Key Molecular Mechanisms in the Pathogenesis of Amyotrophic Lateral Sclerosis (ALS).

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ABSTRACT
Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder with a poor prognosis involving both upper motor neurons (UMN) and lower motor neurons (LMN). ALS is considered a multifactorial disease that involves multifactorial emergence of complex interrelation and mechanisms, therefore, therapeutics for ALS should be able to block or compensate for various aberrant pathological events. Growing evidence suggests that endoplasmic reticulum (ER) stress and the UPR pathway, autophagy and apoptosis may be implicated in the pathogenesis of ALS. This review focuses on the recent findings to elucidate potential molecular mechanisms for ALS. A comprehensive understanding of the pathogenesis of ALS may lead to the discovery of a breakthrough treatment strategy for this disease.

INTRODUCTION
Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a fatal progressive neurodegenerative disease without effective treatments. The disease is characterised by the loss of upper motor neurons in motor cortex and lower motor neurons in the brainstem and spinal cord, that results in progressive paralysis and death due to respiratory failure, within three years of onset of the first symptoms (1). It is also the most common adult-onset motor neuron disorder. The disease usually occurs between the 4th and the 6th decade of life and incidence of ALS diminishes rapidly after the age of 80 (2). Limb-onset ALS, is identified by the coexistence of upper motor neuron (UMN) and lower
motor neuron (LMN) signs in the limbs, is the most predominant form of ALS (3), occurring in 70% of cases, whereas 25% of cases display bulbar-onset ALS which is characterised by spastic dysarthria (slurring of speech) and dysphagia (difficulty in swallowing) with subsequent development of limb features (3). Upper motor neuron involvement is reflected by slow movement, hyperreflexia and increased muscle tone whereas symptoms of lower motor neurons include weakness, muscle atrophy, hyporeflexia and fasciculations (4).

The majority of ALS patients usually die within 2-5 years of symptoms onset due to respiratory failure (5). ALS has a poor prognosis with about 20% of ALS patients surviving between 5 to 10 years after symptoms onset with the respiratory phenotype exhibited the worst prognosis (6). Currently, Riluzole and Edaravone have been approved by the United States Food and Drug Administration (FDA) for the treatment of ALS however, Riluzole only prolongs survival by two to three months (7). Riluzole blocks voltage-gated sodium channel therefore reducing the release of glutamate (8). The causes of ALS and molecular mechanisms involved remain investigated however, ALS is considered a multifactorial disease that involves multifactorial emergence of complex interrelation and mechanisms (9). Aging is a normal process of life that involves cellular senescence. Changes at the cellular levels for examples, mitochondrial dysfunction, oxidative damage, inflammation, telomere shortening, DNA damage and epigenetic alteration, are associated with cellular senescence and programmed cell death. These cellular changes may be involved with the pathogenesis of age-related diseases, including amyotrophic lateral sclerosis, Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, cardiovascular diseases and cancer.

PATHOGENIC MECHANISMS OCCURRING IN ALS

Endoplasmic reticulum (ER) stress and the UPR pathway

The endoplasmic reticulum (ER) is an important organelle that is vital for cell survival and normal cellular functions. The ER maintains intracellular calcium homeostasis and is involves in calcium signalling by releasing sequestered calcium ions through ion channel as a response to second messengers for example, inositol triphosphate (IP₃) and protein kinases. The ER is also responsible for
protein synthesis, folding and maturation of secretory and transmembrane proteins and lipid biosynthesis. Newly synthesized proteins in the rough ER undergo glycosylation and oxidative folding through the Calnexin/Calreticulin chaperone system which involves the ER-resident protein 57 (ERp57) to provide higher stability of the polypeptides (10-12). The correctly folded proteins are exported to the Golgi apparatus for further modifications before being distributed to various sites in the cell. ER stress occurs when the ER-calcium homeostasis is disturbed (13) and the accumulation of misfolded or unfolded proteins in the organelle (14). The misfolded proteins undergo refolding by the ER chaperones, for example, BiP or elimination through ER-associated protein degradation (ERAD) pathway. Under mild ER stress, the unfolded protein response (UPR) in cells is activated to restore the protein homeostasis by increasing protein folding capacity and increasing the degradation of abnormal proteins via ERAD and autophagy (15). However, in chronic or irreversible ER stress in which the cells cannot mitigate the ER stress, UPR results in apoptotic cell deaths (16, 17). ER stress is also associated with ALS pathogenesis.

