Development and validation of a model for investigation of transcellular trafficking of the ALS gene product TDP-43

Erin Lynch

A thesis submitted for the partial fulllment of the requirements for the degree of Master of Research in Advanced Medicine

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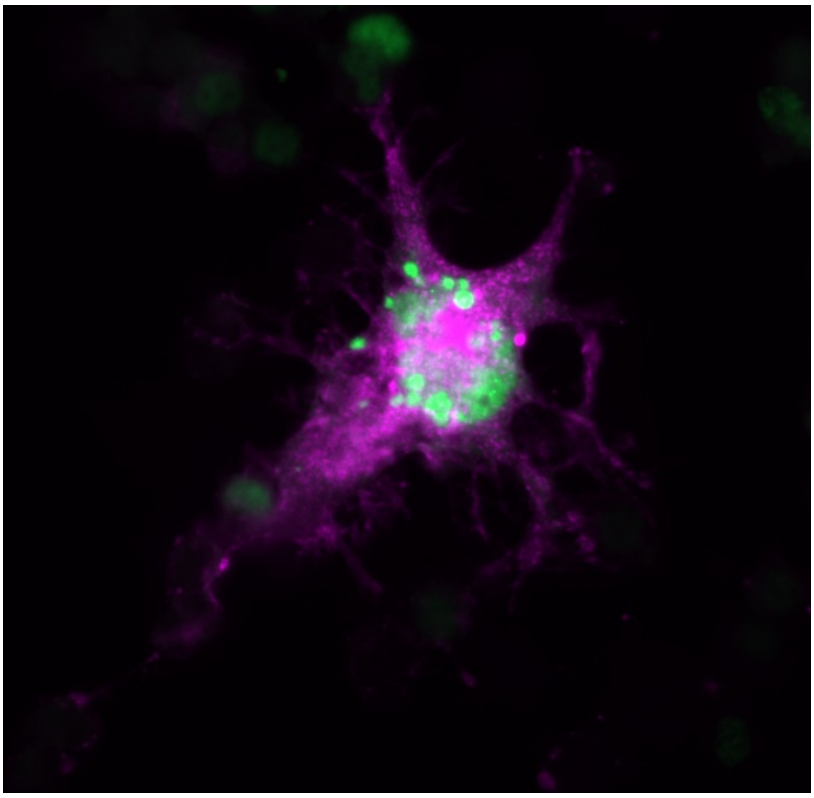

Figure 1 NSC-34 Cell stably expressing a TDP43::GFP fusion protein.

Supervisors: Dr Simon McMullan[1] and Professor Roger Chung[1]

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Declaration of Originality

I certify that the work in this thesis entitled “Development and validation of a model for investigation of transcellular trafficking of the ALS gene product” does not contain, without appropriate knowledge, any material previously submitted for a degree or diploma in any university, except where due reference is made.

I also certify that this thesis does not contain, to the best of my knowledge, any material previously published or written by another person, except where due reference is made.

Erin Lynch

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Abstract

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease that affects the upper and lower motor neurons. ALS is an adult onset disease, which progressively worsens after onset, leading to severe loss of motor function and eventually death. Mutations in the gene encoding TDP-43, an RNA-binding protein, have been identified in patients with heritable ALS. The mutant form of TDP-43 is associated with neuronal degeneration and intracellular aggregate formation *in vitro*. Aggregates have also been identified within patient tissues: it is proposed that mutant TDP-43 drives aggregate formation and that the aggregates play a role in the neuronal dysfunction and eventual death that underlie the clinical features of ALS.

One paradox in the field of ALS research is that neuronal loss is largely restricted to neurons related to motor control, but that degeneration is progressive and occurs at all levels of the neuraxis. A mechanism that could potentially underlie the selective spread of dysfunction through functionally discrete pathways is self-assembly and propagation of cytotoxic proteins. We hypothesize the mutant TDP-43 may drive aggregate formation in affected cells and could potentially move between cells via a prion-like mechanism.

The specific aims of this thesis are to; (1) generate a plasmid construct that encodes a GFP fusion protein of wild-type and mutant TDP-43, allowing the direct visualization of TDP-43 distribution in live cells. (2) To generate vectors that drive stable and permanent integration of those fusion proteins. (3) To test whether GFP is detectable in cells that are co-cultured with TDP-43 mutants.

Introduction

Motor neurone disease (MND) is the term for a large group of disorders characterized by primary degeneration of motor neurons. There are different types of MND however Amyotrophic Lateral Sclerosis (ALS) or Lou Grieg's Disease is the most common form. ALS is an adult onset disease, usually diagnosed between the ages of 40 and 60 years, which progressively worsens, ultimately leading to muscle weakness, atrophy, paralysis and ultimately death by respiratory failure within 3 to 5 years from diagnosis (Kiernan et al., 2011). The overall prevalence of ALS is between 1 and 4 per 100,000, with an increased incidence associated with age. Recent epidemiological data from the United States indicate very low prevalence of ALS in persons under 40 years of age, but a doubling of incidence for every decade until 80 years of age (Mehta et al., 2014). In Australia, the prevalence is 2.75 per 100,000 (AIHW, 2011). The prevalence for Europe in 2.16 per 100,000 in general however the incidence is higher in men 3 per 100,000 compared to 2.4 per 100,000 for women (Logroscino et al., 2010). Although ALS affects individuals of all demographic backgrounds, white males and individuals of European ancestry are significantly more likely to develop ALS.

Although sporadic ALS is common, a family history of ALS is a major risk factor for development of the disease, leading to a concerted research effort to identify genetic markers of the disease. As a result, a large number of candidate genes have been identified. One major challenge in this field is to determine molecular, cellular, and systemic consequences of mutants identified from patient populations. This challenge is the major focus of my research project.

The hallmarks of MND are a combination of upper and lower motor neuron loss (Rowland and Shneider, 2001;Kiernan et al., 2011). Lower motor neurons are located within the ventral horn of the spinal cord and brain stem motor nuclei and directly innervate skeletal muscle fibres. Upper motor neurons are cortical or brainstem neurons that project directly or indirectly to lower motor neurons, driving their activity and ultimately recruiting muscle contraction. Loss of upper motor neurons is characterised by loss of dexterity and slowness of movement, whereas lower motor neuron loss is accompanied by muscle weakness, atrophy, and fasciculation (Bruijn et al., 2004). In most cases, patients present with limb weakness and wasting (>60%: Logroscino et al., 2010). A small percentage present with flaccid or spastic dysarthria and dysphagia indicative of bulbar onset ALS (20%: (Kiernan et al., 2011) and in addition to what are thought of as pure motor symptoms it is now predicted that around 50% of ALS patients will also have some cognitive deficits typically memory and language impairment, executive dysfunction, and some behavioural abnormalities (Hardiman et al., 2011).

There is no definitive clinical test used to diagnose ALS (Rowland, 1998). Some of the clinical test used include; the El Escorial criteria (Brooks et al., 2000), nerve conduction studies (Brownell et al., 1970), electromyography (Thompson et al., 1987), transcranial magnetic stimulation (Claus et al., 1995), motor unit number estimation (McComas et al., 1971;Shefner, 2001) and strength tests(Colombo et al., 2000), Norris Scale(Guiloff, 2002;Mora, 2002), and Appel Scale(Appel et al., 1987) are only a few examples. Each test has its limitations as a diagnostic tool so many clinicians use multiple tests to diagnose ALS (Winhammar et al., 2005).

The major neuropathological features of ALS are; (1) degeneration of the cortico spinal tract and extensive loss of lower motor neurons from the anterior horns of the spinal cord (Brownell et al., 1970;Hughes, 1981). (2) The degeneration and loss of neurons in the primary motor cortex which project axons to the lower motor neurons(Udaka et al., 1986). (3) Reactive gliosis of glial cells within the motor cortex and spinal cord in the areas where there is degeneration (Schiffer et al., 1996).(4) Intracellular inclusions within neurons and glia (Rowland, 1998). (5) Impaired firing of motor neurons (Kiernan et al., 2011).

There are neither current biomarkers nor diagnostic tests that are available for ALS. As recently reviewed by Vucic et al. (2014), a large number of prospective treatments for ALS have been trailed, including cell replacement therapy and gene therapy techniques, with only very limited success. There is only one treatment available; Riluzole which is an anti-glutamatergic compound. However it is an ineffective treatment boasting a modest prolonged survival of patients for 3-6 months (Bensimon et al., 1994;Lacomblez et al., 1996).

Charcot who described motor neuron disease in 1869 first proposed that MND affected only motor neurons and started in one area and then spread to others (J-M Charcot, 1869). Since then there have been 4 hypotheses proposed to explain the pathophysiology of ALS; dying forward, dying backward, independent degeneration and the newest prion-like propagation hypothesis. The dying forward hypothesis proposes that ALS is a disorder primarily of the corticomotor neurons and that the degeneration of the anterior horn neurons is due to anterograde glutamate excitotxicity (Eisen et al., 1992). Conversely the dying back hypothesis is that ALS is primarily a disorder of the lower motor neurons, with the pathogens retrogradedly transported back up to the upper motor neurons and the cortex (Chou and Norris, 1993). Independent degeneration hypothesis suggests that both the upper and lower motor neurons degenerate and that this occurs independently (Rowland and Shneider, 2001) and the newest hypothesis that of the ‘prion-like’ propagation aims to explain the contiguous spread of ALS, that it is via neuron to neuron transmission of pathogenic proteins (Polymenidou and Cleveland, 2011).

The aetiology of ALS remains unclear and what is known to date has been largely determined through the study of genes identified within familial cases (Atkin and Paulson, 2014). Sporadic ALS (SALS) cases represent ~90% of all ALS cases and familial (FALS) cases are the remaining 10% of cases. However the pathology of the familial and sporadic cases are essentially indistinguishable (Andersen and Al-Chalabi, 2011). Electrophysiological studies in humans have shown that there is a hyperexcitiability of the motor system in the early stages of ALS (Daube, 2000) and even pre-symptomatic ALS in mouse models (Kuo et al., 2004). Neuronal aggregates is a hallmark of neurodegenerative diseases and many cellular mechanisms have been implicated in disease pathology in ALS including; protein aggregation, impaired axonal transport, mitochondrial dysfunction, endoplasmic reticulum stress, glutamate excitotoxicity, neuron inflammation and microglia activation, oxidative stress and RNA metabolism(Wood et al., 2003;Shaw, 2005;Sreedharan et al., 2008;Vance et al., 2009;Baloh, 2011;Alami et al., 2014).

One paradox in the field of ALS research is that neuronal loss is largely restricted to neurons related to motor control, but that degeneration is progressive and occurs at all levels of the neuraxis.

Although ALS was first described in 1896 by Charcot progress in elucidating causes of ALS was slowly progressing. That was until 1993, when the first gene to be implicated in familial cases of ALS was identified. Cu/Zn Superoxide Dismutase 1 (SOD1) was the first gene identified by Rosen et al. (1993),and subsequently 160 mutations of the SOD1 gene have been identified (Bruijn et al., 2004). The focus of ALS research was able to switch to molecular mechanisms of ALS pathology particularly with the SOD1 mutation and the subsequent identification that mutant SOD1 can have a toxic effect (Clement et al., 2003;Atkin et al., 2014).

With the refinement of genetic sequencing and techniques 15 other genes have been identified having a link with either FALS or SALS (**Table 1**). One of the most notable genes that has been identified since 1993 is TARDBP (Sreedharan et al., 2008;Van et al., 2008). This started a major breakthrough in linking disease mechanisms. The TARDBP gene codes for a protein TDP-43, that was identified as a component in ALS pathology in 2006 (Arai et al., 2006). This linked a genetic mechanism with a possible pathological mechanism.

Table 1 Genotype-phenotype correlation for ALS and FTD genes

Gene	Chromosome	Phenotype
SOD1	21q22.11	ALS-FTLD
ALS2	2q33.2	ALS
SETX	9q34.13	ALS
VAPB	20q13.33	ALS
CHMP2B	3p12.1	ALS-FTLD
ANG	14q11.1	ALS-FTLD
FIG4	6q21	ALS
TARDBP	1p36.22	ALS-FTLD
FUS	16p11.2	ALS-FTLD
OPTN	10p14	ALS-FTLD

ATXN2	12q23-q24.1	ALS
C9orf72	9p21.2	ALS-FTLD
SIGMAR1	9p13	ALS-FTLD
UBIQULN2	xp11.21	ALS-FTLD
VCP	9p13	ALS-FTLD

Table adapted from Al-Chalabi and Visscher (2014). ALS -Amyotrophic lateral sclerosis. FTLD -Frontotemporal lobar dementia.

Human Trans-activation response (TAR) DNA- binding protein of 43kDa (TDP-43) is encoded by the *TARDBP* gene on chromosome 1 (Cohen et al., 2012). TDP-43 is 414 amino acid protein encoded by 6 exons (Wang et al., 2008),it has highly conserved RNA recognition motifs: RRM1 and RRM2 (Buratti et al., 2005), nuclear localization and nuclear export signal within the N-terminal and a C-terminal tail that is glycine rich mediates protein-to protein interactions (Ayala et al., 2008) (**Figure 2**).There has been great interest in TDP-43 and many efforts to elucidate the physiological functions. It is still unknown what all the processes that TDP-43 is involved in and it is unknown how TDP-43 contributes to ALS. TDP-43 is extensively expressed in nearly every tissue and is highly conserved among both mammals and invertebrates (Baloh, 2011) and is essential for embryonic development (Dewey et al., 2012).

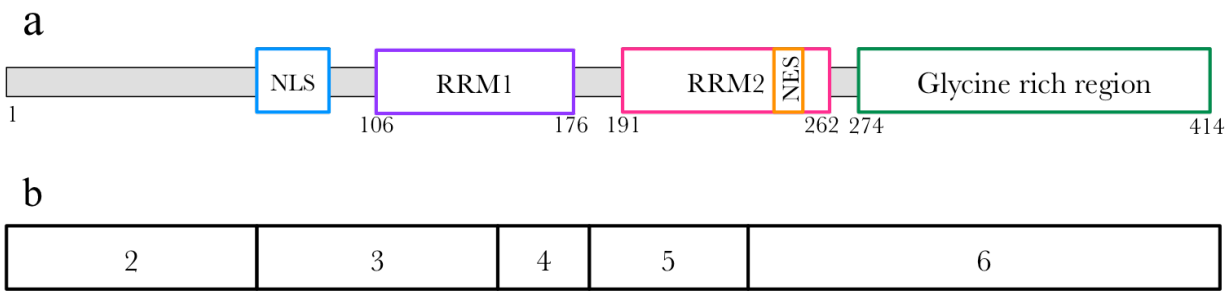


Figure 2 (**A**) Schematic of TDP-43 Protein. The majority of the mutations lie within the glycine rich region, which is also where the prion like domain is. NLS: Nuclear location signal, RRM1: RNA recognition motif 1, RRM2: RNA recognition motif 2, and NES: this is the predicted nuclear export signal. (**B**) Schematic of the TDP-43 exons. Schematic adapted from Lagier-Tourenne et al. (2010)

Mutations in the TARDBP gene have been identified in patients with ALS, in both FALS and SALS cases(Neumann et al., 2006;Kabashi et al., 2008;Sreedharan et al., 2008) and are thought to account for 5% of FALS and 0.5-2% of SALS. Pathological TDP-43 is present in FTLD providing further evidence that ALS may be part of a continuum of neurodegenerative disorders rather than discrete disorders (Arai et al., 2010). Currently

there are ~44 mutations in the TARDBP gene that have been identified as pathogenic (**Table 2**) (Gitcho et al., 2008;Kabashi et al., 2008;Sreedharan et al., 2008;Van et al., 2008;Chen-Plotkin et al., 2010). The majority of these mutations are missense and occur within the glycine rich C-terminal (**Figure 2**).

The TDP-43 protein is normally localized to the nucleus of the cell, however it does move between the nucleus and cytoplasm on a regular basis(Nakashima-Yasuda et al., 2007). All of the functions that TDP-43 is involved in are not yet fully understood nonetheless some of the normal functions have been identified. TDP-43 is structurally similar to heterogeneous ribonucleaicproteins (hnRNPs) and is involved in multiple aspects of RNA/DNA processing including transcription, splicing, transport and translation (Wegorzewska and Baloh, 2011).

Pathological TDP-43 is aggregated within cytoplasmic inclusions in patient motor neurons and their surrounding glial cells(Mackenzie et al., 2010). Pathological TDP-43 is not only aggregated but, phosphorylated, ubiquitinated and abnormally cleaved (Neumann et al., 2006;Kwong et al., 2007;Neumann, 2009). Although it is known that TDP-43 is essential to the normal function of the cell what isn't known is the mechanism behind the pathological TDP-43 and how it affects cells. There are currently three hypotheses; 1.Toxic gain of function, the toxicity caused by the cytoplasmic aggregates of TDP-43 (Johnson et al., 2009) 2. Loss of nuclear function, the mislocalisation of TDP-43 into the cytoplasm impedes the essential nuclear function of the protein (Wang et al., 2008). 3. Prion-like hypothesis, where the protein can move from neuron to neuron causing the neurodegeneration to spread (Polymenidou and Cleveland, 2012)

The theory that protein aggregates are a fundamental causal pathology has been widely debated within the literature(Zhang et al., 2009;Cushman et al., 2010;Polymenidou and Cleveland, 2011;King et al., 2012;Walker et al., 2013). This debate stems from the fact that the evidence of protein aggregation within patients suffering from ALS comes from post mortem tissues. The main point of contention is that the aggregate formation could be the very last thing that occurs within this disease.

We hypothesize that the mutant TDP-43 may drive aggregate formation in affected cells and could potentially move between cells via a prion like mechanism.

The specific aims of this thesis are to; (1) generate a plasmid construct that encodes a GFP fusion protein of wild-type and mutant TDP-43, allowing the direct visualization of TDP-43 distribution in live cells. (2) To generate vectors that drive stable and permanent integration of those fusion proteins. (3) To test whether GFP is detectable in cells that are co-cultured with TDP-43 mutants.

To achieve these aims we will express fluorescent fusion proteins of wild type and mutant TDP-43 in cultured neurons and (a) examine differences in the expression patterns and (b) examine evidence for trafficking of mutant proteins. To this end we have constructed fluorescent fusion proteins of the wild type and mutant gene sequences, and have inserted these sequences into a plasmid vector that will transpose the fusion protein. Our intention is to express this construct in individual cells in cultured conditions and assess the consequence of expression of the mutant gene. We also intend to express our construct in

a homogenous population and determine whether it is of use in a high throughput application.

Table 2 TARDBP mutations and the reported phenotype

Mutation	Exon	Location	Phenotype
A90V	3	N-terminal	SALS
D169G	4	RNA recognition motif 1	SALS
K263E	6	Intron	FTLD
N267S	6	Intron	SALS/FTLD
G287S	6	Glycine rich region	FALS
G290A	6	Glycine rich region	FALS
S292N	6	Glycine rich region	SALS
G294A	6	Glycine rich region	FALS/SALS
G294V	6	Glycine rich region	SALS
G295S	6	Glycine rich region	FALS/SALS/FTLD
G295R	6	Glycine rich region	SALS
G298S	6	Glycine rich region	FALS
M311V	6	Glycine rich region	FALS
A315T	6	Glycine rich region	FALS
A321V	6	Glycine rich region	SALS
A321G	6	Glycine rich region	SALS
Q331K	6	Glycine rich region	SALS

S332N	6	Glycine rich region	FALS
G335D	6	Glycine rich region	SALS
M337V	6	Glycine rich region	FALS/SALS
Q343R	6	Glycine rich region	FALS
N345K	6	Glycine rich region	FALS
G348C	6	Glycine rich region	FALS/SALS
G348V	6	Glycine rich region	FALS
N352S	6	Glycine rich region	FALS
N352T	6	Glycine rich region	FALS
G357S	6	Glycine rich region	SALS
M359V	6	Glycine rich region	FTLD
R316S	6	Glycine rich region	SALS
P363A	6	Glycine rich region	SALS
Y374X	6	Glycine rich region	SALS
G376D	6	Glycine rich region	FALS
N378D	6	Glycine rich region	FALS
N378S	6	Glycine rich region	SALS
S379C	6	Glycine rich region	SALS
S379P	6	Glycine rich region	FALS
A382P	6	Glycine rich region	SALS
A382T	6	Glycine rich region	FALS/SALS

I383V	6	Glycine rich region	FALS
G384R	6	Glycine rich region	FALS
W385G	6	Glycine rich region	FALS
N390D	6	Glycine rich region	SALS
N390S	6	Glycine rich region	SALS
S393L	6	Glycine rich region	FALS/SALS

Table adapted from Janssens et al. (2011). SALS: Sporadic ALS, FALS: Famillial ALS and FTLD:Frontotemporal Lobar Degeneration.

Methods

Experiment 1: Induction of TDP-43 aggregates in NSC-34 cells

Overview: NSC-34 cells were transfected with plasmids containing human TARDBP (wild-type or M337V mutation). The transfected cells were treated with different stressors to determine whether the intracellular localisation of the protein could be altered and in turn whether it was possible to induce intracellular aggregates containing TDP-43 protein in this cell model.

Table 3 Plasmids and the abbreviations used in the aggregation experiments

Plasmid	Abbreviation
pCMV_ac_TADRBP_wt_GFP_pA	CMV_WT
pCMV_ac_TARDBP_M337V_GFP_pA	CMV_M337V

Maintenance of NSC-34 cell culture

NSC-34 cells (Cashman NR et al, Dev Dyn 1992) were incubated in Dulbeccos modified eagle medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin/streptomycin (Sigma), incubated at 37^{o}C and 5% CO_2, and passaged into new flasks when they reached 90% confluence (typically every 3 – 4 days). For passaging, the medium was aspirated and the cells were washed in 5 ml phosphate-buffered saline (PBS: Gibco). The cells were detached from the flask with 1.5 ml Trypsin (Sigma), which was then inhibited with 5 ml DMEM. The 6.5 ml of suspended cells was transferred into a 15 ml falcon tube and centrifuged (Allegra X-15, Beckman Coulter) @1000rpm for 5 minutes to form a pellet. The supernatant was aspirated and the pellet was re-suspended in 5 ml fresh medium and gently agitated to remove clumps. Cells were split at 1/10 density into a new 75 cm^2 flask containing 10 ml DMEM and returned to the incubator.

Amplification and purification of plasmids from glycerol stock

CMV_WT and CMV_M337V plasmids (**Figure 3**) (Origene) containing ampicillin resistance genes were prepared from glycerol stocks of DH5α *E. coli* containing each plasmid (kindly provided by Dr Ian Blair, ASAM, Sydney). To make a starter culture a sterile pipette was used to scrape a sample of frozen cells and incubated for 8 hours at 37^{o}C with shaking at 200 rpm in 5 ml of Luria Bertani (LB) broth containing 100 μg/ml ampicillin. 200 μl of starter culture was then added to 25 ml LB broth containing 100 μg/ml ampicillin and incubated while shaking for 12 hours.

Plasmid DNA was purified using a Bioline miniprep kit according to the manufacturers protocol. The concentration and purity of the plasmid DNA was detected using UV

spectrophotometry. The absorbance of 1.5 µl of plasmid DNA were measured at 260 and 280 nm using a NanoDrop™ (Thermo Fisher Scientific). From this 20 µl samples containing 0.5 µg of plasmid DNA were sent to Macrogen (Korea) for Sanger sequencing on an ABI3730XL (Life technologies). The raw chromatogram trace files were analysed using Sequencher software/ Genious 6.0.3 (Biomatters)

Following the confirmation that the correct plasmids were present large-scale preparation was carried out using a Midi prep kit (Invitrogen) following the manufacturer's instructions, after which the DNA pellet was resuspended in 500 µl dH_20. Plasmids were stored at -20 ^oC until used.

