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Experimental approaches for elucidating co-agonist regulation of NMDA receptor in motor neurons: Therapeutic implications for amyotrophic lateral sclerosis (ALS)

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neuromuscular disease characterised by selective loss of motor neurons leading to fatal paralysis. Although most cases are sporadic, approximately 10% of cases are familial and the identification of mutations in these kindred has greatly accelerated our understanding of disease mechanisms. To date, the causal genes in over 70% of these families have been identified. Recently, we reported a mutation (R199W) in the enzyme that degrades D-serine, D-amino acid oxidase (DAO) and co-segregates with disease in familial ALS. Moreover, D-serine and DAO are abundant in human spinal cord and severely depleted in ALS. Using cell culture models, we have defined the effects of R199W-DAO, and shown that it activates autophagy, leads to the formation of ubiquitinated protein aggregates and promotes apoptosis, all of which processes are attenuated by a D-serine/glycine site antagonist of the N-methyl D-aspartate receptor (NMDAR). These findings suggest that the toxic effects of R199W-DAO are at least in part mediated via the NMDAR involving the D-serine/glycine site and that an excitotoxic mechanism may contribute to disease pathogenesis.

1. The role of co-agonists at the NMDA receptor in mammalian forebrain

The major excitatory transmitter in the central nervous system is glutamate, whose powerful actions in fast conduction and synaptic plasticity are principally mediated through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and N-methyl-D-aspartate (NMDA) receptors respectively. Whilst AMPA and kainate receptors are activated solely by glutamate, NMDA receptors are co-incidence detectors, that require the binding of both glutamate and a co-agonist (D-serine or glycine) to GluN2 and GluN1 subunits respectively, combined with depolarisation to release the magnesium block present under resting conditions. NMDA receptors are heterotetrameric complexes usually composed of two GluN1 subunits and two GluN2A-D subunits, with the GluN1-GluN2A-GluN2B complex being the predominant receptor at hippocampal synapses [1].

There have been substantial advances in the characterisation of the diverse properties of different NMDA receptor subunits and the elucidation of their pivotal involvement in synaptic plasticity. One aspect that has only recently been fully recognised is the important role of the two co-agonists that function at the NMDA receptor, which are essential for operation of the NMDA receptor and differentially regulate receptor function. It is particularly in brain regions such as hippocampus, cerebral cortex and amygdala, that models of synaptic plasticity such as long term potentiation (LTP) have helped to establish the different effects of D-serine and glycine at NMDA receptors [2,3,4,5]. One example is the selective affinity shown by heterotetrameric NMDA receptors containing GluN1 and GluN2A subunits, which have a greater affinity for D-serine compared to the NMDA receptor containing GluN1 and GluN2B subunits [6]. On the other hand NMDA receptors containing GluN1 and GluN2B subunits have a much greater affinity for glycine compared to GluN2A containing receptors [6]. Both GluN2A and GluN2B containing receptors are found at the synapse and elegant work has been carried out to show the association between GluN2B containing receptors and activated calcium and calmodulin-dependent kinase II which is translocated to the synaptic membrane during LTP [7,8]. Current evidence from studies in the forebrain indicate that D-serine is the major co-agonist involved both in NMDA receptor mediated LTP and excitotoxicity [2–5]. NMDAR-mediated currents (EPSCs) are diminished by D-amino acid oxidase (DAO)

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which metabolises D-serine, whereas NMDAR-mediated currents induced by afferent stimulation are diminished by glycine oxidase (GO) and not by DAO.

Co-agonist specificity at NMDA receptors in other CNS regions such spinal cord are less well characterised.

2. The potential importance of D-serine in spinal cord is indicated from the identification of a mutation in D-amino acid oxidase (*DAO*) in amyotrophic lateral sclerosis/motor neuron disease (ALS)

The significance of DAO in spinal cord was only recently highlighted when our group identified a pathogenic mutation in the *DAO* gene that was associated with ALS [9].

Levels of DAO are highly enriched in brain stem, spinal cord and cerebellum in contrast to cerebral cortex [9,10,11,12,13], whereas serine racemase is most abundant in forebrain compared to brain stem [14,15]. These high concentrations of DAO in spinal cord suggest that this region may have a selective vulnerability that requires a tight regulation of D-serine levels carried out in part by DAO though oxidative deamination of D-serine.

ALS is a devastating condition, causing muscle atrophy, paralysis, impaired speech and swallowing which rapidly progresses to death from respiratory failure in 3–5 years. The characteristic pathological features of the disease are loss of motor neurons in spinal cord, brain stem and motor cortex and sclerosis of the descending cortico-spinal tract from motor cortex (lateral crossed and ventral uncrossed). At the cellular level, the hall mark of disease is the presence of ubiquitinated protein inclusions positive for TDP-43 [16].