Several genes that are involved in proteostasis are implicated in FALS including protein disulphide isomerase (PDI) variants (18), UBQLN2 and p62/SQSTM1 (19, 20). PDI is an ER chaperone protein and assists in the rearrangements of disulphide bonds via catalysis of thiol-disulphide exchange (21, 22). PDI proteins are also distributed at the cytosol and mitochondria (22-24) and at high levels due to misfolded proteins, the mitochondrion-associated PDI can induce apoptosis (25). In ALS, overexpression of PDI has been reported to decrease SOD1 aggregates by attenuating aggregation and preventing proteasome disturbance (26). PDI was also observed to be colocalized with FUS and TDP-43 inclusions from the spinal cord derived from ALS subjects (27). p62/SQSTM1 and ubiquilin-2 are important in the early steps of autophagy (28, 29) and mutation in p62/SQSTM1 at L341V that results in defective recognition of light chain 3 beta (LC3B) may interfere with the delivery of autophagic substrates to the autophagosome (30). Treatment with salubrinal, an ER-stress protective agent has also been observed to reduce disease manifestation in transgenic mice (31).

Three major ER transmembrane proteins regulate the UPR; inositol requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)-like ER Kinase (PERK) and Activating Transcription Factor 6 (ATF6) in which both IRE1 and PERK are type I transmembrane proteins with protein kinase activity whereas ATF6 is a type II transmembrane protein (32, 33).

Inositol requiring enzyme 1 (IRE1)

Upon sensing misfolded or unfolded proteins, IRE1 dimerizes and activated by autophosphorylation. Following this, it uses its endoribonuclease activity to splice a 26-intron from X-box binding protein 1
(XBP1) mRNA to form XBP1s (34, 35). The XBP1s encodes a basic leucine zipper (b-ZIP) transcription factor that translocates to the nucleus where it binds to the target sequence of the regulatory region of the UPR genes for upregulations such as genes that function in ERAD for examples, ER-degradation-enhancing-α-mannidose-like protein (EDEM) and protein disulphide isomerase (PDI) that are involved in protein folding (36, 37). IRE1 can also activate apoptosis cell death under high chronic ER stress by activating TNF-receptor-associated factor 2 (TRAF2) which in turn activates the apoptosis-signalling-kinase 1 (ASK 1), following which activated ASK 1 activates c-Jun N-terminal protein kinase (JNK) that is involved in apoptosis by regulating the Bcl-2 family of proteins (38). An increased amount of IRE1 and XBP1s have been observed in the spinal cord samples from the SOD1G93A mice ALS mouse model (39, 40). Increased splicing and nuclear translocation of XBP1 mRNA have also been found in Neuro2a cells expressing G85R-SOD1 (41). Postmortem spinal cord samples from SALS patients also demonstrated increased XBP1s (42). XBP1 has been suggested to be involved in autophagy because knockdown of XBP1 in SOD1G93A mice resulted in reduced SOD1 aggregates and improved cell survival (42).
Double-stranded RNA-activated protein kinase (PKR)-like ER Kinase (PERK)