Lipofectamine transfection of NSC-34 cells

The day before transfection 13 mm glass cover slips were coated with poly-l-lysine (0.01%, Sigma) and cells were seeded at a concentration of 1 x 10^5/ml in cell medium in a 24 well plate. To assess cell density for seeding, cells were counted using an automated cell counter Countess (Invitrogen) that utilizes trypan blue (Sigma).

Transfections were completed using Lipofectamine® 2000 (LifeTechnologies) as per reagents instructions. Briefly, 50 µl/well of OptiMEM (Life Technologies) was combined with DNA (0.5 µg/well) in an Eppendorf tube and incubated for 5 minutes at room temperature. 50 µl/well of OptiMEM was combined with Lipofectamine2000 (1 µl/well) and incubated for 5 minutes in a second Eppendorf tube. The DNA and Lipofectmine®2000 mixes were combined and left for 20 minutes at room temperature. Cell media was then gently aspirated from the cells and replaced by 100 µl of the DNA and Lipofectamine® mixture and 400 µl DMEM containing 10% FBS and no antibiotics. Plates were replaced in incubator for 4 hours after which the transfection media was aspirated and replaced with normal cell media for 24 hours.

Induction of TDP-43 aggregates in NSC-34 cells

To induce TDP-43 aggregate formation, two common experimental stressors were used: 4 hours 50 mM KCl or heat shock at 43^oC for 1 hour. The control was no addition of any stressor. A stock solution of KCl was diluted in normal cell media and 500 µl of this was added per well the final concentration was 50 mM. After the experiment the media was aspirated and half of the cells were fixed immediately in 4% paraformaldehyde (PFA) and the rest were incubated overnight in fresh cell media and subsequently fixed with 4% PFA.

Histological analysis of TDP-43 transfected NSC-34 cells

Coverslips were mounted with Prolong Gold with DAPI nuclear stain (Life Technologies), sealed with clear nail polish, and stored at 4 ^oC. Cells were imaged with a Zeiss fluorescence microscope with Zen acquisition software. Cells (n=100) were scored as to whether they had nuclear or cytoplasmic location of the TDP-43. It was also recorded whether there were large intracellular inclusions (>1 µm) of TDP-43 and their location (ie: nucleus or cytoplasm). Data was analysed using Graphpad prism, Chi-Square test with a Fishers exact was used. Data are from three repeat experiments unless otherwise stated.

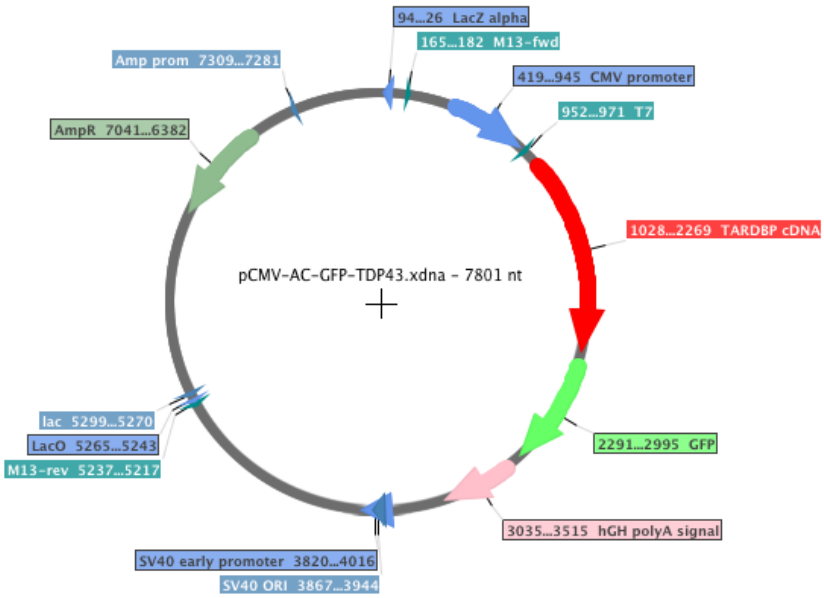


Figure 3 Vector map for CMV:TARDBP plasmids from Dr Ian Blair. The plasmid from Origene and the M337V mutagenesis was completed by the Blair Lab using Agilent Quikchange XL kit according to manufacturer's instructions.

Experiment 2: Generation of Tol2 plasmids for stable expression of TDP-43 variants

Overview

To complement the transient transfection studies undertaken in Experiment 1, experiments were undertaken to generate more permanent transgene expression of TDP43. This was undertaken by generating a plasmid construct that incorporates a Tol2 sequence, resulting in integration of the artificial transgene into the host genome (**Figure 4**).

Table 4. Plasmids generated with Tol2 sites for stable expression

Plasmid	Abbreviation
pTol2P2A_CMV_mCherryCAAX_pA	Tol2_mCH
pTol2P2A_CMV_hTDP-43_wt_EGFP_pA	Tol2_WT
pTol2P2A_CMV_hTDP-43_M337V_EGFP_pA	Tol2_M337V
pCS2FA_transposase	pCS2+

Table 5. Entry clones for Tol2 Plasmids with corresponding att sites

Plasmid	Entry Clone	att sites
pTol2P2A_CMV_mCherryCAAX_pA	p'5_CMV/SP6	attL4, attR1
	pME_mCherry_CAAX	attL1, attL2
	p'3_pA	attL3, attR2
	pDEST_pTol2P2A	attR3,attR4
pTol2P2A_CMV_hTDP-43_wt_EGFP_pA	p'5	attL4, attR1
	pME_hTDP-43_wt	attL1, attL2
	p'3_EGFP_pA	attL3, attR2
	pDEST_pTol2P2A	attR3,attR4
pTol2P2A_CMV_hTDP-43_M337V_EGFP_pA	p'5	attL4, attR1
	pME_hTDP-43_M337V	attL1, attL2
	p'3_EGFP_pA	attL3, attR2
	pDEST_pTol2P2A	attR3,attR4

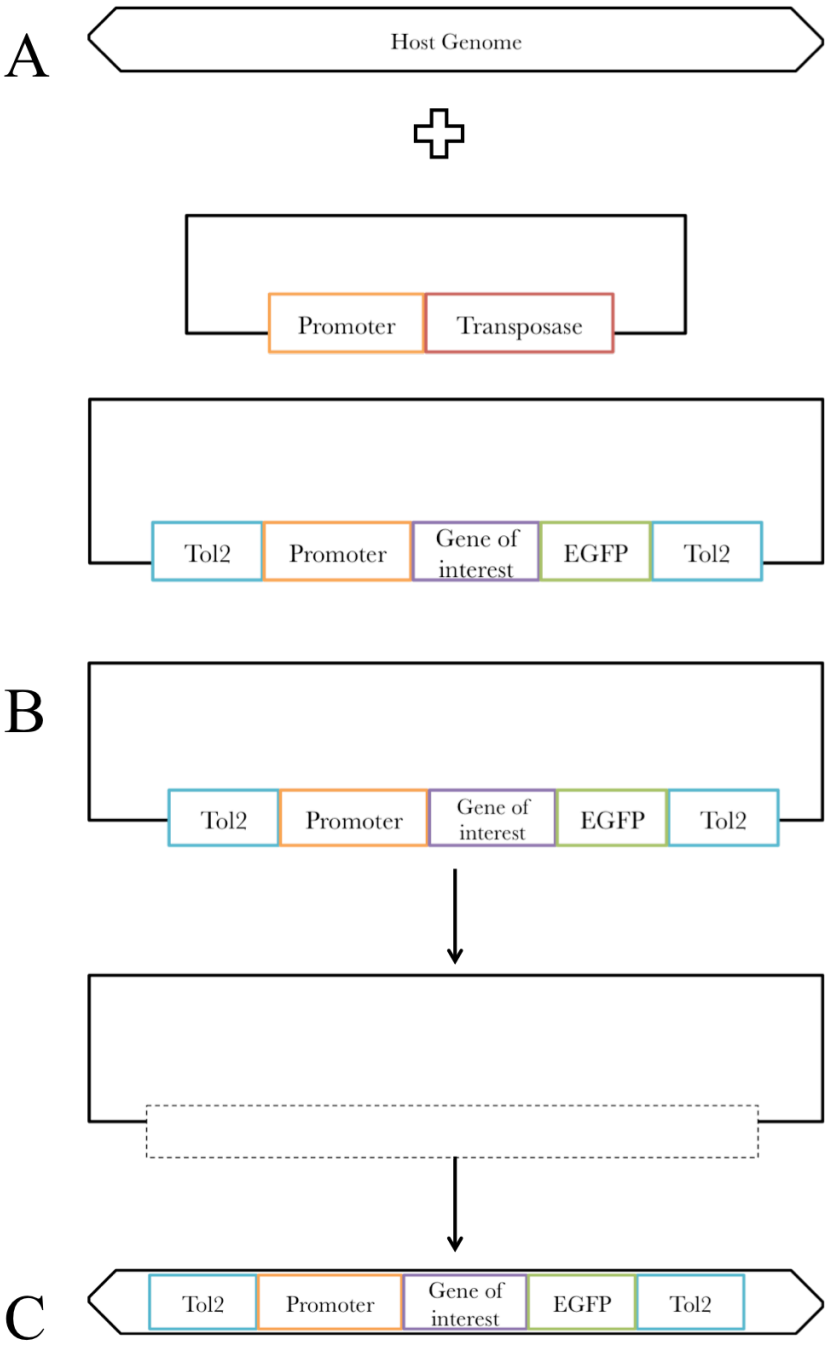


Figure 4 Schematic of Tol2 integration into host genome. (**A**) The Tol2 plasmid and Transposase plasmid are introduced into a cell. (**B**) The transposase plasmid excises the Tol2 plasmid containing our gene of interest. (**C**) This is then integrated into the genome of the cell.(Kwan et al., 2007)

Plasmid generation- LR cloning

Tol2 plasmids were constructed using a multisite gateway cloning technique from Invitrogen. This uses att sites and allows the specific recombination of three plasmids at one time into a single destination vector (**Figure 5**). The specific entry clones used are

listed in **Table 5**. Entry clones for Tol2 Plasmids with corresponding att sites and contained att sites; p5'E contained an attL4 and attR1, pME contained attL1 and attL2 and the p3'E contained an attR2 and attL3. The multisite gateway LR reaction recombination was performed using 60 ng of DNA. In one tube 60 ng of the p5'E, p3'E, pME and pDEST for the selected plasmid was combined with 2 µl of LR Clonase II plus and supplemented with dH_20 for a final volume of 10 µl. The reaction was incubated at 25 ^oC for 24 hours and was inhibited with proteinase K (2 ng/µl) at 37 ^oC for 10 minutes.

The transposable element, which is responsible for the integration of our fusion protein, was a pCS2+ transposase plasmid (**Figure 7**). This was prepared from glycerol stock kindly provided by Dr Nick Cole Lab (ASAM, Sydney) following the same method as mentioned in the above section with 100mg/ml of ampicillin as the selection antibiotic.

Plasmids were then transformed into competent bacteria using the heat shock method as follows: α-Select chemically competent cells (Bioline) were thawed on ice; 2 µl of plasmid DNA was gently added to the cells and incubated on ice for 5 minutes. The cells were immersed in a water bath at 42 ^oC for 30 seconds and immediately returned to ice. 250 µl of room temperature Super Optimal broth with Catabolite-repression (SOC) medium was added to the cells before recovery for 1 hour at 37 ^oC, shaking at 200 rpm. After recovery 200 µl of cells were spread over the surface of an LB agar plate containing 100 mg/ml of ampicillin or 50 mg/ml Kanamycin. Plates were inverted and incubated overnight at 37 ^oC.

A colony was picked from the growth plate with a pipette tip, which was then used to innoculate 5 ml of LB broth containing 100 mg/ml ampicillin for 16 hours at 37 ^oC shaking at 200 rpm. Small-scale preparation of the plasmid DNA was completed as described above. The final plasmid was eluted twice in 30 µl of dH_20 and concentration and purity was detected by UV spectrophotometry.

The plasmids were then assessed with a restriction digest/ diagnostic digest before sequencing. Restriction endonucleases were used to cut plasmid DNA at restriction sites. The digestion reaction for the TDP-43 constructs was as follows; 1 µl EcoR1 restriction endonuclease (New England Biolabs), 2 µl of EcoR1 10x buffer (New England Biolabs), and 500 ng/µl of plasmid DNA were made up to a volume of 10 µl with dH_20. The digestion for the Tol2_mCH was the same as for the TDP-43 however the restriction endonuclease was 1 µl StuI (New England Biolabs) and 2 µl of StuI1 10x buffer (New England Biolabs). This was incubated at 37^0C for 30 minutes before being run out on an agarose gel to separate the digested fragments.

The plasmid DNA fragments cut by restriction digestion were separated using gel electrophoresis. DNA samples were separated in a 1% (w/v) agarose gel in 1x TBE buffer. DNA samples with 2 µl of 1x loading dye were loaded into the wells, with the adjacent well filled with 5 µl of Easy Ladder II (Bioline). Electrophoresis was carried out at 100-130 V for ~30 minutes. DNA bands were visualised under a UV illumitation using a Gel Documentation system (GelDock, BioRad).

Samples were sent of for sequencing to the Australian Genome research facility (AGRF, Melboure) for Sanger sequencing. Using applied biosystems 3730 and 3730xl capillary

sequencers. The raw chromatogram trace files were analysed using Sequencher software/ Genious 6.0.3 software (Biomatters)

Following the confirmation that the correct plasmids were present large-scale preparation was carried out using a Maxi prep kit (Qiagen) following the manufacturer's instructions, after which the DNA pellet was resuspended in 1000 µl dH_20. Plasmids were stored at -20 ^oC until used.

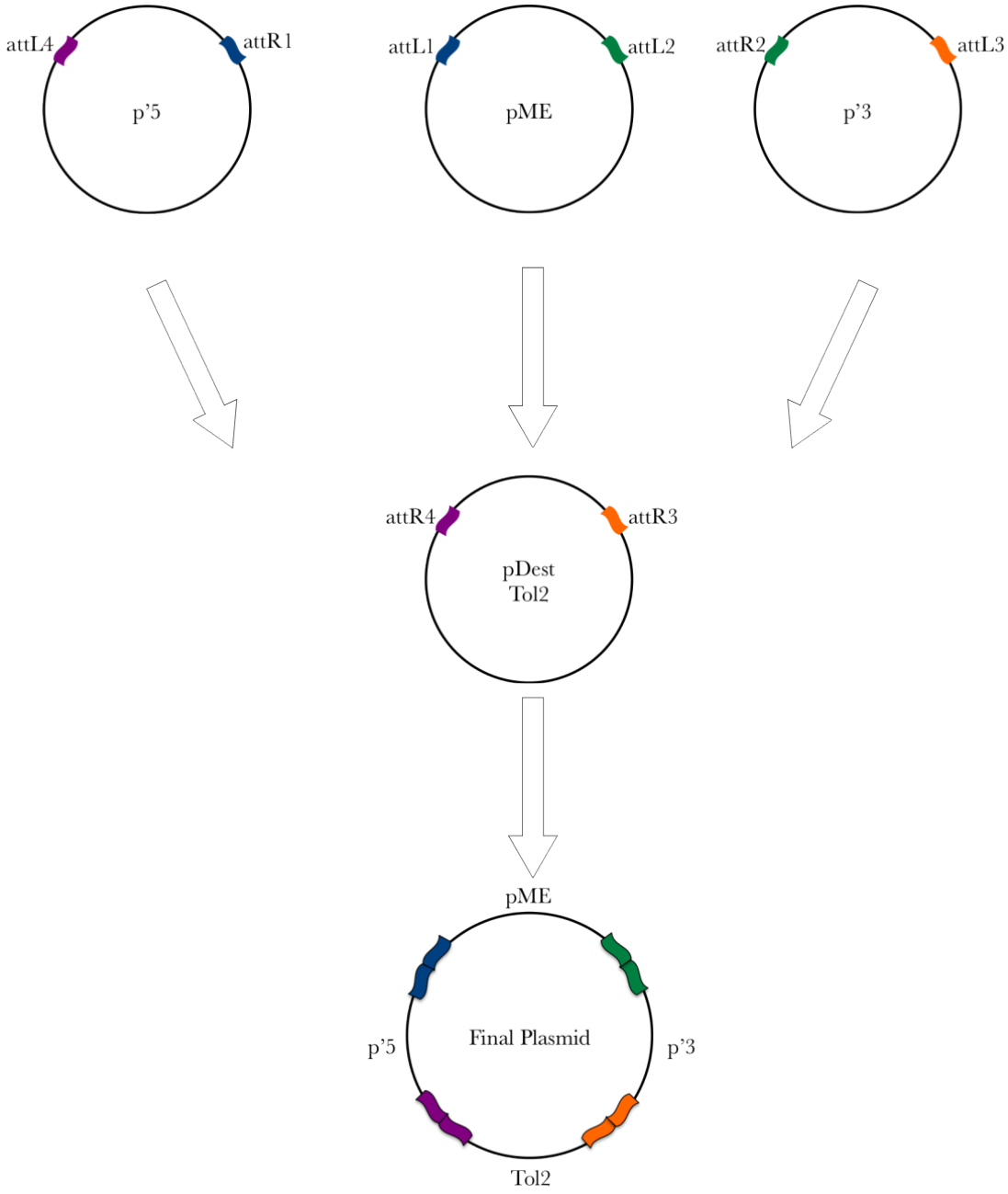


Figure 5 Schematic diagram of LR cloning reaction. The LR reaction combines the complementary attL and attR sites on the entry clones and the destination vector (pDest) to generate the final plasmid, allowing for the recombination of multiple entry clones at the one time (Hartley et al., 2000).

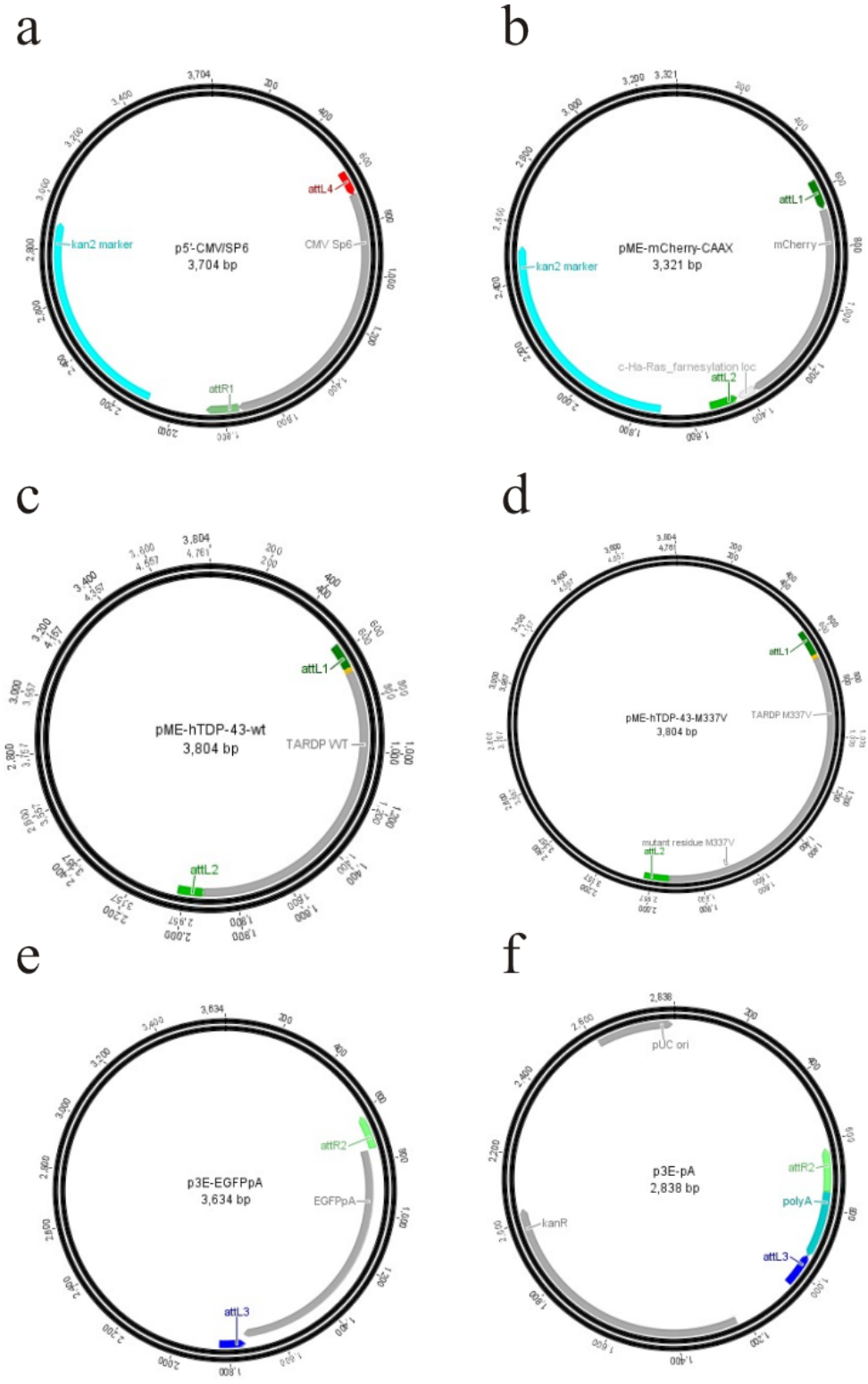


Figure 6 Construct maps of the entry clones used to construct Tol2 plasmids with attL and attR sites. (**A**). p5' CMV entry clone. (**B)** pME with mCherry CAAX. (**C**) pME with hTARBP-wt. (**D**). pME with hTADBP-M337V (**E**) p3' with EGFP_pA. (**F**) p3' with pA only. All Tol2 construct maps were generated with Genious 6.0.3 software.

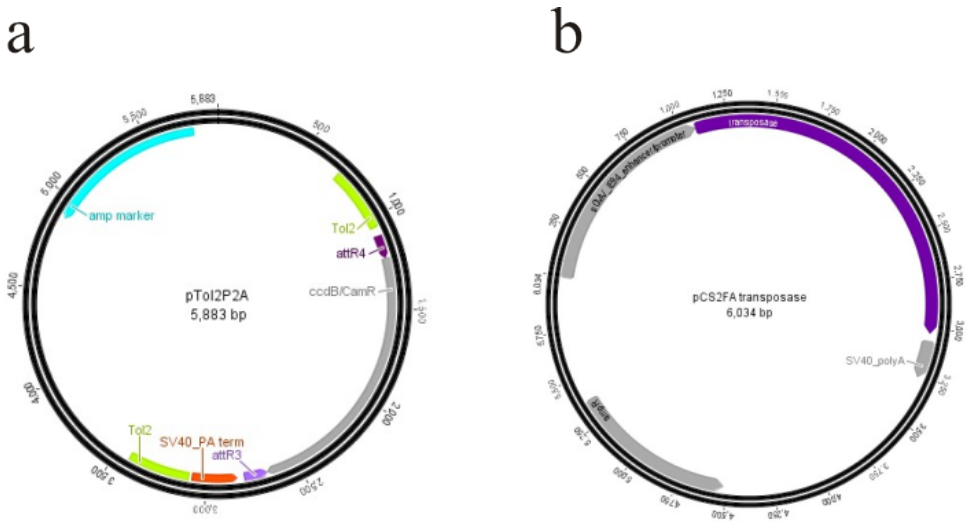


Figure 7 (**A**) Destination vector with Tol2 sites used in the LR reaction to create Tol2 vectors (Kwan et al., 2007) (**B**). Vector map of transposase used with the Tol2 sites to integrate our fusion plasmid into the host genome.

