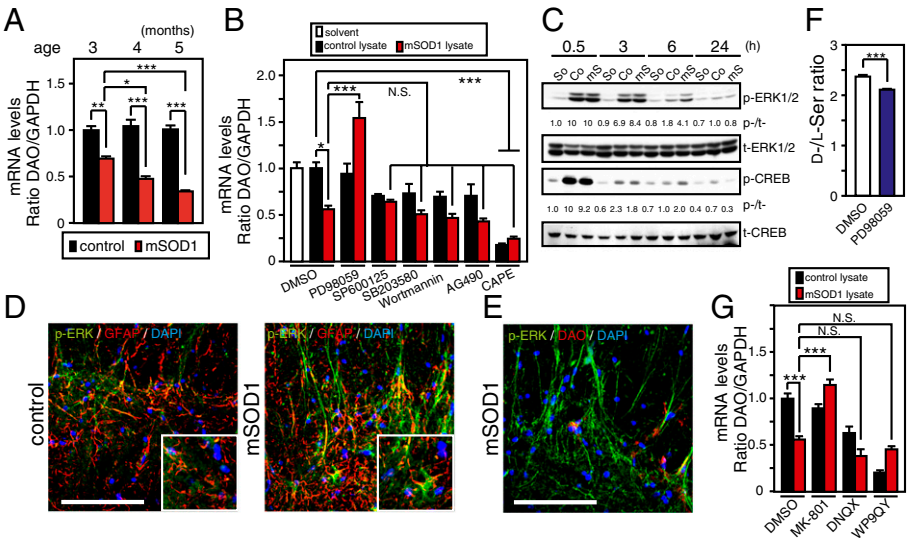

Fig. 4. DAO expression is down-regulated through the NMDAR/ERK pathway in mSOD1 mice. (*A*) mRNA expressions of DAO standardized with GAPDH in spinal cords of control and mSOD1 mice at 3 (preonset), 4 (onset), and 5 (end stage) mo of age were analyzed in real-time PCR. $P < 0.0001$ (one-way ANOVA), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (followed by Tukey's multiple comparison test). (*B* and *G*) Primary cultured glia were treated with spinal cord lysate of control or mSOD1 mice or solvent with/without various inhibitors or dimethyl sulfoxide (DMSO). The mRNA expression of DAO standardized with GAPDH in the cells was analyzed in real-time PCR ($n = 3$). $P < 0.0001$ (one-way ANOVA), $^{*}P < 0.05$, $^{***}P < 0.001$, N.S., not significant (followed by Tukey's multiple comparison test). (*C*) Primary cultured glia were stimulated with spinal cord lysate of control (Co) or mSOD1 mice (mS) or solvent (So) for the indicated time, and Western blotting was performed with phospho (p)-ERK, total (t)-ERK, p-CREB, and t-CREB antibodies. Values indicate relative amounts of p-ERK1/2 or p-CREB standardized with t-ERK1/2 or t-CREB, respectively. (*D* and *E*) Lumbar spinal cords of control and mSOD1 mice (5 mo) were costained with immunofluorescent analysis of phospho-ERK1/2 (green) and GFAP (red, *D*) or DAO enzyme histochemistry (red, *E*). Nuclei were stained with DAPI (blue). Shown regions are lamina IX. Squared areas in *D* are high magnification images. (*F*) Primary cultured glia were incubated with D-serine in the presence of PD98059 for 5 d ($n = 6$). Levels of D-/L-serine in the cultured media were determined by using 2D-HPLC. Data are plotted as the mean ± SEM.

proinflammatory stimuli induce compensatory DAO expression in astrocytes (Fig. S8*A*), the potent activation of ERK1/2 diminishes the action and rather results in reduction of DAO expression (Fig. S8*C*). In ALS, excessive glutamate remains in the synaptic cleft due to loss of a glutamate transporter (EAAT2) (31–33) and is thought to activate ERK1/2 in reactive astrocytes through NMDARs but not AMPARs (Fig. 4*G* and Fig. S9*C*). The notion that activity of NMDARs affects DAO expression is supported by an in vivo study that systemic administration of MK-801 significantly up-regulates mRNA expression of DAO in the rat hindbrain (34). Although research into astrocytic NMDARs is still controversial, some findings support the involvement of astrocytic NMDARs in rodent and human astrocytic cell signaling (35, 36). Whether the NMDARs observed in reactive astrocytes in mSOD1 mice (Fig. S8*G*) are functional and have pathological significance awaits future studies, but our findings shed light on the aberrant DAO regulation in mSOD1 mice.

