

The role of D-amino acids in amyotrophic lateral sclerosis pathogenesis: a review

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Abstract A potential role for D-amino acids in motor neuron disease/amyotrophic lateral sclerosis (ALS) is emerging. D-Serine, which is an activator/co-agonist at the N-methyl-D-aspartate glutamate receptor subtype, is elevated both in spinal cord from sporadic cases of ALS and in an animal model of ALS. Furthermore, we have shown that a mutation in D-amino acid oxidase (DAO), an enzyme strongly localized to spinal cord motor neurons and brain stem motor nuclei, is associated with familial ALS. DAO plays an important role in regulating levels of D-serine, and its function is impaired by the presence of this mutation and this may contribute to the pathogenic process in ALS. In sporadic ALS cases, elevated D-serine may arise from induction of serine racemase, its synthetic enzyme, caused by cell stress and inflammatory processes thought to contribute to disease progression. Both these abnormalities in D-serine metabolism lead to an increase in synaptic D-serine which may contribute to disease pathogenesis.

Keywords Amyotrophic lateral sclerosis (ALS) · Motor neuron disease · Familial ALS · D-Amino acid oxidase (DAO) · Serine racemase (SR)

Introduction

A potential role for D-amino acids in ageing and neurodegenerative conditions such as Alzheimer's disease was first

proposed more than two decades ago (Fisher et al. 1991; D'Aniello et al. 1992). However, it is only recently that strong evidence has emerged to show that D-serine, an activator/co-agonist at the N-methyl-D-aspartate (NMDA) glutamate receptor subtype, not only plays an essential physiological role in long-term potentiation mediated at NMDA synapses (Henneberger et al. 2010) but is also elevated in a second chronic neurodegenerative condition, motor neuron disease/amyotrophic lateral sclerosis (ALS) (Sasabe et al. 2007). More recently, we have shown that a mutation in D-amino acid oxidase (DAO) is associated with familial ALS (Mitchell et al. 2010). DAO plays an important role in regulating levels of D-serine, and its function is impaired by the presence of this mutation and this may contribute to the pathogenic mechanism occurring in sporadic ALS. Similarly, D-serine has been shown to accumulate in ALS spinal cord, indicating that this abnormality may represent a common process occurring in disease. This has stimulated interest in understanding the physiology of D-amino acids in the central nervous system (CNS), their biosynthesis and degradation, and more importantly, the pathogenic mechanisms involved in neurodegeneration. These topics will be addressed in this review.

Amyotrophic lateral sclerosis disease mechanisms and the insight obtained from familial ALS

Amyotrophic lateral sclerosis is the most common adult-onset neuromuscular disease with a life-time risk of 1 in 500. ALS targets the motor system affecting motor neurons in the spinal cord, brain stem and cerebral cortex causing progressive muscle weakness, atrophy and paralysis, leading to severe disability. The condition usually

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progresses rapidly leading to death from respiratory failure within 5 years of onset. What causes this selective vulnerability is unknown but due to their high oxidative activity and strong excitatory drive, motor neurons are thought to be particularly susceptible to reactive oxygen species and impaired regulation of glutamate at spinal cord synapses, the latter being the foundation for the only non-symptomatic treatment of ALS with a drug that reduces glutamate release, riluzole. The most substantial advances in understanding ALS pathogenesis have come from studies of the familial form of the disease which accounts for 5 % of cases and is predominantly autosomal dominant in its mode of inheritance. Familial ALS (FALS) is clinically indistinguishable from sporadic cases of ALS (SALS). To date at least 15 FALS loci and causal genes have been identified, the most common being C9ORF72, a gene of unknown function, copper-zinc-dependent superoxide dismutase (SOD1) and TAR DNA binding protein 43 (TARDBP), which encodes TDP-43 (see review, Ferraiuolo et al. 2011). Cases with mutations in either C9ORF72 or TARDBP exhibit the same TDP-43-positive polyubiquitinated inclusions that are the hallmark of sporadic ALS but lacking in cases with SOD1 mutations. Other less common mutations present in FALS kindred occur in genes involved in the unfolded protein response [e.g. vesicle-associated membrane protein/synaptobrevin-associated membrane protein B gene (VAPB)], the removal of ubiquitinated proteins by the proteasomal system and autophagy [e.g. valosin containing protein (VCP) and ubiquilin-2] further demonstrating that the accumulation of misfolded proteins in ALS is emerging as a key event in ALS (Chen and de Belleroche 2012).

A DAO mutation is associated with FALS

Following a genome screen of an extensive ALS kindred with six affected individuals in three generations, which lacked mutations in any of the known genes causing FALS, we identified a novel FALS locus in a 9.2-cM region on chromosome 12q22-23. Subsequent candidate gene screening identified a coding mutation (R199W) in DAO, a 39.4-kDa protein consisting of 347 amino acids, which was transmitted with disease (Fig. 1a). The age at onset in the index case was 40 years and the duration of illness was 21 months, which is typical of the disease progression seen in familial cases with classical ALS. A similar rapid disease progression was seen in other affected family members, the average age of death for six affected individuals being 46.6 years (range 42–56 years).