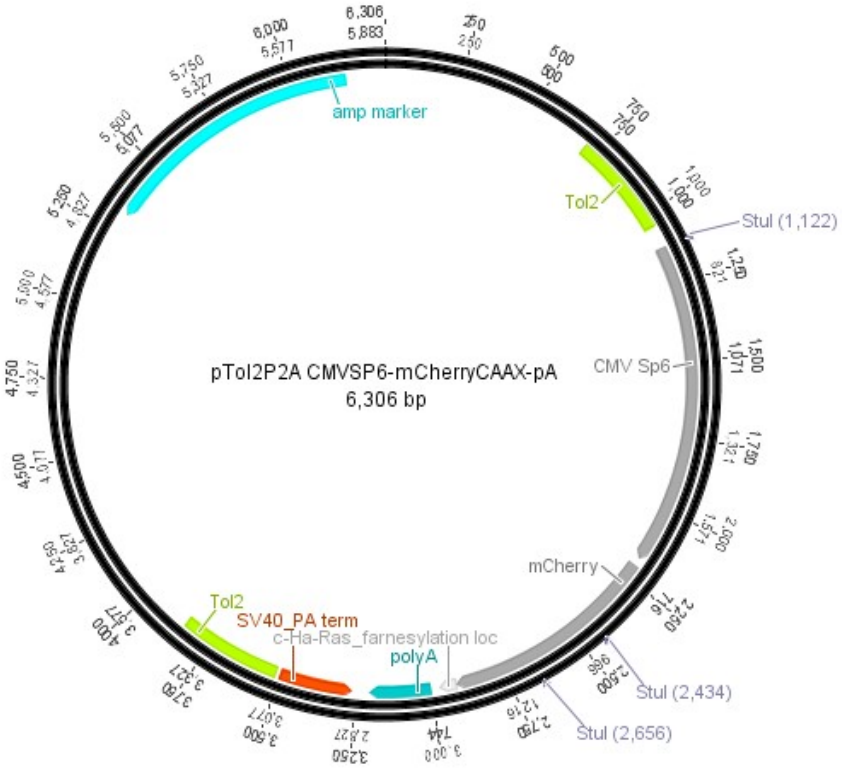


Figure 8 Final construct map for Tol2_mCH plasmid which was generated via the LR reaction. Containing a CMV driven mCherry fluorphore with a CAAX motif.

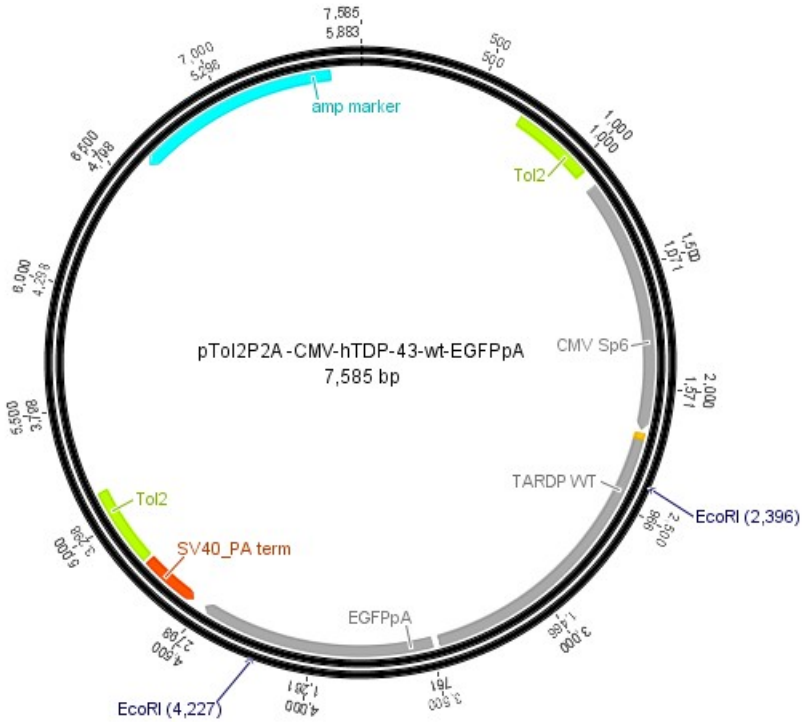


Figure 9 Final construct map for Tol2_WT which was generated via the LR reaction containing the gene encoding human TDP-43 wild-type protein.

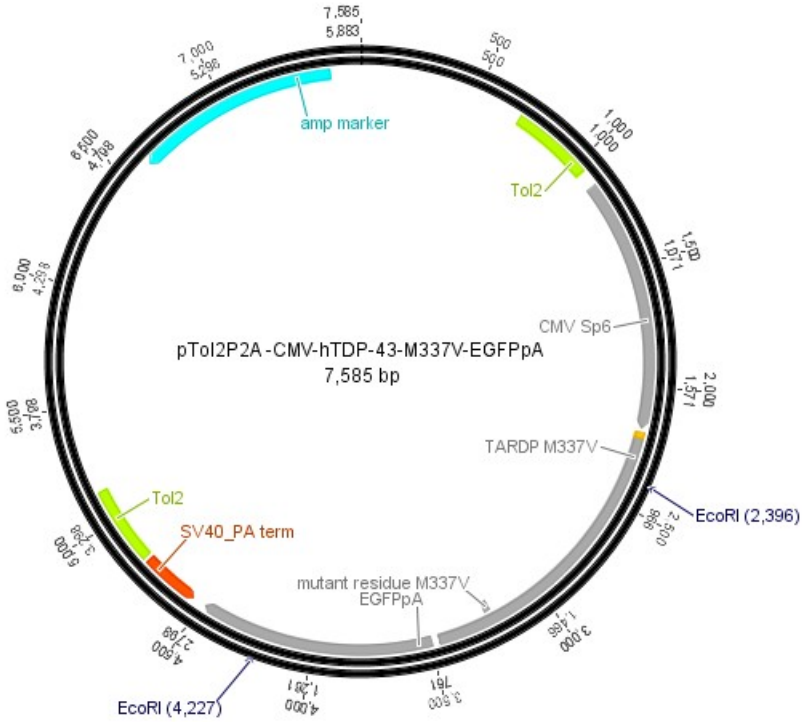


Figure 10 Final construct map for Tol2_M337V which was generated via the LR reaction containing the gene encoding the human TDP-43 M337V protein

Lipofectamine transfection of NSC-34 cells

The day before transfection 13 mm glass cover slips were coated with poly-l-lysine (100%, Sigma) and cells were seeded at a concentration of 1 x 10^5/ml in cell medium in a 24 well plate. To assess cell density for seeding, cells were counted using an automated cell counter Countess (Invitrogen) that utilizes trypan blue (Sigma).

Transfections were completed using Lipofectamine2000 (Life Sciences) as described in experiment 1 with only a minor change. Tol2 DNA and transposase plasmid DNA was used in equal concentrations being 0.5 µg/well bringing the total DNA for a single well to 1 µg. The amount of Lipofectamine®2000 was doubled to 2 µl/well to account for the extra DNA being used. Only 100 µl of the DNA and lipofectamine mixture was added per well and 400 µl DMEM containing 10% FBS and no antibiotics. Plates were replaced in incubator for 4 hours after which the transfection media was aspirated and replaced with normal cell media for 24 hours.

To assess whether there was stable integration of the Tol2 plasmids cells were fixed at three time points; 24 hours, 7 days and 14 days to ensure that there was lasting expression of the EGFP tagged TDP-43.

Induction of TDP-43 aggregates in NSC-34 cells

Transfected cells were in a 24 well plate on coated cover slips three different conditions were used to induce aggregates. They were; 4 hours 50 mM KCl, heat shock for 1 hour at 43^{o}C or overnight incubation (24 hours) in 10 µM MG132 [$C_{26}H_{41}N_3O_5$] (proteasome inhibitor, Sigma). The control was no addition of any stressor. Stock solutions of each compound were diluted in normal cell media and 500 µl of this was added per well. After the experiment the media was aspirated the cells were fixed immediately in 4% paraformaldehyde (PFA).

Histological analysis of TDP-43 transfected NSC-34 cells

Coverslips were mounted with Prolong Gold with DAPI nuclear stain (Life Technologies), sealed with clear nail polish, and stored at 4 ^{o}C. Cells were imaged with a Zeiss fluorescence microscope with Zen acquisition software. Cells (n=100) were scored as to whether they had nuclear or cytoplasmic location of the TDP-43. It was also recorded whether there were large intracellular inclusions (>1 µm) of TDP-43 and their location (ie: nucleus or cytoplasm). Data was analysed using Graphpad prism, Chi-Sqaure test with a Fishers exact was used. Data are from three repeat experiments unless otherwise stated.

Experiment 3: Single-cell transfection of NSC-34 cells

Overview

To assess whether long-term expression of the mutant TDP-43 gene results in trafficking of mutant TDP43 protein from transfected neurons to surrounding non-transfected cells,

we initially attempted to transfect single NSC-34 cells with TDP::GFP fusion proteins in a Tol2 cassette by single cell electroporation. A glass pipette filled with plasmid DNA was placed in gentle contact with target cells and a train of voltage pulses passed through the tip of the pipette, repelling the DNA and simultaneously driving the formation of small pores in the cell membrane. Cells were then incubated for >24 hours and examined for the expression of fluorescent reporter proteins.

Single cell electroporation

The day before the electroporation, 13 mm glass coverslips were coated with poly-l-lysine (100%, Sigma) seeded at a concentration of ~$5x10^5$/ml in normal cell medium, and incubated overnight. For electroporation a coverslip was removed from the incubator and moved into the recording chamber of a patch clamp set-up, covered with normal cell medium, and cells imaged under differential interference contrast optics (**Figure 11**, Supplementary Video 1).

Borosilicate glass (OD 1 mm, ID 0.5 mm) micropipettes with a tip diameter between 1-2 μm were pulled on a P-2000 micropipette puller (Sutter instruments, CA) and filled with plasmids encoding EGFP (pCAG_EGFP, Addgene: 11150)(**Figure 12**), Tol2:mCh, Tol2:WT and Tol2:M337V (400 - 600 ng/μl in 0.9% saline). A fluorescent dextran conjugate (1 – 3 % tetramethylrhodamine dextran, Life Science, MW 3000, anionic in 0.9% NaCl) was used in initial experiments as it provides easy visualization and validation of electroporation (Supplementary Video 1). A silver wire was placed in the micropipette in contact with the DNA solution, and the pipette was mounted on the headstage of an Axoporator 800a (Molecular Devices, USA). The headstage was mounted on a 3 axis micromanipulator (Sutter, MM-200) and maneuvered into gentle contact with the target cell, verified as either a 30% increase in pipette resistance or visualised dimpling of the cell membrane. Electroporation parameters were 10V, 100Hz, 0.5ms pulse with a 1 s train or -10V, 200Hz, 1 ms pulse with 1 s train, for dextran or plasmid DNA respectively(Oyama et al., 2013). A single cover slip was removed from a 24-well plate within a sterile class II laminar flow hood and placed in a 32 mm petri dish with 1500 μl of DMEM. The petri dish was transferred to the electroporation rig, 10 cells were electroporated per cover slip. Once electroporation was completed the cover slip was placed into a new 24 well plate with fresh cell medium and returned to the incubator. The cover slips were out of the incubator on average for 25 minutes.

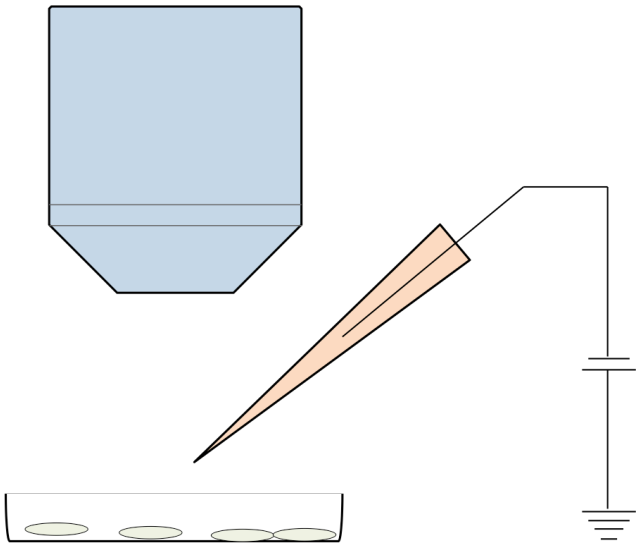


Figure 11 Schematic of the single cell electroporation set up. The objective used was 40x water immersion lens, which was suitable to be immersed in the cell medium.The micropipette had a final tip diameter 2-1 μm, and is filled with the DNA plasmid and 0.9% NaCl. The micropipette is brought into gentle contact with the cell , before an electrical charge is delivered disrupting the cell membrane and driving the plasmid into the cell. The electrode is in the micropipette in contact with the DNA plasmid solution, and is connected to an amplifier which provide the electrical charge.

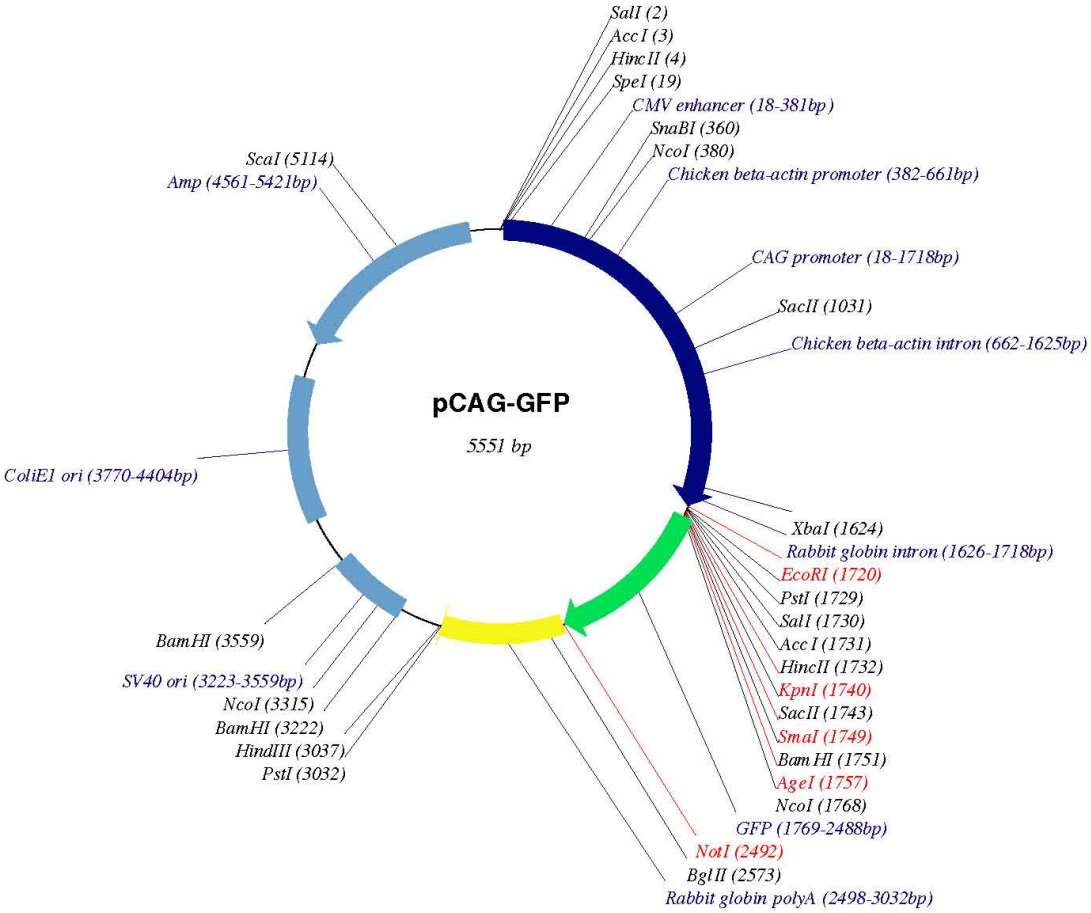
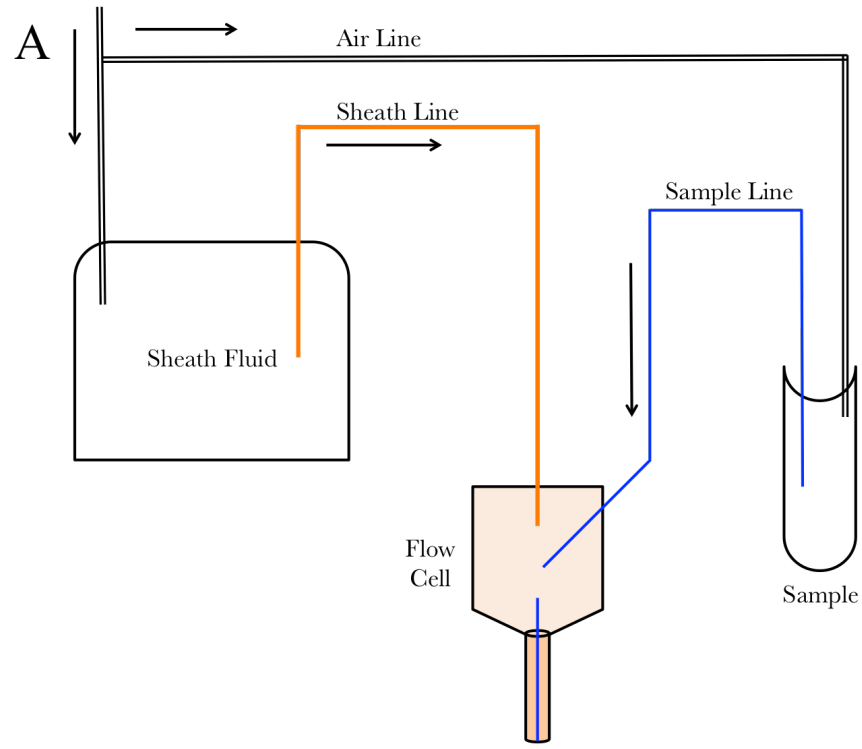


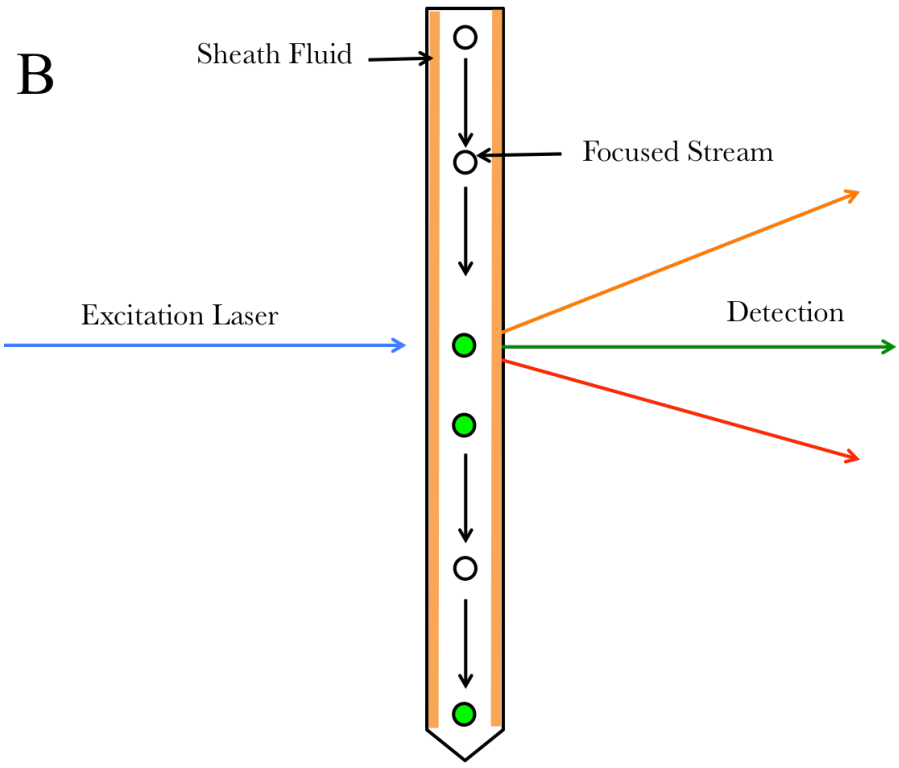
Figure 12 Construct map of GFP used in Single cell electroporation experiements. Plasmid was purchased from Addgene (11150)amplified and purified according to manufacturers instructions.

Experiment 4: Generation of double-transfected cell lines

Outline

Although initially promising, SCE is labour-intensive and ultimately proved poorly suited for transfection with the Tol2 constructs. In order to generate a high-throughput assay to assess the physiological consequences of mutant TDP-43 expression and its intercellular trafficking, we generated cell lines that constitutively expressed two transgenes. First, cells were Lipofectamine -transfected with a Tol2 vector encoding wild-type or mutant TDP-43::GFP fusion proteins. We then sorted GFP-positive cells by FACS (**Figure 13** & **Figure 14**) and transfected them again with a Tol2 vector that drives the expression of mCherry (RFP), allowing identification of originally transfected cells. This double-transfected cell line was then sorted again, leading to the generation of a cell line that stably expresses both RFP and TPD-43::GFP (**Figure 14**).


A
Air Line
Sheath Line
Sample Line
Sheath Fluid
Flow
Cell
Sample


B
Sheath Fluid
Focused Stream
Excitation Laser
Detection

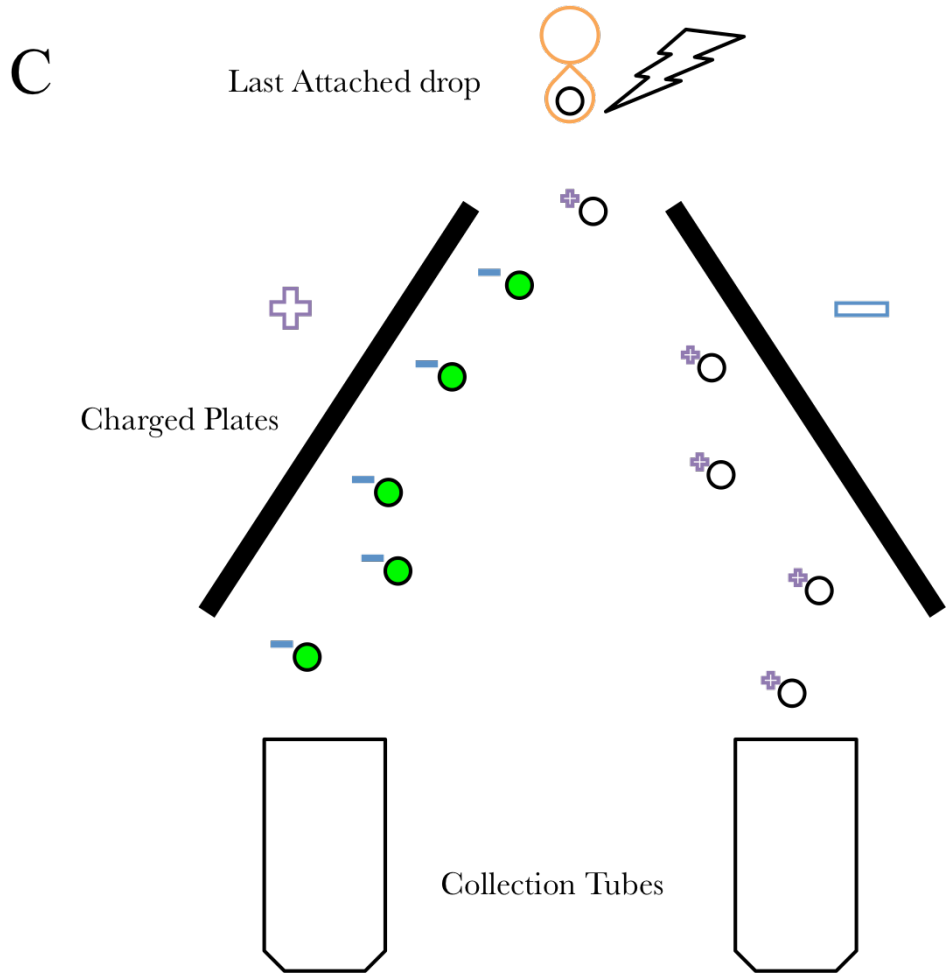


Figure 13 Schematic of Fluorescence-activated cell sorting. (**A**) Fluidics system; Air pressure moves the sample which contains the suspended cells and the sheath fluid into the flow cell. Within the flow cell the sample is hydrodynamically focused single stream with the sheath fluid surrounding. (**B**) The excitation laser focuses onto a single point where the lensed collect the forward scatter and side scatter signal generated when the excitation laser hits a particle. The sample continues down into the nozzle which vibrates in order to create single droplets containing one particle each. (**C**) The last attached drop to this stream is where the charge is applied if a particle is detected within the parameters set. Parameters are set using Accudrop® beads to calibrate the drop delay and the BD FACS software. This now charged particle passes through the deflection plate moving into the collection tube that is appropriate. Figure was adapted from Hawley and Hawley (2004) & Park et al. (2014)

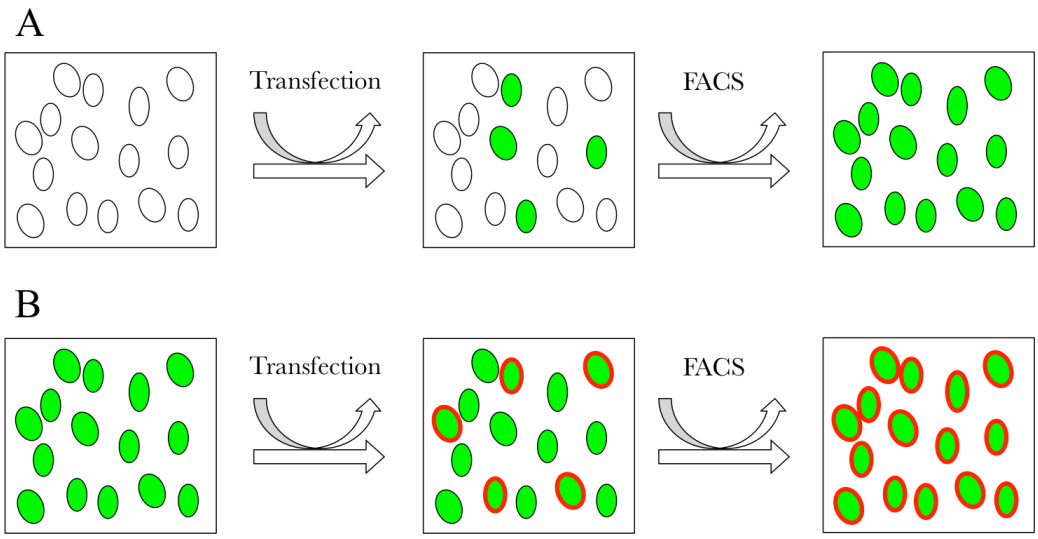


Figure 14 Schematic of experimental procedure to create a double transfected cell line. (**A**) The first transfection via lipofectamine with Tol2 plasmids encoding for wild-type and M337V mutant TDP-43 proteins, the cells are then sorted using FACS to create a stably transfected populations. (**B**). The second round involves transfecting the stably expressing cells from A with the Tol2 plasmid encoding the mCherry fluorescent reporter. These cells were then sorted to create populations that are both GFP and RFP positive.

