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

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Abbreviations

ALS, amyotrophic lateral sclerosis; ALS-PDC, Amyotrophic lateral sclerosis-parkinsonism dementia complex; AMPD3, AMP deaminase-3; AMPK, 5' AMP-activated protein kinase; APP, Amyloid precursor protein; ATP, Adenosine triphosphate; ATXN2, *Ataxin-2*;C9ORF72, chromosome 9 open reading frame 72; CaBPs, calcium binding proteins; CAMK, Ca2+ /calmodulin-dependent protein kinase; CDK5, Cell division protein kinase 5; CHMP2B, charged multivesicular body protein 2B; COX-2, cyclooxygenase-2;CSF, cerebrospinal fluid; DCTN1, dynactin subunit 1; DYNC1H1, dynein cytoplasmic 1 heavy chain 1; ER, endoplasmic reticulum; ERBB4, receptor tyrosine-protein kinase erbB-4; ERK, extracellular-signal-regulated kinase; FBP2, fructose-1,6-bisphosphatase 2; Fig4, factor-Induced gene 4; FUS, fused in Sarcoma; GABA, gamma-aminobutyric acid; GH, Growth hormone; GLT, glutamate transporter; GLAST, glutamate-aspartate transporter; GLUT4, Glucose transporter 4; Hex, Hexosaminidase; HFE, IGF, Insulin-like growth factor; IL-1β, Interleukin-1β; IL-6, Interleukin 6; IL-8,Interleukin 8; INF-γ, Interferon gamma; KCC2, potassium-chloride cotransporter 2; LMN, lower motor neurons; MAPK, Mitogen-activated protein kinase, MMP-9, metalloproteinase-9; mtDNA, mitochondrial DNA; NF-κB, Nuclear factor-κB; NOX, NADPH oxidase; NFT, neurofibrillary tangles; NMDA, N-methyl-D-aspartate; MCT, monocarboxylate transporter; PGE2,prostaglandin E2; PKC, Protein kinase C; PP2A, Protein phosphatase 2A; QUIN, Quinolinic acid; RABA7A, Ras-related protein Rab-7a; ROS, reactive oxygen species; SMA, spinal muscular atrophy; SMN1, survival of motor neuron 1; SOD1, Superoxide dismutase-1; SQSTM1,sequestosome 1; TARDBP, tAR DNA-binding protein; Tbc1d, TBC1 domain family member 1; TNF-α, Tumor necrosis factor alpha; TLR4, toll-like receptor 4; UCP, uncoupling protein; UMN, upper motor neurons; UPR, unfolded protein response; UPS, ubiquitin proteasome system; VCP, Valosin-containing protein; VEGF, vascular endothelial growth factor;Xbp1, X-box binding protein 1.

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   ammoniagenesis— 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.

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1. Introduction

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

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

ALS Aetiology: An enduring enigma

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

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2. ALS: A multi organ disease

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

2.1 Role of Skeletal muscle

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

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

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

2.2 Liver: An emerging locus of ALS

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

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

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

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

3. Aims

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

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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$^{\delta/\delta}$ 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.

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Impaired muscle glycogen and glucose homeostasis in ALS

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

ALS resistance of Extraocular muscles (EOMs): Role of glycolysis

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

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5. Ammonia: the elusive pathological factor

Notably, defective glycolysis, which reduces ATP levels, in fast twitch skeletal muscle activates catabolic reactions of adenine nucleotides (i.e. purine nucleotide cycle) and amino acids (fig 1) [80, 81]. Intriguingly, such catabolic reactions produce ammonia—a neurotoxin1000 times more toxic than ethanol at equimolar concentration [82]. Since ammonia is toxic, it is obligatorily removed—mainly through hepatic urea cycle which transforms ammonia into urea [83]. Notably, when the urea cycle is impaired, as it occurs in fatty liver disease, increased ammonia production from skeletal muscle or from dietary sources can cause chronic hyperammonia (>35-50 μM) and consequent neurodegeneration and motor impairments (fig 1-2) [33, 84-88]. Indeed, in many liver diseases—including fatty liver disease, which commonly occurs in ALS—because of impaired urea cycle-mediated ammonia removal, hyperammonia frequently leads to corticospinal hyperexcitability, myelopathy and spasticity— features strikingly reminiscent of neurophysiological phenotypes of ALS symptoms[89-93].

5.1 Ammonia neurotoxicity: Hypothesis and evidence

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

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

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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 ammoniagenesis 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, Iłzecka 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, analternative 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 ammoniagenesis[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 ammoniagenesis[106, 107]. Conversely,

20 research showed that ammonia-counteracting compounds such as phenylbutyrate, ariginine, 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 β-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

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

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

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

6. Mechanisms of ammonia's neurotoxicity

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

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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, includeaxon swelling, blood brain

6 barrier breakdown andastrogliosis and microgliosis[131-133]. The molecular pathological features,

7 explained by ammonia's toxicity, include formation of inclusion bodies such as bunia 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. Alkalisation-induced impaired macroautophagy-endolysosomal system**