DAO (EC.1.4.3.3) is a peroxisomal flavin adenine dinucleotide (FAD)-dependent oxidase, which catalyses

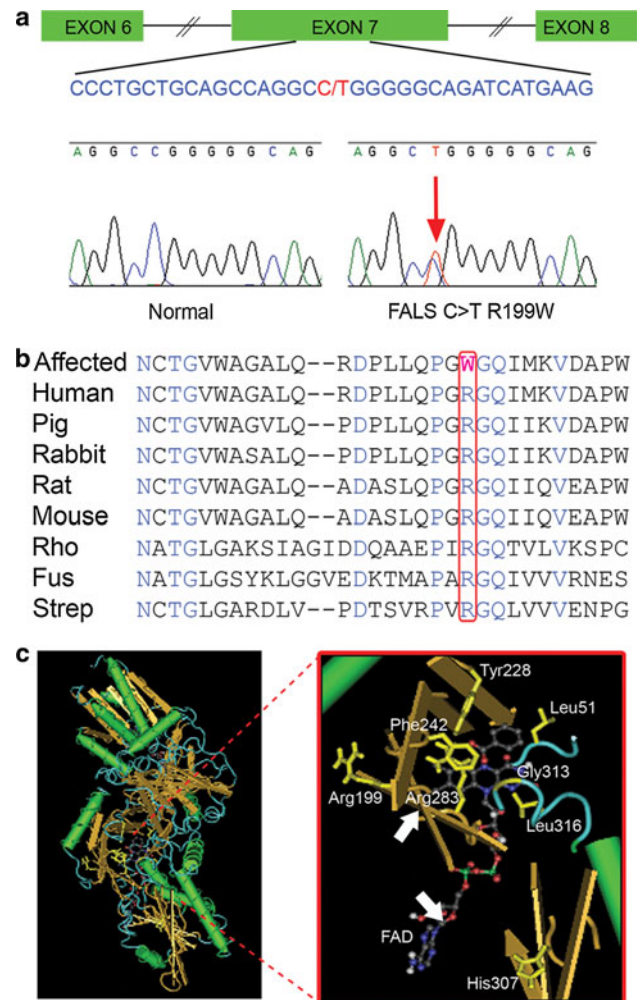


Fig. 1 Detection of a R199W mutation in DAO and a protein model. **a** Graphical representation of DAO exons 6–8 showing an expansion of part of exon 7. The R199W point mutation is indicated in red together with the corresponding “normal” and “mutated” sequence electrograph. **b** DAO amino acid conservation of the mutated region among eight species. The position of Arg199 (boxed) is indicated in red and variant amino acids in black. Conserved residues are shown in blue. Rho, *Rhodotorula gracilis*; Fus, *Fusarium Solanii*; Strep, *Streptomyces Avermitilis*. **c** Ribbon representation of porcine DAO homodimer (PDB:1AA8, MMDB:5815) and an expanded view of the DAO active site showing the position of Arg199 and FAD (arrows) in relation to key residues including Tyr228, His307, Leu51, Phe242, Arg283, Gly313, and Leu316. Data taken from Mitchell et al. (2010) (colour figure online)

the oxidative deamination of D-alanine, D-serine and D-proline to their corresponding keto-acids. Arg199 is highly conserved across species, being found in mammals, yeast, fungi and bacteria (Fig. 1b) and lies close to the FAD and substrate binding sites (Fig. 1c). DAO is strongly localized to peroxisomes (Fig. 2a) as demonstrated following transient transfection of COS-7 cells using GFP-tagged wild-type or R199W DAO and transduced with a