Preparation of Cells for FACS sorting

NSC-34 cells were seeded at a density of $5.57x10^5$ cells/ml in $75cm^2$ flasks the day prior to transfection in normal cell medium following the same protocol as above.

Lipofectamine transfection

NSC-34 cells were transfected with Tol2:WT and Tol2:M337V plasmids using Lipofectamine2000 (Life Sciences) as follows: 350 µl of OptiMEM (Sigma) was combined with DNA (10 µg) in an Eppendorf tube and incubated for 5 minutes at room temperature. 350 µl of OptiMEM was combined with Lipofectamine2000 (50 µl) and incubated for 5 minutes in a second Eppendorf tube. The DNA and Lipofectmine2000 mixes were combined and left for 20 minutes at room temperature. Cell media was then gently aspirated from the cells and replaced by ~850 µl of the DNA and lipofectamine mixture and 6.5 ml DMEM containing 10% FBS and no antibiotics. Flasks were replaced in incubator for 5 hours after which the transfection media was aspirated and replaced with normal cell media for 24 hours

FACS cell sorting

The day prior to sorting the Influx FACS (BD) was cleaned with successive washes under the normal working pressure; bleach (1.25%), detergent and warm water then 40% (v/v) Ethanol was run through the system and left to soak overnight. The day of sorting 30 minutes prior 1x PBS (autoclaved), which was used as sheath fluid was run through to clean any leftover ethanol out. A 0.1 µm (Millipore) filter was used to prevent DNA contamination between the sample and sheath fluid.

Sphero ® 8 peak beads (BD Biosciences), which are hard dyed micro particles that exhibit 8 different distinct intensities were used to calibrate the Influx. Droplet sorting was calibrated with Accudrop® beads (6 um, BD biosciences) which were used to set the drop delay for the 100 μm nozzle. To ensure that the last attached drop would have the charge applied to it.

The populations corresponding to the cells was resolved from the background using a forward vs. Side- scatter plot using BD software. Fluorescent EGFP expressing cells were discerned from non-EGFP expressing cells using a 200 mW 488 nm laser source on bioplots of forward scatter versus EGFP fluorescence detected using a 510/17 nM bandpass filter.

Cells were detached from the flask and resuspended at a concentration of 5 x 10^5 cells/mL in DMEM. Cell numbers were equalised between the three populations to be sorted; NSC-34 cells, NSC-34 cells transfected with Tol2:WT and NSC-34 cells transfected with Tol2:M337V. Cells were sorted into 15 ml falcon tubes containing 2 ml DMEM, at the conclusion of sorting cells were plated into 25cm^2 flasks with 5 ml of fresh cell medium and returned to the incubator (37^{o}C , 5% CO_2). Cell medium was changed to fresh cell medium 24 hours later. Cells were monitored for any sign of contamination and were plated into a 75cm^2 flask when they reached ~90% confluence in the 25cm^2 flask.

Secondary Transfection of the Sorted Cells

NSC-34 cells that had been sorted to be GFP positive with the Tol2 plasmids were transfected for a second time with another Tol2 plasmid. This is the Tol2:mCh. Cells were seeded at a density of 5.57x10^5/ml in a 75cm^2 flask the day before transfections. Transfections were completed as above.

Secondary FACS

Cells were sorted according to the protocol used above; hierarchical gating was used the second time around as the final cell population needed to be positive for two flurophores EGFP and mCherry. Hierarchical gating was set up during calibration so that EGFP was detected first using a forward vs. Side-scatter plot with the 488 nM laser. These EGFP positive cells were then distinguished between mCherry expressing cells from non-expressing cells using a 610/30 nM bandpass filter.

Only cells that were EGPF positive and mCherry positive were sorted into the 15 ml falcon tube containing 2 ml of cell medium. Cells were plated into 25cm^2 flasks with 5 ml of fresh cell medium and returned to the incubator (37^{o}C , 5% CO_2). Cell medium was changed to fresh cell medium 24 hours later. Cells were monitored for any sign of contamination and were plated into a 75cm^2 flask when they reached ~90% confluence in the 25cm^2 flask.

Co-culture of TDP-43 stably transfected cells with naïve cells

Stably transfected NSC-34 cells with the TDP-43 plasmid and mCherry plasmid present were plated out in a 24 well plate with un-transfected NSC-34 cells in equal parts, these were cultured and fixed with 4% PFA at 2day, 4day and 6 day time points.

Cells were imaged using Leica TCS SP5X confocal microscopes. Cells were scored as to whether there was red or green fluorescence

Experiment 5: Membrane potential Assay

Outline

Our ultimate objective is to use cell lines that express wild-type or mutant TDP-43::GFP fusion proteins to investigate the cellular and physiological consequences of mutant TDP-43. Here we determined the feasibility of this approach by conducting membrane potential assays of cell lines generated in Experiment 4. Utilising a microplate reader that detects a change in propriety fluorescent dye either an increase or decrease indicating depolarisation or hyperpolarisation of the cells respectively. This will provide a high throughput method of detecting whether there is a change in membrane potential in response to a stimulus such as a drug or potassium. A variety of compounds will be used to assess whether there are enough receptors on the cells to pick up a significant change in the fluorescence.

Preparation of cell lines

Four different cell lines were used for the membrane potential assay. NSC-34 cells and Neuro2a cells that were not transfected were used for the initial assessment of the assay. Stably transfected NSC-34 with SOD1 wild-type and SOD1 A4V mutation (kindly from Dr Julie Atkin, ASAM, Sydney) were assessed as were the stably transfected NSC-34 cells with the TDP-43 fusion constructs for the wild-type and the M337V mutant. NSC-34 cells and Neuro2a (Klebe, 1969)cells were maintained as mentioned above, there is no difference in cell medium between the two lines. The SOD1 stably transfected cells had 0.4 mg/mL of the Geneticin antibiotic (G418, Sigma) in the cell culture medium.

The day before the assay cells at 90% confluency in a 75 cm^2 flask were detached with trypsin (Sigma) and then resuspended in 9 ml Leibovitz's L-15 media supplemented with 1% FBS (Sigma), 1% penicillin/streptomycin (Invitrogen), and 15 mM glucose. Of the resuspended cells 90 µl was plated per well in a black walled clear bottom 96 well micro plate (Corning, NY) and were incubated overnight at 37 ^oC with ambient CO_2.

Compounds

[D-Ala^2, N-$MePhe^4$, Gly-ol]-enkephalin acetate (DAMGO) a synthetic Mu-Opioid receptor agonist, was purchased from AusPep (Tullamarine, Australia). WIN 55,212-2 a non-selective cannabinoid receptor agonist was purchased from Sapphire Bioscience (Sydney, Australia), Veratridine a neurotoxin which activates Na Channels was purchased from the National measurement institute (Pymble). Somatostatin (SST14) was purchased form AusPep (Tullamarine, Australia).Potassium used was potassium chloride (KCl, MW 74.56) from Sigma. All compounds were diluted in the Low K HBSS buffer.

Flex assay

The membrane potential of cells were measured using a commercially available FLIPR ® membrane potential assay kit (Blue) from molecular devices (Sunnyvale, CA). The dye was reconstituted with HBSS (Low-K); 90 µl of dye solution was added to each well of

the microplate without the removal of the L15, bringing the total volume to 180 µl in each well. The plate with the dye was then incubated in the dark at 37 ^{o}C with ambient CO_2 for 45 minutes, to allow for equilibration. Fluorescence was measured using a FlexStation3 (Molecular Devices) microplate reader with an excitation wavelength of 530 nM and emission was read at 565 nm. The background fluorescence of cells with no dye and dye only was measured prior to the initial assay to gauge a baseline. Initial readings were taken at every 2 s for 120 s at which time the drug was then added to the cells at a volume of 20 µl and readings were continued to be taken for another 480 s.

Statistical analyses

The concentration response curves were obtained by fitting a 4-point linear model with GraphPad Prism 4 (Graphpad Software). Statistical differences between the curves was assessed with a 2-way ANOVA with multiple comparison

Results

Experiment 1-

NSC-34 cells were transiently transfected with plasmids containing human TARDBP (wild-type or M337V mutation). The transfected cells were treated with different stressors to determine whether the intracellular localisation of the protein could be altered and in turn whether it was possible to induce intracellular aggregates containing TDP-43 protein in this cell model.

Transfections were completed using lipofectamine, 24 hours after the lipofectamine and DNA was removed, cells were checked using fluorescence microscope (Zeiss). Successfully transfected cells could be identified by GFP fluorescence and there was a transfection rate of around ~15% for both the wild-type and mutant protein. Fluorescence would last 72hours from transfections before there was a decline in the number of GFP positive cells.

The control condition, which had no treatment, the GFP was predominantly located within the nucleus of the cells (**Figure 15** & **Figure 17**). There was no difference between the two control conditions between the wild-type (mean= 77 SEM± 0.577) and the M337V mutation (82±1.668) (Fischer's exact p=0.4839).

Potassium alters the location of the TDP-43 protein.

The cells transfected with the wild-type TADRBP and treated with 50mM of KCL did have significant cytoplasmic location of the TDP-43 protein (43±0.577) compared to the wild-type control (23±0.333) (*p=0.0041*). The M337V mutation also had significantly more cytoplasmic location of the TDP-43 protein (41±1.528) compared to the mutant control (18±0.882)(*p=0.0006*). There was no significant difference between the wild-type location under the KCL condition and the M337V location under the KCL condition (p=0.8861)

Heat treatment at 43°C alters the location of mutant TDP-43 protein

The cells transfected with the wild-type TADRBP and heat shocked for 1 hour did not have significant cytoplasmic location of the TDP43 protein (21±1.067) compared to the wild-type control (23±0.333) (p=0.8646) and remained in a nuclear location. The M337V mutation did have significantly more cytoplasmic location of the TDP-43 protein (35±0.333) compared to the mutant control (18±0.882) (*p=0.0100*). There was a significant difference between the cellular location of the wild-type protein and the M337V mutation in the Heat treatment (*p=0.0401)*

Intracellular aggregation was not induced in response to stressors

None of the cells in the KCl treatment group showed any formation of aggregates in either the wild-type or the M337V TDP-43 protein. Within the heat shock treatment the wild-type protein saw 16/300 cells (5%) form aggregate inclusions. The mutant protein was only slightly higher with 22/300 cell (7%) forming aggregate inclusions. This was not statistically significant (p=0.7673)

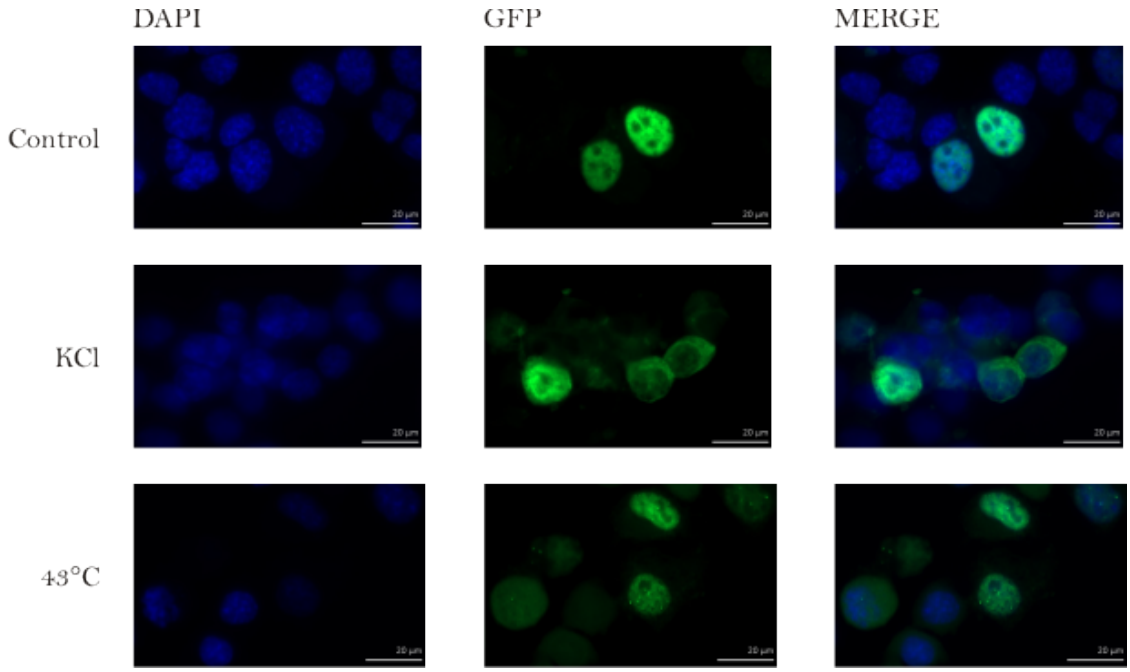


Figure 15 NSC-34 cells transfected with CMV_WT via Lipofectamine®. The control showed a predominantly nuclear location of the protein (GFP). The KCL treatment significantly altered the location of the TDP-43 plasmid from the nucleus to the Cytoplasm (*p=0.0041)* where as the Heat treatment at 43°C did not significantly alter the location of the TDP-43 wild-type protein (p=0.8646) Scale = 20 μm

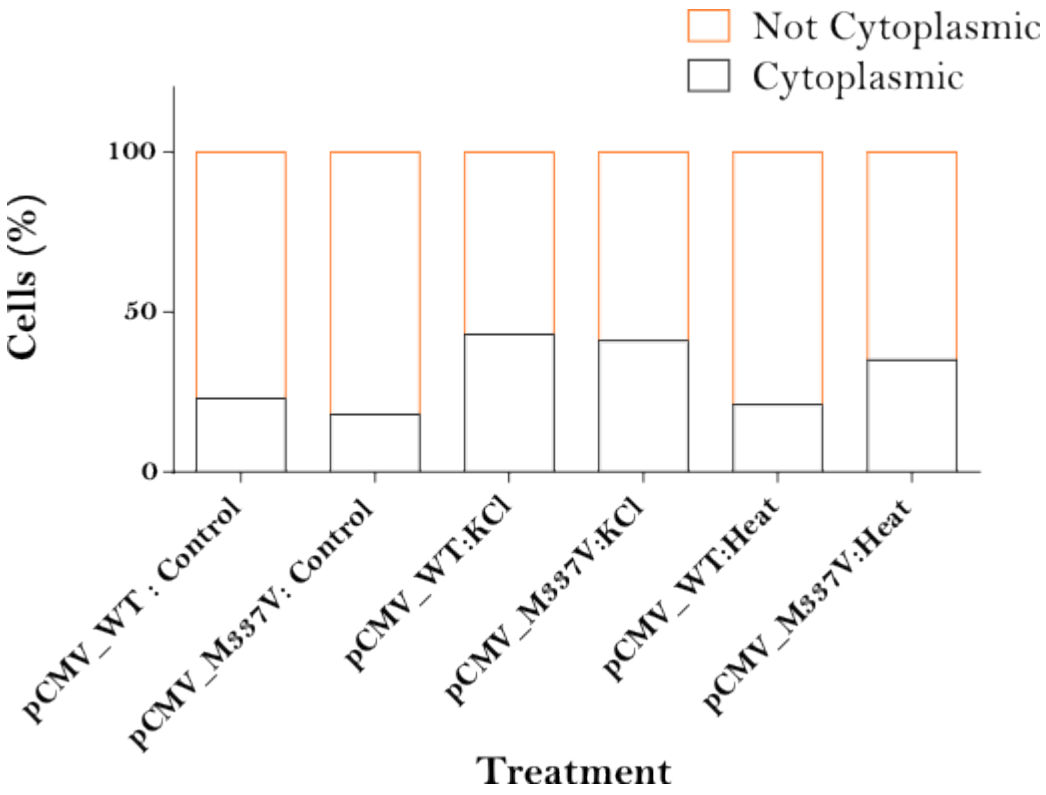


Figure 16 Proportion of cytoplasmic location of NSC-34 cells transiently transfected with the wild-type and mutant TDP-43 protein. Values are from 100 cells per experiment and a total of three experiments.

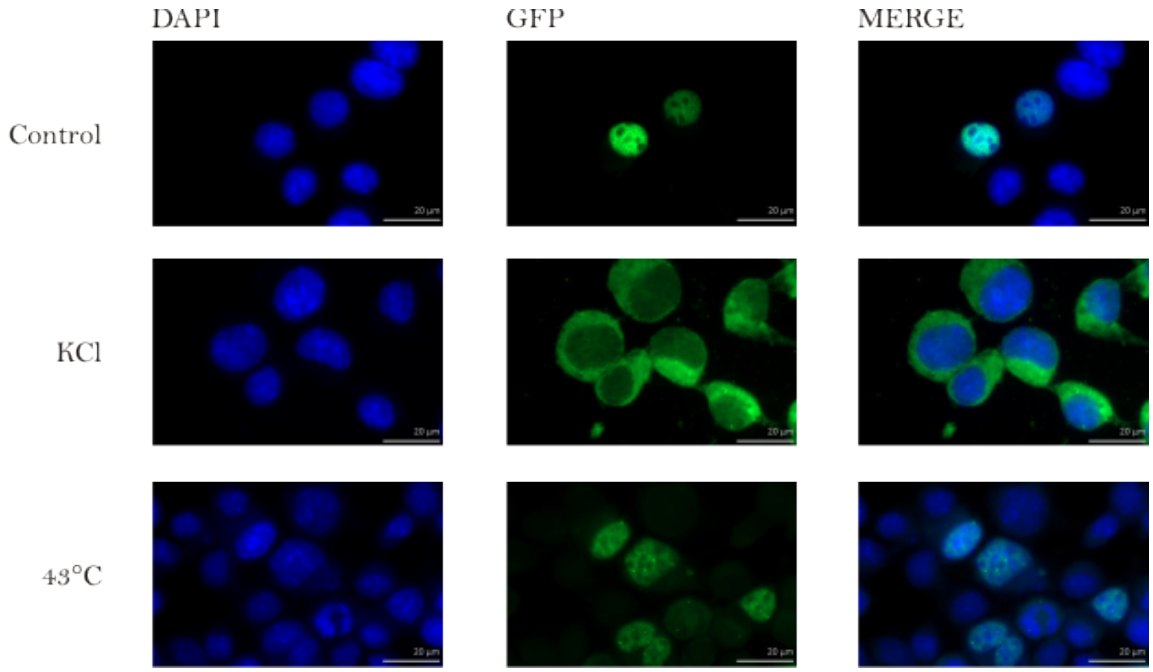


Figure 17 NSC-34 cells transfected with CMV_M337V via Lipofectamine®. The control showed a predominantly nuclear location of the protein (GFP). The KCL treatment significantly altered the location of the TDP-43 plasmid from the nucleus to the

Cytoplasm (*p=0.0006)* additionally the Heat treatment at 43°C did significantly alter the location of the TDP-43 wild-type protein (*p=0.0100*). Scale = 20 μm

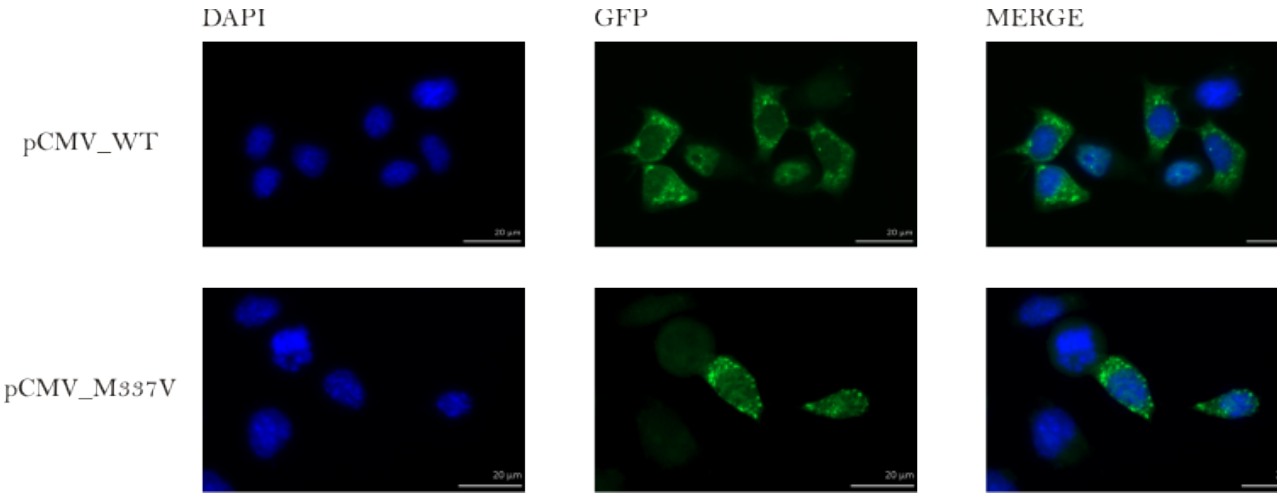


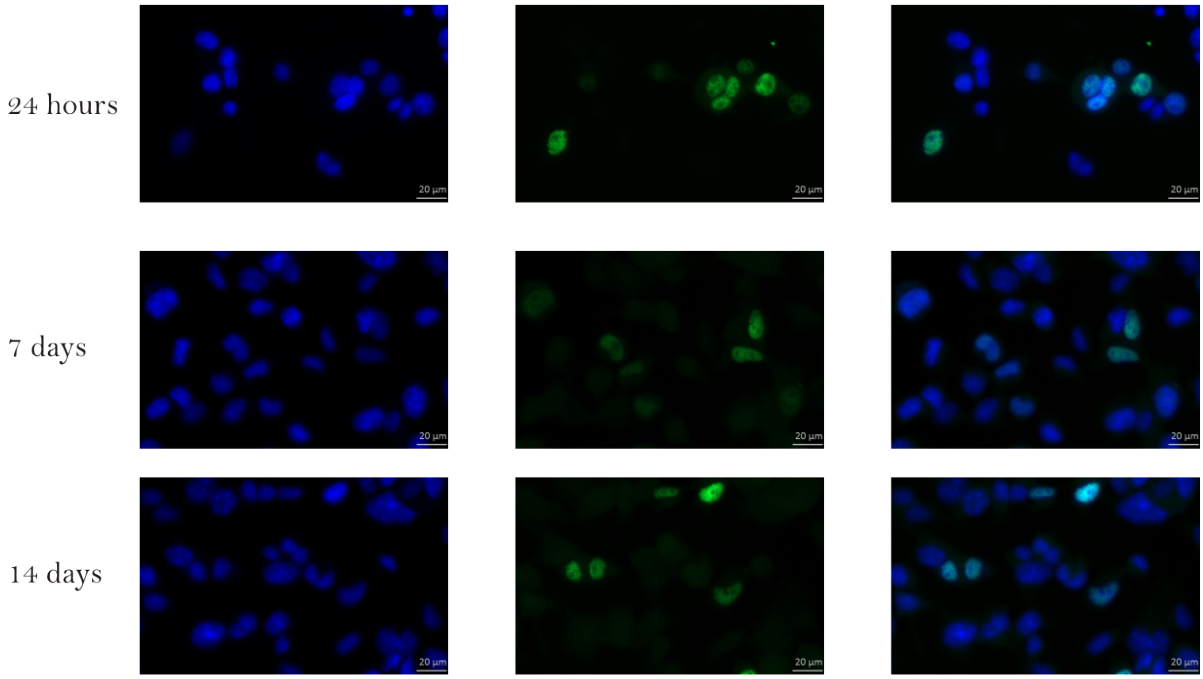
Figure 18 Possible aggregate formation was observed in the heat shock treatment group. The TDP-43 protein in cells with aggregate like appearance is predominantly localised to the cytoplasm.The aggregate formation was not significant with only 5% of wild-type cells and 7% of mutant TDP-43 cells forming aggregates (p=0.7673) scale = 20 μm

Experiment 2- Tol2 Aggregate formation

To complement the transient transfection studies undertaken in Experiment 1, experiments were undertaken to generate more permanent transgene expression of TDP43. The Tol2 plasmids were generated using Multi-site Gateway® LR recombination reaction (Hartley et al., 2000).

