PERSPECTIVE

Novel small molecule TRVA242 targets neuromuscular junction in amyotrophic lateral sclerosis

Research over the past decade has enabled a deeper understanding of the pathophysiology of amyotrophic lateral sclerosis (ALS). While 10% of all ALS cases have been reported to be familial with a clear Mendelian inheritance, clinically, sporadic and familial forms of ALS cannot be distinguished (Robberecht and Philips, 2013). Presently there are only two Food and Drug Administration approved treatment options for ALS - riluzole and radicava (also known as edavarone). Riluzole is mostly known to delay the onset of ventilator dependence and extends the life span by 2–3 months; edavarone on the other hand has been reported slow disease progression at all stages in ALS (Jaiswal, 2019). However, given the multifaceted nature of ALS, there is an urgent need to identify more molecules with a strong therapeutic potential.

The four major genes that have emerged bearing the most number of mutations are chromosome 9 open reading frame 72 (C9ORF72), transactive response binding DNA element (TARDBP), superoxide dismutase 1 (SOD1) and fused in sarcoma (FUS). Interestingly, the same genes have been implicated in frontotemporal dementia-another neurodegenerative disease with cognitive and behavioural impairments. Often ALS and frontotemporal dementia are considered as opposite ends of the spectrum of disorder. Nonetheless, it is amply clear that no single genetic causality can be attributed for ALS; ALS rather results from defects in multiple cellular processes arising from mutation in these genes e.g., excitotoxicity, RNA processing defects, mitochondrial dysfunction, accumulation of protein aggregates, axonal transport defects, oxidative stress, etc. (Robberecht and Philips, 2013). Infact, edvarone has been reported to act by scavenging peroxyl radicals, inhibiting motorneuron death and reducing oxidative stress (Ito et al., 2008). However, where and how the motor neuron (MN) deficits arise and what factors aggravate them is still debatable. Whether motor dysfunction starts in a die-forward (anterograde) mechanism involving degeneration of MNs in the cortex (possibly resulting from excitotoxic mechanisms) followed by distal neuromuscular junction (NMJ) defects or a die-back (retrograde) path starting distally at the neuromuscular junction or nerve terminal and progressing towards the cell body is still debatable (Moloney et al., 2014). Nonetheless both these hypotheses have highlighted the importance of the NMJ and it has emerged as a key player in the pathophysiology of ALS.

The NMJ is envisioned as a tripartite synapse comprising the presynaptic MN, the postsynaptic muscle cell and surrounding glial cells; their perfect, co-ordinated dynamics assure efficient transmission of action potentials from the MNs to the muscle cells. Defects in any of the three components can lead to NMJ disassembly and there is mounting evidence of early NMJ dysfunction in ALS. The most accepted notion of ALS pathology is that early deficits arise at the NMJ and precede neuronal degeneration, MN loss and manifestation of clinical symptoms. The progress of this pathology has been most extensively studied in a murine model of ALS - the humanized SOD1 G93A transgenic mouse. These animals show denervation at the NMJ, followed by MN axonal deficits and loss of MN cell bodies before progressive paralysis and death (Fischer et al., 2004). Additionally, the fast-fatigable MN subtype displays enhanced vulnerability to synaptic deficits compared to other subtypes suggesting an even more selective defect that arises at the NMJ before MN degeneration (Pun et al., 2006). Murine models of FUS also point to an early time-point of NMJ dysfunction wherein conditional expression of the human mutation in FUS (FUS P525L) leads to a significant decrease in synaptic vesicle numbers and axonal retraction in fast-fatigable MN (Sharma et al., 2016). Similarly, in C9ORF72 BAC transgenic mice, RNA foci and di-peptide repeat expansions precede MN degeneration (Ciura et al., 2013). These studies provide crucial insights to the fundamental understanding of ALS pathophysiology, and reaffirm the importance of targeting early dysfunction at the NMJ as a potential therapeutic approach.

