

1 **A(a)LS: Ammonia-induced amyotrophic lateral sclerosis**

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## 1 **Abbreviations**

2 ALS, amyotrophic lateral sclerosis; ALS-PDC, Amyotrophic lateral sclerosis-parkinsonism dementia  
3 complex; AMPD3, AMP deaminase-3; AMPK, 5' AMP-activated protein kinase; APP, Amyloid  
4 precursor protein; ATP, Adenosine triphosphate; ATXN2, *Ataxin-2*;C9ORF72, chromosome 9 open  
5 reading frame 72; CaBPs, calcium binding proteins; CAMK, Ca<sup>2+</sup> /calmodulin-dependent protein kinase;  
6 CDK5, Cell division protein kinase 5; CHMP2B, charged multivesicular body protein 2B; COX-2,  
7 cyclooxygenase-2;CSF, cerebrospinal fluid; DCTN1, dynactin subunit 1; DYNC1H1, dynein cytoplasmic  
8 1 heavy chain 1; ER, endoplasmic reticulum; ERBB4, receptor tyrosine-protein kinase erbB-4; ERK,  
9 extracellular-signal-regulated kinase; FBP2, fructose-1,6-bisphosphatase 2; Fig4, factor-Induced gene 4;  
10 FUS, fused in Sarcoma; GABA, gamma-aminobutyric acid; GH, Growth hormone; GLT, glutamate  
11 transporter; GLAST, glutamate-aspartate transporter; GLUT4, Glucose transporter 4; Hex,  
12 Hexosaminidase; HFE, IGF, Insulin-like growth factor; IL-1 $\beta$ , Interleukin-1 $\beta$ ; IL-6, Interleukin 6; IL-  
13 8,Interleukin 8; INF- $\gamma$ , Interferon gamma; KCC2, potassium-chloride cotransporter 2; LMN, lower motor  
14 neurons; MAPK, Mitogen-activated protein kinase, MMP-9, metalloproteinase-9; mtDNA, mitochondrial  
15 DNA; NF- $\kappa$ B, Nuclear factor- $\kappa$ B; NOX, NADPH oxidase; NFT, neurofibrillary tangles; NMDA, N-  
16 methyl-D-aspartate; MCT, monocarboxylate transporter; PGE2,prostaglandin E2; PKC, Protein kinase C;  
17 PP2A, Protein phosphatase 2A; QUIN, Quinolinic acid; RABA7A, Ras-related protein Rab-7a; ROS,  
18 reactive oxygen species; SMA, spinal muscular atrophy; SMN1, survival of motor neuron 1; SOD1,  
19 Superoxide dismutase-1; SQSTM1,sequestosome 1; TARDBP, tAR DNA-binding protein; Tbc1d, TBC1  
20 domain family member 1; TNF- $\alpha$ , Tumor necrosis factor alpha; TLR4, toll-like receptor 4; UCP,  
21 uncoupling protein; UMN, upper motor neurons; UPR, unfolded protein response; UPS, ubiquitin  
22 proteasome system; VCP, Valosin-containing protein; VEGF, vascular endothelial growth factor;Xbp1,  
23 X-box binding protein 1.

24

1 **Abstract**

2 Amyotrophic lateral sclerosis (ALS) is a dreadful, devastating and incurable motor neuron  
3 disease. Aetiologically, it is a multigenic, multifactorial and multiorgan disease. Despite intense  
4 research, ALS pathology remains unexplained. After the literature review, this paper posits a  
5 new integrative explanation. This framework proposes that ammonia neurotoxicity is a main  
6 player in ALS pathogenesis. According to this explanation, a combination of impaired ammonia  
7 removal— mainly because of impaired hepatic urea cycle dysfunction—and increased  
8 ammoniogenesis— mainly because of impaired glycolytic metabolism in fast twitch skeletal  
9 muscle—causes chronic hyperammonia in ALS. In the absence of neuroprotective calcium  
10 binding proteins (calbindin, calreticulin and parvalbumin), elevated ammonia—a neurotoxin—  
11 damages motor neurons. Ammonia-induced motor neuron damage occurs through multiple  
12 mechanisms such as macroautophagy-endolysosomal impairment, ER-stress, CDK5 activation,  
13 oxidative/nitrosative stress and neuroinflammation. Furthermore, the regional pattern of calcium  
14 binding proteins' loss, owing to either ER stress and/or impaired oxidative metabolism,  
15 determines clinical viabilities of ALS. Most importantly, this new framework can be generalised  
16 to explain other neurodegenerative disorders such as Huntington's disease.

17

## 1 **1. Introduction**

2 Amyotrophic lateral sclerosis (ALS) is the most feared, frequent, flummoxing and fatal motor neuron  
3 disease [1-3]. It is a biphasic disease which starts insidiously, later followed by relentless progression  
4 once symptomatic [4]. Although rare, it is a grim and demeaning illness: it slowly cripples and confines  
5 its victims in their own body, ultimately killing them by breathing failure within 3–5 years after onset [2,  
6 5]. No cure exists[1]. Ever since Charcot's description of ALS (1869), a classical view defines ALS as an  
7 adult-onset neurodegenerative disease of upper and lower motor neurons [6, 7]. However, ALS is  
8 clinically characterised by variability about the type and degree of motor neuron and non-motor neuron  
9 involvement[8].

10 ALS pathology involves an interaction of multiple genes and environmental factors. Indeed, ALS is  
11 mainly a polygenic disease (70%-90); and the heritable form (familial) ALS contributes to merely 30% of  
12 total ALS cases [3, 9]. Remarkably, mutant C9ORF72, TARDBP, FUS, and SOD1 genes account for  
13 70% of all familial ALS cases [10]. Evidence shows that environmental factors such as intense physical  
14 activity, cigarette smoking, viral infections, and the ingestion of non-protein amino acids (i.e.  $\beta$ -N-  
15 methylamino-L-alanine) play a role in ALS [5, 11, 12].

### 16 **ALS Aetiology: An enduring enigma**

17 Despite nearly 150 years of research, ALS remains an enigma [13]. Although, at the cellular, molecular  
18 and metabolic levels, a staggering and ever expanding list of pathogenic mechanisms have been linked to  
19 ALS [13, 14]. These include protein aggregation, mitochondrial dysfunction, oxidative/nitrosative stress,  
20 endoplasmic reticulum (ER) stress, axonal transport defects, glutamate excitotoxicity, impaired  
21 macroautophagy, impaired glycolysis, neuroinflammation, and glucose and fat metabolism impairments  
22 [13-19]. However, hitherto no hypothesis exist that effectively links all these mechanisms to a singular  
23 central cause [3]. Hence, despite steadily accumulating knowledge about ALS, a key question still lingers:  
24 what cause ALS?

## 1 **2. ALS: A multi organ disease**

2 Since ALS is manifestly a neurological disorder, researchers have long embraced an intuitive neurocentric  
3 view of ALS, assuming that intrinsic neuronal pathology causes ALS [7, 20]. Against this view, however,  
4 growing evidence suggests that ALS pathology extends well beyond neuronal cells and involves multiple  
5 organs [7, 14, 21]. Unsurprisingly, ALS is now deemed as a systems disease [20, 22]. Not only that,  
6 evidence increasingly shows that primary pathological events, inherited or acquired, within these organs  
7 may act as distal cause of ALS [14, 21, 22]. Such evidence is reviewed below.

