Review

Synaptic dysfunction and altered excitability in C9ORF72 ALS/FTD

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is characterized by a progressive degeneration of upper and lower motor neurons, resulting in fatal paralysis due to denervation of the muscle. Due to genetic, pathological and symptomatic overlap, ALS is now considered a spectrum disease together with frontotemporal dementia (FTD), the second most common cause of dementia in individuals under the age of 65. Interestingly, in both diseases, there is a large prevalence of RNA binding proteins (RBPs) that are mutated and considered disease-causing, or whose dysfunction contribute to disease pathogenesis. The most common shared genetic mutation in ALS/FTD is a hexanucleotide repeat expansion within intron 1 of C9ORF72 (C9). Three potentially overlapping, putative toxic mechanisms have been proposed: loss of function due to haploinsufficient expression of the C9ORF72 mRNA, gain of function of the repeat RNA aggregates, or RNA foci, and repeat-associated non-ATG-initiated translation (RAN) of the repeat RNA into toxic dipeptide repeats (DPRs). Regardless of the causative mechanism, disease symptoms are ultimately caused by a failure of neurotransmission in three regions: the brain, the spinal cord, and the neuromuscular junction. Here, we review C9 ALS/FTD-associated synaptic dysfunction and aberrant neuronal excitability in these three key regions, focusing on changes in morphology and synapse formation, excitability, and excitotoxicity in patients, animal models, and in vitro models. We compare these deficits to those seen in other forms of ALS and FTD in search of shared pathways, and discuss the potential targeting of synaptic dysfunctions for therapeutic intervention in ALS and FTD patients.

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1. Introduction

The mutation found in the C9ORF72 (C9) gene is characterized by an expanded GGGGCC (G4C2) hexanucleotide repeat expansion (HRE) in its non-coding region on chromosome 9p21 and represents the most common genetic abnormality in frontotemporal dementia (FTD; 10–30%) and amyotrophic lateral sclerosis (ALS; 20–50%) to date (DeJesus-Hernandez et al., 2011; Renton et al., 2011). This discovery strengthened the already known-yet largely neglected-symptomatic and genetic overlap between a uniformly fatal motor neuron disease and the second most common form of early-onset dementia. Extensive research over the past six years on the role of the (G4C2) n repeat expansion in C9ORF72, has led to the proposal of different disease-causing mechanisms for mutant C9: (1) protein loss-of-function due to haploinsufficient

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expression of C9orf72 mRNA (Ciura et al., 2013; Therrien et al., 2013; Xiao et al., 2015); (2) toxic RNA gain-of-function (Almeida et al., 2013; Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Lee et al., 2013; Sareen et al., 2013; Zhang et al., 2015); and (3) toxicity caused by repeat-associated non-ATG initiated (RAN) translation, which leads to the accumulation of dipeptide repeat (DPR) proteins (Ash et al., 2013; Gendron et al., 2015; Jovicic et al., 2015; Kwon et al., 2014; May et al., 2014; Mizielinska et al., 2014; Mori et al., 2013; Tran et al., 2015; Wen et al., 2014; Yang et al., 2015; Zhang et al., 2014; Zu et al., 2013). Ongoing studies are investigating the cellular consequences of these causative pathways. As an example, a multiple recent studies suggest that there are deficits in nuclear-cytoplasmic trafficking of RNAs and/or proteins due to C9 RNA toxicity or DPR formation (Boeynaems et al., 2016; Freibaum et al., 2015; Jovicic et al., 2015; Khosravi et al., 2017; Liu and Taylor, 2017; Xiao et al., 2015; Zhang et al., 2015). A major consensus of these studies is that the C9 repeat expansion leads to aberrant RNA-processing events, critical for overall cellular health, but also important for the organization of the synapse and its cytoskeletal components, as well as for the regulation of scaffolding and signaling complexes that modulate synaptic strength and function (Iacoangeli and Tiedge, 2013; Liu-Yesucevitz et al., 2011).

The primary physiologic function of neurons is the transmission of information and commands via electrical potentials created by ion gradients. The propagation of those potentials from one neuron to another occurs at the synapse, where a variety of excitatory and inhibitory inputs combine to drive a tightly regulated release of neurotransmitters. Synapse homeostasis is maintained in part by glia, which contribute to neuronal function by mediating neurotransmitter concentrations, eliminating damaged synapses, and maintaining myelin insulation (Barres and Barde, 2000; von Bernhardi et al., 2016). Disruption of the electrochemical gradient has a negative effect not only on the proper propagation of information and neuronal network activities, but on the health of neurons themselves, eventually leading to motor and memory deficits and neuronal cell death.