DAO inactivation in mice results in pathological reflex (Fig. 1*A*), spinal motoneuron degeneration (Fig. 1 *B–D*), and abnormal locomotor activity (14). Although more detailed studies are required for full characterization of the motor phenotype in the ^{B6}DAO$^{-/-}$ mice, the ^{B6}DAO$^{-/-}$ mice may bear a pathophysiological resemblance to the classical adult onset familial ALS associated with R199W-DAO, in which DAO activity is at trace level. Given that reduced DAO activity in the motoneuron input is crucial for motoneuron degeneration, undegraded D-amino acids might reasonably be associated with the mechanism of the degeneration. Moderate loss of DAO activity increases exclusively D-serine in the spinal cord, whereas its complete loss concomitantly affects D-alanine (Fig. S6*A*). Both D-serine and D-alanine, albeit a lower affinity ligand than D-serine, bind to a coagonist site in NR1 subunit of NMDARs (37); therefore, the increase of both D-amino acids in the motoneuron input leads to elevating the occupancy of the coagonist site. Because coagonist binding is not only essential for NMDAR activity, but also increases the receptor's affinity for glutamate (38) and decreases its desensitization (39), the high occupancy of the site is assumed to elevate motoneuron excitability. Thus, D-serine homeostasis in the spinal cord is considered to be physiologically important in motoneuronal excitability, and our findings give rise to the view that inactivity of DAO is pathologically relevant to the vulnerability of motoneurons to excitotoxicity in ALS.

The reduced DAO activity observed commonly in mSOD1 mice and familial ALS with R199W-DAO may not represent the whole ALS pathology because DAO activity is not suppressed in mTDP-43 mice. However, together with the findings in our earlier study that D-serine is increased in both sporadic and familial ALS with A4T-SOD1 (10), it is likely that a group of sporadic ALS and familial ALS with DAO and SOD1 mutations share the pathology of increased D-serine.

In conclusion, this study provides a unique understanding of the role of DAO and D-serine in motoneuron physiology as well as in ALS pathophysiology as a putative enhancer of motoneuron excitability. Our data also stress the potential use of regulators of DAO activity or D-serine antagonists as a therapeutic strategy in ALS.

Materials and Methods

Materials. For information about the materials used in this study, please refer to *SI Materials and Methods*.

Animals. All experiments on animals were carried out in accordance with institutional guidelines. The study protocol was approved by the Animal Experiment Committee of KEIO University. Animals used in this study are detailed in *SI Materials and Methods*.

Histological Analysis. Histological analysis is detailed in *SI Materials and Methods*.

Motoneuron Counts and Diameter Measurement. Please refer to *SI Materials and Methods* for details regarding motoneuron counts and diameter measurement.

Enzyme Assay of DAO. DAO activity was determined as reported by Watanabe et al. (40) with some modifications. Please see *SI Materials and Methods* for additional details.

2D-HPLC. Amino acids in tissues were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) (Tokyo Kasei), subjected to HPLC (NANOSPACE SI-2 series; Shiseido), separated into each amino acid by a reversed-phase column, and further separated into enantiomers by an enantioselective column. The fluorescence intensity was detected at 530 nm with excitation at 470 nm. Please see *SI Materials and Methods* for additional details.

RNA Isolation and Real-Time Quantitative PCR. RNA isolation and real-time quantitative PCR are detailed in *SI Materials and Methods*.

Cloning, Mutagenesis, and Transfection. See *SI Materials and Methods* for additional details.

Primary Culture of Glia. Primary cultured glia were prepared from cerebellum of E16 mouse embryos and treated with various inhibitors or spinal cord lysate or both. Please refer to *SI Materials and Methods* for additional details.

Western Blot Analysis. Western blot analysis is detailed in *SI Materials and Methods*.

Statistical Analysis. All values in the text and figures of this study indicate means ± SEM. Statistical analyses for the experiments were performed with two-tailed Student's *t* test or one-way ANOVA followed by Tukey's multiple comparison test, in which $P < 0.05$ was assessed as significant. All analyses were performed by using Prism 5 (GraphPad Software).

ACKNOWLEDGMENTS. We thank K. Yamashita, N. Suzuki, A. Gotoh, and S. Hayashi for invaluable assistance with animal work; D. Wylie for expert opinion on the manuscript; and T. Yoshida-Nishimoto and M. Yamamoto for indispensable support. This work was supported by Grant-in-Aid for Scientific Research (A) (to S.A.), Grant-in-Aid for Young Scientists (B) (to J.S.), the Nakabayashi Trust for ALS Research (to J.S.), and Takeda Science Foundation (to J.S.). We appreciate Shiseido Co. Ltd. (Tokyo, Japan) for technical support.

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