### 8 **2.1 Role of Skeletal muscle**

9 ALS starts and spreads from skeletal muscle [14, 23]. Indeed, some early symptoms of ALS involve  
10 neuromuscular system: muscle atrophy, cachexia (wasting), weakness, and fasciculation (twitches). In  
11 fact, cachexia reduces survival of ALS patients [19]. Reinforcing such observations, data from animal  
12 models of ALS showed neuromuscular dysfunction precede motor neurons loss [14]. For instance, Frey et  
13 *al.* showed selective loss of fast-fatigable neuromuscular synapses of SOD1G93A mice by 6 weeks of  
14 age—2 month before symptomatic phase [24]. Cogently, a study showed that the expression of mutant  
15 gene (SOD1G93A) exclusively in skeletal muscle of transgenic mice caused cachexia, neuromuscular  
16 denervation, paresis, and motor neuron degeneration [25].

17 Skeletal muscle possesses mainly two types of muscle fibres: fast twitch and slow twitch [26]. Lately,  
18 evidence suggests that in ALS fast twitch muscle motor units are selectively damaged before over  
19 symptoms; whereas slow twitch motor units show damage after overt symptoms [27, 28]. For example, a  
20 set of studies showed a rapid motor unit loss during the presymptomatic phase (5 weeks of age) in fast—  
21 but not slow-twitch muscles of the SOD1 G93A mouse [27]. Accordingly, fast-twitch muscle appears to  
22 be more susceptible to damage in ALS patients [28]. Therefore, fast twitch muscle pathology appears to  
23 be the distal cause of ALS.

1 Together, those findings have led to the “dying-back” hypothesis[14].It holds that ALS is a distal  
2 axonopathy in which pathological changes first arise distally at the neuromuscular junction and progress  
3 backward toward the spinal cord cell body [14]. That said, however, recent and prior research mandates  
4 refinement of this hypothesis. Recently, experiments in the SOD1G93A mice showed independent and  
5 parallel degeneration of both upper and lower motor neurons at early stage, hinting at a common  
6 pathological mechanism [29]. Consistent with this, recent neuroimaging studies showed early stage  
7 involvement of UMN in ALS patients[30]. In fact, Gower (1886), Charcot’s contemporary, suggested  
8 simultaneous and independent degeneration of upper and lower motor neurons in ALS[31].Thus, a  
9 common but hitherto unidentified pathological factor emanating from skeletal muscle appears to damages  
10 both upper and lower motor neurons.

## 11 **2.2 Liver: An emerging locus of ALS**

12 Aside from skeletal muscle, mounting evidence suggests that liver dysfunction commonly occurs in ALS.  
13 Indeed, literature on the liver pathology in ALS has existed for over a half century [32, 33]. Earlier,  
14 researchers showed a range of liver abnormalities in ALS patients including the disturbance of  
15 unconjugated bilirubin metabolism, mitochondrial defects, and copper accumulation in hepatic lysosomes  
16 [32]. More recently, clinical studies suggest that hepatic steatosis (fatty liver degeneration) is a common  
17 and unique phenomenon in motor neuron diseases including ALS [22, 34, 35]. Nodera *et al.* found that  
18 hepatic steatosis was present in 76% of ALS patients [22]. In line with this, studies showed reduced  
19 growth hormone/Insulin-like growth factor-1 (GH/IGF-I) levels, which induce hepatic steatosis, in ALS  
20 [21, 36]. Moreover, research showed that ALS-associated environmental factors such as virus infection  
21 (i.e. retrovirus virus and HIV) and cigarette smoking cause hepatic steatosis [12, 37, 38]. Furthermore,  
22 viral hepatitis, which causes hepatic insufficiency and frequent fatty liver degeneration, has been linked to  
23 motor neuron disease [39-42]. Finally, Reye-like syndrome, associated with fatty liver degeneration, has  
24 been associated with spinal muscular atrophy (SMA), a lower motor neuron disease[35].

1 A number of genetic findings also support this notion. Iron dysregulation disorders such as HFE gene-  
2 related hemochromatosis and hyperferritinemia—which induces hepatic steatosis—frequently (30%)  
3 occurs in ALS [43, 44]. Additionally, mutant cholesterol and lipid pathways genes such as CREB, TDP-  
4 43 ATXN2, paraoxonase and CYP7A1, implicated in hepatic steatosis, have been linked to ALS [45-52].  
5 Moreover, an interaction between disturbances in hepatic mitochondrial function and ER homeostasis  
6 causes hepatic steatosis; and investigators discovered morphological changes in ER structure and  
7 mitochondria in the liver of ALS patients [32, 53]. These findings accord well with the fact that mutant  
8 ER-stress regulating genes such as XBP1, SigR1, VCP, TDP-43, FUS, SOD1, and VAPB have been  
9 linked to ALS [54, 55]. Furthermore, SMN gene, implicated in ALS and SMA, have been shown to  
10 regulate the development and function of liver[35].

11 Finally, hepatic steatosis is linked to the metabolic syndrome, characterised by hyperglycaemia,  
12 hyperglucagonemia, insulin resistance and altered serum triglycerides; and such findings have been  
13 reported in ALS [56-62]. In this regard, it is interesting to note that damage to fast twitch skeletal muscle,  
14 the main site of glucose disposal and the largest reservoir of glycogen in humans, leads to hepatic  
15 steatosis [63].

16 Notably, Li et *al.* showed exendin-4, which counteracts hepatic steatosis, ameliorated motor neuron  
17 degeneration partly by correcting this systemic metabolic alteration[64, 65]. This clearly suggests that,  
18 much like skeletal muscle, liver pathology is not merely an innocent bystander, but rather a premorbid  
19 condition, which plays an active role in ALS pathogenesis.

### 20 **3. Aims**

21 Thus, (i) identifying skeletal–muscle produced unknown pathological factor, (ii) unravelling its nexus and  
22 synergism with hepatic steatosis,(iii) understanding the mechanisms by which this pathology factor  
23 causes motor neuron damage, and (iv) revealing the cause(s) of clinical heterogeneities would fully untie  
24 the Gordian knot of ALS pathology—allowing the development of predictive and prognostic biomarkers

1 as well as potent drugs [3, 13]. Hence, by taking a systems view, this paper aims to fill these knowledge  
2 gaps. Moreover, by fusing these separate pieces together, this paper presents a full picture of ALS  
3 pathology.

#### 4 **4. Impaired glycolysis in fast twitch muscle: one of the pathological triggers of ALS**

5 Evidence suggests that defective energy deficit in skeletal muscle triggers ALS. Investigators found  
6 impaired skeletal muscle metabolism, characterised by low ATP levels and hypermetabolism, causes  
7 neuromuscular dysfunction in ALS mouse model [66, 67]. Conversely, metabolic interventions such as  
8 high-calorie diets and reducing hypermetabolism improved survival and alleviated symptoms in ALS.  
9 However, the functional link between skeletal muscle metabolic impairment and ALS remains nebulous.  
10 Instructively, since ALS begins from fast twitch muscle—which relies on anaerobic glycolysis for energy  
11 (i.e., ATP), this immediately suggests that impaired anaerobic glycolysis produces the unknown  
12 pathological trigger.