After the sequencing confirmation of the Tol2 plasmids cells were transfected using lipofectaine and GFP expression was checked at 24hours, 7days and 14days to ensure that the plasmids were being integrated and stably expressed (**Figure 19**). The cellular location of the proteins was predominantly nuclear for both the wild-type (Mean = 90 SEM ± 0.316) and the mutant (88±0.667) and there was no significant different between the two proteins (p=1.000). Following the confirmation that the Tol2 plasmids were stably expressed, cells were transfected again with the TDP wild-type or M337V mutant and treated with the same stressors as the transiently transfected cells.

A



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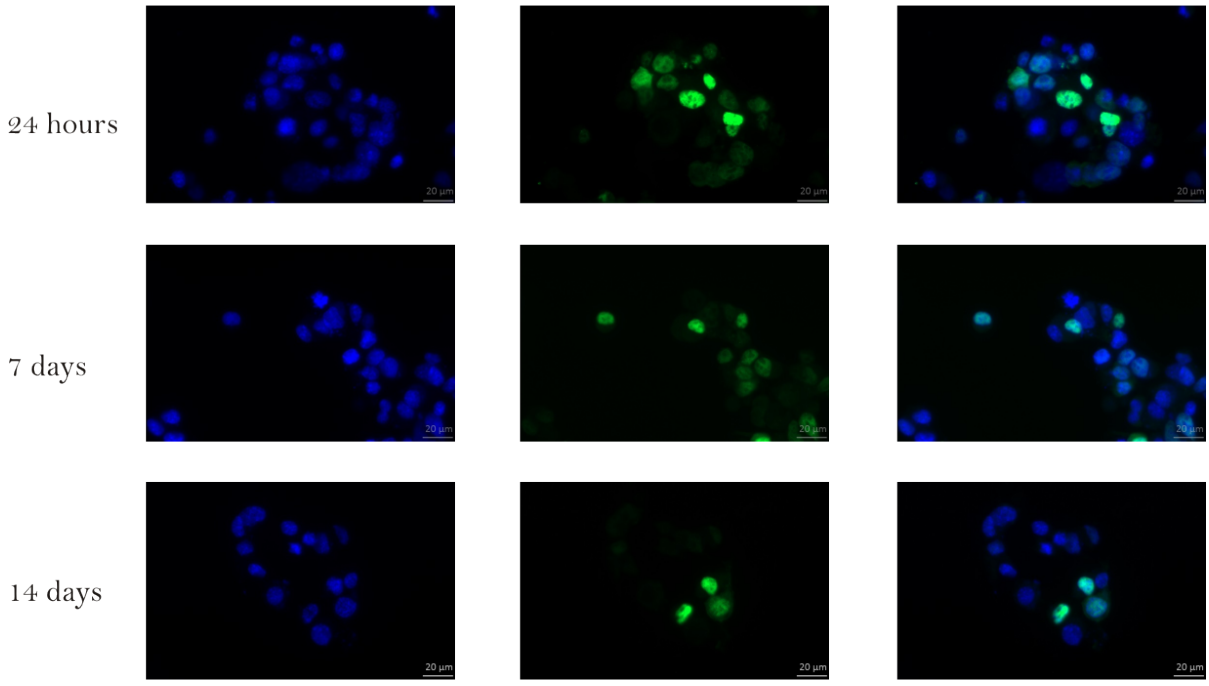


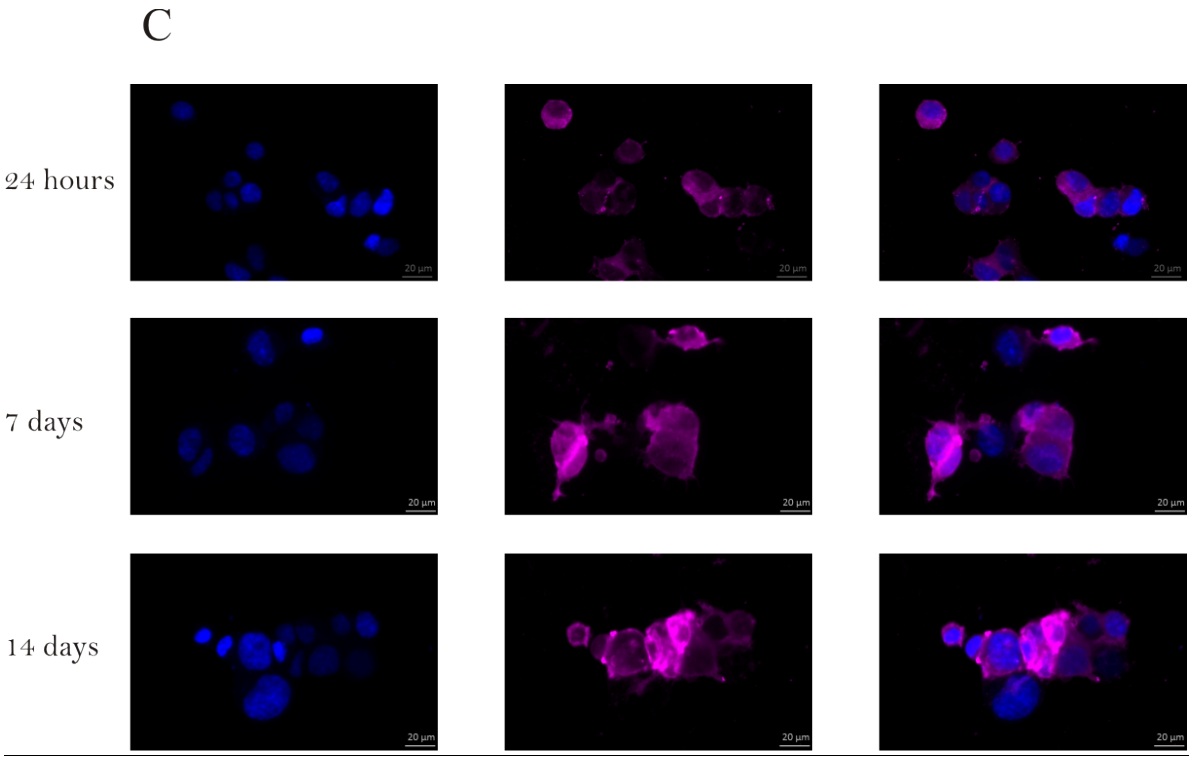


Figure 19 The stable integration of NSC-34 cells transfected with Tol2 plasmids via lipofectamine. (**A**) The wild-type TDP-43 tol2 plasmid had a nuclear localisation of the GFP and GFP positive cells were seen 24 hours, 7 and 14 days after the initial transfection with lipofectamine. (**B**) The mutant TDP-43 tol2 plasmid likewise had nuclear localisation of GFP fluorescence and GFP positive cells were seen 24 hours, 7 and 14 days after the transfection with lipofectamine. (**C**)The Tol2 plasmid encoding the mCherry-CAAX was localised to the endo- membrane of the cell. RFP positive cells were also seen 24 hours, 7 and 14 days after transfection with lipofectamine. Scale bar = 20 μm

Potassium alters the location of the TDP-43 protein.

The cells transfected with the wild-type TADRBP and treated with 50mM of KCL did have significant cytoplasmic location of the TDP-43 protein (33±1.502) compared to the wild-type control (10±0.667) (*p=0.0001*). The M337V mutation also had significantly more cytoplasmic location of the TDP-43 protein (37±0.333) compared to the mutant control (12±0.667)(*p=0.0001*). There was no significant difference between the wild-type location under the KCL condition and the M337V location under the KCL condition (p=0.6567)

Heat treatment at 43^{o}C does not alter the location of the TDP-43 protein

The cells transfected with the wild-type TADRBP and heat shocked for 1 hour did not have significant cytoplasmic location of the TDP43 protein (12±1.202) compared to the wild-type control (10±0.667) (p=0.8217) and remained in a nuclear location. The M337V mutation did not have significantly more cytoplasmic location of the TDP-43 protein (17±1.557) compared to the mutant control (12±0.667) (p=0.3083). There was no significant difference between the cellular location of the wild-type protein and the M337V mutation in the Heat treatment (p=0.4222)

MG132 treatment alters the location of the TDP-43 protein

The cells transfected with the wild-type TADRBP and treated with MG132 overnight had significant cytoplasmic location of the TDP43 protein (23±1.667) compared to the wild-type control (10±0.667) (*p=0.0212*) which was predominantly nuclear. The M337V mutation did have significantly more cytoplasmic location of the TDP-43 protein (30±0.557) compared to the mutant control (12±0.667) (*p=0.0001*). There no significant difference between the cellular location of the wild-type protein and the M337V mutation in the MG132 condition (p=0.3364).

Intracellular aggregation was not induced in response to stressors

There was no significant aggregation of the TDP-43 proteins in response to any of the stressors for either the wild-type protein or the mutant proteins. Interestingly there was a large number of cells with very small and bright concentrations of the TDP-43 protein within the nuclear area, for cells treated with the heat shock (**Figure 20** & **Figure 21**) Although the exact location could not be determined using microscope.

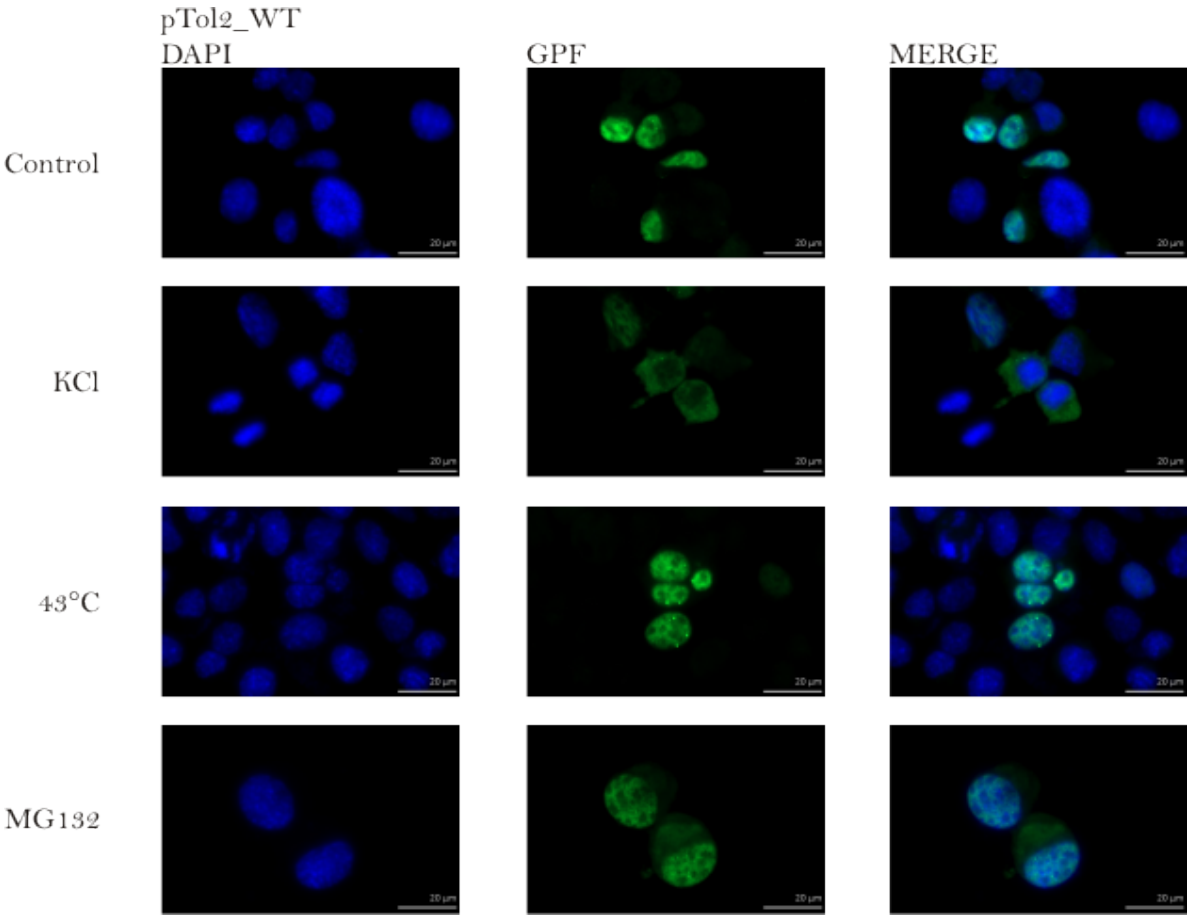


Figure 20 NSC-34 cells lipofectamine transfected with Tol2_WT plasmids, then exposed to three different treatments. The control was no treatment and the TDP-43 protein remained nuclear. The KCl treatment had a significant cytoplasmic localisation of the wild-type protein (*p=0.0001*). There was no significant mislocalisation after heat

treatment (p=0.8217), conversely there was significant cytoplasmic localisation of the TDP-43 after treatment with MG132 (p=*0.0212*). Scale= 20 μm

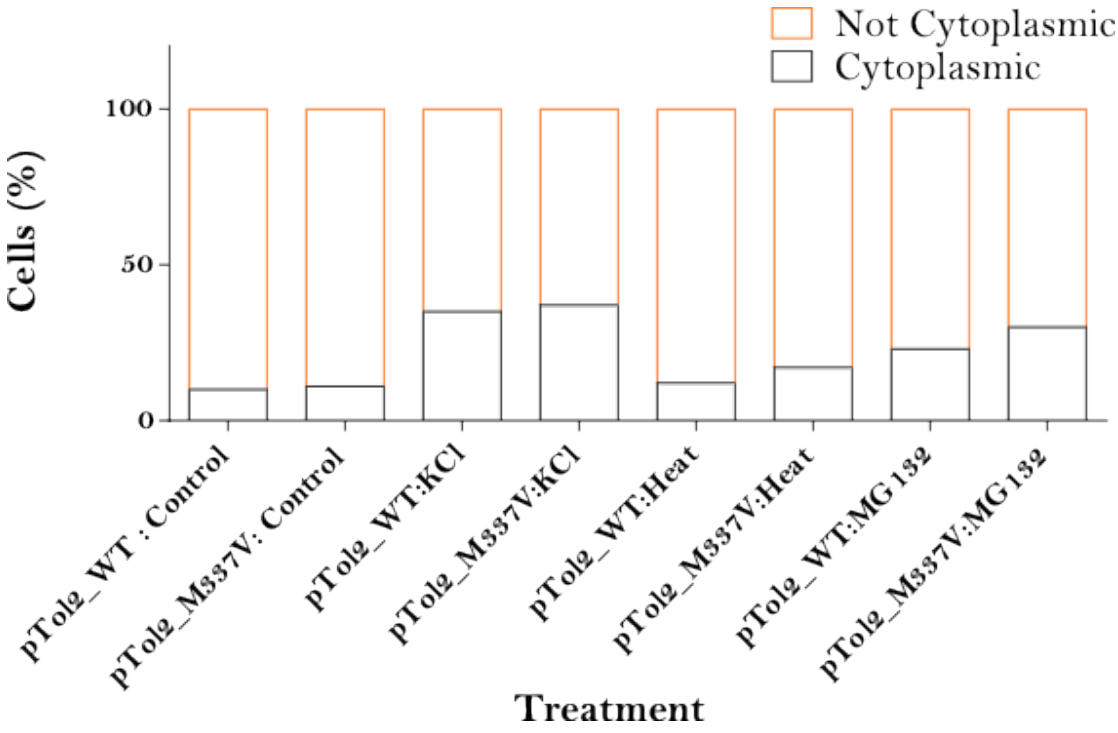


Figure 21 Proportion of cytoplasmic location of NSC-34 cells stably transfected with Tol2 plasmids. Values are from 100 cells per experiment and a total of three experiments.

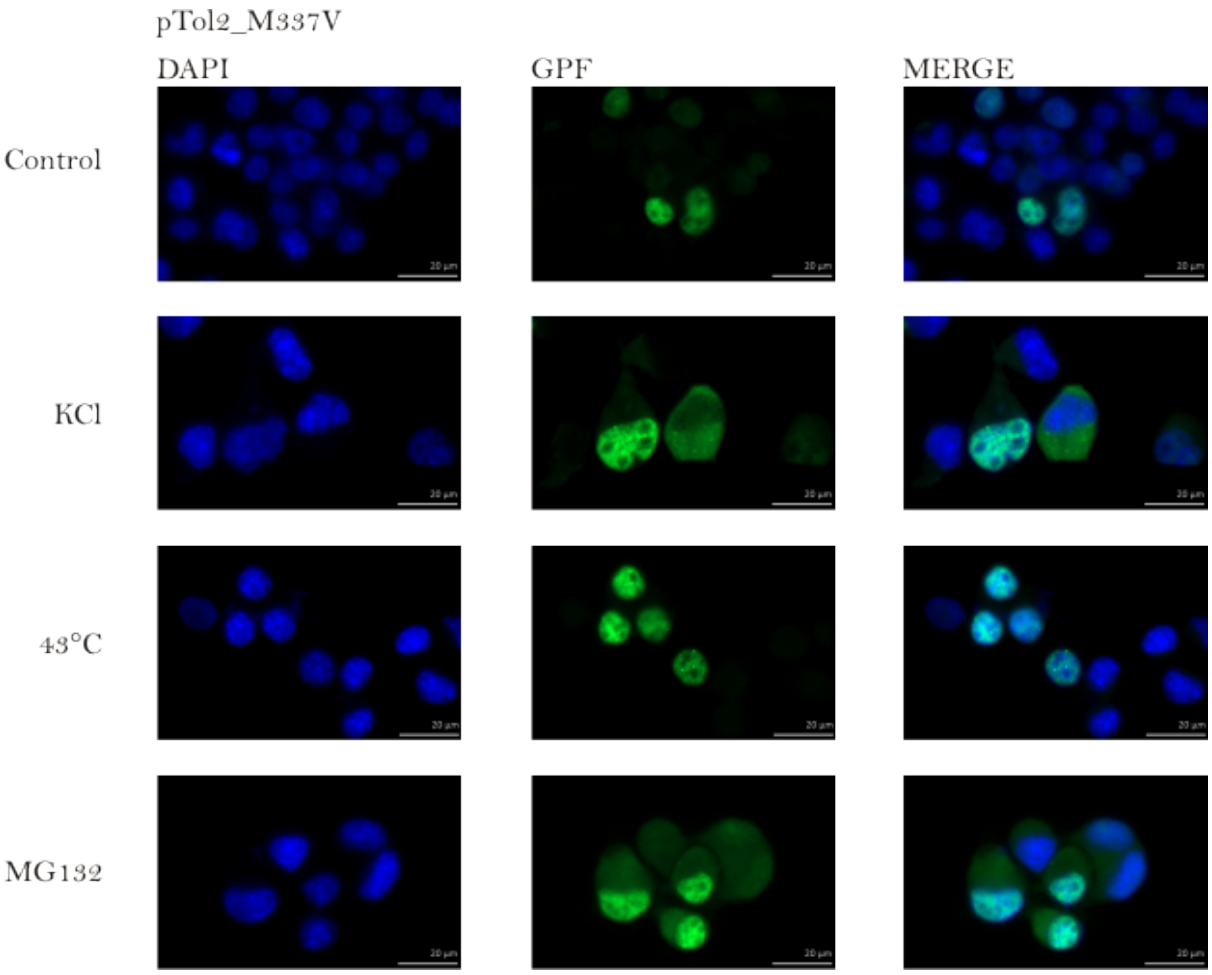


Figure 22 NSC-34 cells lipofectamine transfected with Tol2_M337V plasmids, were then exposed to three different treatments. The control cells had nuclear TDP-43 protein. The KCl treatment had a significant cytoplasmic localisation of the wild-type protein (*p=0.0001*). There was no significant mislocalisation after heat treatment (p=0.3083), alternatively there was significant cytoplasmic localisation of the TDP-43 after treatment with MG132 (p=*0.0001*) Scale bar = 20 μm

Experiment 3- Single cell electroporation of Tol2 plasmids

To assess whether long-term expression of the mutant TDP-43 gene results in trafficking of mutant TDP43 protein from transfected neurons to surrounding non-transfected cells, we initially attempted to transfect single NSC-34 cells with TDP::GFP fusion proteins in a Tol2 cassette by single cell electroporation. A glass pipette filled with plasmid DNA was placed in gentle contact with target cells and a train of voltage pulses passed through the tip of the pipette, repelling the DNA and simultaneously driving the formation of small pores in the cell membrane. This method enables the controlled transfection of a single cell at a time and doesn't affect surrounding cells (Supplementary Video 2)

Pilot experiments to determine whether the single cell electroporation technique was compatible with the NSC-34 cell line were completed using TMR dextran. Reproducible single cell labelling was obtained with trains of 1ms pulses delivered at 50hz for 0.5sec

with 240/314 (76%) cells labelled on the first attempt (Supplementary Video 1 & **Table 6**).