13 Ammonia-induced alkalisation 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 alkalisation and lysosomal enzyme leakages occur, impairing the lysosomal hydrolysis of

17 proteins, lipids and carbohydrates (fig 2). Second, ammonia alkalises 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 alkalisation-induced toxicity is especially relevant to ALS because macroautophagy-

24 endolysosomal dysfunction causes motor neuron degeneration[143]. Indeed, mutantgenesof this pathway

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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 neurofilamentaggregation [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 intraneuronallysosomal inclusion bodies) in motor neurons—a

16 hallmark of ALS (fig 2) [134, 150].Since cathepsin D mediates lipofuscin and α-synuclein clearance, and

17 since downregulation of cathepsin D occurs in ALS, this explains frequently observed deposits of

18 lipofuscin granules and α-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 GM2ganglioside, 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 scantly 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 explains 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 α-glucosidase—a glycogen degrading lysosomal enzyme—partly causes glial and

19   neuronal glycogen accumulation in ALS, and experiments showed that ammonia leaks α-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**

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Ammonia toxicitycould explain Golgi apparatus fragmentation in ALS, an early and frequently observedevent [135]. Sun et *al.* showed that CDK5 activation fragments Golgi apparatus [174]. Interestingly, Cagnon and Braissant showed that ammonia activatesCDK5. They also showed that CDK5 activation led to neuronal cell death and impairment of axonal outgrowth [129]. Apparently, p25-induced mislocalization and deregulation of CDK5 activity occurs in ALS (fig 2) [137, 175]. In fact, Nguyen et *al.* reported that an attempted re-entry of motor neurons into the G1-S phase of the cell cycle—subsequent to Cdk5 deregulation—is a critical step of neurodegeneration in ALS [175].

6.3 Increased oxidative/nitrosative stress and MAPK expression

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

6.4 Neuronal hyperexcitability

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

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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 excitotoxicty. 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-κB, IL-1β,and PGE2, leading to neuroinflammation and degeneration (fig 2)

23   [192].

17

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,

18

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

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

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

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

The role of respiratory chain complex subunits

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

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

9. Biomarkers and therapeutics

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

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

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

10. Summary

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

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

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

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

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 mutifactorial 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-1β, NF-κB, and PGE2) in astrocytes (iii) TLR-4 activation and (iv) neutrophil burst derived NADPH

20   oxidase (NOX)-induced oxidative stress.