#### 13 **Impaired glycolysis in ALS**

14 Compelling evidence suggest that muscle glycolysis is impaired in ALS. Valosin-containing protein  
15 (VCP), a gene linked to ALS, causes defective muscle glycolysis and reduced ATP levels. Dupis et al.  
16 linked upregulation of mitochondrial uncoupling proteins UCP1 and UCP3—which suppresses glycolysis  
17 and causes hypermetabolism—to muscle denervation in ALS [68]. Bernardini et al. showed low  
18 expression of glycolysis genes such as FBP2 and enolase3 in the skeletal muscles of ALS patients [69].  
19 Brockington et al. uncovered down regulation of glycolytic enzyme lactate dehydrogenase 1 in the  
20 VEGF<sup>δ/δ</sup> mouse model of ALS [70]. Moreover, experiments showed that the gain-of-interaction of the  
21 SOD1 G93A mutant with cytosolic malate dehydrogenase induces glycolytic impairments. Dunckley et  
22 al. linked variants of FLJ10986, a protein linked to glycolysis, with the susceptibility of sporadic ALS  
23 [71]. Collectively, these findings clear show impaired glycolysis in skeletal muscle of ALS patients and  
24 mouse model.



## 1 **Impaired muscle glycogen and glucose homeostasis in ALS**

2 Notably, fast twitch skeletal muscle glycolysis depends on muscle glycogen storage and glucose  
3 transporter 4 (GLUT4)-mediated muscle glucose uptake [26, 72]. Accumulating evidence suggests  
4 defective muscular glycogen metabolism and impaired GLUT4-mediated muscular glucose uptake in  
5 ALS. Derave et *al.* discovered diminished muscle ATP and glycogen accumulations in SOD1 G93A mice  
6 [27]. Smittkamp et *al.* revealed impaired insulin-stimulated glucose uptake exclusively in fast-twitch  
7 skeletal muscle in middle-stage SOD1 G93A mice [73]. Accordingly, fast twitch skeletal muscle fibres of  
8 TDP-43 transgenic mice show defective insulin-induced GLUT4 translocation and glucose uptake.  
9 Moreover, in the mutant TDP-43-linked ALS mice, Perera et *al.* reported decreased AMPK, which  
10 mediates muscle contraction-induced glucose entry and glycogen synthesis [72, 74, 75]. Conversely,  
11 AMPK activator drugs (i.e. latrepirdin) delayed ALS in SOD1G93A mice [76]. Furthermore, muscle  
12 contraction facilitated glucose uptake involving Ca<sup>2+</sup>/calmodulin-dependent GLUT4 translocation  
13 appears to be defective in ALS. For example, investigators linked mutant neuregulin-ERBB4 gene,  
14 involved in calcium-induced glucose uptake during muscle contraction, to ALS [75, 77]. Thus, it is  
15 obvious that ALS involves impaired carbohydrate metabolism that support muscle glycolysis.

## 16 **ALS resistance of Extraocular muscles (EOMs): Role of glycolysis**

17 Finally, the metabolic characteristics of—ALS-resistant—extraocular muscles (EOMs) further  
18 consolidate this notion [78]. Two fundamental differences exist between EOMs and skeletal muscle  
19 metabolism [79]. First, compared to skeletal muscles, EOMs have high glycolysis capacity, evident by the  
20 overexpression of glycolytic enzymes (e.g. lactate dehydrogenase, enolase) [79]. Second, owing to their  
21 high vascularity, EOMs rely more on instantaneous glucose uptake— less on glycogen storage and  
22 GLUT4-mediated muscle glucose uptake [79]. All in all, these three sets of findings point that defective  
23 glycolysis causes ATP deficits in fast twitch skeletal muscle of ALS patients. Hence, the unknown  
24 pathological factor emanating from skeletal muscle appears to have direct connection with defective  
25 muscle glycolysis. How?

## 1 **5. Ammonia: the elusive pathological factor**

2 Notably, defective glycolysis, which reduces ATP levels, in fast twitch skeletal muscle activates catabolic  
3 reactions of adenine nucleotides (i.e. purine nucleotide cycle) and amino acids (fig 1) [80, 81].  
4 Intriguingly, such catabolic reactions produce ammonia—a neurotoxin 1000 times more toxic than ethanol  
5 at equimolar concentration [82]. Since ammonia is toxic, it is obligatorily removed—mainly through  
6 hepatic urea cycle which transforms ammonia into urea [83]. Notably, when the urea cycle is impaired, as  
7 it occurs in fatty liver disease, increased ammonia production from skeletal muscle or from dietary  
8 sources can cause chronic hyperammonia (>35-50  $\mu\text{M}$ ) and consequent neurodegeneration and motor  
9 impairments (fig 1-2) [33, 84-88]. Indeed, in many liver diseases—including fatty liver disease, which  
10 commonly occurs in ALS—because of impaired urea cycle-mediated ammonia removal, hyperammonia  
11 frequently leads to corticospinal hyperexcitability, myelopathy and spasticity— features strikingly  
12 reminiscent of neurophysiological phenotypes of ALS symptoms[89-93].

### 13 **5.1 Ammonia neurotoxicity: Hypothesis and evidence**

14 Together, these findings provide a compelling rationale for a new hypothesis. That ALS pathology  
15 involves not only skeletal muscle-induced increased ammonia production, because of impaired glycolysis,  
16 but also impaired ammonia removal, secondary to hepatic steatosis-induced faulty urea cycle, leading to  
17 chronic hyperammonia and consequent progressive motor neuron degeneration (figure 1-3).  
18 Astonishingly ammonia's role has seldom been directly investigated. Nonetheless, supporting this  
19 hypothesis, diverse lines of data, obtained from clinical and animal studies, show that hyperammonia,  
20 increased ammoniogenesis, and decreased ammoniaremoval occur in ALS.

21 A clinical study showed elevated ammonia level in motor neuron disease patients—with ammonia levels  
22 inversely correlated to disease duration[94]. These investigators also found a causal relationship between  
23 ammonia and ALS by noting that infusion of amino acids, which causes ammoniogenesis, aggravates  
24 ALS[94]. Moreover, dietary supplements of branched chain amino acids was one of the factors associated

1 with the early onset of ALS (45 years) in Italian soccer players[95]. Accordingly, other investigators  
2 reported accelerated skeletal muscle protein catabolism ALS [96]. This chimes with the fact that hepatic  
3 steatosis-induced glucagon secretion, which occurs in ALS, increases ammoniogenesis through protein  
4 degradation[97]. Consistent with this, as noted above, intense or prolonged physical exertion— an  
5 ammoniogenic activity—is an ALS risk factor [11, 98].

6 Beside a link between hepatic steatosis and a faulty urea cycle, other lines of clinical evidence further  
7 implicate the impaired urea cycle in ALS [46, 99]. For example, Ilzecka et *al.* showed decreased arginine  
8 levels, an amino acid required for liver urea cycle function, in ALS patients [100]. Additionally, research  
9 showed that metabolic acidosis, which impairs urea cycle, occurs in ALS [57, 101]. Impaired hepatic urea  
10 cycle activates glutamine synthetase, an alternative ammonia detoxification pathway; and researcher also  
11 found increase in glutamine synthetase expression in blood platelets of ALS patients [102, 103].