Utilising the same approach cells were electroporated with a GFP plasmid (**Figure 12**), as a plasmid was being electroporated the parameters were altered slightly. NSC-34 cells were electroporated with -10V trains of 0.5ms pulses delivered at 100Hz for 1s. When electroporating the GFP plasmid there was a 15% (43/280) success rate of cells recovered after one labelling attempt (**Table 6**).

Single cell electroporation was then attempted with the Tol2 plasmids. In each attempt equal parts pCS2+ transposase plasmid was combined with the Tol2 plasmid and 0.9% NaCl. As there were no recoveries of 400 cells, different electroporation parameters were then trialled (Steinmeyer and Yanik, 2012) to no avail (**Table 6** & **Table 7**)

Table 6 Summary of the results of the Single cell electroporation of the NSC-34 cells

Construct	Cells Zapped	Cells Recovered	%
Dextran	314	240	76.43
GFP	280	43	15.36
Tol2:mCherry	180	0	0
Tol2:wt	367	0	0
Tol2:M337V	207	0	0

Table 7 Summary of the different electroporation parameters used with the Tol2 plasmids

Voltage	Train	Frequency	Width
-10	1 s	50 Hz	0.5 ms
-10	1 s	200 Hz	0.5 ms
-10	1 s	200 Hz	1.0 ms
-10	1 s	1 kHz	1.0 ms

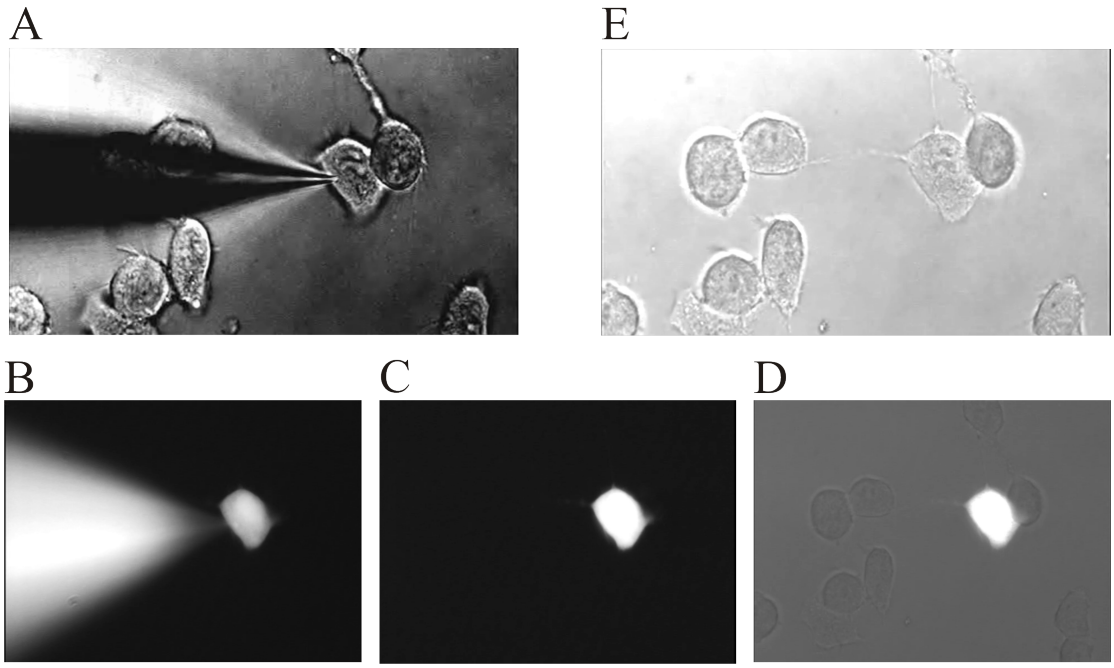


Figure 23 Single cell electroporation of NSC-34 cell. (**A**) The pipette tip filled with plasmid and 0.9% saline solution is brought down until it is just touching the membrane of the cell. (**B**) A current of 10V at 100 Hz for 0.5 s is applied Now viewing under epifluorescence we can see the pipette filled with dextran and the NSC-34 cell is filled with dextran. (**C**) The pipette is moved gently out of contact with the cell and we see that the cell is still filled with dextran. (**D**). Only one cell was filled with dextran and the surrounding cells were not affected. (**E**). Under DIC we can see that all of the cells look normal and none of them were adversely affected by the single cell electroporation. (Images were taken from the supplementary video 1)

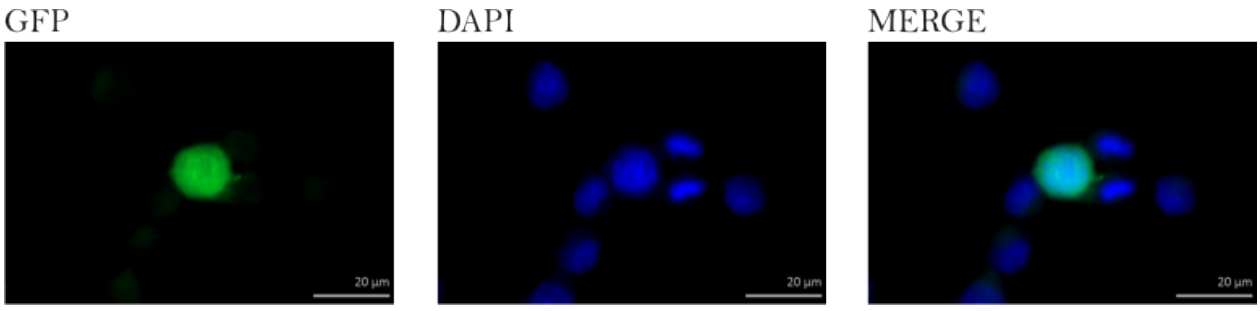


Figure 24 Successful Single cell electroporation of NSC-34 cells using pCAG_GFP plasmid. This cell was fixed ~24 hours after electroporation. Scale = 20 µm

Experiment 4- FACS

In order to generate a high-throughput assay to assess the physiological consequences of mutant TDP-43 expression and its intercellular trafficking, we generated cell lines that constitutively expressed two transgenes. First, cells were Lipofectamine -transfected with a Tol2 vector that drives the expression of either the TDP-43 wild-type or TDP-43 mutant. We sorted these GFP positive cells by FACS and transfected them a second time with the Tol2 vector that drive mCherry (RFP). The RFP allowing identification of originally transfected cells, we then sorted RFP-positive cells by FACS. This double-

transfected cell line was then sorted again, leading to the generation of a cell line that stably expresses both TPD-43::GFP and RFP.

Although initially promising, SCE is labour-intensive and ultimately proved poorly suited for transfection with the Tol2 constructs. In order to generate a high-throughput assay to assess the physiological consequences of mutant TDP-43 expression and its intercellular trafficking, we generated cell lines that constitutively expressed two transgenes. First, cells were Lipofectamine -transfected with a Tol2 vector encoding wild-type or mutant TDP-43::GFP fusion proteins. We then sorted GFP-positive cells by FACS (**Figure 26**) and transfected them again with a Tol2 vector that drives the expression of mCherry (RFP), allowing identification of originally transfected cells. This double-transfected cell line was then sorted again,(**Figure 28**) leading to the generation of a cell line that stably expresses both RFP and TPD-43::GFP.

NSC-34 cells stably transfected with Tol2_WT and Tol2_M337V plasmids (**Figure 25**) were sorted using hierarchical gating set for GFP fluorescence with the 488nm laser (**Figure 26**). The cells recovered fully with no signs of contamination.

NSC-34 cells were further transfected a second time with the Tol2_mCh vector (**Figure 27**) and a second round of FACS was completed using the hierarchical gating of GFP, then RFP (**Figure 28**). The cells recovered and were viable.

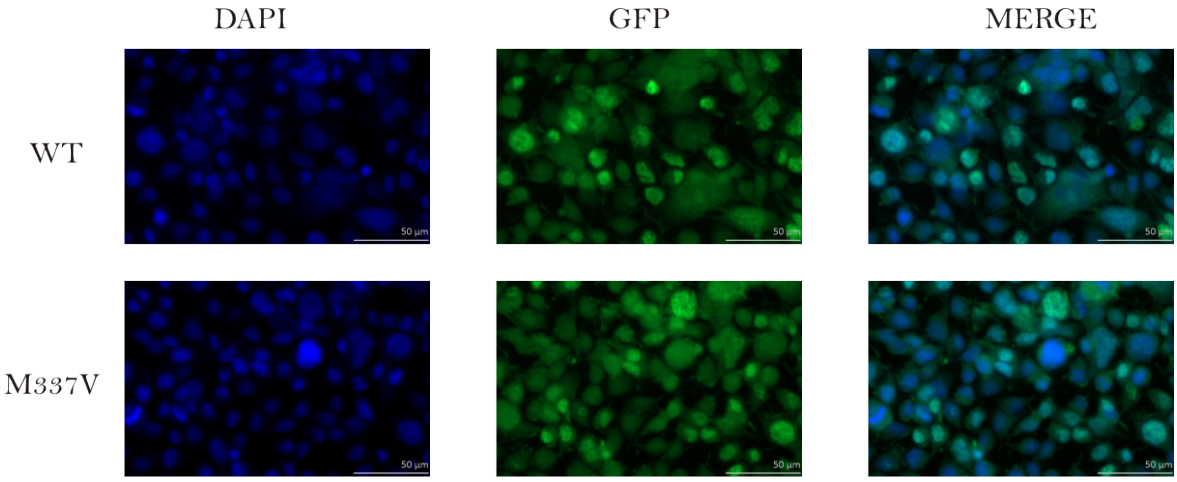


Figure 25 NSC-34 cells stably transfected with Tol2 plasmids and sorted using a BD influx. All cells are positive for GFP fluorescence. Some cells are showing a cytoplasmic location and others are nuclear in location, this is normal as TDP-34 shuttles between a nuclear and cytoplasmic location. Gating was set so that the probability of a false positive event occurring was <0.001%, 1 in 100,000 cells had a chance of being a false positive. Scale bar = 50 μm

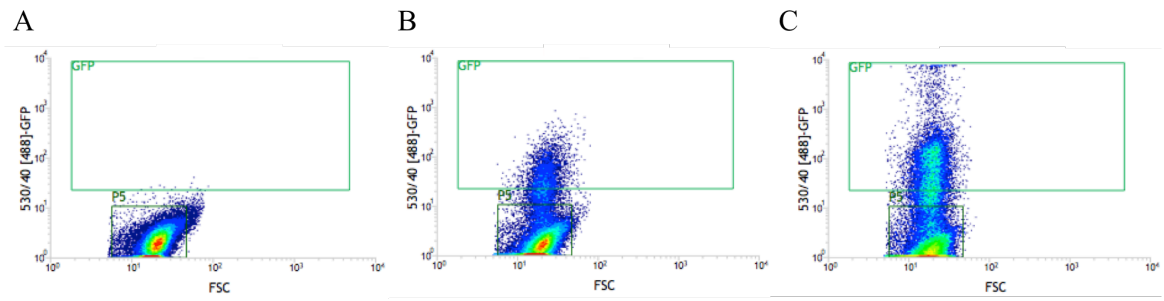


Figure 26 Scatter plots of the parameters used for sorting the NSC-34 cells. The green box indicates the gate set for GFP positive cells. If cells fall within this gates area they will be collected as GFP positive cells. The x-axis is the forward scatter and the y-axis is the side scatter, 530/40[488 nM] GFP (**A**) Control cells (**B**) NSC-34 cells transfected with the wild-type TDP-43 protein (**C**) NSC-34 cells transfected with mutant TDP-43 protein.

Co-culture of cells-

The stably transfected NSC-34 cells that were positive for EGFP and RFP fluorescence were then seeded with transfected NSC-34 cells at equal concentrations. They were seeded in a 24 well plate with 13mm cover slips incubated then fixed at 2 days, 4 days and 6 days. Using the confocal microscope, 2 random fields of view from 4 coverslips per condition were imaged using the 20x oil immersion lens. Cells were then counted and scored as being GFP only positive or both RFP and GFP positive. Only cells that had a DAPI nuclear stain were included.

The amount of GFP only cells was significantly different between the wild-type protein and the mutant (*p=0.0002*)

Multiple comparisons revealed that the mutant protein after being co-cultured for 6 days had significantly more GFP only positive cells when compared to the wild-type at 6 days (*p=0.0002*). The mutant at 6days was furthermore significant compared to the mutant control (p=0.*001*), 2days (p=0.*001*), and 4 days (*p=0.035).*

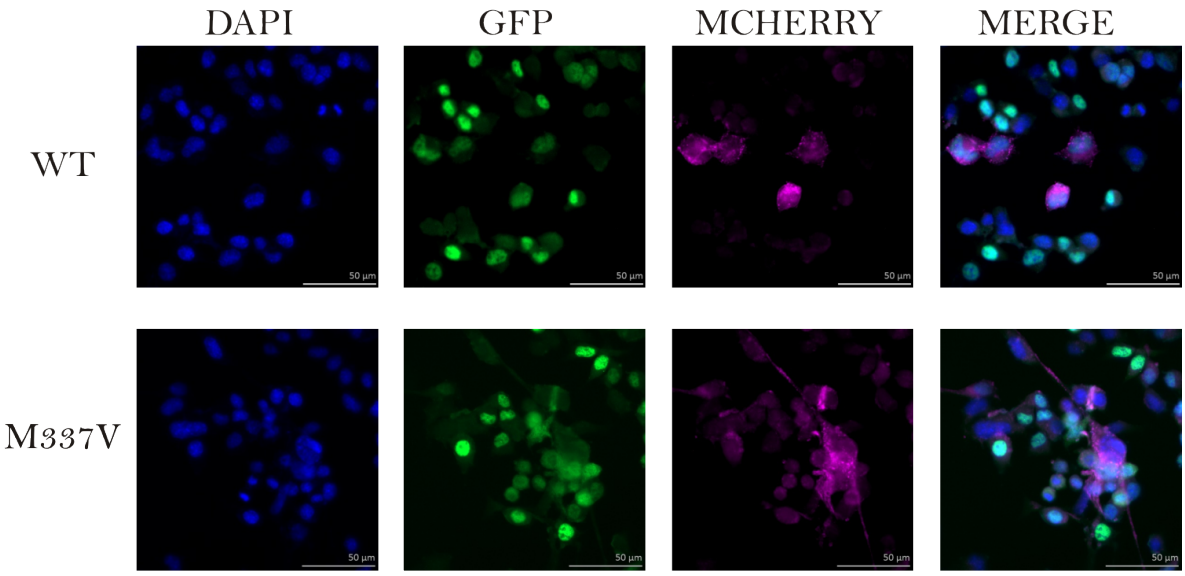


Figure 27 NSC-34 cells stably transfected with Tol2 TDP-43::GFP plasmids were transfected a second time with Tol2_mCH and sorted using a BD influx. All cells are positive for GFP fluorescence. There is a lack of mCherry positive cells, indicating that there is an issue with the methodology or tools used. The merged image should have displayed colocalisation between all three channels. Gating was set so with the probability of a false positive event occurring was <0.001%, 1 in 100,000 cells had a chance of being a false positive. Scale bar = 50 μm50 μm

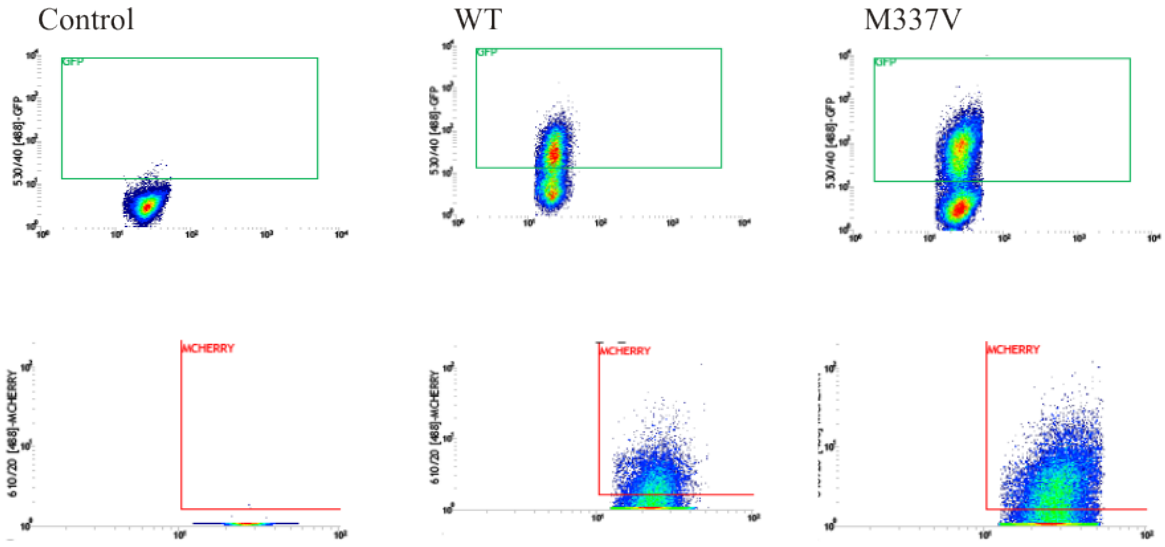


Figure 28 Scatter plots of the parameters used for sorting the NSC-34 cells. The green box indicates the gate set for GFP positive cells. If cells fall within this gated area they will be collected as GFP positive cells. The x-axis is the forward scatter and the y-axis is the side scatter, 530/40[488 nM] GFP. The second row of Scatter plots are the mCherry gates, the y-axis for this graph was 610/20 [488] mCherry.

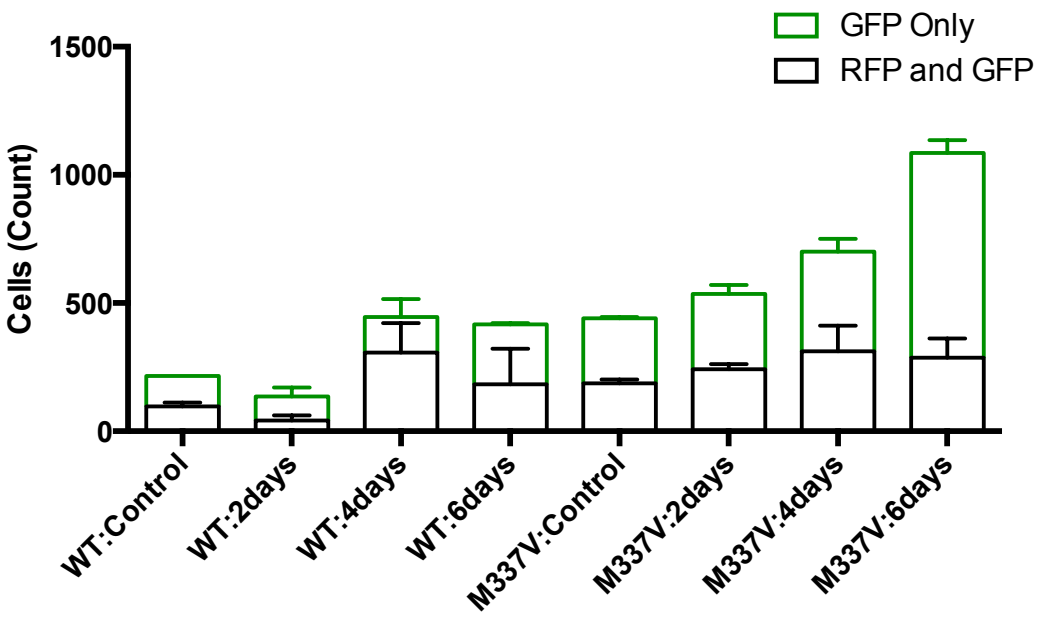
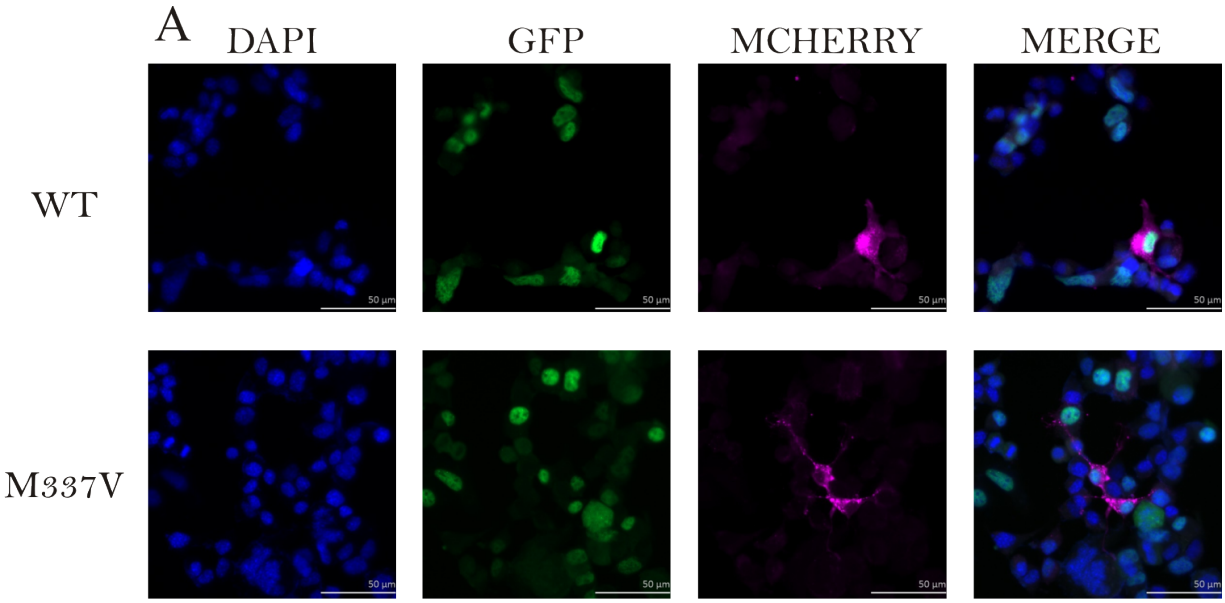


Figure 29 Cell counts from the co-cutler experiment. The proportion of GFP only positive cells to both RFP and GFP positive cells is displayed. The only significant GFP group was the TDP-43 M337V mutant protein at 6 days Values are the mean with SEM from 4 random fields of view, from one experiment. Cells were only included if they were DAPI positive.