Upon sensing of ER stress, double-stranded RNA-activated protein kinase (PKR)-like ER Kinase (PERK) dimerizes and autophosphorylated. The activation of PERK phosphorylates Ser51 on the α-subunit of Eukaryotic Initiation Factor 2 (eIF2α) (43). The phosphorylation of eIF2α prevents the formation of ribosomal initiation complexes therefore inhibiting general mRNA translation thus reducing the ER workload for adaptive response to reduce the accumulation of unfolded proteins in the ER (43) and also prevents cells from ER stress mediated-apoptotic cell death (44). Paradoxically, phosphorylation of eIF2α is required for the translation of Activating Transcription Factor 4 (ATF4) or also known as growth arrest and DNA damage inducible gene (GADD153) that is a basic leucine zipper (b-ZIP) transcription factor that regulates expression of several transcription factors for example, C/EBP homologous protein (CHOP) (44-47). CHOP upregulates several pro-apoptotic BH3 domain-only proteins genes for example, BIM and downregulates the synthesis of the anti-apoptotic Bcl-2 family of proteins to induce apoptosis (48). Studies have reported that there were elevated amounts of phospho-PERK-PERK (31, 39) and phospho-eIF2α (31) in SOD1G93A mouse model and Neuro2A cells transfected with mutant SOD1. A study has also shown that SOD1G93A transgenic mice treated with Guanabenz, a drug for hypertension treatment have less accumulation of mutant SOD1 proteins and an elevated phosphorylation of eIF2α, contributing to a significant amelioration of ALS with a delay in the onset and prolongation of the early phase of disease and survival (49).

Activating Transcription Factor 6 (ATF6)

Upon ER stress, Activating Transcription Factor 6 (ATF6) translocates to the Golgi apparatus where it is cleaved by site 1 (S1) and site 2 (S2) proteases forming a b-ZIP transcription factor whose cytosolic domain can translocate to the nucleus and activate UPR target genes (34, 35, 50-52). Cleavage and translocation of ATF6 have been verified in Neuro2A cells transfected with mutant SOD1 (41) whereas knocking down of ATF6 in NSC-34 cells transfected with mutant SOD1 has been shown to increase SOD1 accumulation (53). Increased amounts of ATF6 have also been reported in ALS patients (Atkin et al., 2008). It has also been observed that both native and mutant VAPB interact with ATF6 and decreasing the capability to promote the expression of XBP1 with mutant VAPB being more potent to inhibit ATF6 than native VAPB (54).
Fig. 1: The unfolded protein response (UPR). Severe chemical and physiological inducers may induce ER stress in cells. UPR is controlled by three master regulators: IRE1, PERK and ATF6. IRE1α activation leads to the mRNA splicing of a 26-nucleotide from X-box binding protein 1 (XBP1) that upregulates the expression of UPR-dependent genes. PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α) results in global protein translation attenuation. However, some mRNAs are preferentially translated such as ATF4. ATF6 is directed to the Golgi, where it is proteolytically cleaved and the active ATF6 fragment activates the transcription of UPR genes involved in protein folding, processing and degradation.
Autophagy

Autophagy (‘self-eating’) is a lysosome degradation pathway of long-lived proteins or organelles and these sequestered cytoplasmic materials are delivered to lysosomes using double membrane vesicles for degradation (55). Autophagy has a key role in maintaining cell homeostasis, keeping the metabolic balance between synthesis and degradation and also the turnover of cytoplasmic proteins in a stressful condition (56). Autophagic vacuoles (AVs) are autophagy-related vesicular structures that are widely present in the affected neurons of neurodegenerative disorders, although autophagy is considered as a neuroprotective response to eliminate pathogenic proteins, aberrant AVs induce neuronal cell death in these disorders (56). Macroautophagy and chaperone-mediated autophagy (CMA) are the two different types of autophagy presence in neurons. As depicted in Fig.2 overleaf, in macroautophagy, the phagophore engulfs cytoplasmic proteins and forms a double membrane vesicle (autophagosome) and is delivered to lysosomes where the autophagosome is fused with hydrolytic enzymes for degradation (57). In CMA, soluble proteins are recruited by chaperone proteins, for example hsc70 and following that, the chaperone complex interacts with LAMP-2A, a receptor protein and the protein is translocated across the lysosome membrane assisted by the luminal chaperone (Lys-hsc70) and is then degraded in the lysosomal lumen (58). In ALS, the accumulation of autophagosomes has been found in the spinal cord of SALS individuals which indicates autophagy dysfunction in the disease (59). Furthermore, in the mutant SOD1 ALS mouse model, axonal transport is impaired and autophagosome transport process is affected, causing autophagy dysfunction (60, 61), therefore, mutant SOD1 clearance by autophagy is beneficial for motor neuron loss in ALS (62).
Fig.2: **Overview of autophagy.** This diagram illustrated a simplified version of autophagy. The initiation stage (vesicle nucleation) is activated by phosphorylation of ULK1 complex thereby promoting the movement of PI3K class III complex to the pre-autophagosomal structure. The pre-autophagosomal structure is formed from the phagophore that surrounds and eventually engulfs substrates for degradation. Receptors such as p62 and optineurin proteins bind to their cargo selectively. The completed autophagosome is then transported by dynein motors along the microtubule to fuse with the lysosome. Lysosomal degradative enzymes (lysozymes) that require an acidic environment to function then degrade the autophagic cargo.