Cognitive dysfunction observed during normal ageing is known to parallel selective loss of synapses and changes in spine density and morphology (Morrison and Baxter, 2012). Progressive synaptic loss and dysfunction (i.e. synaptopathy) is believed to occur early in disease progression of cognitive disorders including Alzheimer’s disease (AD) (DeKosky and Scheff, 1990; Selkoe, 2002; Terry et al., 1991) and Frontotemporal Dementia (FTD) (Gong and Lippa, 2010; Petkau et al., 2012), and in other neurodegenerative diseases such as Huntington’s disease and Parkinson’s disease (Day et al., 2006; Picconi et al., 2012; Raymond et al., 2011; Villalba et al., 2015)). It is therefore no surprise that synaptic dysfunction is also implicated in ALS, the most common motor neuron disease (Belzil et al., 2016; Cleveland and Rothstein, 2001). It is now believed that ALS and FTD exist on the same spectrum (Belzil et al., 2016; Cleveland and Rothstein, 2001). These diseases are ultimately defined by the degeneration of neurons; in ALS, the motor neurons of the motor cortex and spinal cord, and, in FTD, the cortical neurons of the frontal and anterior temporal lobes. The plasticity of synapses has led to the belief that synaptic loss may be reversible, which raises the possibility of therapeutic intervention early in disease progression prior to neuronal cell death, and warrants studies aimed at identifying the underlying mechanisms of synaptopathy in conjunction with the discovery of novel therapeutic targets (Freeman and Mallucci, 2016).

Here, we review observations regarding synaptic dysfunction in the C9orf72 ALS-FTD spectrum. We will discuss changes of overall neuronal morphology, including dendritic arborization and spine density, as well as deficits in membrane excitability, such as hyperexcitability. We will examine how these alterations lead to neuronal dysfunction of both cortical and motor neurons, the two major neuronal subtypes affected by degeneration in FTD and ALS. We will compare these findings to reports of synaptic deficits in non-C9 ALS/FTD to see whether there are potential overlapping mechanisms between subsets of ALS and FTD patients. Finally, we will briefly discuss putative therapies targeted towards modulating synaptic deficits in ALS/FTD.

2. Aberrant neuronal morphology and synapse formation

The length and complexity of dendrites are common measurements of neuronal health and maturity, yet few studies to date have examined the effects of the C9 repeat expansion on neuronal morphology. Overexpression of poly-GA DPRs in primary mouse cortical neuron cultures led to decreased dendritic arborization, caused by the co-aggregation of the transport factor Unc19, which supports the concept of synaptic dysfunction in C9 disease pathogenesis (May et al., 2014). In a second study, overexpression of a short hexanucleotide repeat construct (5′(G4C2)6−MS2) in rat spinal cord neurons reduced the number of primary dendritic branches specifically for those neurons that showed neuritic expression of repeat RNA foci as determined by RNA FISH analysis (Schweizer Burguete et al., 2015). In the same report, similar dendritic branching deficits were also found in a Drosophila model of (G4C2)6, supporting the hypothesis of synaptic dysfunction as a result of the C9 repeat expansion.

As for non-C9 ALS, a very recent study reported significant synaptic loss in the prefrontal cortex of sporadic ALS patients using high-resolution imaging of postmortem brain tissue samples (Henstridge et al., 2017). Most interestingly, the degree of synaptic loss correlated with the severity of cognitive impairment of the individual patients, and was not due to cortical atrophy, further supporting the idea that synaptic loss occurs before neurodegeneration. This is in strong agreement with observations of synaptic loss in the ventral thalamus of a mouse model deficient of the FTD gene progranulin (Grn−/−) (Lui et al., 2016). Further, mutant superoxide dismutase 1 (SOD1)G370S mature mouse cortical neurons and spinal motor neurons showed reduced dendritic length and complexity, as determined by Sholl analysis, in addition to a reduced spine density (Fogarty et al., 2016; Fogarty et al., 2017; Sgobio et al., 2008; Spalloni et al., 2011). In contrast, early postnatal SOD1G85Q mouse spinal cord motor neurons elongate and branch more rapidly than control motor neurons (Filipchuk and Durand, 2012) suggesting differing synaptopathic mechanisms between the two mutations, or age dependent morphological aberrations, do exist. Interestingly, overexpressing an ALS-associated profilin 1 mutation, PFN1C71G, in mouse hippocampal neurons increases dendritic length, dendritic arborization and spine density (Brettie et al., 2015) while TDP-43Δ357ST mouse cortical neurons show increased bleeding of dendrites without changes in length (Zhang et al., 2016). Genetic manipulations of other disease-associated proteins in different in vitro models support a loss of synaptic integrity in ALS/FTD. For example, mutations of the mitochondrial protein coiled-coil-helix-coiled-helix domain-containing 10 (CHCHD10) have been linked to sporadic and familial ALS/FTD spectrum disease (Bannwarth et al., 2014). Knockdown or overexpression of mutant CHCHD10 in primary hippocampal neurons reduced expression of synaptic marker proteins synaptophysin and drebrin (Woo et al., 2017). Transgenic mice overexpressing mutant fused in sarcoma (FUS)G92A, a well-characterized RNA binding protein with
known mutations causing ALS and FTD (Kapeli et al., 2017), have severely reduced dendritic arborization in spinal motor neurons and reduced spine density in cortical layers IV-V (Sephton et al., 2014). Similar changes were observed in a transgenic FTD mouse model overexpressing ANLS-FUS, which exhibits decreased dendritic spine and synapse density in the frontal cortex in addition to a decrease in synaptic protein expression (Shiihashi et al., 2017). Similarly, knockdown of FUS in primary hippocampal neurons leads to decreased spine density and spine maturity, and reduces the subcellular synaptic expression of post-synaptic density protein 95 (PSD-95) and its interacting protein SynGAP (Yokoi et al., 2017). Similar to FUS, mutations in ubiquilin2 (UBQLN2) can cause both ALS and FTD-like symptoms. Consequently, a transgenic mouse overexpressing mutant UBQLN2 (UBQLN2P497H) showed behavioral cognitive impairments and significant morphological synaptic deficits as demonstrated by protein aggregates in dendritic spines of the hippocampus and frontal cortex and decreased spine density in the molecular layer of the dentate gyrus (Gorrie et al., 2014). Furthermore, there is reduced synapse formation in mutant valosin-containing protein (VCP) ALS patient-derived, induced pluripotent stem cells differentiated into spinal motor neurons (iPSC-MNs) (Hall et al., 2017). These examples highlight the complexity of synaptic dysfunctions and suggest that dendritic phenotypes may differ between subtypes of ALS, between ALS and FTD and between different brain regions and neuronal cell types. More qualitative assessments of dendrite morphologies may be important to discerning specific disease mechanisms (Fig. 1).