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4

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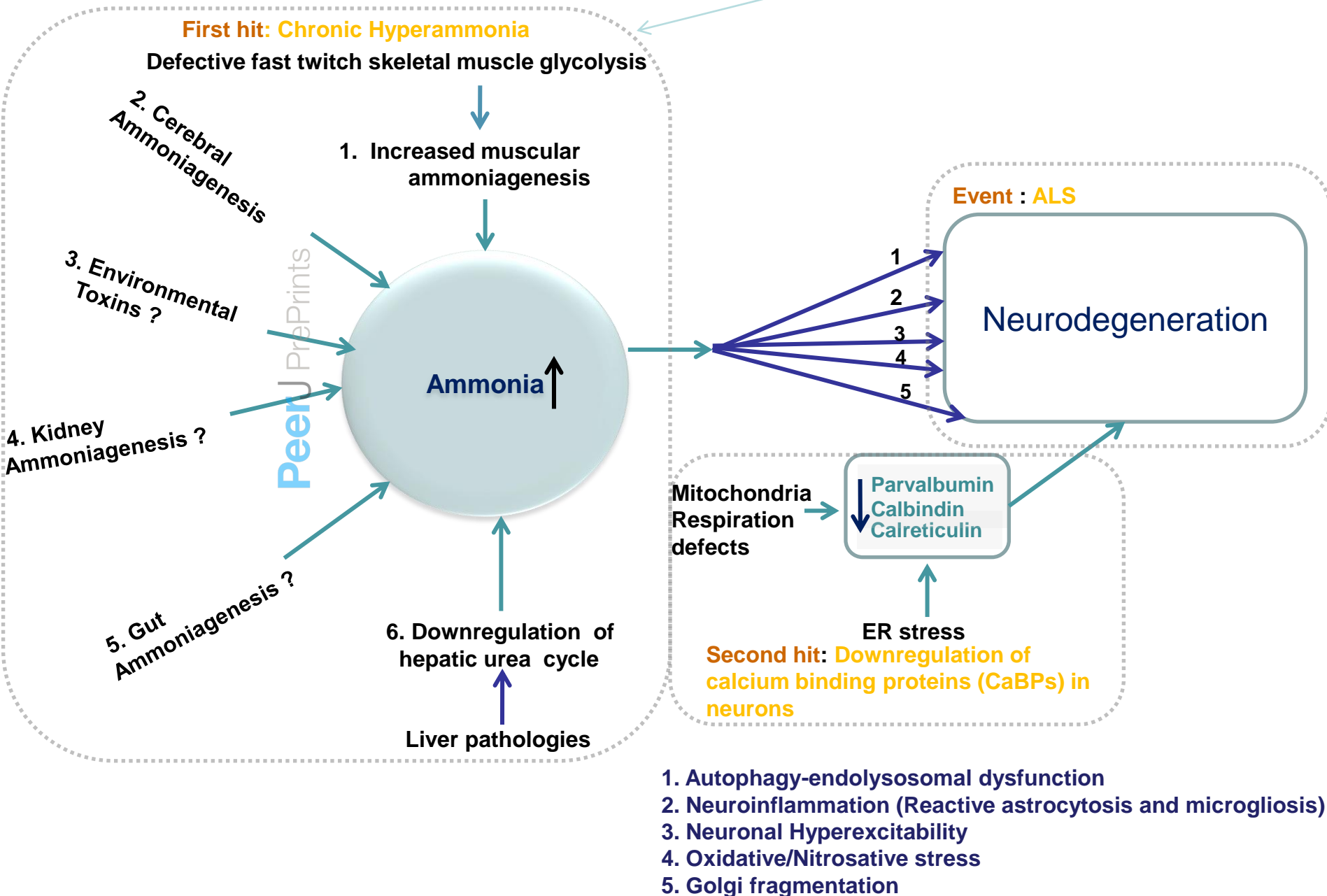
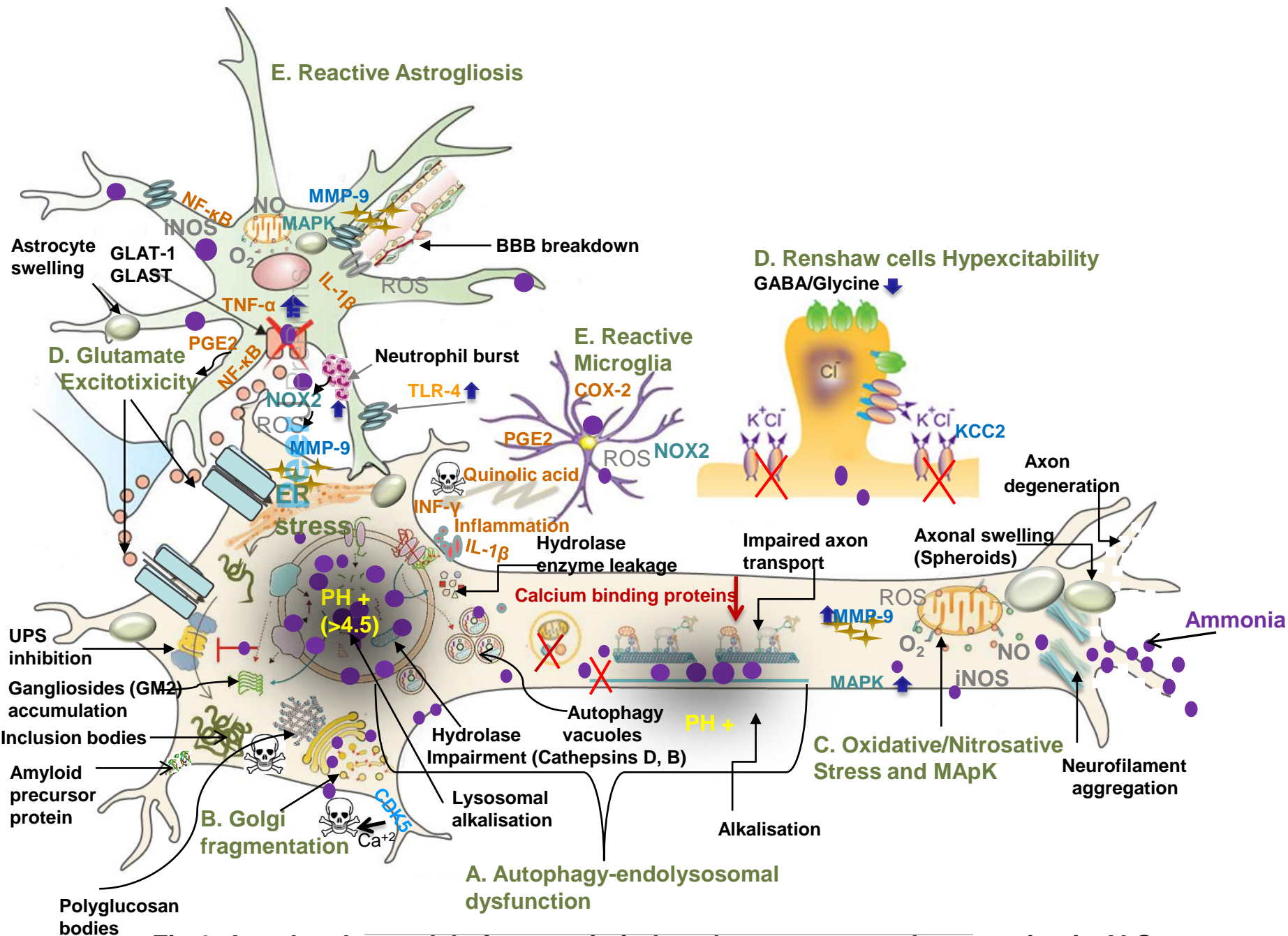Fig 1. Line diagram: Mechanisms of motor neuron damage in ALS.

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