12 Animal models of ALS further cement this ammonia hypothesis. Investigators showed hyperammonia  
13 and impaired urea cycle in 50 day old SOD1G93A mice compared to wild type mice of the same age  
14 [104]. Moreover, these investigators showed increased glutamine, a precursor of ammonia, in  
15 SOD1G93A mice [104]. Additionally, in the mutant SOD 1 G86R mice, de Aguilar et al. showed early  
16 (3 months of age) muscle denervation along with increased AMP deaminase-3 (AMPD3)—an enzyme of  
17 purine nucleotide cycle involved in ammoniogenesis[105]. Furthermore, a set of studies showed increased  
18 arginine vasopressin release in the SOD1 mice, and independent research showed that arginine  
19 vasopressin causes muscle protein degradation and consequent ammoniogenesis[106, 107]. Conversely,  
20 research showed that ammonia-counteracting compounds such as phenylbutyrate, arginine, resveratrol  
21 and l-carnitine alleviated symptoms and enhanced survival in the ALS mouse model [93, 108-112].

22 Further supporting this hypothesis, experiments have shown that environmental neurotoxins implicated in  
23 ALS causes ammonia toxicity. Dietary intake of  $\beta$ -N-methylamino-L-alanine, a non-protein amino acid  
24 linked to Guam's ALS epidemic, causes liver damage and ammonia toxicity [113, 114]. Similarly, the

1 ingestion of *Lathyrus sativus* seeds, implicated in neurolathyrism (an upper motor neuron disease), causes  
2 liver dysfunction, urea cycle impairment, and chronic ammonia toxicity [115, 116]. Finally, animal  
3 studies showed that the pesticide pyrethroid, which causes an ALS-mimicking syndrome, leads to protein  
4 catabolism ammonia toxicity [117, 118].

5 Yet another line of evidence bolsters the ammonia neurotoxicity hypothesis. Interestingly, reports showed  
6 that motor neuron disease could be one of Huntington's disease (HD)'s presenting features [119, 120]. In  
7 fact, aside from genetic overlap with ALS, HD shares many pathophysiological characteristics with ALS:  
8 skeletal muscle atrophy, hepatic steatosis, hyperglycaemia, and adipose tissue dysfunction [121, 122].  
9 Tellingly, although often regarded as curious findings rather than telltale observation, impaired urea cycle  
10 as well as hyperammonia occurs in HD [121]. Strikingly, data from mouse models of HD showed that  
11 protein-restricted diets not only reduced hyperammonia but also prevented the motor deterioration [86].  
12 This suggests that ammonia could be a common culprit in range of neurodegenerative conditions,  
13 especially affecting motor system.

14 In addition to muscles and the liver, ammonia metabolism involves other organs, including the gut, the  
15 kidneys and the brain (fig 1) [85]. Hence, this hypothesis does not preclude a role of these organs.  
16 Although no evidence has yet emerged to implicate the gut and kidneys in ALS, some data at least  
17 suggests a role of cerebral ammoniogenesis in ALS. Studies showed increased deamination of  
18 catecholamine—which causes cerebral ammoniogenesis—in ALS, evident by the overactivity of  
19 catecholamine oxidising enzymes such as MAO-B and aldehyde oxidase [123-125]. Put together, these  
20 findings implicate ammonia neurotoxicity in ALS.

## 21 **6. Mechanisms of ammonia's neurotoxicity**

22 When hyperammonia occurs, ammonia enters into the brain, leading to neurotoxicity. Ammonia exerts  
23 pleiotropic neurotoxic effects by activating an array of cellular mechanisms—which are the proximal  
24 causes of ALS (fig 1-2). These mechanisms include: 1) alkalinisation-induced impairment of

1 macroautophagy-endolysosomal system, 2) Golgi impairment, 3) increased oxidative/nitrosative stress  
2 and MAPK up-regulation 4) neuronal hyperexcitability and 5) neuroinflammation[85, 126-130].

3 As described below, taken together, these five mechanisms not only explain several frequent cellular and  
4 molecular histopathological hallmarks of ALS but also neurophysiological features of ALS (fig 1 and 2).  
5 The cellular histological features, explained by ammonia's toxicity, include axon swelling, blood brain  
6 barrier breakdown and astrogliosis and microgliosis[131-133]. The molecular pathological features,  
7 explained by ammonia's toxicity, include formation of inclusion bodies such as Bunina bodies and Lewy  
8 bodies, gangliosides accumulation, glycogen aggregation, neurofilament derangement, Golgi  
9 fragmentation, and reduced glutamate transporters[57, 134-139]. Moreover, ammonia toxicity explains a  
10 key neurophysiological feature of ALS: neuronal hyperexcitability [17]. Finally and most importantly,  
11 ammonia toxicity explains why ALS is mainly a motor neuron disease.

### 12 **6.1. Alkalisiation-induced impaired macroautophagy-endolysosomal system**

13 Ammonia-induced alkalisiation impairs the macroautophagy-endolysosomal system—one of the main  
14 cellular garbage disposal systems. This occurs at least in two ways. First, ammonia, a weak base,  
15 preferentially accumulates in lysosomes because of their low acidity (pH~4.5) [128]. Consequently, intra-  
16 lysosomal alkalisiation and lysosomal enzyme leakages occur, impairing the lysosomal hydrolysis of  
17 proteins, lipids and carbohydrates (fig 2). Second, ammonia alkalisies acidic membranous compartments  
18 of axon terminals, jamming membrane microtubules and thereby blocking the anterograde-to-retrograde  
19 transport of endosomes [140]. Consequently, impaired fusion of endocytic compartments with lysosomes  
20 occurs, causing defective autophagy of endocytosed material (fig 2) [85, 140, 141]. As a result, toxic  
21 accumulation of protein aggregates, glycolipids, and carbohydrates occurs[85]. In turn, these toxic by-  
22 products activate the apoptosis programme, causing cell death [142].

23 Ammonia's alkalisiation-induced toxicity is especially relevant to ALS because macroautophagy-  
24 endolysosomal dysfunction causes motor neuron degeneration[143]. Indeed, mutant genes of this pathway

1 such as SOD1, FIG4, CHMP2B, SQSTM1, DCTN1, DYNC1H1, and RAB7A have been linked to  
2 ALS[143]. Consistent with this interpretation, research showed impaired dynein-dependent retrograde  
3 axonal transport, required for autophagosome-lysosome fusion, causes motor neuron degeneration [144,  
4 145]. Furthermore, consistent with lysosomal enzyme leakage, investigators reported increased lysosomal  
5 enzyme levels (i.e. acid phosphatase, Cystatin C) in the CSF and plasma of ALS patients [146, 147].

## 6 **Impaired lysosomal proteolysis**

7 Ammonia-induced impaired lysosomal proteolysis explains key histopathological hallmarks of ALS  
8 including formation of inclusion bodies (fig 2). Ammonia-induced alkalinisation in lysosomes impairs the  
9 activities of protease enzymes including cathepsin B and cathepsin D [128, 148]. Strikingly, investigators  
10 showed downregulation of cathepsin B and cathepsin D in ALS [149, 150]. Notably, defective lysosomal  
11 proteolysis causes swollen axonal dystrophy (spheroids), with histological features such as ubiquitinated  
12 and non-ubiquitinated inclusion bodies, amyloid precursor protein, and neurofilament aggregation [151].  
13 In keeping with this, research revealed such findings in ALS [138, 139, 150, 152-155].