**Mechanisms of Autophagy**

Mammalian (or mechanistic) target of rapamycin complex 1 (mTORC1) is the main regulator for the initiation and maturation stages of autophagy [63]. It has been reported that mTORC1 inhibition increases autophagy, whereas the stimulation of mTORC1 decreases autophagy [64]. The activation of mTORC1 results in the inhibition of Unc-51-like-kinase 1 (ULK1) resulting in the inhibition of autophagy initiation [65, 66]. AMP-activated protein kinase (AMPK) inhibits mTORC1 and activates the ULK1 complex therefore, initiating autophagy [67]. Autophagy can also be induced by the accumulation of aberrant proteins, damaged and dysfunctional organelles and the formation of reactive oxygen species which all lead to the activation of ULK1 and the inhibition of mTORC1.
The activated ULK1 complex in turn activates the Beclin1-VP534 complex and ATG16L (68). Beclin1-VP534 acts as a class III PI3K to produce phosphatidylinositol-3-phosphate (PI3P). Double FYVE domain-containing protein 1 (DFCP1) and WD-repeat protein interacting with phosphoinositides (WIPI) are recruited to PI3P to generate phagophore, a premature membrane structure of an autophagosome (69-72). Class III PI3 kinase complex is required for the elongation of the phagophore membrane. Furthermore, WIPI proteins also activate ATG16L which in turn forms a complex with ATG12 and ATG5 (69). The ATG16-ATG12-ATG5 complex conjugates phosphatidylethanolamine (PE) to microtubule-associated protein 1 light chain 3 beta (LC3B) to elongate and form an autophagosome from a phagophore (73). LC3B recruits damaged organelles and aggregated protein into an autophagosome (74). Autophagosome then fuses with the lysosome to form autolysosome in which the lysosomal enzymes degrade the protein aggregates and damaged organelles. The fusion of autophagosome and lysosome is facilitated by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (75-77), Rab7 (78, 79), UV radiation resistance-associated gene protein (UVRAG) (80), the homotypic fusion and protein sorting (HOPS) complex (81), LC3 (82) and γ-aminobutyric acid-type-A receptor–associated proteins (GABARAPs) (83). This maturation stage of autophagy can be inhibited by the phosphorylation of UVRAG by mTORC1 (84).

Autophagy is reported to be upregulated in ALS as shown by the increased levels of the autophagy initiation proteins, Beclin-1 and the Atg5–Atg12 complex in SALS subjects (Hetz et al., 2009) and an increase in autophagosomes in spinal cords from SALS individuals (59, 85). Furthermore, mutant SOD1 interacts with Beclin-1 and destabilizes Beclin-1-Bcl-xL complex thus interfering with the initiation of autophagy and may be associated with neurotoxicity in the ALS mouse model (86). Substrate recognition in which the ubiquitinilated cargoes are recognised by the autophagy receptors such as p62 and OPTN for engulfment is an important process that is prone to disruption in neurodegenerative disease (87, 88). Missense mutations in p62 that is encoded by the gene SQSTM1 have been reported in SALS and FALS. Further, p62 is involved in the degradation of SOD1 mutant and TDP-43 aggregates through autophagy–lysosome degradation pathway (89, 90). Mutations in OPTN have been observed to interfere with the autophagy-mediated clearance of protein aggregates and damaged mitochondria through mitophagy (91, 92).