Fig. 1. Schematic depiction of morphological deficits contributing to synaptic dysfunction in ALS/FTD. Key features include alterations of dendritic arborization and changes in spine density and spine shape. These deficits have been commonly observed in cortical neurons, hippocampal neurons, and spinal motor neurons of ALS, FTD, and ALS/FTD models.
ical deficits vary with age and muscle location (Tremblay et al., 2017). The authors suggest that reporting methods must be standardized to truly determine the significance of structural changes at the NMJ.

Mouse models of C9orf72 ALS/FTD have shown considerable variation in their ability to recapitulate motor disease. No structural changes or denervation of the neuromuscular junction were observed in either of two mouse models carrying a bacterial artificial chromosome (BAC) containing human C9ORF72 with expanded repeats (O’Rourke et al., 2015; Peters et al., 2015); however, Liu and colleagues report that a third BAC model shows substantial NMJ denervation in mice with fast-progressing disease and subtle morphological abnormalities in mildly affected mice (Liu et al., 2016). Mice injected with an AAV9 virus containing 102 interrupted hexanucleotide repeats show cytoskeletal abnormalities and decreased postsynaptic acetylcholine receptor expression (Herranz-Martin et al., 2017). Finally, NMJs remain intact despite inducible knockout of C9orf72 in neuronal and glial cells of adult mice (Koppers et al., 2015).

Drosophila C9 models more consistently capture motor dysfunction. Most overexpress only the hexanucleotide repeat, which is sufficient to disrupt NMJ structure and function. Developmentally expressing 30 or 58 repeats in motor neurons causes a reduction in the number of active zones (Freibaum et al., 2015; Zhang et al., 2015). 58 repeats also decreased bouton counts and total muscle area (Freibaum et al., 2015; Perry et al., 2017). In addition to the development of structural abnormalities, HRE overexpression can disrupt functional NMJ homeostasis. In the NMJ of ALS fly models expressing mutant TDP-43CAG58 or (CAG58) repeats, expansion, expression of the synaptic vesicle trafficking protein Hsc-70 is post-transcriptionally decreased, resulting in impaired FM1-43 dye uptake, which suggests a common mechanism of synaptic dysfunction between TDP-43 and C9 ALS (Coyne et al., 2017). Flies overexpressing the poly-GR DPRs without the presence of repeat RNA show even more severe NMJ structural deterioration than do flies expressing repeat RNA, and also show increased levels of postsynaptic glutamate receptor subunits (Perry et al., 2017). Thus, flies reveal potential structural and functional mechanisms of NMJ deterioration in C9 ALS.