14 In regard to non-ubiquitinated inclusion bodies, Kikuchi *et al.* showed that decreased cathepsin B generates  
15 Bunina bodies (small eosinophilic intraneuronally lysosomal inclusion bodies) in motor neurons—a  
16 hallmark of ALS (fig 2) [134, 150]. Since cathepsin D mediates lipofuscin and  $\alpha$ -synuclein clearance, and  
17 since downregulation of cathepsin D occurs in ALS, this explains frequently observed deposits of  
18 lipofuscin granules and  $\alpha$ -synuclein aggregation in ALS patients [142, 156-158]. Moreover, reduced  
19 cathepsin B activity induces APP accumulation, and Bryson *et al.* showed increased APP level in the  
20 SOD1 G93A mouse, which contributed to motor neuron damage [152]. As for impaired proteolysis-  
21 induced ubiquitinated inclusion bodies, ubiquitin inclusion aggregates such as 'lewy body-like inclusions'  
22 and 'skein-like inclusions' have been found in ALS (fig 2) [139, 159]. This finding accords with the  
23 observations that inhibition of macroautophagy impairs the ubiquitin proteasome system (UPS) [160].

1 Finally, neurofilament aggregation and spheroid formations have been found in the ALS mouse model  
2 and in patients (fig 2) [155].

### 3 **Impaired lysosomal ganglioside clearance**

4 Gangliosides are complex sialylated glycosphingolipids, particularly found in the CNS [161]. Notably,  
5 GM2 ganglioside is a main ganglioside in motor neurons [162]. Accumulation of GM2 ganglioside, owing  
6 to impaired lysosomal Hexosaminidase (Hex) enzymes, frequently causes motor neuron disease [163-  
7 165]. For example, Banerjee et al. reported slow accumulation of GM2 ganglioside, primarily in motor  
8 neurons, in patients with progressive motor neuron disease associated with partial Hex A and no Hex B  
9 activity [165]. By implication, this suggests that accumulation of gangliosides including that of GM2  
10 occurs in ALS and that ammonia increases GM2 ganglioside levels. Indeed, although scantily investigated,  
11 some investigators reported increased ganglioside levels in ALS including GM2 ganglioside [136, 166,  
12 167]. In line with ammonia's role in ganglioside metabolism, Perez et al. showed that ammonia causes  
13 leakage of Hexosaminidase A (Hex A), indicating GM2 accumulation [168, 169]. Thus, ammonia-induced  
14 GM2 accumulation could partly explain the heightened vulnerability of motor neurons in ALS (fig 2).

### 15 **Impaired lysosomal carbohydrate clearance**

16 Animal and clinical studies reported neuronal and glial glycogen accumulation and polyglucosan bodies  
17 (branched chained glycogen aggregates) in ALS (fig 2) [57, 170, 171]. Notably, Dodge et al. showed that  
18 decreased level of  $\alpha$ -glucosidase—a glycogen degrading lysosomal enzyme—partly causes glial and  
19 neuronal glycogen accumulation in ALS, and experiments showed that ammonia leaks  $\alpha$ -glucosidase  
20 from lysosome [57, 172]. Thus, ammonia-mediated lysosomal dysfunction explains yet another  
21 histological feature of ALS. Of note, this fits with the observations that upper and lower motor neuron  
22 lesions frequently arise in polyglucosan body diseases [173].

## 23 **6. 2 Impaired Golgi function**

1 Ammonia toxicity could explain Golgi apparatus fragmentation in ALS, an early and frequently  
2 observed event [135]. Sun *et al.* showed that CDK5 activation fragments Golgi apparatus [174].  
3 Interestingly, Cagnon and Braissant showed that ammonia activates CDK5. They also showed that CDK5  
4 activation led to neuronal cell death and impairment of axonal outgrowth [129]. Apparently, p25-induced  
5 mislocalization and deregulation of CDK5 activity occurs in ALS (fig 2) [137, 175]. In fact, Nguyen *et al.*  
6 reported that an attempted re-entry of motor neurons into the G1-S phase of the cell cycle—subsequent to  
7 Cdk5 deregulation—is a critical step of neurodegeneration in ALS [175].

### 8 **6.3 Increased oxidative/nitrosative stress and MAPK expression**

9 Additionally, data suggested that ammonia induces oxidative/nitrosative stress and MAPK expression,  
10 frequently found pathological features of ALS (fig 2) [126]. Research showed that oxidative/nitrosative  
11 stress and mitogen-activated protein kinase (MAPK) increases extracellular matrix degrading enzymes  
12 such as urokinase-type plasminogen activators and MMP-9 [176]. Unsurprisingly, experiments found  
13 increased levels of these extracellular matrix degrading enzymes occur in ALS [177]. Strikingly, Kaplan *et*  
14 *al.* observed overexpression of MMP-9 increased the vulnerability of fast fatigable limb-innervating  
15 motor neuron [178]. MMP-9 appears to exert neurotoxicity mainly through up-regulation of ER stress (fig  
16 2) [178]. Moreover, Skowrońska, *et al.* showed that increase in MMP-9, which degrades the extracellular  
17 matrix, destroys the blood brain barrier (BBB). Predictably, Nicaise *et al.* showed impaired blood-brain  
18 and blood-spinal cord barriers in mutant SOD1-linked ALS rodents [132]. Additionally, since MAPK  
19 regulates cytoskeletal homeostasis, ammonia-induced MAPK activation explains why cytoskeleton  
20 abnormalities such as intermediate filaments accumulation occur in ALS [154, 179].

### 21 **6.4 Neuronal hyperexcitability**

22 Furthermore ammonia intoxication explains neuronal hyperexcitability in ALS—a cardinal characteristic  
23 of ALS [16]. By decreasing potassium-chloride cotransporter KCC2, located in the brain and spinal cord,  
24 ammonia increases chloride levels in neurons (fig 2) [180]. Increased neuronal chloride levels in turn



1 suppress GABA and Glycine-mediated inhibitory neurotransmission, causing neuronal hyperexcitability  
2 (fig 2) [181]. In keeping with this, Fuchs et *al.* discovered decreased KCC2 expression in ALS-vulnerable  
3 motoneurons in spinal cord and hypoglossal nuclei of SOD1-G93A mice but not in EOMs [182].  
4 Concordantly, researchers reported spinal motor neuron hyperexcitability and degeneration in ALS  
5 patients [183]. In fact, Hübner et *al.* showed that KCC2 knockout mice died after birth owing to motor  
6 deficits that caused respiratory failure, a feature similar to ALS[181].

7 Furthermore, ammonia causes glutamatergic excitotoxicity. By MAPK activation and increasing oxidative  
8 stress, ammonia decreases the glutamate transporter EAAT2 (GLT-1) and glutamate-aspartate transporter  
9 (GLAST) (EAAT-1) in astrocytes (fig 2) [127, 184]. Consequently, decreased transporters impair  
10 astrocyte-mediated high affinity glutamate uptake and clearance, leading to defective glutamatergic  
11 neurotransmission and excitotoxicity [127, 184, 185]. In line with this, decreased GLT-1 and GLAST has  
12 been found in the spinal cord of SOD1 G93A mice and ALS patients [186-188]. Interestingly, increased  
13 CSF glutamate was associated with a spinal onset of the disease and with severity of the symptoms in  
14 41% of ALS patients [188].