During autophagy maturation in which autophagosomes fuse with lysosomes, vesicle trafficking is required for the retrograde transport of autophagosomes from axons to the cell body (93) that occurs in a microtubule- and dynein-dependent manner (94, 95). Dynactin-dynein complex is involved in the autophagosome movement in which dynactin functions as an adaptor to link dynein with cargo along the microtubule track (96). Mutations in the dynein light chain impair autophagosomes movement to
lyosomal and reduced life span in a mouse model of ALS (97) whereas point mutations for the p150 subunit of dynactin have been implicated in FALS (98). Mutant SOD1 may also interfere with the autophagy-lysosome pathway as evident from the accumulations of p62 is in the spinal cord suggesting that the autophagic flux might be impeded in ALS (99, 100).

Dysregulations of TDP-43 and FUS may also result in autophagy impairment (101, 102). TAR DNA-binding protein 43 (TDP-43) and Fused in sarcoma (FUS) are ubiquitously expressed RNA-binding proteins that mainly localize in the nucleus but mutant TDP-43 and mutant FUS are sequestered in the cytoplasm of ALS-affected motor neurons and this condition is exacerbated by ER stress (103).

At present, more than 30 autophagy-related proteins (Atgs), which are well conserved from yeast to mammals have been discovered (104). Mutant TDP-43 destabilises and reduces the level of ATG7 mRNA causing autophagy impairment (101) because ATG7 is important in the formation of the autophagosome (105). FUS inhibits the formation of the autophagosome but overexpression of Rab1 restores autophagosome formation (102). FUS regulates Rab1 by binding to the GTPase to affect the stability of the mRNAs (106). C9ORF72 is predicted to contain a ‘Differentially Expressed in Normal and Neoplasia’ (DENN)-like domain, a highly conserved GDP/GTP exchange factor that activates RAB GTPases and hence is likely to be involved in RAB7A regulation of membrane trafficking events (107, 108). Human RAB7A localizes primarily to lysosomes and is found predominantly in endosomes in later/matured stages of multivesicular bodies (MVB) (109) and is involved in the transport of endosomes from early to late endocytic compartments of the cell. C9ORF72 is a Rab1 effector and depletion of C9ORF72 inhibits the translocation of the activated ULK1 complex to the phagophore (110).
Apoptosis

Apoptosis is a programmed cell death that is identified by cell shrinkage, cell surface blebbing, organelle contraction, apoptotic bodies formation, chromatin condensation and nuclear fragmentation (111). As ALS is characterised by the loss of motor neurons, it is suggested that apoptosis is the cause of motor neuron cell death (111). Apoptosis is detected in the brain and spinal cord of SALS individuals by TUNEL staining (112). Cell apoptosis is also increased in SOD1 mutant mice (113, 114). Mutant SOD1 transgenic mice also are able to activate pro-apoptotic proteins, for examples caspase 1 (115) and caspase 3 (116), whereas Bcl-2, an anti-apoptotic protein has been found to delay the disease onset and prolongs SOD1 mutant mice survival (117). Furthermore, elevated levels of AIF (apoptosis induction factor) and cytochrome c in motor neurons of the spinal cord of the SOD1 mouse model have also been observed (116). Nuclear localization of AIF and cyclophilin A have been observed to cause motor neuronal cell death in the SOD1 mouse model (118).