As for other non-C9 genetic models of ALS, knockdown of the TDP-43 fly-homolog, TBPH, in Drosophila leads to NMJ structural deficits, which can be rescued by inducible TBPH expression at specific time points (Romano et al., 2014). In addition, Drosophila NMJs expressing mutant FUS revealed disorganized presynaptic active zones via electron microscopy, but did not show gross changes in morphology when analyzed via confocal microscopy (Shahidullah et al., 2013). At the same time, mutant and wild type FUS overexpression in mice induces denervation of the NMJ (Septon et al., 2014) and decreased dendritic spine and synaptic density in the frontal cortex, which was observed before neuronal loss had occurred (Shiibashi et al., 2017). Similarly, mutant Ubiquilin 2 (UBQLN2) mice, a model for both ALS and FTD, show decreased spine density and impaired synaptic plasticity (Gorrie et al., 2014) while mutant VAPB mice NMJs show increased bouton counts, increased fragmentation, and increased total area (Larroquette et al., 2015). Interestingly, synaptic overgrowth at the NMJ is also observed in a Drosophila model of ALS/FTD, overexpressing Charged Multivesicular Body Protein 2B (CHMP2B) Intron5, which was rescued by overexpression of the small endosomal GTPase Rab8 (West et al., 2015). Mutations in CHMP2B are found in both ALS and FTD patients (van Blitterswijk et al., 2012), and a transgenic mouse overexpressing mutant CHMP2B (Intron5) in neurons shows both ALS and FTD behavioral phenotypes, including a decreased number of fully innervated NMJs, albeit no significant spinal motor neuron loss (Vernay et al., 2016).

3. Excitotoxicity

Excitotoxicity is broadly defined as toxicity induced by excessive exposure of the brain to the excitatory neurotransmitter glutamate (Olney, 1969). It is considered a synaptic event during which excess glutamate in the synaptic cleft leads to overactivation of post-synaptically localized glutamate receptors, which in turn triggers intracellular cell death cascades. Excitotoxicity is one of the major disease mechanisms ascribed to contributing to the degeneration of motor neurons in ALS (Rothstein et al., 1991; Taylor et al., 2016) (Fig. 2), while less is known about excitotoxic mechanisms in FTD.

One of the underlying mechanisms of excitotoxicity in ALS is the loss of glial glutamate transporters, which, under normal physiological conditions, are responsible for maintaining a tightly controlled glutamate homeostasis in the synaptic cleft, ensuring fast neuronal synaptic transmission (Danbolt et al., 1994; Danbolt et al., 1994). Rothstein and colleagues initially observed increased glutamate levels in ALS patient-derived cerebral spinal fluid and were then able to show selective loss of glutamate transporter EAAT2 in postmortem motor cortex brain tissue of ALS patients when compared to healthy control subjects (Rothstein et al., 1990; Rothstein et al., 1995). The authors proposed that dysregulation of glutamate transporters leads to increased extracellular glutamate levels, which in turn triggers excitotoxic cell death of motor neurons, without the actual loss of the glia cell itself. Subsequent in vitro studies confirmed that the loss of astroglia glutamate transporter function alone was sufficient to cause motor neuron degeneration (Rothstein et al., 1992). The concept of non-cell autonomous disease mechanisms in ALS via astrocyte dysfunction has since been demonstrated in numerous in vivo and in vitro studies, and has revolutionized the way ALS disease is studied and treated (see Therapeutics section, below; (Arbour et al., 2017; Boillee et al., 2006; Darman et al., 2004; Haidet-Phillips et al., 2011; Ilieva et al., 2009; Madill et al., 2017; Marchetto et al., 2008; Nagai et al., 2007)). While most of these studies were done working with mouse models or primary cultured cells from mutant SOD1 transgenic mice, recent data support the idea of astrocyte-mediated motor neuron cell death in other cases of familial ALS, as well as sporadic ALS (Pehar et al., 2017). For example, treatment of spinal motor neurons with mutant TDP-43-expressing astrocyte conditioned media increases levels of reactive oxygen species, which can be reversed using sodium channel blockers, such as riluzole (Rojas et al., 2015).