## 15 **6.5 Neuroinflammation**

16 Ammonia extensively affects the function of astrocytes and microglia (fig 2). Through several  
17 mechanisms including (1) Quinolinic acid (QUIN) production, (2) NADPH oxidase (NOX) activity-  
18 induced reactive oxygen species (ROS) generation, (3) Toll-like receptor 4 (TLR-4) activation, and (4)  
19 extracellular-signal-regulated kinase (ERK) pathway stimulation, ammonia induces a transition from a  
20 resting state into reactive astroglia and microglia phenotype (fig 2) [108, 189-191].Consequently, reactive  
21 astroglia and microglia increase oxidative stress and stimulate the release of a range of proinflammatory  
22 cytokines including NF- $\kappa$ B, IL-1 $\beta$ ,and PGE2, leading to neuroinflammation and degeneration (fig 2)  
23 [192].

1 Emerging data indicate a role of QUIN in ALS[193]. Chen et al. detected overproduction of serum  
2 tryptophan, kynurenine and QUIN in the CSF of ALS patients compared to controls, concomitant with  
3 microglial activation and neuroinflammation (fig 2) [133]. Similarly, experiments showed increased  
4 microglial and neutrophil-derived NOX activity correlated with fast ALS progression[194]. In keeping  
5 with increased ammonia-induced inflammation, investigators showed TLR-4 activation, and elevated  
6 levels of various pro-inflammatory cytokines in ALS [195, 196].

## 7 **7. Clinical heterogeneities in ALS: The role of Calcium-binding proteins (CaBPs)**

8 However, the postulated ammonia neurotoxicity as the sole cause of ALS raises an awkward question. If  
9 ammonia damages both upper and lower motor neurons equally, then why does ALS often deviate from  
10 its classical pattern, manifesting as either the upper or lower motor neuron dominant subtype [8]?  
11 Moreover, why it is a relatively rare disorder? These questions clearly indicate that a protective factor  
12 exists that counteracts ammonia toxicity, and that anatomic-region specific loss of this factor causes the  
13 clinical heterogeneities in its presentation.

14 One such neuroprotective factor identified in ALS is the ER family of calcium binding proteins (CaBPs)  
15 (fig 1-3) [197]. By regulating voltage-gated calcium ion channels, CaBPs reduce calcium overload and  
16 cytotoxicity, thus protecting neurons from cell death [197]. The CaBPs involved in motor neuron  
17 protection include calreticulin, parvalbumin, and calbindin which are distributed in anatomic region  
18 specific manner within motor neurons [198, 199]. Calreticulin expression mainly occurs in limb-  
19 innervating lower motor neuron regions such as the lumbar spinal cord area and fast-fatigable  
20 motoneuron; whereas calbindin and parvalbumin are expressed in both lower and upper motor neurons  
21 [198, 199].

22 Differential anatomic region-specific distribution of CaBPs in the CNS partly explains different patterns  
23 of motor neurodegeneration [198, 200]. In the SOD1 G93A ALS mouse model, during the  
24 presymptomatic stage, fast-fatigable motoneuron denervation mainly accompanies calreticulin loss [200,

1 201]. By contrast, investigators showed that loss of calbindin and parvalbumin correlated with both upper  
2 and lower motor neuron damage [198].

### 3 **8. Region specific regulation of CaBPs: ER stress and bioenergetics**

4 How do neurons lose different CaBPs in different anatomic regions of the CNS? Research showed that  
5 ER stress downregulates calreticulin in limb-innervating lower motor neurons motor. In fact, calreticulin  
6 co-localises with the endoplasmic reticulum [201]. This fits with the fact the ER stress is particularly  
7 linked to lower motor neuron diseases (i.e. SMA and Kennedy's diseases). This also accords with the  
8 finding that neuronal MMP-9—which enhances ER stress—selective damages fast fatigable lower motor  
9 neurons. Additionally, since androgens modulate ER genes, this explains why sexual dimorphism occurs  
10 in limb-innervating motor neuron damage. Interestingly, research revealed increased ER stress and  
11 reduced calreticulin in Alzheimer's disease (AD) [201]. This explains why AD occasionally co-exists  
12 with motor neuron diseases [202].

13 As for the causes of reduced parvalbumin and calbindin expression in ALS, research implicates impaired  
14 oxidative metabolism secondary to defective mitochondrial electron transport (the respiratory chain)  
15 system [203, 204]. Indeed, of the five protein complexes of the mitochondrial respiratory chain, research  
16 has frequently showed reduced respiratory chain complex I and IV activity in sporadic ALS patients [197,  
17 203]. Within these two complexes, complex IV appears to be particularly involved in ALS. This chimes  
18 well with the fact that 90% of all parvalbumin and calbindin-immunoreactive cells showed dense staining  
19 for respiratory complex IV (cytochrome c oxidase) [205]. Furthermore, hyperhomocysteinaemia, found to  
20 be highly prevalent in ALS, damages mitochondria and suppresses respiratory complex IV activity [206,  
21 207]. Revealingly, compared to skeletal muscle, the EOMs have slow metabolism characterised by low  
22 complexes I and IV activities (~50%) yet elevated mitochondria density with increased complex I and IV  
23 levels (30% to 2 times)—explaining why parvalbumin and calbindin levels remain relatively unaffected  
24 in EOMs [208, 209].

25

## 1 **The role of respiratory chain complex subunits**

2 Interestingly, alterations in mitochondrial respiratory chain complex subunits also partly determine the  
3 spectrum of motor neuron damage. Investigators reported that altered Cytochrome c oxidase subunit Vb  
4 caused spinobulbar muscular atrophy, whereas Cytochrome c oxidase subunit I microdeletion induced  
5 upper motor dominant motor neuron damage [210, 211]. Furthermore, deficiency of complex I involved  
6 lower motor neuron damage involving spinal and bulbar areas [212]. This fits with the findings that  
7 anatomic region-specific differences in mitochondrial respiration contribute to the localized  
8 neurodegeneration [213].

9 In summary, these findings suggest that the regional loss of CaBPs expression, dependent on ER stress  
10 and defective mitochondrial respiration in the brain determines the anatomically variable manifestation of  
11 ALS. Collectively, it is also clear that motor neuron degeneration depends not only on postulated  
12 ammonia neurotoxicity but also on deficits of CaBPs within motor neuron.

## 13 **9. Biomarkers and therapeutics**

14 From this insight about ALS pathogenesis, diagnostic, disease monitoring and therapeutic measures  
15 emerge—fostering real hopes that ALS can be halted or even cured. Because ammonia is a volatile  
16 organic compound, excreted from breath and skin, an ammonia breath test presents a simple, reliable,  
17 robust, inexpensive and non-invasive tool for diagnosis and monitoring of ALS[214]. This ammonia  
18 breath test would prove invaluable in expediting drug discovery process. Aside from ammonia,  
19 gangliosides (e.g. Sialosylglobotetraosylceramide) and serum lysosomal enzymes can also serve as  
20 reliable adjuvant biomarkers of ALS[136].