Molecular Pathways of Apoptosis

Fig. 3: Molecular pathways of apoptosis. Three different apoptosis pathways have been identified: the mitochondrial pathway (intrinsic pathway), the death receptor pathway (extrinsic pathway) and the ER pathway.
Three main apoptosis pathways have been recognised, which are the mitochondrial pathway (intrinsic pathway), the death receptor pathway (extrinsic pathway) and the ER-stress pathway. As illustrated in Fig.3, in the mitochondria-dependent apoptotic pathway, the translocations of Bax and Bid from the cytosol to mitochondria induce cytochrome c release from mitochondria to the cytosol to initiate apoptosis. Bid is a BH3-domain-only pro-apoptotic protein and although both the full length and truncated form of Bid (t-Bid) translocate to mitochondria, t-Bid is the most biologically active form of Bid. Cytochrome c then activates caspase-9 in the presence of apoptotic protease-activating factor-1 (Apaf-1) to activate downstream executioner caspases namely caspase-3, caspase-6 and caspase-7. This pathway can be inhibited by the anti-apoptotic protein, Bcl-2 and X-linked inhibitor of apoptosis protein (XIAP) that inhibits the caspase activity of caspase-3, caspase-6 and -7.

Caspases involved in apoptosis are categorised by their mechanism of action and are either initiator caspases (caspase-8 and -9) or executioner caspases (caspase-3, -6 and -7) (119). In the death receptor pathway, the binding of the death receptor ligands for examples, TNF or CD95-ligand to their corresponding death receptors namely, TNF receptor (TNFR)-1 or Fas results in the recruitment of the monomeric procaspase-8 protein and this in turn results in dimerization and activation of caspase-8 (120). Consequently, activated caspase-8 activates downstream executioner caspases, caspase-3, caspase-6 and caspase-7 either directly or indirectly through the cleavage and activation of Bid protein by caspase-8 (121). Chronic and prolonged ER-stress due to an imbalance of calcium homeostasis and the accumulation of aberrant proteins in the ER can cause apoptosis. In the ER-stress apoptotic pathway, caspase-12 can activate executioner caspases, caspase-3, caspase-6 and caspase-7 resulting in motor neuron cell death. The activation of caspase-12 does not depend on the plasma membrane and mitochondria-targeted apoptotic signals. On the other hand, the activation of caspase-1 which is primarily responsible for activation of IL-1, which in turn activates downstream executioner caspases, caspase-3, caspase-6 and caspase-7 and is also partly responsible for the cleavage of Bid to activate the mitochondrial apoptosis pathway. In all pathways, the activation of effector caspases means that the apoptosis event is inevitable therefore, inhibiting the downstream effector caspases do not have any neuroprotective effects on the cells (122).

**Caspases in the ALS neurodegenerative processes**

Caspases are a family of cysteine-aspartate proteases that cleave substrates at specific aspartic acid residues and to date 14 mammalian caspases have been identified as effectors of apoptosis (120). Caspases are synthesised as inactive zymogens (procaspases) and proteolytic cleavage by other caspases is required for caspase activation. Caspase-8 and caspase-9 both cleave procaspase-3 to generate active caspase-3. Caspases can be categorised according to their functions.

The first subfamily consists of caspases-1,-4,-5,-11,-12 and -14 and plays an important role in cytokine maturation. Procaspase-1 is widely distributed in the spinal cord motor neurons and it has been shown that in SOD1 transgenic mice, the activation of caspase-3 occurs concurrently with the loss of motor neurons.
neurons and the glial response (115, 123-125). It has also been observed there was a delay in disease progression and mortality in ALS mice treated with a caspase inhibitor (126). Caspase-4 is localized in the ER membrane and is activated following ER stress (127). Caspase-4 cleaves after Asp174 to form a C-terminal fragment of 25 kDa (CTF25) and although the function of CTF25 is still under studies, it has been demonstrated that inhibition of caspase-4 reduces the activities of downstream caspases, caspase-3 and caspase-7 which, in turn reduce the fragmentation of CTF25 and the clearance of TDP-43 that will affect the neuronal survival (128). Caspase-12 which is also activated upon ER stress was detected in the spinal cord of transgenic SOD1G93A and SOD1G85R mice but not in wild types (40). Further, the motor neurons that are immunopositive for caspase-12 are observed to be condensed, shrunken and vacuolized (129). Caspase-11 regulates the activation of caspase-1 and caspase-3 and is upregulated in the spinal cord of SOD1G93A transgenic mice at the disease onset and remains at the high levels throughout the disease (130). To date the functions of caspase-5 and caspase-14 in ALS are still under investigation.