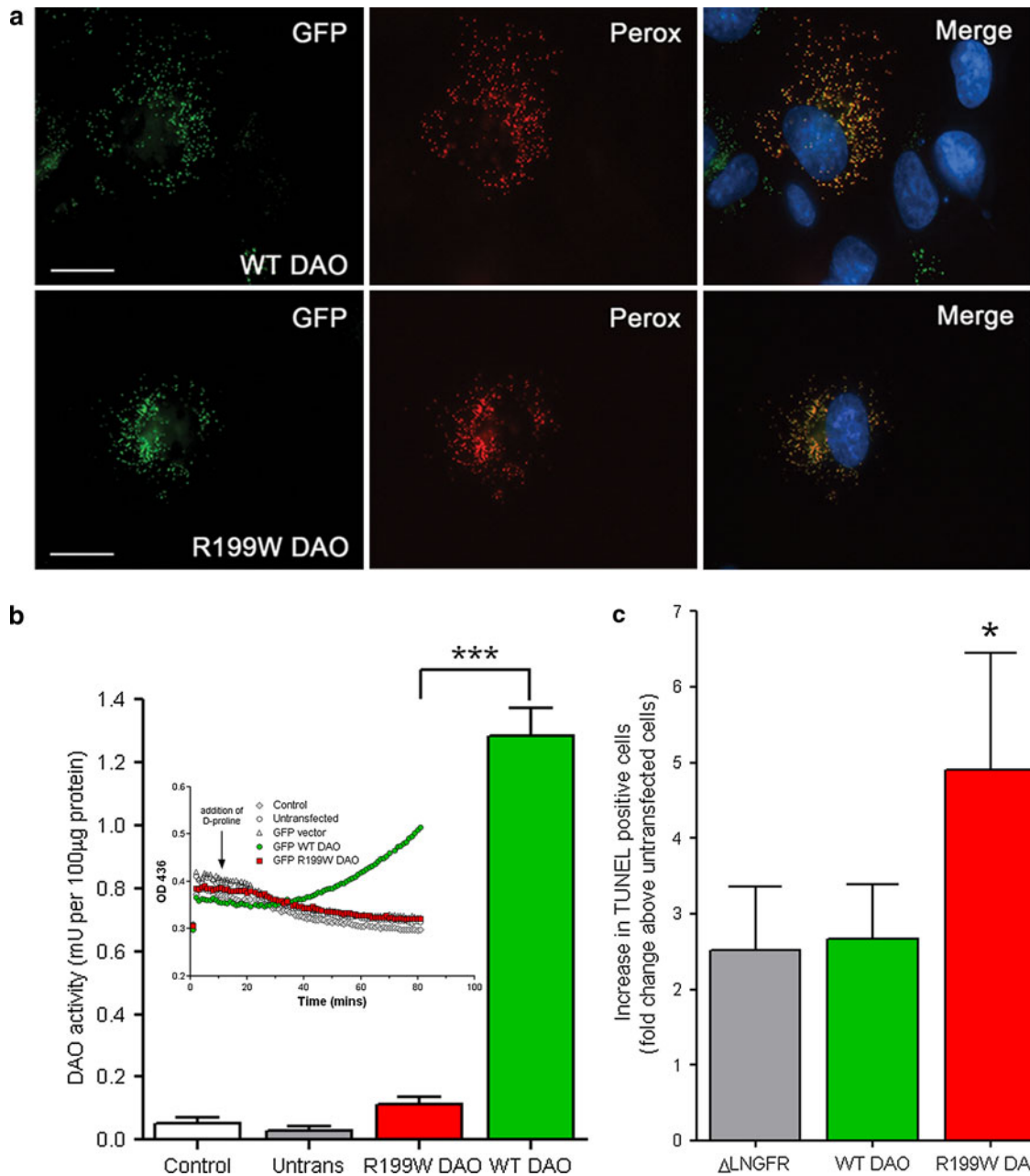


Fig. 2 Localization and functional characterization of WT and R199W DAO. **a** Localization of GFP-tagged wild-type (WT) and R199W DAO in peroxisomes. COS-7 cells were transiently transfected with GFP-tagged DAO and transduced with peroxisomal organelle lights. GFP-tagged WT and R199W DAO (GFP), peroxisomal (Peroxisome marker, Perox) GFP organelle lights and merged (Merge) images with DAPI nuclear staining. Scale bars 20 µm. **b** Effect of mutation on enzyme activity determined using untagged DAO constructs in COS-7 cells. One-way ANOVA $P < 0.0001$ with Bonferroni post hoc test indicated $***P < 0.001$. Values are mean \pm SEM, $n = 6$. Inset

shows a representative experiment using GFP-tagged constructs. **c** Effect of R199W DAO in lentiviral-transduced primary rat motor neurons. Motor neurons were transduced with the following lentiviruses at DIV1: WT DAO, R199W DAO or a negative control, Δ LNGFR and TUNEL-positive cells quantified at DIV3 (fold change over TUNEL-positive cells in untransfected cells). One-way ANOVA $P = 0.043$ with Bonferroni post hoc test value indicated compared to Δ LNGFR and WT DAO, $*P < 0.05$. Values are mean \pm SEM, $n = 4$. Data taken from Mitchell et al. (2010)

peroxisomal signal peptide. Enzyme activity is virtually abolished by the presence of R199W when transfected into COS-7 cells (Fig. 2b) and furthermore, DAO enzyme

activity in spinal cord from a case expressing the mutation was substantially reduced compared to control or sporadic ALS cases (Mitchell et al. 2010).