Additional contributing factors to excitotoxicity in ALS are thought to be via intrinsic neuronal deficits at the synapse caused by altered glutamate receptor expression or receptor dysfunction, leading to excessive calcium influx and subsequent neuronal cell death. Many studies addressing this aspect of excitotoxicity have focused on the expression, distribution, and function of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) in the spinal cord of ALS patients. The AMPARs are ionotropic glutamate receptors responsible for fast synaptic transmission in the central nervous system (CNS) (Henley and Wilkinson, 2016). Functional properties of AMPARs depend greatly on the composition of their subunits, GluA1-4, which determines their effect on synapse formation, stabilization and synaptic plasticity (Huganir and Nicoll, 2013). Research has focused on the GluA2 subunit because of its ability to regulate the Ca2+-permeability and the trafficking of AMPARs (Bennett et al., 1996; Hollmann et al., 1991; Wright and Vissel, 2012). GluA2 renders GluA2-containing AMPARs Ca2+-impermeable, and it controls constitutive and activity-dependent AMPAR endocytosis and synaptic plasticity (Malinow and Malenka, 2002). Although most AMPARs in the adult brain contain the GluA2 subunit and thus are Ca2+-impermeable,
the Ca\(^{2+}\)-permeable AMPARs in mature neurons are most likely regulated during synaptic plasticity (long term potentiation (LTP) and long term depression (LTD)) and under stress (Henley and Wilkinson, 2016; Man, 2011; Sanderson et al., 2016; Shepherd, 2012). Most AMPARs become Ca\(^{2+}\)-permeable by the removal of GluA2 (Wenthold et al., 1996). However, numerous reports suggest that GluA2-containing AMPARs become Ca\(^{2+}\)-permeable due to unediting of GluA2 RNA at the Q/R site (Melcher et al., 1995; Melcher et al., 1996; Nishikura, 2010; Sommer et al., 1991; Washburn et al., 2014). GluA2 editing at this site is accomplished by the editing enzyme ADAR2, a member of the adenosine deaminase family acting on RNA (ADAR) protein family (Keegan et al., 2004; Seeburg et al., 1998). ADARs deaminate adenosine in double-stranded RNA transcripts, thus changing the nucleotide from an adenosine to an inosine (Bass and Weintraub, 1987; Kim et al., 1994; Melcher et al., 1995; O’Connell et al., 1995). Under basal physiological conditions, GluA2 is fully edited, but under certain physiological or pathological conditions, it is unedited (Wright and Vissel, 2012). Lack of GluA2 editing and the subsequent increase in Ca\(^{2+}\) permeability of AMPARs has been associated with vulnerability of neurons to cell stressors, such as ischemia, and in neurodegenerative diseases, such as AD and ALS (Gaisler-Salomon et al., 2014; Grosskreutz et al., 2010; Hideyama et al., 2012b; Kawahara et al., 2004; Kwak and Kawahara, 2005; Peng et al., 2006; Takuma et al., 1999).

Kwak and colleagues have been instrumental in elucidating the role of ADAR2 function and GluA2 Q/R editing deficiencies in ALS and were able to show that, in laser-captured spinal motor neurons of sporadic ALS patients, GluA2 Q/R has reduced editing efficiency, which is correlated with reduced levels of the editing enzyme ADAR2 (Kawahara et al., 2003; Takuma et al., 1999). Similar results were obtained in a recent study analyzing spinal motor neurons from a single mutant FUS ALS patient (Aizawa et al., 2016). Follow-up studies using conditional ADAR2 knockout mice and human tissue propose that reduced GluA2 Q/R editing and subsequent increase in AMPAR calcium permeability is responsible for motor neuron deficits in the spinal cord, as well as TDP-43 pathology due to calcium-activated, calpain-induced fragmentation of full length TDP-43 (Aizawa et al., 2010; Hideyama et al., 2010, 2012a; Yamashita and Kwak, 2014). An alternative hypothesis proposed that calcium influx via NMDA receptors leads to calpain-mediated ADAR2 cleavage and subsequent changes in GluA2 Q/R editing efficiencies, which in turn increases AMPAR calcium permeability and neuronal cell death (Mahajan et al., 2011). Increased AMPAR calcium permeability was further suggested to be due to relatively lower levels of GluA2 mRNA, as well as the limited calcium buffering capacity of motor neurons in general (Carriedo et al., 1996; Van Damme et al., 2002; Virgo et al., 1996). Ongoing studies in our laboratory and by other research groups are examining the role of AMPARs in C9ORF72 ALS/FTD and will hopefully provide additional information on this important excitotoxic pathway. Pervious studies in our laboratory using C9 patient derived hiPSC motor neuron cultures showed increased susceptibility to glutamate toxicity, supporting a contribution of AMPARs to excitotoxic cell death in C9 ALS/FTD (Donnelly et al., 2013). Similar findings have been confirmed in a recent study looking at increased neuronal cell death upon specific AMPA-receptor activation using patient derived C9 ALS iPSCs with corresponding isogenic control lines (Selvaraj et al., 2018). Interestingly, this study shows that the C9 mutation leads to increased mRNA and protein expression of the GluA1 subunit of AMPA receptors compared with increased Ca\(^{2+}\) permeability of C9 iPSC motor neurons. No GluA2 Q/R editing deficits were found in these neurons, suggesting that the increased Ca\(^{2+}\) permeability and increased susceptibility to AMPA-mediated cell death is due to an increase in GluA2 lacking AMPA receptors. Using RNAscope imaging technology the authors confirmed increased GluA1 transcript expression in C9 ALS spinal cord autopsy tissue, but not C9 patient prefrontal cortex tissue samples, suggesting that this change in AMPA receptor subunit composition is specific to motor neurons and might explain motor neuron susceptibility in ALS.