The second subfamily of caspases includes caspases-2,-3,-6,-7,-8,-9 and -10 and it can be further categorised into initiator and effector caspases. The initiator caspases consisted of procaspases-2,-8,-9 and -10. The deletion of caspase-2 in SOD1G93A transgenic mice did not have an effect on the expression of ALS suggesting that the role of caspase-2 in ALS may be unimportant compared to other initiator caspases (131). The second caspase initiator, procaspase-8 is activated by Fas receptor in the plasma membrane via an adaptor protein, Fas-associated death domain protein (FADD/MORT1) and activated caspase-8 is released into the cytosol to activate downstream executioner caspases, caspase-3 and caspase-7 (121). In transgenic SOD1 mice, activation of procaspase-8 is only observed in the spinal cord at the end-stage of the disease suggesting that caspase-8 may contribute to the late degenerative process (132). Furthermore, caspase-8 levels are significantly increased in the cerebrospinal fluid (CSF) of ALS patients compared with healthy controls and in CSF of ALS patients with limb-onset of ALS but not in bulbar-onset of ALS compared with healthy subjects (133).

Caspase-9, another caspase initiator has an important role in the mitochondria-dependent apoptosis pathway. During intrinsic apoptosis pathway, cytochrome c is released from the mitochondria to the cytosol inducing the activation of caspase-9 in the presence of adaptor protein, apoptotic protease activating factor-1, Apaf1 (134) to form the heptameric backbone of the apoptosome complex causes the recruitment and activation of caspase-9 through dimerization (135, 136). Active caspase-9 initiates apoptosis by cleaving consequently, the activation of executioner caspases-3,-6 and -7 (119). It has also been observed that the caspase-9 levels are significantly increased in the serum derived from ALS subjects compared to controls (137).
Cytochrome c is an evolutionarily conserved peripheral protein, that contains 104 amino acids in mammals and it has a specific function in the transfer of electrons between complex III (ubiquinol: cytochrome c oxidoreductase) and complex IV (cytochrome oxidase) (138). In postmortem of healthy human spinal cords and non-transgenic mice, cytochrome c is confined in the mitochondrial intermembrane/inter-cristae spaces however, it is mislocalized to the cytosol of spinal cords of both ALS patients and SOD1 transgenic mice (123). It has also been reported that in SOD1G93A mice, the translocation of cytochrome c occurs concurrently with the translocation of the pro-apoptotic protein, Bax, thus activating procaspase-9 (129). Studies have shown that caspase-3 and caspase-7 are activated in spinal cords of SOD1 model mice in a time-dependent manner that is concomitant with the onset of the neurodegenerative process (124, 125).

**Bcl-2 family of oncoproteins**

The Bcl-2 family of proto-oncogenes regulates apoptosis and can be classified into three subfamilies. The first subfamily consists of anti-apoptotic genes which are bcl-2, bcl-xL, bcl-w and Mcl-1 that contains all four Bcl-2 homology (BH) domains. The second subfamily consists of pro-apoptotic genes, bax, bak, bax and bcl-xL, which contain BH1 and BH2 domains. The third subfamily also consists of pro-apoptotic genes. This subfamily contains BH3 only domain and its subfamily members include Bim, Bid, Bad, death protein 5/hara-kiri (DPS/Hrk), Puma and Noxa. BH3-only proteins can be further hierarchically categorised into ‘killers’ in which Bid, Bim and Puma directly activate Bak- and Bax-dependent apoptosis, whereas other BH3-only proteins for examples, Bad and Noxa are defined as ‘sensitizers’ which modulate the pathway by sequestrating anti-apoptotic proteins (139). Puma, Bim and Bid bind with high affinity to anti-apoptotic Bcl-2 proteins and this may be related to their relatively high potency in terms of inducing apoptosis compared to other BH3-only proteins (140).