Effect of FALS-associated DAO mutation on motor neuron function

The presence of the FALS-associated mutation in DAO, R199W DAO, has a marked effect on cell viability. Using primary rat motor neuron cultures transduced with lentiviral vectors expressing wild-type and R199W DAO, a significant increase in the number of TUNEL-positive cells is seen in motor neurons expressing R199W DAO compared to cells expressing either the vector or wild-type DAO (Fig. 2c). A similar effect is also seen when untransduced motor neurons are co-cultured with primary astrocytes transduced with lentiviral vectors expressing wild-type DAO, R199W DAO or vector, where there is a significant increase in the number of TUNEL-positive motor neurons detected in co-cultures with astrocytes infected with R199W-DAO compared to wild-type DAO. This finding highlights the importance of astrocytes in mediating the toxic effects of the R199W-DAO mutation on the motor neuron, which remain to be established. However, one potential mediator of these effects, which is induced and released from astrocytes, is D-serine (Wolosker et al. 1999). Indeed, the effect of glial-derived D-serine on the postsynaptic NMDA receptor is known to be critical to the induction of long-term potentiation in hippocampus (Henneberger et al. (2010).

Further analysis of the effect of this mutation using the motor neuron cell line, NSC-34, demonstrated that cells expressing R199W DAO showed a variety of abnormal features including atrophy and loss of projections, compared to cells expressing wild-type DAO. A prominent feature of R199W DAO expressing cells was the presence of ubiquitinated protein aggregates (0.5 μm or greater in diameter), which were increased in abundance threefold compared to untransfected cells and cells expressing either the GFP-vector or wild-type DAO (Fig. 3a, b). The number of atypical shrunken atrophic cells lacking projections was also increased in R199W DAO compared to wild-type and control cells (Fig. 3b).

In order to investigate the effect of R199W DAO in cells exposed to ER stress, aggregate formation was quantified in the presence of the ER stress inducer, tunicamycin. The number of aggregate containing cells expressing R199W DAO increased to over 40 % in the presence of tunicamycin (Fig. 3b) indicating that the effects of R199W DAO on large aggregate formation were potentiated by ER stress.

Similar findings to those obtained with R199W DAO have been demonstrated for another DAO mutation that lacks DAO enzyme activity and was originally detected in a mouse line (ddY/DAO⁻) and later shown to be due a natural point mutation in DAO (G181R which is equivalent to human G183R). This mouse line shows abnormal locomotor activity (Almond et al. 2006) and has

subsequently been backcrossed with C57BL/6J mice and maintained as homozygotes (DAO^{-/-}) in order to study the effect of the mutation on motor phenotype in more detail (Sasabe et al. 2012). These mice developed an abnormal limb reflex in which hind-limbs are retracted towards the body when lifted up, rather than showing a normal extension of the hind-limbs. This was accompanied by a significant loss of motor neurons in lumbar spinal cord (24 %) and the presence of ubiquitin-positive aggregates, evident at 15 months.

DAO is enriched in brain stem nuclei and spinal cord

In human brain, DAO is present in pyramidal cells of cerebral cortex, hippocampal neurons (CA1–4) and in both glial and neuronal cells in cerebellum (Verrall et al. 2007). A similar distribution is seen in the rat brain (Moreno et al. 1999), which further highlights the more intense labelling seen in hindbrain and brain stem nuclei. This is clearly demonstrated in rat cerebellum, where DAO is intensely labelled in Bergmann glial cells and Purkinje cells (Fig. 4a) and in the gigantocellular reticular and facial nuclei of the brainstem (Fig. 4b and expanded in Fig. 4c). In human spinal cord, motor neurons are strongly stained for DAO and less intense staining is seen throughout the spinal cord grey matter (Mitchell et al. 2010). A similar pattern of distribution is found in mouse spinal cord (Fig. 5a) which is punctate and indicative of peroxisomal localization.

D-Serine is abundant in rat forebrain including cortex and hippocampus with moderate labelling in midbrain and hindbrain (Williams et al. 2006). D-Serine is expressed in the grey matter of adult mouse lumbar spinal cord including large motor neurons, although some staining is also evident in smaller surrounding cells that are assumed to be glia (Fig. 5b). Transporters have been demonstrated on neuronal and glial cells that allow both uptake and release of D-serine from the synapse, such as the high affinity D- and L-serine transporters, alanine-serine-cysteine transporter, Asc-1 and ASCT2. Asc-1 shows a prominent neuronal localization (Matsuo et al. 2004) and ASCT2 is found in both neurons and glia (see review by Verrall et al. 2010). In addition, D-serine has been shown to be released from neurons in response to depolarisation, which is mediated through a calcium-independent mechanism (Rosenberg et al. 2010).