Despite significant advances in our knowledge of ALS pathophysiology, another question that still remains open is whether mutations in ALS related genes lead to gain- or loss-of-function mechanisms? As human pathology can only be studied at end stages, much of our understanding on this has come from studies involving small animal models. These models have enabled us to appreciate the variety of phenotypes associated with specific genetic mutations and discern to a certain degree whether a particular genetic mutation leads to a gain- or loss-of-function or what we see as a manifested clinical symptom can arise from both. For example, embryonic motor behaviour which manifests as early as 12–15 hours in the form of spontaneous coiling has been found to be increased or decreased in zebrafish overexpressing mutant human forms of SOD1 or TARDBP respectively (Benedetti et al., 2016; Bose et al., 2019b). Similar overexpression of mutant human forms of TARDBP result in a compromised fidelity of synaptic neurotransmission at the NMJ, whereas overexpression of mutant human forms of SOD1 or zebrafish carrying a mutation in the SOD1 gene leads to an increased synaptic neurotransmission at the NMJ in 48 hours post-fertilization (hpf) zebrafish embryos. Upon complete removal of *tardbp* and *tardbpl* a drastic decrease in synaptic transmission at the NMJ is observed (Bose et al., 2019a). Nonetheless, at 48 hours hpf, both overexpression of mutant human forms of TARDBP or complete removal of *tardbp* and *tardbpl* leads to defects in locomotor activity suggesting that zebrafish models of ALS serve as strong hypothesis-generating platforms especially for early deficits at the NMJ.

Information from rodent animal models are still considered the benchmark for preclinical validation of disease mechanisms. Nonetheless, small animal models are gaining an increasing popularity owing to their ease of genetic manipulation and amenability to small molecule library screening. However, very few studies adopt a strategy that effectively encompass multiple ALS deficits spanning multiple models thereby limiting the success of a treatment strategy in a sporadic population. The study by Patten et al. (2017) has adopted such an approach to test the neuroleptic efficacy of pimozide in multiple animal and genetic models of ALS. Pimozide is a drug that was originally approved by the US Food and Drug Administration for schizophrenia and is a D2 dopamine receptor antagonist but is known to have other targets as well. Pimozide was found to rescue locomotor deficits in multiple animal models of ALS possibly by antagonizing T-type calcium channels, independently of the genotype, suggesting it could be effective in sporadic ALS. A small randomized clinical trial with sporadic ALS patients confirmed the stabilization of motility and target engagement at the NMJ.

Using a similar strategy, a second study was done utilizing the NMJ as a biomarker to assess the potential of a pimozide derivative-TRVA242 (Boseet al., 2019b). From a high throughput screening in *C. elegans* of 3756 novel small molecules derived from or structurally related to pimozide, 11 hits were confirmed in a TDP-43 gain-of-function zebrafish model and TRVA242 was identified as the most potent compound in improving locomotor activity. Thereafter, TRVA242 was subject to exhaustive analysis in multiple C. elegans, zebrafish and a mouse model of ALS (**Figure 1**). TRVA242 was found to rescue spontaneous coiling deficits in zebrafish overexpressing mutant human TARDBP or SOD1 by increasing and decreasing (respectively) the coiling frequency to WT levels. It was also found to recover locomotor deficits and spinal MN outgrowth deficits in these zebrafish models at 48 hpf. In a zebrafish loss-of-function model, in which both orthologues of TARDBP (*tardbp* and *tardbpl*) had been deleted (*tardbp*$^{-/-}$*tardbpl*$^{-/-}$), TRVA242 recovered deficits in small distance movement at 48hpf in double mutant embryos. Additionally, a C9ORF72 zebrafish model of ALS characterized by reduced locomotor activity (at 5 days post-fertilization) and aberrant motorneuron outgrowth, also showed improvement upon chronic treatment with TRVA242.

To enable a deeper understanding of the engagement of TRVA242 at the NMJ, electrophysiological recordings were ob-