During apoptosis, cytochrome c is released from mitochondria to the cytosol through membrane channels consisting of Bax pro-apoptotic protein (141). The opening of the mitochondrial voltage-dependent anion channel (VDAC), which is also implicated in the release of cytochrome c, is accelerated by pro-apoptotic proteins Bak and Bax (142).

Additionally, anti-apoptotic proteins such as Bcl-2 inhibit cytochrome c release by interfering with membrane insertion and pore formation by Bax or by Bcl-x, that regulates mitochondrial membrane potential and volume (143). Bcl-xL also interacts with caspase-9 and Apaf-1 and inhibits the activation of Apaf1-dependent caspase-9 activation (144). Earlier studies have reported that Bcl-2 mRNA level is significantly decreased and Bax mRNA level is significantly increased in the lumbar cord of human ALS cases and affected transgenic SOD1 model mice (125, 145). Further studies in human ALS cases and
symptomatic transgenic SOD1 mice have also observed that Bcl-2 and Bcl-xL proteins are either unchanged (112, 146) or decreased (147, 148). Further, a study has reported that the expression of Bcl-2 and SOD1 proteins were significantly decreased in lymphocytes from SALS patients than in lymphocytes from age and sex matched controls (149).

On the other hand, the pro-apoptotic Bax and Bad proteins are increased (146-148). Most recently, motor neurons differentiated from human embryonic cells showed increased expression of pro-apoptotic proteins, Bax and caspase 9 after a 48-hours exposure to cerebrospinal fluid (CSF) from ALS patients (150). Bim is a potent activator of apoptosis and directly activates Bax in neurons (151). Further study has observed an upregulation in the mRNA level of the proapoptotic BH3-only, Bim in the spinal cord of post-symptomatic SOD1G93A transgenic mice and only a slight increase in the mRNA levels of Bid and noxa but on the contrary, an upregulation in the mRNA levels of Bcl-2 and Bcl-xL was demonstrated (152). These results are in contrast with the previous findings in which Bcl-2 and Bcl-xL levels were decreased in the mice model of ALS and this may be due to analysis at different stages of the disease or variability in mouse genetic background (153). Another BH3-only protein, Puma, was shown to be significantly upregulated in motor neurons of SOD1G93A mice during early stage of the disease and genetic deletion of Puma significantly improved motor neuron survival and delayed disease onset and motor dysfunction but no significant effect on lifespan was observed in SOD1G93A mice (154).

CONCLUSION

In summary, here we describe a detailed review of the pathogenic mechanisms involved in ALS. The incidence of familial and sporadic ALS is predicted to significantly increase during the next few decades due to aging populations. Thus, understanding the disease mechanism/s leading to motor neurons loss in ALS is paramount. Further, there are lack of effective treatments to prevent or ameliorate ALS and a further understanding of the mechanisms leading to the pathogenesis of the disease would, are essential to develop effective therapies for ALS.
DECLARATIONS

Acknowledgements

This work is dedicated to the memory of Jackie de Belleroche, an intelligent woman with passion and dedication who has contributed to scientific progress in the study of ALS. She was a mentor to all of us in her lab and she will always be. Special thanks to Alex Morris, Midhat Salman and Luigi Montibeller from the Neurogenetics Group for their support and to Majlis Amanah Rakyat (MARA) Malaysia for the PhD financial assistance.

Contribution

IB wrote the manuscript.

Consent for publication

Not applicable.

Competing interest

The author declare that she has no competing interest.

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