The primary source and synthesis of D-serine is enzymatic racemization of L-serine by serine racemase (SR). Although initially observed in glial cells, SR is abundantly expressed in mouse forebrain where it is predominantly localized in pyramidal neurons of the cerebral cortex and hippocampal CA1 region (Miya et al. 2008). Weaker

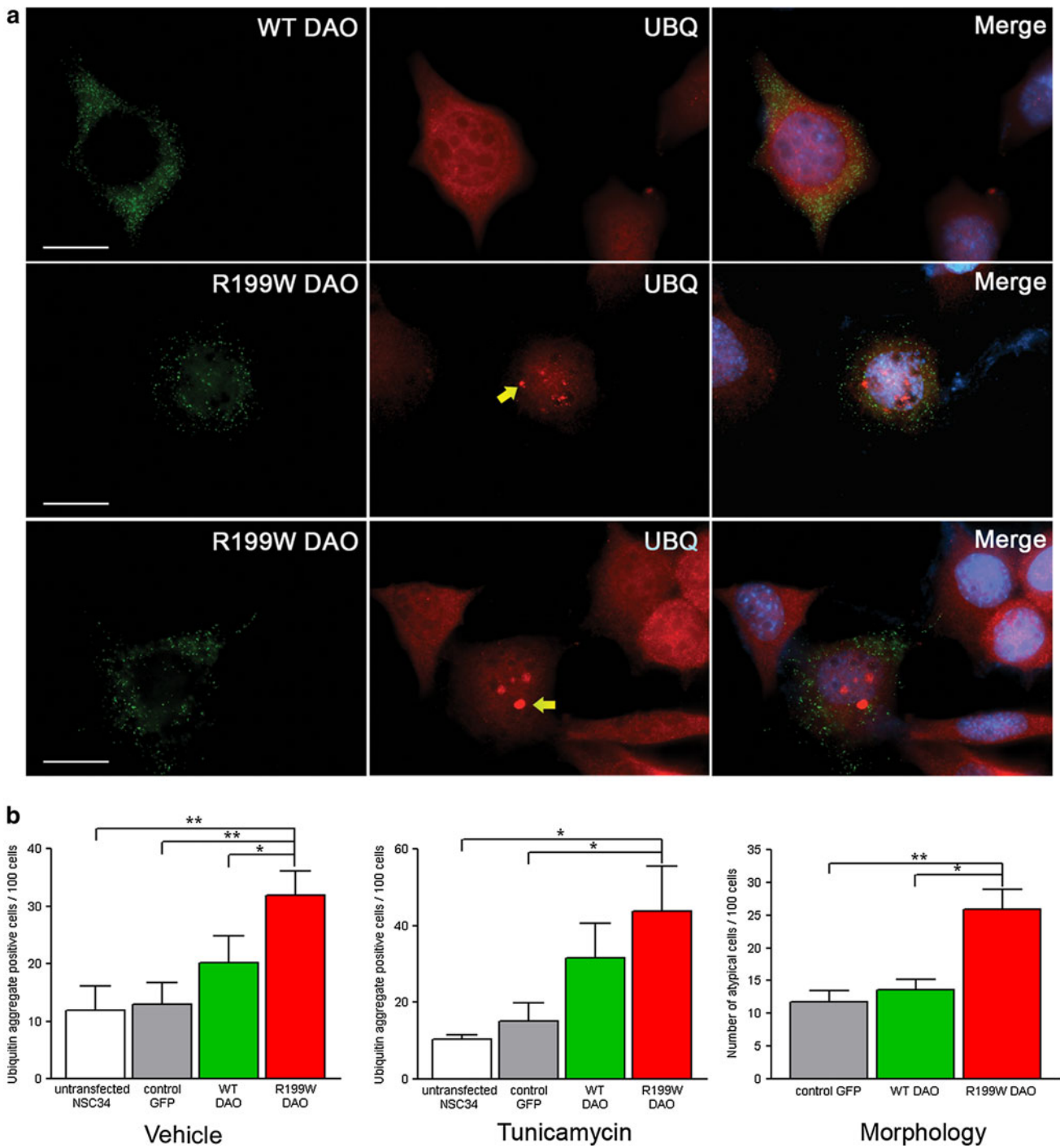
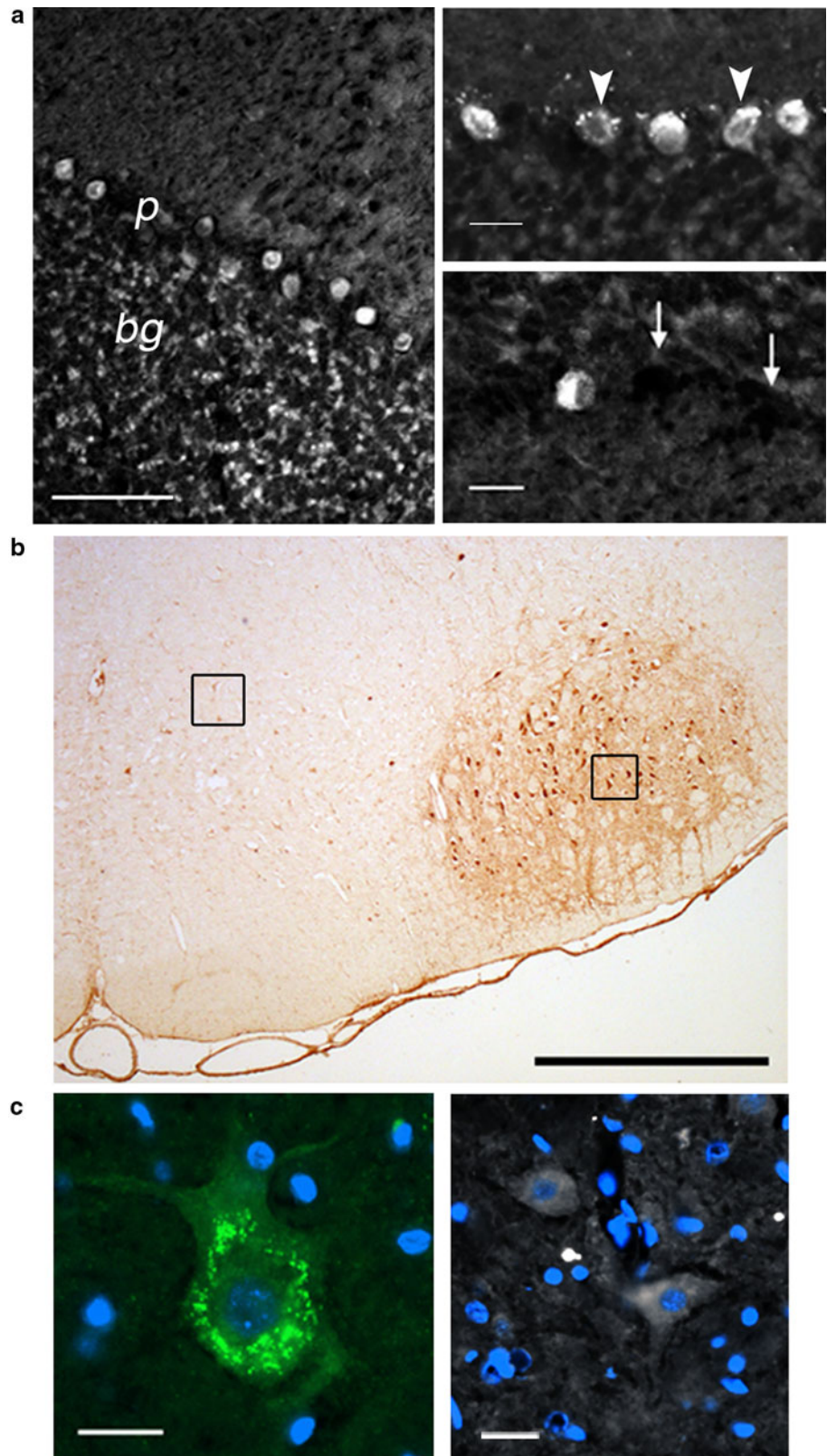