Additional studies support a role of AMPA receptors and excitotoxicity in ALS as well as FTD. For example, the gene linked to juvenile-onset, autosomal recessive ALS (ALS2), the ALS2 gene, binds to the glutamate receptor interacting protein 1 (GRIPI). Loss of ALS2, mimicking ALS2 disease, leads to decreased plasma membrane expression of GluA2 and subsequent increased neuronal susceptibility to glutamate excitotoxicity (Lai et al., 2006). Similarly, phosphatase and tensin homolog (PTEN), a negative regulator of the rapamycin pathway, is thought to be involved in motor neuron survival. PTEN knockdown decreases GluA1, GluA2, and GluA3 expression in induced pluripotent stem cells differentiated into...
spinal motor neurons and thereby decreases AMPA-induced toxicity (Yang et al., 2014). Misfolded SOD1 in SOD1<sup>G93A</sup> mouse spinal cord binds to Na<sup>+</sup>/(K+)/ ATPase-α<sub>3</sub>, leading to decreased GluA2 expression and increased size of cholinergic synapses (Ruegsegger et al., 2016). Altered Na<sup>+</sup>/(K+)/ATPase-α<sub>3</sub> expression was confirmed in spinal cord tissue of both sporadic and familial ALS patients (Ruegsegger et al., 2016). Changes in AMPA receptor subunit composition were also found in a mouse model of FTD, generated by forebrain-specific expression of mutant CHMP2B (Gascon et al., 2014). In a time-dependent manner and correlated to FTD-like behavioral and pathological features, the mice showed increased mRNA and protein levels of GluA2, GluA3 and GluA4, but not GluA1, a finding that was confirmed in behavioral variant FTD patient-derived iPSC cortical neurons. Via electrophysiological examination of acute mouse brain slices, the authors then showed that this altered AMPA receptor subunit composition led to more Ca<sup>2+</sup>-impermeable AMPA receptors in the mouse prefrontal cortex. These data strongly suggest that synaptic AMPA receptor function might be altered differently in motor neurons from ALS patients versus cortical neurons from FTD patients. It will be of great interest to understand whether such differences are present in C9 ALS/FTD patients, where both motor neurons and cortical neurons are affected.

Despite its high Ca<sup>2+</sup> permeability and significant involvement in excitotoxic events in other neurodegenerative diseases, especially stroke, the N-methyl-D-aspartate (NMDA) receptor has not been discussed to the same extent as AMPA receptors in ALS or FTD related disease mechanisms (Spalloni et al., 2013), and no studies have been published to date to link NMDA receptor dysfunction to C9-mediated disease pathogenesis. Initial studies by Courratier and colleagues suggested that differences in expression of the GABA receptors GABA<sub>A</sub> receptor α<sub>1</sub> and α<sub>2</sub> between spinal motor neurons and oculomotor neurons in ALS patients may indicate pathological differences in inhibitability, which might explain selective neurodegeneration of motor neurons (Comley et al., 2015). In addition, voltage gated calcium channel subunit mRNAs and high voltage activated calcium channels were found to be upregulated in SOD1<sup>G93A</sup> mouse spinal cord (Chang and Martin, 2016). Knocking down FUS in primary mouse neurons leads to reduced levels of GluA1 mRNA, which in turn reduced AMPA receptor surface expression and basal synaptic transmission as well as a reduction in mature mushroom-shaped spines, suggesting that loss of function of FUS greatly impacts synaptic function (Udagawa et al., 2015).

### 4. Altered neuronal excitability

The culmination of synaptic deficits is a change in the neuron's ability to receive and propagate electrical signals. Therefore, alterations on the overall neurophysiological level are to be expected in diseases characterized by synaptopathy. Here we will summarize studies supporting the presence of altered intrinsic neuronal excitability and its contribution to disease pathogenesis in C9 ALS and FTD. Almost all ALS patients, familial and sporadic, present with neurophysiological alterations described as hyperexcitability, which is detected early during disease progression and often recedes at later stages of the disease (Bae et al., 2013b; Kiernan, 2009). Hyperexcitability in neurons is often described as a condition during which the neuron is unusually or excessively excitable, frequently reducing the threshold to fire action potentials upon stimulation. The mechanisms leading to hyperexcitability are multifactorial, and include altered inhibitory inputs from GABAergic interneurons, intrinsic changes of neuronal sodium (Na<sup>+</sup>) and potassium (K<sup>+</sup>) channel function, and extrinsic impairments of the extracellular potassium homeostasis due to the dysfunction of perisynaptic astrocytes (Do-Ha et al., 2017; Geervasinga et al., 2016). Cortical hyperexcitability is measured with the use of transcranial magnetic stimulation (TMS; Hallett, 2000) and can be detected in both sporadic and familial ALS patients, including C9ORF72 patients, as well as some subsets of FTD patients, suggesting that divergent mechanistic pathways lead to altered neuronal physiological function shared among all patients on this spectrum disorder (Bae et al., 2013a; Bae et al., 2013b; Geervasinga et al., 2015c; Vucic et al., 2008; Wainger and Cudkowicz, 2015). Interestingly, while cortical hyperexcitability was confirmed via TMS to be present in symptomatic C9ORF72 ALS patients, it was absent in asymptomatic C9ORF72 expansion carriers.
repeat carriers (Geevasinga et al., 2015b), similar to what had been reported for SOD1 ALS patients (Vucic et al., 2010). Furthermore, cortical hyperexcitability detected by TMS was only associated with C9orf72 ALS patients but not with C9 behavioral variable FTD (bvFTD) or C9 ALS-FTD patients (Schanz et al., 2016). These data suggest that TMS measures of cortical excitability could be used to detect early onset of ALS disease and could potentially be used to facilitate detection of disease onset of asymptomatic familial patients. In addition, these studies provides evidence that, despite their commonalities, alterations in cortical excitability may differ between ALS and FTD, even in familial cases sharing the same genetic cause (Fig. 3).