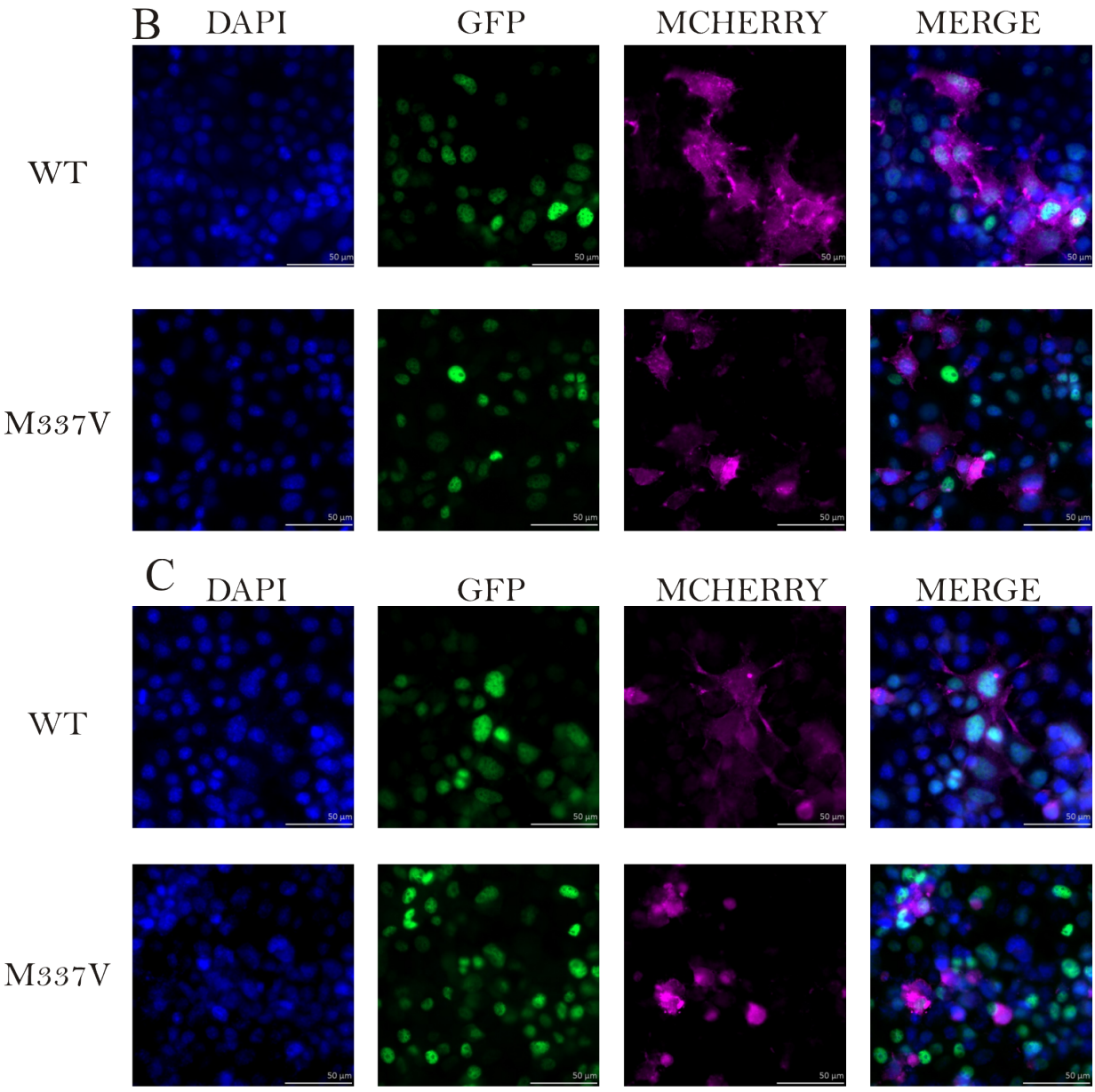


Figure 30 NSC-34 cells that were un-transfected were plated in equal densities with the NSC-34 cells that had been transfected twice with the TDP-43**::**GFP and the mCherry-CAAX. These cells were then fixed at 2 day intervals and imaged (**A**) Cells after two days. There is no significant difference between the amount of GFP only expression. This image is similar to the control condtion (Figure 26) highlighting the lack of RFP positive cells. (**B**) Cells after 4 days showed a slight increase in the amount of GFP only cells but this was not significant for the wild-type or mutant protein. (**C**) Cells after 6 days. There is a significant difference in the amount of GFP only cells for the mutant protein at 6 days. (*p=0.0002)* Scale bar = 50 μm

Experiment 5- Flex Station Assay

A stably transfected cell line has the potential to be utilised in many different high throughput techniques. Here we determined the feasibility of this approach by conducting membrane potential assays of cell lines generated in Experiment 4. Utilising a microplate

reader that detects a change in propriety fluorescent dye either an increase or decrease, indicating depolarisation or hyperpolarisation of the cells respectively. This will provide a high throughput method of detecting whether there is a change in membrane potential in response to a stimulus such as a drug or potassium. A variety of compounds will be used to assess whether there are enough receptors on the cells to pick up a significant change in the fluorescence.

NSC-34 Pilot/characterization Assay

In un-transfected NSC-34 cells loaded with the membrane potential dye the addition of DAMGO (MOR agonist) produced a decrease in fluorescence (**Figure 31**) consistent with the hyperpolarization of the Cells. There was also a decrease in fluorescence when WIN 55,212 (Cannabanoid receptor agonist)(**Figure 31**) was added as well as SST14 (Somatostatin peptide 14, which binds to somatostatin receptors)(**Figure 31**). The addition of KCl saw an increase in fluorescence consistent with the depolarization of the cells (**Figure 32**). The addition of Veratridine (opens voltage dependent sodium channels and prevents their inactivation) to the cells saw an initial decrease in fluorescence however at the larger dose there was a latent increase in fluorescence consistent with the depolarization of the cells (**Figure 31**). Two different doses were used in this initial assay and the responses of the cells do appear to be concentration dependent, this data is from two experiments, which were duplicate experiments.

Neuro2a Characterisation

In the un-transfected Neuro2a cells loaded with membrane potential dye, addition of DAMGO, WIN and SST14 produced similar results to that of the NSC-34 cells. There was a decrease in fluorescence (**Figure 31**), consistent with the hyperpolarization of the cells. The addition of KCl saw an increase in fluorescence consistent with the depolarization of the cells (**Figure 32**). The addition of Veratridine saw an decrease in fluorescence consistent with the hyperpolarization of the cells when the concentration was 1 um with the addition of 100 nm of Veratridine there was an increase in fluorescence at time as well as a decrease (**Figure 31**). This response also appears to be concentration dependent, however this is only pilot data from two experiments, which were duplicates and only two doses.

The responses to the difference compounds appeared to be consistent between the two cell lines. A 2-way ANOVA comparing at the responses between cell lines was not significant (mean= 99.746 SEM± 0.198) $p=0.1356$, indicating that either cell line would be appropriate for use in this assay.

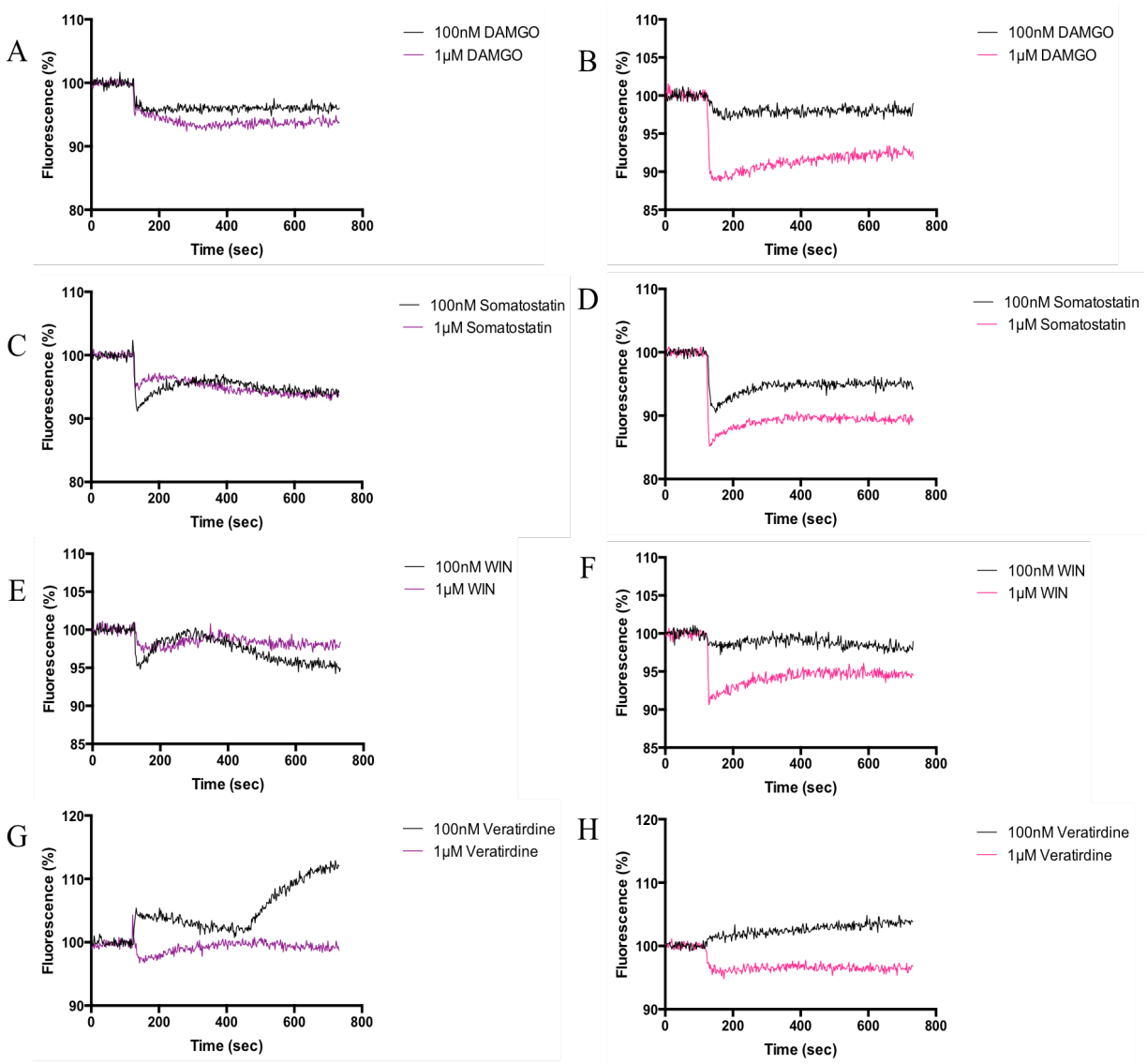


Figure 31 Raw traces of responses to different compounds, fluorescence is normalised to the base line with and without dye. The change in fluorescence indicates an alteration in the cells membrane potential. The increase in relative fluorescence indicates the depolarisation of the cell, a decrease in relative fluorescence indicates the hyperpolaristion of the cell. Graphs **A,C,E** and **G** are the responses seen from the NSC-34 cells. Graphs **B,D,F** and **H** are the responses seen from the Neuro2a cells.

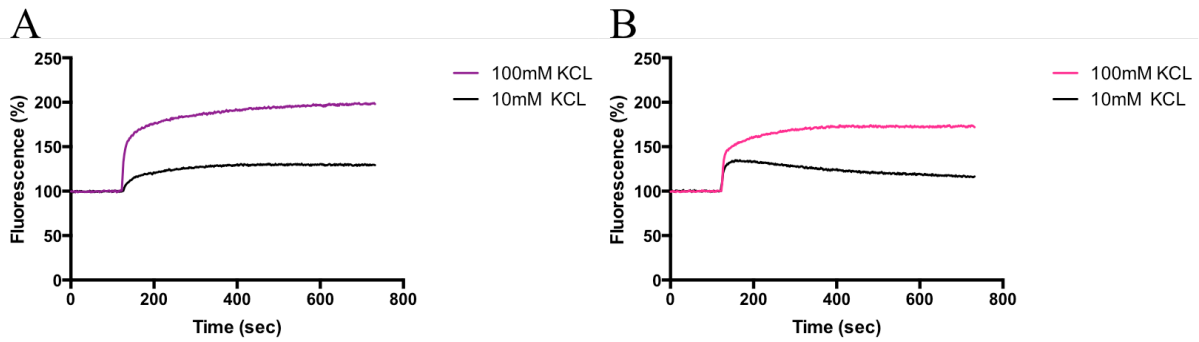


Figure 32 Raw example traces of the response to the addition of KCL. There is an increase in fluorescence indicating the depolarisation of the cell. (**A**) NSC-34 cells respond to the addition of KCl in a dose dependent manner. (**B**) Neuro2A cells respond to the addition of KCl in a dose dependent manner.

Membrane potential assay of stably transfected cells

For the preliminary investigation of possible differences between cells in a stably transfected cell line KCl will be used, as there is a large concentration dependent response (**Figure 32**). Different concentrations between 300mM and 1mM of KCl were added to two different NSC-34 cell lines; NSC-34 cells stably transfected with the wild-type and A4V mutant of the SOD1 gene (**Figure 33**) and cells stably transfected with TDP-43 wild-type and M337V mutant proteins (**Figure 34**). A 2way ANOVA was completed to assess the differences between the wild type and mutant genes stably expressed within the cells. With a multiple comparisons to assess whether a specific concentration of KCl had a statistically significant effect. The data analyzed was from three duplicate experiments.

SOD1 wild type and A4V.

Over all there was a significant difference between the wild type response and the A4V response to the same concentrations of KCl (*p 0.0001)* (**Figure 35**). The A4V mutation had a larger response to the KCl than the wild-type. Multiple comparisons of the concentration revealed that 300mM (*p<0.05)*, 100mM (p<0.*05*), 30mM (*p<0.05)* and 10mM (p<0.*05*) were significantly different between the wild type and A4V cells. There was no significant difference of the responses to the 1mM and 3mM concentrations (p>0.05).

TDP-43 wild type and M337V.

There was a significant difference between the responses of cells stably transfected with the wild type and the M337V mutant to the concentrations of KCl (*p<0.001)* (**Figure 35**). The response of the cells expressing the mutant TDP-43 protein was lower than the wild-type protein. Multiple comparisons revealed that only concentrations of 100mM (*p<0.05)* and 10mM (*p<0.05)* were statistically significant differences.

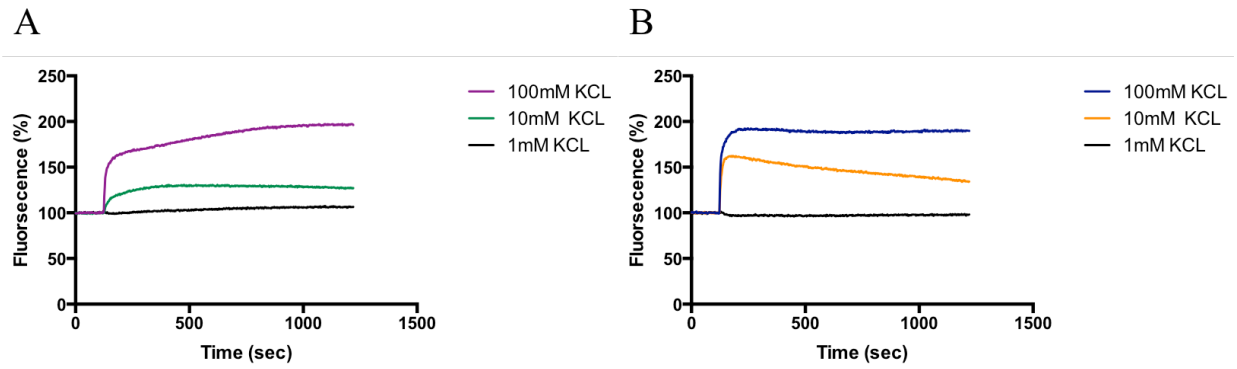


Figure 33 Raw fluorescnece traces of NSC-34 cells stably expressing SOD1 wild-type and A4V mutation. Both cell lines exhibit a concentration dependnt response to KCl. (**A**) responses of cells expressing wild-type SOD1. (**B**) Responses of cells expressing A4V SOD1

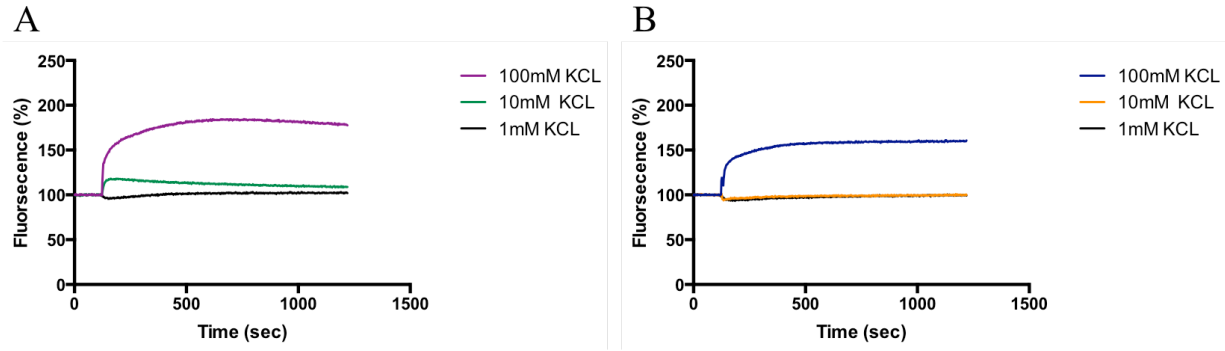


Figure 34 Raw fluorescnece traces of NSC-34 cells stably expressing wild-type and mutant TDP-43 proteins which were created in tehe previous experiment. Both cell lines exhibit a concentration dependnt response to KCl. (**A**) responses of cells expressing wild-type TDP-43. (**B**) Responses of cells expressing the M337V TDP-43 mutation.

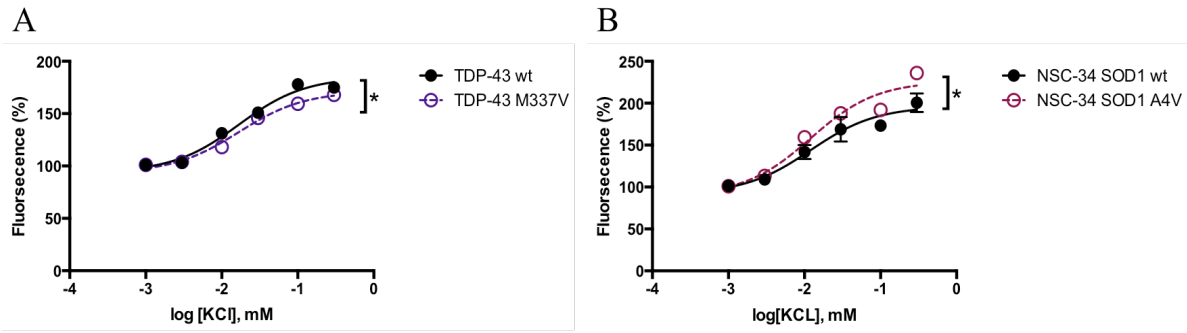


Figure 35 Concentration response cureves for the two cell lines.(**A**) NSC-34 cells expressing the mutant TDP-43 protien have a decreased response to KCl compared to the Wild-type TDP-43 protein. (**B**) NSC-34 cells expressing SOD1 A4V mutation have an increased response to the addition of different KCl concentrations compared to cells expressing the SOD1 wild-type.

Discussion

ALS is a neurodegenerative disorder that affects the upper and lower neurons. The identification of TDP-43 as a component of the pathological inclusions within patient tissue and the subsequent identification of mutations in the TARDBP gene that encodes this protein highlighted a possible mechanistic link (Sreedharan et al., 2008). Under normal conditions TDP-43 is localized to the nucleus of cells, it is a protein that is ubiquitously expressed and important for the survival and function of that cell(Ling et al., 2013). In ALS pathological TDP-43 is found in the cytoplasm, phosphorylated ubiquitinated and unusually cleaved.

One of the hypotheses as to how TDP-43 contributes to this degeneration is that it becomes prion-like and moves between neurons. Causing the progressive spread of neurodegeneration that is observed clinically in patients (Polymenidou and Cleveland, 2011)

The goals of this study were three fold. First we wanted to stably introduce protein in the form of a fluorescent fusion protein to our cells. Secondly we wanted to use a technique that would allow single cellular resolution and control over which cells we transfected with our protein and thirdly we wanted to create a stably transfected cell line for possible use in high throughput screening assays that could assess differences in function

Experiment 1- Induction of TDP-43 aggregates in NSC-34 cells

NSC-34 cells were transiently transfected with plasmids containing human TARDBP (wild-type or M337V mutation). The transfected cells were treated with different stressors to determine whether the intracellular localisation of the protein could be altered and in turn whether it was possible to induce intracellular aggregates containing TDP-43 protein in this cell model.

Potassium mislocalisation

The cytoplasmic localization of the TDP-43 protein was a novel finding as to the best of our knowledge KCl has not been used as a treatment before. The majority of the current literature uses stressors that induce oxidative stress (sodium arsenite) (Duan et al., 2010) or inhibit proteasome function (MG132) (Bence et al., 2001) as just a couple of examples to facilitate the movement of the TDP-43 protein from a nuclear location to a cytoplasmic location. The use of KCL was based on a method described by Follett et al. (2013) who used KCl within a Parkinson's disease model. KCl when added to the HEK239 cells and SH-SY5Y neuroblastoma cells lead to the formation of alpha-synuclein aggregates within the cytoplasm of these cells. We did not see any aggregation formation however it took 48 hours for the alpha-synuclein to start to form aggregates from when the KCl was removed (Follett et al., 2013).

Heat shock and aggregation

Mislocalisation of the TDP-43 protein was not achieved using the Heat shock method, at 43^{o}C for one hour (Shang and Taylor, 2011)This temperature may have been too much of a stressor causing cell death or it was not robust enough to induce any mislocalisation of the TDP-43 proteins.

The lack of aggregate formation was a surprising finding as using the heat shock treatments have been successful in inducing inclusions within the a cell culture model (Bence et al., 2001;Ross and Poirier, 2004;Ferreira et al., 2007;Volkening et al., 2009)Some cells did show what looked like cytoplasmic aggregates. Although this was not a statistically significant finding, it does demonstrate that TDP-43 in our cells could form aggregates but different stressors may be more appropriate and have a stronger effect.

Limitations of this model

One limitation of the experiments completed with the pCMV plasmids is that endogenous TDP-43 was not measured or quantified in the cells. Although the focus of the experiments was on mislocalisation and aggregation of the human TDP-43 protein that was transfected into the cells, it could have been beneficial to determine the location of the endogenous TDP-43. TDP-43 is ubiquitously expressed in all cells and the treatments could have had an effect on the endogenous TDP-43. An over expression of TDP-43 in cells can cause endogenous TDP-43 to mis-localise (Ling et al., 2010)and even form aggregates (Zhang et al., 2009). There is a possibility that there was aggregate formation however it was not detected.

The transient transfection meant that there was a time limit to which the cells were expressing the TDP-43 protein and this limited the scope of the experiments completed, as a singular treatment could be given.

Lipofectamine can be toxic to the cells but there was no noticeable increase in dead cells or debris after the transfections, however the low transfection efficiency meant that there were not many GFP positive cells possibly skewing results. Further compounding this is the method of scoring of the location of the fluorescent TDP-43. This was done manually using the Zen software and simply counting cells, which means there is a lot of possible bias. Using a more advanced program that could also quantify the fluorescence would provide more meaningful and accurate data.

The use of the transiently transfected cell line was beneficial in demonstrating that the location of human TDP-43 protein could be altered in the presence of KCl but it was not an appropriate method to use for further investigation of TDP-43 protienopathies.

Experiment 2- Tol2 plasmids for stable expression of TDP-43 variant

To complement the transient transfection studies undertaken in Experiment 1, experiments were undertaken to generate more permanent transgene expression of TDP-43. This was undertaken by generating a plasmid construct that incorporates a Tol2 sequence, resulting in integration of the artificial transgene into the host genome via a transposase. After the construction of the plasmids, transfected cells were exposed to different stressors to determine whether the intracellular localisation could be altered and if this led to the aggregation of the TDP-43 protein.

Stable integration of the TPD-43 fusion protein

This is the first time to our knowledge that Tol2 plasmids have been made with CMV reporter and the hTDP-43 protein for use in mammalian cells. The TDP-43**::**GFP remained predominantly nuclear when there was no stressor added. Sustained expression for 14 days after transfection with all three Tol2 plasmids was observed.

Tol2 is a transposon-based gene vector, which provides a controlled method to introduce our gene of interest(Kawakami et al., 2000). The Tol2 transposons employ a cut and paste mechanism that involves the excision of the element of interest from the plasmid and the subsequent integration of that element into a genome of a cell (as one example) (Grabundzija et al., 2010).

Transposase systems are an alternative to a viral vector. Viruses are obligate intracellular parasites that depend on the incorporation of their own genes into a host cell and the subsequent expression for survival. To this end viruses have adapted many different mechanisms, which allow them to pass through the cell membrane and into the cytoplasm(Bouard et al., 2009) or through the nuclear envelope to the nucleus of the host cell (Naldini, 1998).Viral vectors exploit viruses' ability to integrate genetic material into a host cell making them an effective method of gene delivery. However the viral vectors give a variable copy number affecting the transduction of the gene of interest. Not to mention the time consuming task of creating the vector and getting the viral titer correct.