Fig. 3 Effect of R199W DAO on ubiquitin aggregates. **a** NSC-34 cells expressed GFP-tagged WT or R199W DAO 72 h after transfection. Ubiquitin (UBQ) staining with aggregates in GFP-positive cells is indicated by *arrows* and merged (*Merge*) images with DAPI nuclear staining. *Scale bars* 20 μ m. **b** Effect of R199W DAO on number of ubiquitin aggregates and NSC-34 cell morphology. Cells containing ubiquitin aggregates ($\geq 0.5 \mu$ m in diameter) were quantified after treatment with DMSO vehicle control ($n = 4$)

(*Vehicle*) and 1 μ g/mL tunicamycin ($n = 3$) (*Tunicamycin*), giving one-way ANOVA values of $P = 0.0057$ and $P = 0.044$, respectively. Cells with atypical morphology (rounded, shrunken, and with fewer processes) were quantified (*Morphology*). One-way ANOVA $P = 0.0128$ ($n = 4$) with Bonferroni post hoc test indicated, $*P < 0.05$; $**P < 0.01$. Values are mean \pm SEM. Data taken from Mitchell et al. (2010)

Fig. 4 Localization of DAO in cerebellum and brain stem.

a Rat cerebellum. DAO immunoreactivity in Purkinje cells (*p*) and Bergmann glial cells (*bg*). Note that while most Purkinje cells clearly show peroxisomal localization (*arrowheads*), some cells show no immunoreactivity (*arrows*). Scale bars: *left* 100 μm , *right* 25 μm . **b** Rat brain stem at level of facial nucleus. Anti-choline acetyl transferase staining in brain stem to identify regions (*boxes*) used for DAO immunoreactivity in (**c**): gigantocellular reticular neurons (*left, green*) and facial nerve nuclei (*right*). DAPI nuclear counterstain is shown in *blue*. Scale bars: **b** 1 mm, **c** 25 μm . Data taken from Mitchell et al. (2010) (colour figure online)