The most important and momentous advances in ALS research have come from the identification of mutations in genes that are responsible for the familial form of the disease which accounts for 5–10% of all cases. To date 18 ALS genes have been identified, the most prevalent FALS gene is *C9orf72* [17,18] followed by *SOD1*, *TARDBP* and *FUS* [19,20,21] and these account for ~70% of all cases in our Imperial College cohort of 208 families, which is consistent with other UK, US and European cohorts. Outstanding FALS genes are currently emerging from exome capture/resequencing approaches. The functional effects of these genes provide valuable clues about disease mechanisms which fit into 3 main categories, RNA binding and processing, protein quality control and excitotoxicity. The DNA/RNA binding proteins are TDP-43 and FUS encoded by *TARDBP* and *FUS*, respectively. These are nuclear proteins but they mislocalise to the cytoplasm in disease and accumulate in protein inclusions. *C9orf72* is a gene containing an intronic hexanucleotide repeat of less than 30 units in controls which expands substantially to 500–2400 repeat units in ALS cases. Hexanucleotide expansions in *C9orf72* account for 38% of FALS cases in UK, Europe and USA, but are more abundant in Scandinavia and rare in Asia. These expansions are also causal in ALS cases with fronto-temporal lobar degeneration (FTLD), familial FTLD and sporadic FTLD. Despite the different sites of pathology and phenotype, common cellular features are present. Most surprising, is the relatively high prevalence of hexanucleotide expansions in *C9orf72* found in sporadic ALS cases (8%) indicating low penetrance of disease.

The second mechanism affected in ALS is proteostasis, mutations being found in genes functional in the unfolded protein response, ER stress, protein degradation pathways, carried out by the proteasome and autophagy, VAPB, p62, optineurin, ubiquilin2 [22]. Interestingly, VAPB is also significantly reduced in sporadic cases [23]. In cell culture, VAPB mutations cause endoplasmic reticulum (ER) fragmentation, protein aggregates and apoptotic cell death [24].

Now we come to D-amino acids and the third mechanism, excitotoxicty. This finding arose from linkage analysis carried out in an extended FALS kindred which showed significant association with disease for markers on chromosome 12. Subsequent sequencing of genes in this locus identified a pathogenic mutation in D-amino acid oxidase (DAO) that segregated with disease. The mutation occurred in codon 199 and caused a non-synonymous change from arginine to tryptophan (R199W DAO) [9]. Furthermore, this arginine residue is highly conserved across species from Man to Fungi and Bacteria and the presence of this mutation severely impairs the kinetic characteristics of this enzyme. As DAO is known to catalyse the oxidative deamination of D-serine, an essential co-agonist at the NMDA subtype of glutamate receptor, enhanced levels of D-serine could potentiate NMDA responses and could implicate excitotoxity in disease pathogenesis.

DAO is known to be localised to specific regions of the CNS, showing a strong enrichment in motor nuclei of the brain stem, such as the facial nerve nucleus. We carried out an extensive study of the distribution of DAO, D-serine and serine racemase (SR), the enzyme responsible for D-serine synthesis from L-serine, in human spinal cord from control cases compared to ALS cases [25]. In spinal cord, there is a prominent expression of DAO, D-serine and SR in large motor neurons present in the anterior horn cell region of spinal cord in control cases (Fig. 1). In addition, DAO immunoreactivity is widely present in neuronal fibres and small glial-like cells fibres present in the grey matter. In ALS cases, there is a substantial depletion of the motor neuron pool as shown by loss of motor neuron markers such as choline acetyl transferase (ChAT) and vesicle associated membrane protein associated protein B (VAPB) which is accompanied by ~90% loss of DAO, SR and D-serine staining [25]. This further substantiates the localisation of D-serine in motor neurons together with enzymes involved in their synthesis and metabolism and their depletion in ALS.

3. Functional effects of DAO

3.1. *In vivo studies of DAO deficient models: determination of D-serine*

Extensive work carried out by Dr Konno's group has characterised a naturally occurring mutation in DAO (G181R) found in mouse that reduces DAO activity and has proved to be extremely valuable in characterising behavioural effects of this mutation [26]. Using ddY/DAO$^-$ mice backcrosed with C57BL/6J, a homozygous mouse line (DAO$^{-/-}$) was obtained which exhibited marked effects on motor phenotype. At 8 months, abnormal reflexes characterised by retraction of hind limbs, similar to that found in the ^{G93A}SOD mouse model of ALS, were seen accompanied by a significant reduction of 24% in motor neuron number [27]. By 15 months, increased axonal degeneration with muscle atrophy was detected [27].

Furthermore, this group has also explored the role of D-serine and DAO in the ^{G93A}SOD1 mouse model of ALS and shown that DAO activity in lumbar spinal cord is decreased by 42%, which is accompanied by reduced DAO protein expression. The magnitude of this decrease was comparable to that found in DAO$^{(+/-)}$ heterozygotes. The effect of reduced DAO enzyme activity on D-serine levels was assayed using a highly selective and sensitive 2D-HPLC method and showed an elevation in D-serine levels which increased with disease progression [27]. This confirmed earlier findings from this group, where D-serine was measured using a chemiluminescence assay, in which hydrogen peroxide generated in the presence of DAO and peroxidise was detected using luminol [28]. In the latter study, Sasabe et al. [28] also presented preliminary results from immunohistochemical analysis, that D-serine was elevated in sporadic (two out three studied) and one familial ALS case (A4V SOD1).