21 As for therapeutics, since ammonia toxicity appears to be a major player in ALS, ammonia-removal  
22 strategies seem to be the most effective strategy for ALS treatment [215]. Many existing ammonia-  
23 lowering agents including those that act on the hepatic urea cycle can be employed [216, 217]. These  
24 could include salbutamol, conclevan, neomycin, sodium benzoate, ornithinephenyl acetate and L-

1 ornithine aspartate [215, 218, 219]. Moreover, since impaired fast-twitch skeletal muscle glycolysis plays  
2 a role in ALS, improving muscle glycolysis through various existing drugs such as serotonin agonists and  
3 AMPK agonists (e.g. D-xylose) is another promising pharmacological strategy[220, 221]. Additional  
4 therapeutic strategies could involve correcting system metabolic defects such as hyperglucagonemia and  
5 acidosis [57, 65]. Moreover, other potent therapeutic targets could involve MAPK inhibitors, K-Cl co-  
6 transporters, and hexosaminidase agonists (e.g. Pyrimethamine) [184, 222, 223]. Finally, interventions  
7 that restore the levels of CaBPs should also be simultaneously applied for effective treatment.

## 8 **10. Summary**

9 ALS is a ghastly and incurable disease. Despite increasing wealth of data, ALS remains poorly  
10 understood. By analysing existing literature, this paper has not only identified important knowledge gaps  
11 in ALS aetiopathology but also filled them and tied them together. In doing so, this paper postulate a new  
12 integrative explanation of ALS and suggests potent therapeutic measures to treat ALS. Central to this  
13 explanation is the notion that ALS is a neurological disease of metabolic origin—resembling  
14 hepatocerebral degeneration [215]. This explanation posits that ALS pathology involves theinterplay of  
15 two critical factors: 1) chronic hyperammonia caused by imbalanced interogan ammonia metabolism,  
16 mainly due to muscle and liver pathology (fig 1) and 2) altered CaBPs homeostasis, mainly due to  
17 increased ER stress and impaired mitochondrial respiration (fig 2).

18 Putting all these together in sequence, impaired fast twitch skeletal muscle carbohydrate metabolism  
19 activates purinergic and amino acid catabolism, leading to a release of ammonia, a neurotoxin.  
20 Alternatively, ammonia toxicity can also be induced or exacerbated by other endogenous (e.g. cerebral  
21 deamination, intestinal ammoniagenesis) and exogenous sources (i.e. neurotoxins). Owing to concurrent  
22 liver pathology (e.g. hepatic steatosis) in ALS, impaired hepatic ammonia detoxification occurs.  
23 Consequently, ammonia levels progressively builds up, leading to chronic hyperammonia. Since ALS  
24 pathology also involves loss of neuroprotective CaBPs (i.e. calbindin, calreticulin and parvalbumin),

1 ammonia neurotoxicity in the absence of CaBPs leads to ALS. Ammonia damages motor neurons through  
2 a range of pathways. These pathways include impaired macroautophagy-endolysosomal impairment,  
3 Golgi fragmentation, oxidative/nitrosative stress and reactive microglial and astrogliosis. These  
4 mechanisms explain a range of histopathological and neurophysiological hallmarks of ALS such as bunia  
5 bodies and neuronal hyperexcitability. Finally, since ALS appears to be associated with HD, dementia  
6 and Parkinson's disease this framework can be generalised to explain these disorders [33, 85, 86].

7

1 **Fig 1. Line diagram: Mechanisms of motor neuron damage in ALS.** Mechanism of motor neuron  
2 degeneration in ALS involves two main factors: (i) ammonia neurotoxicity and (ii) down regulation of  
3 neuronal calcium binding proteins (CaBPs). Owing to imbalanced interorgan ammonia  
4 metabolism, ammonia, a well-known neurotoxin, accumulates in neurons. Among the five organs (brain,  
5 skeletal muscle, gut, liver and kidney) involved in ammonia metabolism, ALS appears to mainly involve  
6 the role of liver and skeletal muscle in that confluence of impaired ammonia removal—owing to impaired  
7 hepatic urea cycle—and increased muscular ammoniagenesis—owing to impaired glycolysis in fast  
8 twitch skeletal muscle—leads to chronic hyperammonia in ALS. In the brain, ammonia activates several  
9 neurodegenerative pathways such as (1) autophagy-endolysosomal dysfunction (2) neuroinflammation (3)  
10 oxidative stress (4) Golgi fragmentation and (5) neuronal hyperexcitability. In the absence of neuronal  
11 calcium binding proteins (CaBPs) such as parvalbumin, calbindin, calreticulin, activation of these  
12 degenerative pathways lead to motor neuron damage. Notably, decrease in calreticulin, because of increased  
13 ER stress, leads to lower motor neuron damage, whereas the downregulation of parvalbumin and  
14 calbindin, because of defective mitochondrial respiration, leads to upper motor neuron damage.

15

1 **Fig 2. A molecular model of ammonia-induced motor neuron degeneration in ALS** (Modified from  
2 [224]). Ammonia intoxication directly damages motor neurons through five multifactorial pathological  
3 mechanisms: 1) alkalisation-induced impairment of macroautophagy-endolysosomal system, 2) Golgi  
4 impairment, 3) increased oxidative/nitrosative stress and MAPK up-regulation 4) neuronal  
5 hyperexcitability and 5) neuroinflammation. These mechanisms explain frequently found cellular,  
6 molecular and neurophysiological phenotypes of motor neuron damage in ALS. **A.** Owing to ammonia-  
7 induced alkalisation, impairment of macroautophagy-endolysosomal system induces several key  
8 molecular histopathological features of ALS including : (i) ubiquitinated (lewy and skein body-like  
9 inclusions) and non-ubiquitinated inclusion bodies (i.e. bunia bodies) formation, (ii) amyloid precursor  
10 protein (APP), (iii) gangliosides accumulation (i.e. GM2), (iv) autophagy vacuoles, (v) neurofilament  
11 aggregation and axonal swelling. **B.** Ammonia activates CDK5 which in turn leads to frequently observed  
12 Golgi fragmentation. **C.** Ammonia-induced oxidative/nitrosative stress and MAPK-upregulation lead to  
13 multiple cellular and molecular pathological features such as: (i) blood brain barrier (BBB) breakdown,  
14 and (ii) MMP-9-induced ER stress. **D.** Ammonia causes neuronal hyperexcitability by (i) downregulating  
15 astrocyte glutamate transporters (GLAT-1 and GLAST) and (ii) lowering potassium-chloride co-  
16 transporter KCC2 level which suppresses GABA and Glycine-mediated inhibitory neurotransmission. **E.**  
17 Ammonia leads to neuroinflammation secondary to reactive microglial and astrogliosis. This occurs  
18 because of (i) quinolic acid release from microglia (ii) up-regulation of pro-inflammatory cytokines (TNF,  
19 IL-1 $\beta$ , NF- $\kappa$ B, and PGE2) in astrocytes (iii) TLR-4 activation and (iv) neutrophil burst derived NADPH  
20 oxidase (NOX)-induced oxidative stress.