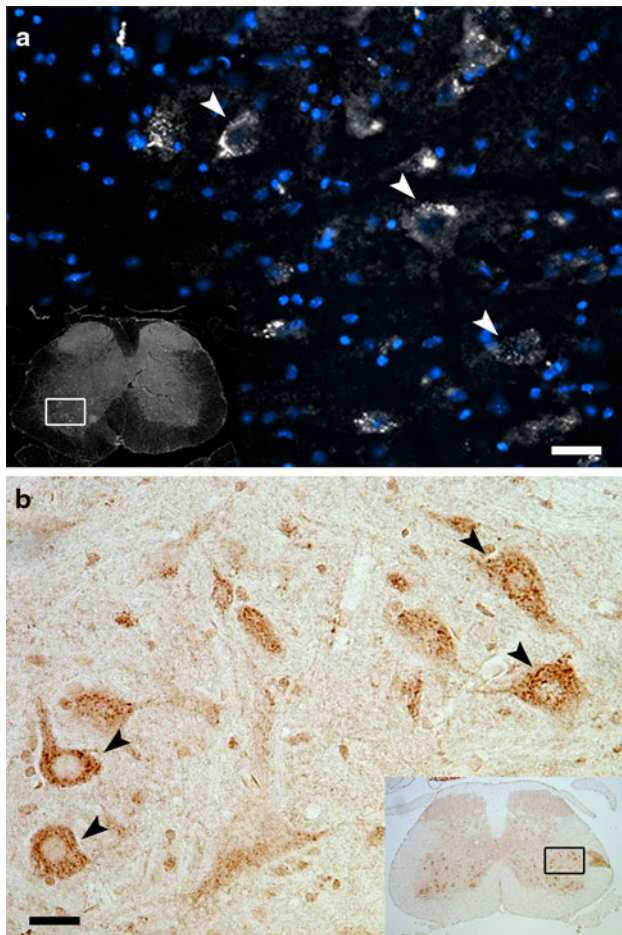


Fig. 5 Localization of DAO and D-serine in mouse lumbar spinal cord. *Insets* show regions (*boxes*) of spinal cord that are enlarged to show **a** DAO (taken from Mitchell et al. 2010) and **b** D-serine immunoreactivity which is widespread in grey matter including motor neurons (*arrowheads* in the enlarged images). DAPI nuclear counterstain is shown in *blue*. *Scale bar* 50 μm (colour figure online)

staining is observed in cerebellum and brainstem. In mouse ventral spinal cord, SR and DAO do not co-localize (Sasabe et al. 2012).

The role of DAO in G93A SOD1-mediated pathogenesis

Using DAO enzyme histochemistry, DAO activity was found to be markedly decreased in lumbar spinal cord and in brain stem reticular formation of G93A SOD1 mice, a widely used model of ALS expressing the human FALS mutation, and specifically this decrease was seen in quiescent astrocytes that were double stained for the astrocytic marker GFAP and DAO enzyme activity (Sasabe et al. 2012). These results strongly point to the selective involvement of DAO in the reticulospinal tract, which plays a major role in regulation of motor neuron excitability in the rodent compared to primates.

The role of D-serine in ALS pathogenesis: a putative model

Increasing evidence supports the role of D-serine in ALS, D-serine levels were found to be elevated in ALS spinal cord using a small sample of cases (Sasabe et al. 2007, 2012) and levels were also elevated during disease progression in the G93A SOD1 mouse model of ALS (Sasabe et al. 2012). This is consistent with the finding that a mutation in DAO, that abolishes enzyme activity, is associated with FALS in an extended kindred (Mitchell et al. 2010) and furthermore, DAO enzyme activity is also markedly reduced in the mouse model of ALS. Both of these effects on DAO activity would reduce D-serine metabolism, which could theoretically contribute to the elevated levels of D-serine seen during motor neuron degeneration. In addition, this disease-associated mutation in DAO, R199W, also impairs cell viability, promotes the formation of ubiquitinated aggregates and increases apoptosis in neuronal cells. Increased apoptosis was also observed when motor neurons were co-cultured on transduced astrocytes expressing R199W indicating that motor neuron cell death induced by this mutation could be mediated by both cell autonomous and non-cell autonomous processes. This is of particular relevance to DAO which is known to be expressed both in neuronal and glial cells and hence both cell types may contribute to the initiation of cell death in FALS cases expressing the R199W mutation. At this stage, it is not possible to know whether the neurotoxic effect of R199W DAO results from the accumulation of the aberrant protein or whether the impaired enzyme activity causes a build-up of synaptic D-serine, a co-agonist at the glycine site of the NMDA glutamate receptor enhancing glutamate transmission. Elevated levels of D-serine in the CNS caused by lack of DAO in the spontaneous mutant G181R DAO, ddY/DAO^- show profound modulation of NMDA transmission as demonstrated by enhanced hippocampal long-term potentiation, improved performance in spatial learning tests (Maekawa et al. 2005) and enhanced NMDA-receptor-mediated excitatory post-synaptic currents in spinal cord dorsal horn neurons (Wake et al. 2001).