In addition to cortical hyperexcitability, there is also axonal excitability, which is often referred to as peripheral nerve excitability, or peripheral hyperexcitability (Do-Ha et al., 2017; Park et al., 2017). Measurements of axonal excitability are performed via the generation of compound muscle action potential (CMAP) responses and the identification of a threshold necessary to produce a specific target response (Park et al., 2017). Changes in axonal excitability are known to occur in both familial and sporadic ALS patients and are thought to contribute to clinical symptoms such as fasciculation and muscle cramps (Layzer, 1994). Increased axonal excitability has been reported in symptomatic C9 ALS patients, similar to sporadic ALS patients, but was unchanged in asymptomatic C9 ALS patients, similar to what had been reported for cortical hyperexcitability (Geevasinga et al., 2015a).

In vitro studies using hiPSC-derived motor neurons confirmed the presence of altered neurophysiological properties in familial forms of ALS and validated the use of patient-derived neurons to study mechanisms of aberrant neuronal excitability. Specifically, hyperexcitability was found in young hiPSC-MNs derived from familial ALS patients (SOD1, FUS, C9orf72) (Waigner et al., 2014), and decreased excitability was detected in C9ORTF72 hiPSC-MNs at an older age of differentiation (Sareen et al., 2013). These early findings were confirmed in a study of the physiological properties of hiPSC-MNs at different time points of differentiation that showed initial hyperexcitability followed by progressive loss of action potential output and decreased synaptic activity in both C9 and mutant TDP-43 hiPSC-MNs (Devlin et al., 2015). However, no apparent changes in basal cell survival were found, which further supports the notion that changes in excitability indicate early functional loss of motor neurons prior to cell death. This hypothesis was further confirmed by an in vivo study of 140 ALS patients comparing compound muscle action potentials (CMAP), as a measure of motor neuron loss to nerve excitability indices (Iwai et al., 2016).

Mouse models of C9orf72 haploinsufficiency have failed to recreate motor dysfunction or altered excitability at the NMJ. C9orf72 homozygous and hemizygous knockout mice do not differ from wild-type mice in the CMAP of the foot flexor muscle (O’Rourke et al., 2016), nor in resting EMGs and myogenic motor evoked potentials of the hind limbs (Jiang et al., 2016). Alternatively, C9 fly models overexpressing the C9 hexanucleotide repeat expansion show alterations in evoked junctional potentials (Zhang et al., 2015), indicating impaired neurotransmitter release at the NMJ, and overexpression of poly-GR DPRs suppresses both spontaneous and evoked responses (Perry et al., 2017). This suggests that a toxic gain of function of RNA foci or DPRs impairs neuromuscular junction transmission in C9 ALS.

Changes in neuronal excitability can also be detected in non-C9 ALS disease models. SOD1G93A mouse cortical neurons show impaired long term potentiation (LTP) (Spalloni et al., 2011), and, although hyperexcitability in this model is not specific to corticospinal neurons, increased calcium permeability is (Kim et al., 2017). SOD1G93A mouse diaphragm muscle NMJs were also found to be hyperexcitable pre-symptomatically (Rocha et al., 2013). This may be attributable to the same mechanisms by which oligomeric mutant SOD1 increases excitability through suppression of potassium currents in Aplysia californica ganglion (Zhang et al., 2017). An alternative cause of synaptic dysfunction at the muscle is that increased muscarinic sensitivity of perisynaptic Schwann cells in
the SOD1<sup>C37R</sup> mouse soleus impairs NMJ plasticity and repair (Arbour et al., 2015). Similar to what was found in regards to the fact that NMJ morphological deficits vary with age and muscle location, changes in electrical propagation vary spatially and temporally (Tremblay et al., 2017). TDP-43<sup>A315T</sup> mouse cortical neurons also show disinhibition and hyperexcitability (Zhang et al., 2016), while Drosophila motor neurons expressing mutant FUS or FUS knockouts show enhanced firing; however, transmission across the NMJ is severely decreased (Shahidullah et al., 2013). Interestingly, hippocampal CA1 pyramidal cells examined in brain slices obtained from one month old UBQLN2<sup>F408H</sup> transgenic mice exhibited hyperexcitability (Radzicki et al., 2016), highlighting the fact that different brain regions may show different forms of aberrant neurophysiological properties and synaptic dysfunction. It would be interesting to see whether, in this model of FTD, cortical hyperexcitability or axonal excitability exists in addition to hippocampal hyperexcitability. Overall, more mechanistic studies are required to understand what leads to these neurophysiological changes and whether the mechanisms are shared between subtypes of ALS and FTD patients.