21



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4

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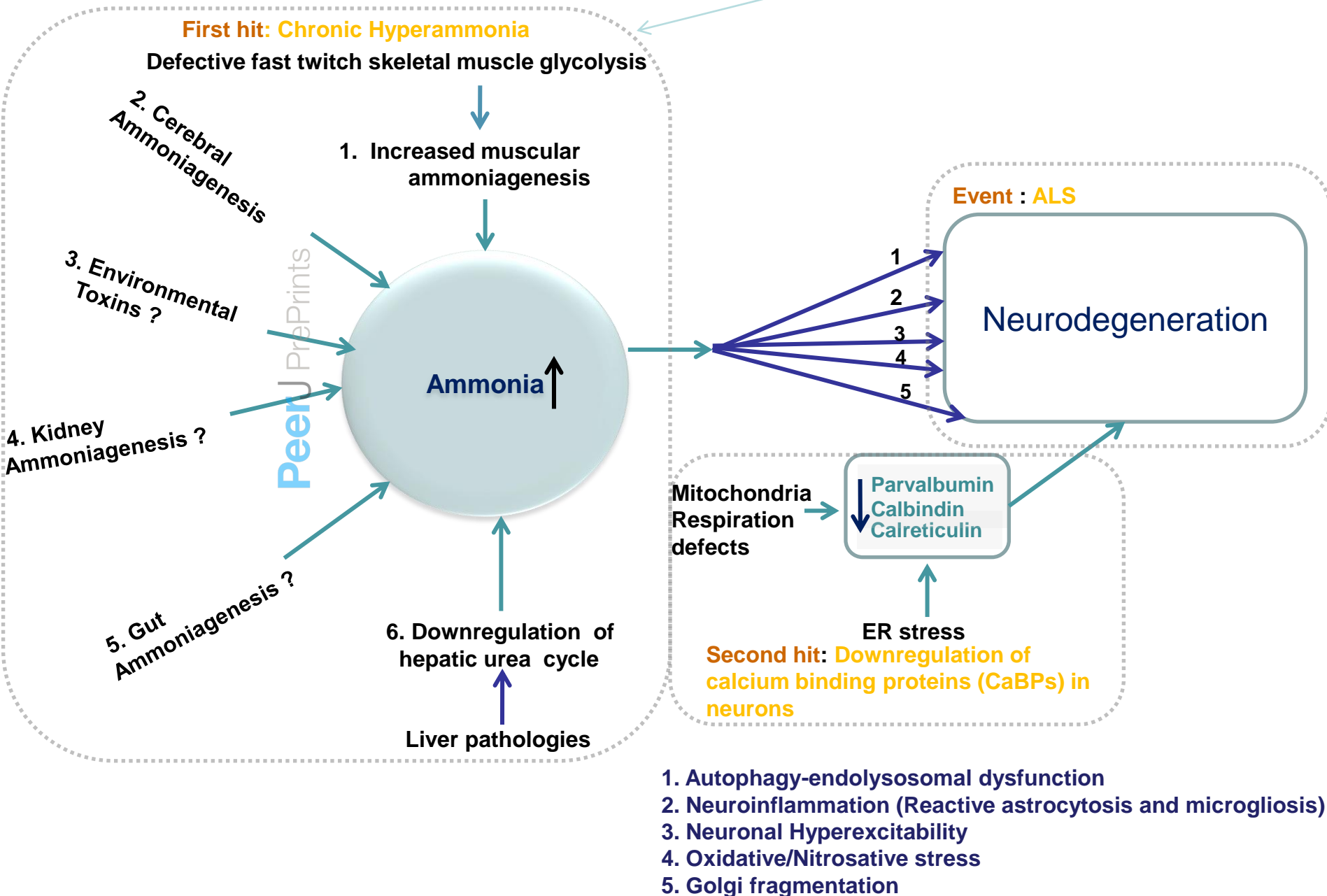
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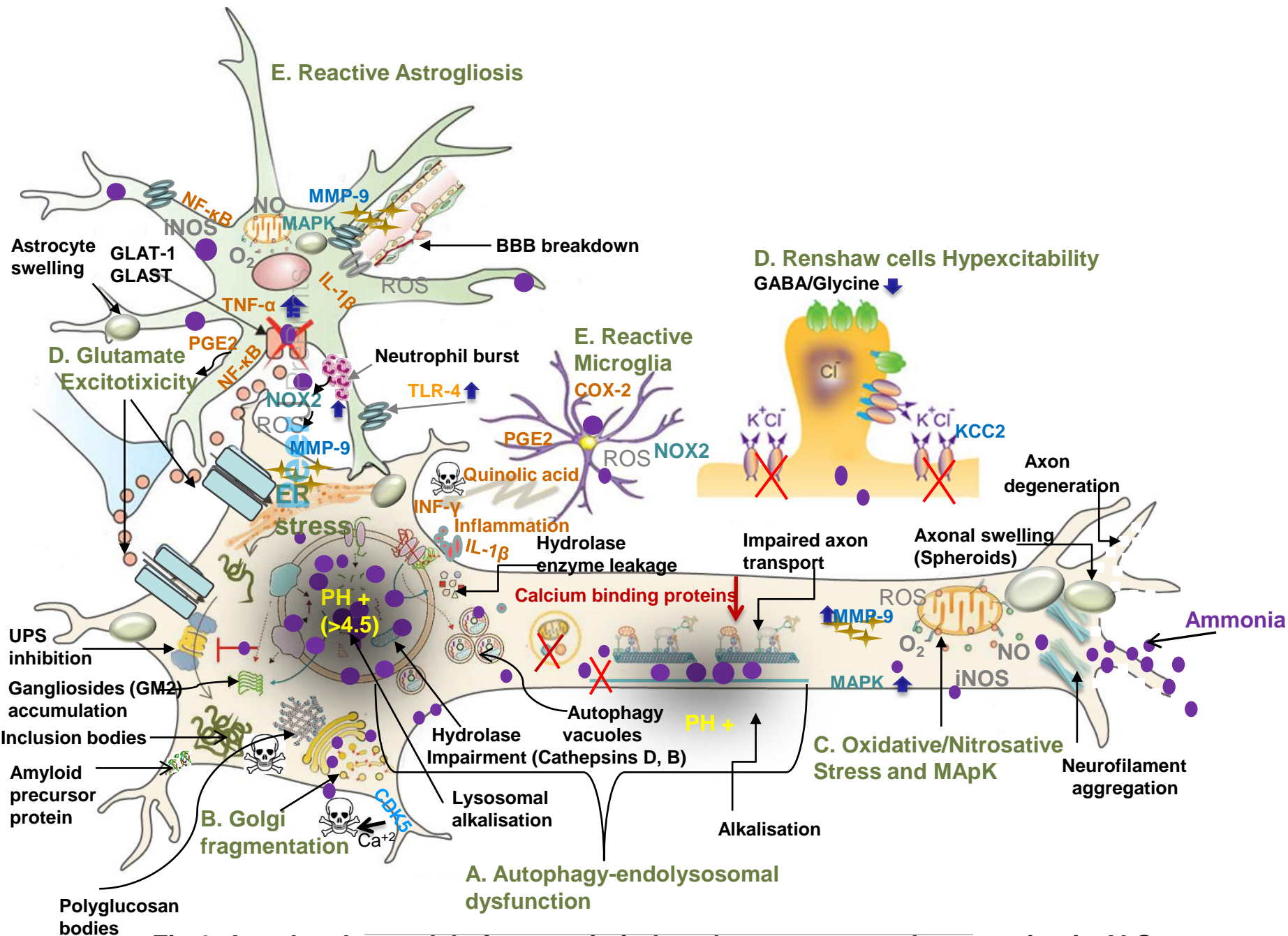
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# Imbalanced interorgan ammonia metabolism



**Fig 1. Line diagram: Mechanisms of motor neuron damage in ALS.**





**Fig 2. A molecular model of ammonia-induced motor neuron degeneration in ALS.**