D-Serine is elevated in a small number of sporadic cases of ALS and this is associated with an increase in the number of SR expressing cells (Sasabe et al. 2007). In G93A SOD1 mice, SR induction has been demonstrated to occur in activated microglia, particularly in the later stages of disease (Sasabe et al. 2007), which is likely to be triggered by the accumulation of mutant SOD1 protein and inflammatory mediators, known to induce SR through the c-Jun-N-terminal kinase pathway (Wu et al. 2004). The fact that SR is induced by a number of factors including inflammatory mediators, oxidative stress and amyloid beta (Wu and Berger 2004) and the more recent finding that

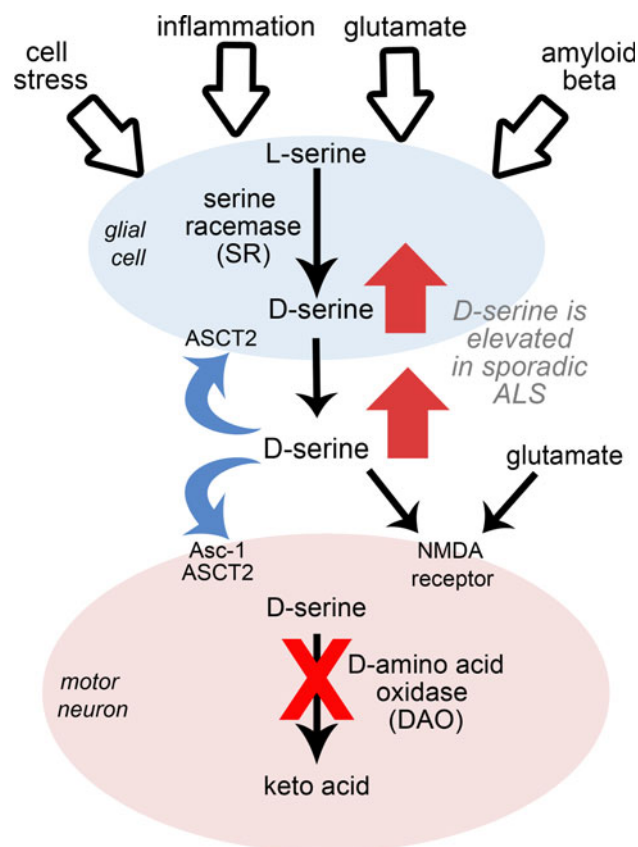


Fig. 6 D-Serine mediated ALS pathogenesis-proposed model. Cellular D-serine is elevated in ALS which can arise either from an increase in serine racemase (SR) caused by cell stress, inflammation, glutamate or amyloid beta, or result from a decrease in D-amino acid oxidase (DAO). In glial cells, increased SR activity leads to elevation of D-serine which is released into the synapse. D-Serine and glutamate activate NMDA receptors on motor neurons potentially leading to excitotoxicity and eventually cell death. D-Serine can be transported back into cells through alanine–serine–cysteine transporters (Asc-1, ASCT2) located on both glial cells and motor neurons

NMDA and amyloid beta toxicity are attenuated in serine racemase knock-out mice (Inoue et al. 2008) suggest that D-serine-mediated toxicity may contribute to other neurodegenerative conditions where these factors are implicated, such as Alzheimer's disease and Parkinson's disease.

Conclusion

There is increasing evidence that synaptic D-serine may play a major role in motor neuron degeneration in ALS pathogenesis. The build-up of cellular D-serine may be due to either reduced DAO activity in the spinal cord and the reticular system, or SR induction caused by cell stress, inflammation, glutamate or amyloid beta (see model in Fig. 6). D-Serine may be released into the synapse from astrocytes, microglia or neurons at different stages of

disease and could potentially facilitate NMDA receptor-mediated excitotoxicity. The role of different cell types in disease progression is also clearly seen in the SOD1 mouse model of ALS where onset and progression of disease are determined by motor neurons and microglia, respectively. The involvement of multiple cells may explain the paradoxical effects of SR knockout in the G93A SOD1 mouse which accelerates onset of disease but increases survival (Thompson et al. 2012). Whether these effects are directly linked to protein aggregation and apoptosis needs to be firmly established. Future studies are in progress to establish the role of R199W DAO in the pathogenesis of ALS using in vivo models and to determine the potential of DAO as a molecular target in other age-related neurodegenerative diseases.

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