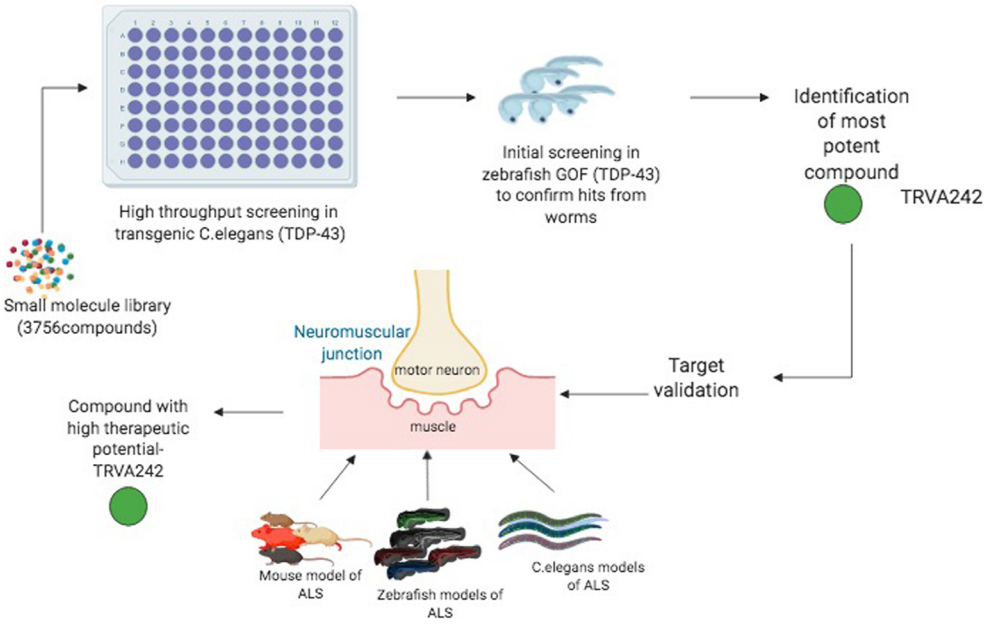

Figure 1 Schematic showing the multi animal model inclusion for screening and identification of small molecules (TRVA242).
Small molecule libraries (3756 compounds) were first screened in a *Caenorhabditis elegans* (*C. elegans*) TDP-43 model through a high throughput screening assay and positive hits were tested on zebrafish models of ALS. Individual *C. elegans* TDP-43 worms were put in 96 multiwell plates containing test compounds and incubated for 6 hours (*C. elegans*) or 24-well plate and incubated overnight (zebrafish). Eleven compounds were identified that improved motor behavior (motility) phenotypes in mutant TDP-43 (mTDP43) *C. elegans*. These positive hits were then tested on mTDP-43 zebrafish at various concentrations (1–20 μM). Eleven active small molecules were identified in our zebrafish model, and the most potent compound was found to be TRVA242, which was active at 5 μM. For details see Bose et al. (2019b). ALS: Amyotrophic lateral sclerosis; GOF: gain-of-function; TDP-43: transactive response DNA-binding protein 43 kDa.

tained from fast twitch muscle cells from both TARDBP or SOD1 overexpressing zebrafish animals at 48 hpf. TARDBP overexpressing animals displayed a reduced cholinergic neurotransmission at the NMJ and TRVA242 treatment significantly restored the deficits. Interestingly, SOD1 overexpressing animals displayed significantly increased neurotransmission at the NMJ. Previous studies suggest a form of hyperexcitability in SOD1 overexpressing models and riluzole-mediated inhibition of the persistent pacemaker sodium current enhanced motor unit functioning by reducing interneuron and motorneuron hyperexcitability (Benedetti et al., 2016). Nonetheless, we found, TRVA242 significantly recovered both synaptic abnormalities, suggesting two hypotheses of target engagement at the NMJ. Either TRVA242 engages a target that is common to both SOD1 and TDP-43 mediated pathology in zebrafish models of ALS or acts at multiple cellular targets in individual pathogenic pathways associated with either TDP-43 or SOD1. A similar NMJ stabilizing effect of TRVA242 with improved efficacy compared to Pimozide in the SOD1 mouse model suggests that the actions of TRVA242 are conserved across species and that TRVA242 could also target multiple distinct mechanisms of dysfunction in ALS spanning multiple animal models.

Since TRVA242 is structurally related to pimozide, it could present a class of compounds targeting a wide range of cellular substrates. Such compounds have an increased propensity of success in a more sporadic population presenting a range of genetic mutations and varied forms of the disease. Albeit, before going to the clinic further exhaustive studies involving pharmacokinetic, pharmacodynamics, behavioral outcomes and other evaluations of TRVA242 are needed. Nonetheless, TRVA242 presents as a strong therapeutic consideration for NMJ tailored therapies in ALS.

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