5. Therapeutic approaches aimed at synaptic dysfunction

Targeting synapse loss and synaptic dysfunction in clinical trials in ALS have mostly focused on the excitotoxic mechanisms of the disease. Riluzole, a sodium channel blocker, is one of only two FDA approved treatments for ALS to date. Although it does not reverse any symptoms, on average it delays disease progression and prolongs life span by approximately three months. Riluzole, which has been ascribed many different routes of therapeutic action, has been extensively reviewed (Bellingham, 2011).

Other potential targets in this pathway were unsuccessfully tested in clinical trials as disease modifiers: memantine (NMDA receptors; (de Carvalho et al., 2010)), topiramate (brain vasculature; (Cudkowicz et al., 2003)), lamotrigine or mexiletine (sodium channels; (Ryberg et al., 2003)(Shibuya et al., 2015)) and gabapentin (GABA receptors; (Miller et al., 2001)). A phase 2 trial of the AMPA receptor antagonist talampanel was initially positive in slowing disease, but a larger phase 3 trial showed no benefit (Pascuzzi et al., 2010). Perampanel, an approved AMPAR antagonist, was effective in halting disease progression and reversing pathology in conditional ADAR2 knockout mice (Akamatsu et al., 2016) and is currently being tested in a clinical Phase 2 trial for sporadic ALS (https://clinicaltrials.gov/ct2/show/NCT03019419). Activation of the glutamate transporter was unsuccessful in improving disease progression when studied as a potential therapy using the FDA-approved beta lactam antibiotic ceftriaxone in a Phase 3 clinical trial, despite showing significant protection in pre-clinical studies by upregulation of glutamate transporter protein (Berry et al., 2013; Rothstein et al., 2005). The search for ALS therapeutics has extended to other types of ion channels. Retigabine, a Kv7 channel opener, has been shown to correct hyperexcitability and prolong survival in C9ORF72, SOD1, and FUS familial ALS patient-derived iPSC-MNs (Wainger et al., 2014). Based on these data, Retigabine is currently being tested in ALS patients in a Phase 2 trial (https://clinicaltrials.gov/ct2/show/NCT02450552).

In addition to targeting receptors or ion channels, recent clinical trials have been approved to study excitability in ALS patients, either as a diagnostic marker of the disease or as a potential biomarker to accompany pharmacological clinical trials. As an example, patients are currently being recruited to measure excitability of spinal neurons by measuring electromyogram (EMG) activity in combination with electrical and/or magnetic stimulation of peripheral nerve and/or cortical structures (https://clinicaltrials.gov/ct2/show/NCT02429492?cond=ALS&draw=1&rank=8; (Turner and Kiernan, 2012)). Finally, a recent study described the use of a positron emission tomography (PET) ligand to quantify synaptic densities in patients with temporal lobe epilepsy (Finnema et al., 2016). The radioligand, which was targeting synaptic vesicle glycoprotein 2A (SV2A; [(11)C]UCB-J), showed good imaging properties and was sensitive enough to detect changes in the patient population. One could imagine that such pre-symptomatic approaches could assist as diagnostic disease biomarkers detecting early neuronal dysfunction before any behavioral symptoms appear.

6. Concluding remarks

In conclusion, possibly due to the renewed awareness of the genetic, pathologic, and clinical overlap between ALS and FTD, more attention has been given to mechanisms of synaptic dysfunction and neuronal excitability in ALS/FTD patients, in particular those carrying the C9ORF72 hexanucleotide repeat expansion mutation. Other shared genetic mutations, including mutations in C9orf72, FBXW7, SQSTM1, UBQLN2 and CHMP2B have already, or will likely in the near future, demonstrated overlapping disease mechanisms leading to cortical neuronal dysfunctions and/or altered axonal excitability of motor neuron axons connecting to the muscle via the NMJs. These dysfunctions are likely to occur early during disease progression, before any significant neuronal degeneration or loss. Because of this early appearance, a better understanding of the underlying mechanisms and a search for tools to detect and monitor these deficits will provide new opportunities to develop novel early stage-interfering therapeutics and to clinically diagnose ALS/FTD patients before the presence of any behavioral symptoms, respectively.

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