There are three similar transposon systems that we could choose from that employ the simple cut and paste insertion. The three different transposon systems differ in both the species they originate from and their biological properties(Grabundzija et al., 2010). *PiggyBac* was isolated from the Cabbage Looper moth (Feschotte, 2006), *Sleeping beauty* was isolated from a Salmonid (teleost) (Ivics et al., 1997)and *Tol2* was isolated from the Medaka fish (Kawakami et al., 2000).

Sleeping beauty was the first major advancement in non-viral vectors that integrate- it was synthesised from inactive copies of TC1/mariner like elements from fish(Urasaki et al., 2006). Sleeping beauty has been successfully used in a wider range of vertebrate species however it uses a non-autonomous transposase. The non-autonomous transposase has deleterious effects which can be quite major and can only be mobilized in the presence of an autonomous transposase (Grabundzija et al., 2010). Size limitation of Sleeping beauty is ~5kb (Izsvak and Ivics, 2004) which was not appropriate for our needs. A secondary issue is over production inhibition, that is there is too much transposase it inhibits the transposition and in turn there is low expression of the plasmid (Huang et al., 2010).The PiggyBac system integrates at TTAA chromosomal sites (Rosenzweig et al., 1983) and has a comparatively efficient transposition level when compared to Tol2 and Sleeping beauty systems (Huang et al., 2010). The PiggyBac system has a preference for inserting in cellular gene or promoter sequences of the target cell, that causes insertional genotocxicity and mutagenic effects (Bire et al., 2013).

Tol2 system has predominantly been used within Zebrafish systems as they are of the same species that the system was isolated, but has subsequently been used in frog, chicken and mouse cells successfully (Kawakami and Noda, 2004;Sato et al., 2007). The Tol2 system uses an autonomous transposase element, which is functional on its own and

does not require another element(Kawakami et al., 1998). Tol2 integrates a single copy through cut and paste mechanism, it does not cause any rearrangement or modification at the target site except for the creating of an 8bp duplication (Kawakami et al., 1998;Kawakami, 2007). Because of the 8 bp duplication, 8 bp direct repeats are always seen adjacent to the integrated Tol2 elements in the target genome (Kawakami et al., 2000). The tol2 transposase only uses minimal *cis* sequences which are necessary for successful transposition, recently it has been shown that transposon constructs need to only be 200bp of DNA from the left end and 150 bp from the right, making it a very minimal transposase (Urasaki et al., 2006). The maximium size of DNA that can be used within a Tol2 vector is around 11 kbp currently, making it suitable for our needs.

The creation of Tol2 plasmids is very simple. To create the entry clones a BP reaction with PCR is used to create entry clones that contain att sites. Then multi-site gateway cloning (Invitrogen) is used in the form of an LR reaction to create the final plasmid. This system means that the four entry clones are combined in one reaction. The Multi-site gateway LR reaction that is used to create the Tol2 plasmids is of large benefit as it does not take long and it allows for multiple recombination's in one reaction.

Overexpression of a gene such as TDP-43 is not a physiologically realistic mechanism to investigate the pathological mechanism behind TDP 43. Patient tissues from FALS and SALs cases show that there has not been seen a increase in the genes amount (Ihara et al., 2013). Tol2 only inserts a single copy making it a more accurate model to study the possible pathogenic mechanisms.

Localisation and Aggregation

Treatment with 50 mM of KCl for 4 hours induced mislocalisation of both the wild-type and mutant TDP-43 protein in the stably transfected NSC-34 cells. Similarly to the earlier experiments this method of treatment did not induce aggregates, as we mentioned above it took 48 hours for aggregates to form in a model of Parkinson's disease (Follett et al., 2013). Treating the NSC-34 cells with heat (43^{o}C) for an hour did not cause mislocalisation of the TDP-43 protein's of the wild-type or the mutant, indicating that the 43^{o}C for one hour may not have been enough of a stressor to cause mislocalisation or subsequent aggregation of the protein.

The stable transfection of the TDP-43::GFP fusion protein meant that a longer treatment could be applied with out the worry of losing too many GFP positive cells. MG132 a proteasome inhibitor was used for 24 hours (10 μM), on the transfected cells and caused localisation of the TDP-43 protein into the cytoplasm of the cells for both the wild-type and the mutant form. Although it has reported that MG132 causes the formation of aggregates of the TDP-43 protein (Walker et al., 2013) there was no GFP positive aggregates identified.

Limitations of this Method

Similarly to the experiments completed with the transiently transfected NSC's the amount of endogenous TDP-43 was not detected in any of the treatment groups. This is a definite next step as the stably introduced TDP-43 could cause the endogenous TDP-43 to be more prone to mislocalisation under the different stressors. And likewise the endogenous

TDP-43 could have been sequestered into cytoplasmic aggregates within the treatment conditions, however this was not examined.

The stable introduction of the TDP-43 could possibly help the survival of the cells, although this is unlikely the transposon could have had a mutagenic effect that confers survival.

The low transfection rate in NSC-34 cells when lipofectamine is utilised can make it hard to identify GFP positive cells, as when the TDP-43 transfers to the cytoplasm, the GFP is not as strong making it harder to identify cells. The method of scoring the 100 cells per experiment was flawed. The cells were chosen at random, but they were manually scored for location of GFP. Utilising a program for the cell counting, including defining fluorescence parameters, will remove some of the bias and provide a more accurate result.

Future directions

We have established that the use of a Tol2 plasmid vector with a transposase will facilitate stable expression of our TDP-43 fusion protein. Building upon the results of the induced localization and aggregate experiments, these could be repeated using the same treatments but altering the concentration, the period of time, increasing the number of treatments, and importantly the time point at which the cells are fixed.

The use of live cell imaging could be another option, as the protein is GFP tagged enabling the location to be visualized. A system such as the IncuCyte Zoom® (EssenBioscience), works within a cell incubator and will image cells with fluorescence at adjustable intervals. Providing data on the time period in which the protein moves and also when aggregation occurs in response to different treatments.

Future experiments should also look at the location of endogenous TDP-43 and whether this is altered in any way.

Experiment 3- Single- cell transfection of NSC-34 cells

To assess whether long-term expression of the mutant TDP-43 gene results in trafficking of mutant TDP43 protein from transfected neurons to surrounding non-transfected cells, we initially attempted to transfect single NSC-34 cells with TDP::GFP fusion proteins in a Tol2 cassette by single cell electroporation. A glass pipette filled with plasmid DNA was placed in gentle contact with target cells and a train of voltage pulses passed through the tip of the pipette, repelling the DNA and simultaneously driving the formation of small pores in the cell membrane. Cells were then incubated for >24 hours and examined for the expression of fluorescent reporter proteins.

SCE Methodology

Electroporation is a technique that uses an electrical current to deliver genes. An electrical energy is used to temporally disrupt the cell membrane and iontophoretically drive charged molecules into the cell (James and Yu, 1996). Electroporation is sometimes referred to as batch electroporation as it is performed on a number of cells at once. Single cell electroporation is an adaptation of the original technique. However single cell electroporation is a highly focused disruption of a single cell's membrane(Rae and Levis,

2002). Single cell electroporation is one of the more recently developed techniques first published in 2002 by Rea et al. It enables the targeted delivery of macromolecules including DNA plasmids into individual living cells.

The electroporation of the fluorescent dextran dye molecule was successful (79%) in the NSC-34 cells as was the single cell electroporation with the GFP plasmid, although the success rate was lower (15%) this involved electroporation a plasmid into the cell that is larger than Dextran. The plasmid once electroporated into the cell requires translation and transduction, two processes which are reliant on the cell recovering from the electroporation and remaining viable.

The technique requires a microelectrode, which is a small glass pipette (0.5-0.3μm) (**Figure 11**)The microelectrode contains the plasmid to be delivered and a microscope is used to guide the pipette tip to the cell. When the pipette tip is right on the cell membrane a voltage (200mV-10V) is applied. The cell membrane will break down, forming pores which facilitates the movement of the plasmid into the cell. The pipette is moved away, the cell is left to recover and the plasmid has now been transferred to the single cell.

As a result of the application of an electrical current; pores will form in the membrane (Weaver and Chizmadzhev, 1996), these pores form on a time scale of nanoseconds(Chang, 1989), macromolecules will be able to enter the cell via these pores(DeBruin and Krassowska, 1999), in the absence of an external electrical field the pores will close (Smith et al., 2004),and the time course of the closure of pores can vary from seconds to minutes(Krassowska and Filev, 2007).

Limitations of SCE

Once the plasmid is electroporated into the cell, it will be within the cells cytoplasm. For the gene to be properly transposed and integrate, the plasmid needs to be stable when in the cytoplasm, it needs to reach the nucleus of the cell with out being completely degraded, it then needs to be integrated into the host genome so it can be replicated and then you want reliable expression of the transgene. This is quite a process and it is possible that the transposase plasmid or the Tol2 plasmid was degraded therefore preventing the integration into the host genome. An alternative is to use a transposase mRNA. Transfections that used a transposase mRNA have been shown to improve the efficiency by ~50% (Kawakami et al., 2004) and mRNA transposase is regularly used with Tol2 plasmids in Zebra fish (Clark et al., 2011). A second option would be to use a transposase protein eliminating the possible issues of plasmid degradation. There is mRNA available in the Tol2kit (Kawakami, 2007) that could be utilised but as of yet no Tol2 transposase protein available.

The use of a separate transposase protein is meant to provide control over the transposase process, such as when the excision occurs and by regulating the amount of transposase it allows that optimisation of the transfection rate. Nonetheless for the SCE we do not necessarily need the separate transposase plasmid, and instead could use a plasmid that contains both the transposase and our Gene of interest with Tol2 sites.

The exact mechanisms behind SCE are still not fully understood, leaving a large scope when troubleshooting. The Tol2 vector has the capacity to carry a DNA insert as large as

11kb without reducing transpositional activity (Kawakami, 2007) and has been used in SCE experiments successfully (Oyama et al., 2013). The parameters we used were modelled on the work completed by Oyama et al. (2013) and (Bestman et al., 2006). papers, conversely Steinmeyer and Yanik (2012) had a success rate of ~20% with these parameters.

Single cell electroporation provides control over not only which neuron the gene is delivered but also the number of neurons targeted in an individual experiment. The technique is relatively easy to learn but does require some practice like most new skills(Kitamura et al., 2008). The equipment can be expensive if every component from the high-powered microscope to the micromanipulator is purchased. Reagent cost is similar to that of the viral reagents, however the plasmid preparation is a lot quicker. Unlike viral vectors there is no associated toxicity with this technique and it is suitable for cell culture or tissue(Olofsson et al., 2003).

Single cell electroporation is a time consuming technique and one major limitation of single cell electroporation is that the DNA is delivered into the cytoplasm of the cell, not directly into the nucleus(Bestman et al., 2006). Nuclear delivery is necessary for the expression of the DNA. It is up to the design of the plasmid and the use of appropriate promoters to get the DNA integrated the genome of the cell(Haas et al., 2001).

The major benefit of using single cell electroporation as a technique to introduce our gene of interest is the amount of control it provides. These initial pilot experiments were attempted to determine whether the Tol2 plasmids would be viable and integrate permanently, to offer a means to investigate the prion-like propagation of the TDP-43 protein. Single cell electroporation used in conjunction with organotypic brain slice preparation would have provided a powerful model. The organotypic brain slice is a 300 um slice of live brain tissue that is then kept alive using a Millipore membrane and artificial cerebro-spinal fluid, in an incubator at 37^{o}C with 5% CO_2. The benefit of using a slice preparation like this is that they last for 7-14 days (Stoppini et al., 1991) and all the cells are in situ. So not only does it provide the opportunity to look at how the protein moves within neurons, but also how it interacts and possibly moves within glial cells. As there is evidence that glial cells play a role in ALS disease progression, with TDP-43 aggregates found in glial cells from patient tissue to name one example (Lobsiger and Cleveland, 2007)

Methodological alternatives

Possible techniques that could be used for the above mentioned experiment with the Tol2 plasmids would be utilising microinjection. Microinjection is a physical gene delivery technique. To circumvent the difficulty of getting the DNA through the plasma membrane A fine glass capillary is used to hold the cell, while a micro needle injects the DNA plasmid. The plasmid can be injected into specific parts of the cell like the nucleus or the cytoplasm. However as this is a physical method it means puncturing the cell membrane and causes a very low survival rate.

One of the major benefits of this technique has over SCE is the ability to choose the cellular location of gene delivery with every injection. Other benefits include the ability to quantify the exact amount of DNA that has been injected, there is no size limit for the

DNA and only a single cell is injected at one time providing control over the amount of cells. Microinjections are labor intensive(Lappe-Siefke et al., 2008) require a high amount of skill (Washbourne and McAllister, 2002) and can require some optimization of the pressure that the DNA is delivered at. The physical nature of the technique means there can be the possibility the injection compromises the function of the cell and sometimes kills the cell entirely(Washbourne and McAllister, 2002).

Experiement 4- Generation of a stably double-transfected cell lines

Although initially promising, the single cell electroporation with our Tol2 constructs was time consuming and proved poorly suited for succsessful transfection. In order to generate a high-throughput assay to assess the physiological consequences of mutant TDP-43 expression and its intercellular trafficking, we generated cell lines that constitutively expressed two transgenes. First, cells were Lipofectamine -transfected with a Tol2 vector encoding wild-type or mutant TDP-43::GFP fusion proteins. These were sorted via FACS. Once recovered were transfected a second with a Tol2 vector that drives the expression of mCherry (RFP), and subsequently sorted a second time. The double transfection was to allow identification of originally transfected cells. To hopefully determine whether our TDP-43 protein moved between cells.

Stable cell line with TDP-43**::**GFP

The Tol2 plasmid that was used did not contain mammalian antibiotic resistance; it only contained the bacterial antibiotic resistance for ampicillin. Establishing a stably transfected population of cells via selective pressure with an antibiotic was not possible.

FACS was an elegant solution to our problem, we needed a population of cells to all be GFP positive containing out TDP-43 protein but we did not want it to be too time consuming. Fluorescence activated cell sorting is a within the discipline of flow cytometry, instead of just quantifying cells and cell types a laser is used to detect and categorize cells based on their fluorescence emission (For review Lanier (2014)). The cells are hydrodynamically focused so that there is a single stream and distance between cells (Galbraith et al., 1999;Hawley and Hawley, 2004). As the cell passes though an excitation laser, the side scatter and forward scatter are detected. A vibrating mechanism causes the stream to break apart in droplets, a charge added to the last attached droplet according to the cell detected that is contained within. The charge causes the cell to be deflected into the correct collection tube when it passes through the charged plates at the end. The whole process of sorting the three samples takes approximately four hours, that includes the initial set up and calibration. The BD Influx was used as it is a closed system, keeping the cells sterile and the medium they were sorted into could be kept at 37^{o}C.

After sorting the cells recovered with no contamination, and were all GFP positive. This stably transfected cell line does not require any form of antibiotic to maintain the expression of a fusion protein, and was quick to establish. This cell line can be used for any number of high through put assays, as long as the GFP does not interfere.

This cell line was not created from a single clone, subsequently there is the possibility that the population of cells is mixed and genetically undefined. The fusion protein that was transfected in could have degraded; a simple western blot for GFP and TDP-43 needs

to be completed to ensure that the full fusion protein is expressed. Conversely PCR or linker mediated PCR can be used to analyse the integration sites. The Tol2 system only creates a single copy insertion with no deletions nor gross rearrangements of the host genome around the integration site(Kawakami, 2005;Suster et al., 2009). The level of expression of the TDP-43 protein compared to the levels of endogenous TDP-43 protein likewise needs to be quantified as this could skew results.

A major benefit of this stably transfected cell line is that all of the TDP-43 mutations could be stably transfected and utilized in an assay. This encourages very broad comparisons of all the mutations before more labour intensive assays are used. Some of the assays this cell line could be used for include the membrane potential assay, calcium assay, screening of drugs or new compounds is also possible.

Limitations of a double transfection

After the second round of sorting of the NSC-34 cells containing TDP-43::GFP fusion protein and the mCherry; a quick visual confirmation that the NCS-34 cells contained GFP and RFP was completed and the cells recovered to ~90% confluence before immediately being plated out for the co-culture assay. It was only after the completion of the co-culture assay that the cells were fixed, mounted and imaged properly.

There was not a consistent amount of double-labelled cells that was expected, the cells were all GFP positive and only some had RFP fluorescence as well. This could be due to a number of factors. The sorting it self is stressful on the cells, as is lipofectamine that can be toxic in large amounts. There is the possibility that the Tol2_mCHerry plasmid was not stably integrated and when the cells were initially checked it was transient expression of the mCherry. Alternatively the mCherry CAAX could have a toxic effect on the cells, although this is not as likely given that the CAAX motif target the endo-membrane and has been used within cells and zebrafish (Hancock et al., 1991).

Using a separate transposase plasmid could be a key issue in the design of this cell line. There is a chance that the pCS2+ plasmid remained as episomal DNA, further compounded by the fact we introduced a second amount. This pCS2+ remains and goes through multiple cycles within the cells causing damage to the chromosomal targets (Bire et al., 2013). Further more as Tol2 transfection may have been compromised as it had already been completed one, the second might not have successfully integrated into Tol2 sites.

Co-culture of stably transfected cells with naïve cells

The co-culture experiment was only a pilot study to determine whether the TDP-43 protein would move between cells with no external stimuli. It appears that after 6 days, TDP-43 may move into un-transfected cells. But there were major issues with our methodology- apart from the fact that the double cell line didn't work other issues that affect our interpretation of the results include; there are obviously more cells as you increase the number of days they were co-cultured, by day 6 the cover slips were very confluent. The confluency issues could have been ameliorated by co-culturing the cells within a flask, and passaging and plating them out on to a cover slip as needed- which removes the possibility of over crowding as you can determine the concentration when

you fix and image them. The amount of cells counted for each condition were not normalised. Cells grow exponentially, the Mutant condition already started out with a larger amount of GFP positive cells than the wild-type protein, there is a possibility that the result simply reflects this fact and not that of the protein actually moving.

Confocal images were taken of random fields of view but they were only taken on one plane paired with the over crowding there is the possibility that GFP or RFP positive cells were missed (as the RFP is within the cytoplasm/membrane and the GFP is mostly within the nucleus). The fluorescence was not quantified; the count was simply completed on the "bright" cells that could be seen. The different conditions were simply counted using Leica AF software, there is an inherent bias in one person completing the counting as there will be a different result each time. More stringent criteria and the use of better software program could remedy this in the future.

Another alternative to using microscopy as the method of detecting the RFP positive cells and GFP positive cells and the multitude of issues to do with bias and counting, would be to use a cell sorter. Following similarly on the principle used to develop the double-labelled cell line with the use of hierarchical gating.

Alternative methods to a double transfection

The rational behind using a cell line expressing two flurophores was to identify these was the "original cells" in an attempt to determine whether the TDP-43 protein moved between cells. An alternative would have been to transfect NSC-34 cells with only the Tol2_mCherry plasmid and sorting to create a cell line that is RFP positive. To co-culture this with the TDP-43 GFP cell line would be another option, as the identification of RFP and GFP positive cells could indicate GFP movement. Conversely different plasmids could be used, that contain a flurophore for the initial cell transfected and a secondary flurophore for the GFP.

Experiement 5- Membrane potential assay

Our ultimate objective is to utilise the stably transfected cells lines express wild-type or mutant TDP-43::GFP fusion proteins, to investigate the cellular and physiological consequences of mutant TDP-43. Here we determined the feasibility of this approach by conducting membrane potential assays of cell lines generated in Experiment 4. Utilising the Flex Station (molecular devices) a microplate reader that detects a change in propriety fluorescent dye either an increase or decrease indicating depolarisation or hyperpolarisation of the cells respectively. We exposed the cells to a variety of compounds and determined in real time, whether there was a change in fluorescence indicating a response.

This will provide a high throughput method of detecting whether there is a membrane potential in response to a stimulus such as a drug or potassium. A variety of compounds will be used to assess whether there are enough receptors on the cells to pick up a significant change in the fluorescence.

NSC-34 and Neuro2a cells can be used within a Flex station

The first cell lines that were used were un-transfected. We used NSC-34 cells and Neuro2a cells, neither of which have been used in a Flex Station assay to the best of our knowledge. The results from the initial membrane potential assay on the NSC-34 cells and the Neuro2a cells demonstrated that there are enough receptors within the cell membrane to detect a response to an agonist.

Due to the preliminary nature of the data more stringent tests need to be completed, namely with the use of proper controls. Each compound is an agonist for a corresponding receptor; DAMGO is a synthetic Mu-Opioid Receptor (MOR) agonist, WIN 55,212-2 is a Cannabinoid receptor 1 (CB1) agonist, SST14 a somatostatin receptor agonist and Veratridine binds to Na channels. Appropriate controls that could be used in further studies would be Naloxone or PTX for the MOR,BIM 23627 for the somatostatin receptors, P2A for potassium channels and tetrodotxin for Na channels (Narahashi, 2001;Cawston et al., 2013;Knapman and Connor, 2015).

Veratridine, is a steroid derived alkaloid which can function as a neurotoxin. Within the literature it is reported that Veratridine acts by binding to sodium channels causing persistent activation and action potentials (Barnes and Hille, 1988). When large concentrations of Veratridine were added to the NSC-34 cells on a few occasions there was a delayed but significant increase in fluorescence indicating that the cell had become depolarized. This occurred after the initial hyperpolarization. This could be due to many reasons; the compound did not bind properly for one, or it could be a real result within this cell line. Further assays with a range of concentrations of Veratridine with appropriate controls such as tetrodotoxin (Narahashi, 2001), will need to be conducted before any conclusions are made.

Cannabanoids have been posited as a beneficial as a neuroprotectant agent in ALS(Moreno-Martet et al., 2012). The neuroprotective properties of cannabinoid compounds have been studied in different neurodegenerative disorders (Raman et al., 2004;Fernandez-Ruiz et al., 2010) The rational behind cannabinoids as a neuroprotectan in neurodegenerative diseases is associated with their ability to decrease excitotoxicity, microglial activation, neuroinflammation and oxidative stress (Fernandez-Ruiz et al., 2010;Moreno-Martet et al., 2012). The administration of a cannabinoid compound Δ^9-Teterahydrocannabinol (THC) in the SOD1 mouse model (mutation G93A) was shown to actually delay the motor impairment and prolong the survival (Raman et al., 2004) this result has similarly be shown with the CB1 agonist WIN 55,212-2 (Bilsland et al., 2006). CB1 receptors have been identified within NSC-34 cells via RT-PCR, western blots and immunocytochemistry (Moreno-Martet et al., 2012) and our results confirm these findings. It is also important to note that the cells were undifferentiated at the time of the assay. NSC -34 cells can have a 5-fold increase/ in the amount of CB1 receptors present after differentiation (Moreno-Martet et al., 2012).

The membrane potential assay is just one type of functional assay that the stably transfected cell lines could be used with. The Flex assay does have some benefits, the major benefit being that it is a real time assay, as the assay is running you can see the real time response on the computer as it happens (Knapman et al., 2013). The flex assay is a no wash assay, which removes the possibility of loosing cells, however this limits the

usability of the flex. There is no bleed through of fluorescence as one well is read at a time, also providing a large amount of data that can be obtained. Multiple additions of compounds can be administered within the one assay allowing more complex questions to be answered, such as the effect of priming a cell, or which receptor is responding, or the binding affinity of the agonist within the cell line. The flex station can also be used with Ca^{2+} assays, as there is a proprietary dye available for that purpose. The clear bottom 96-well plate additionally means that cells can be imaged after the assay, allowing possible morphological or protein changes to be visualized.

In summary the results from the membrane potential assay demonstrated that the cells are an applicable tool to study the possible functional effects of the mutations within the functional membrane potential assay. However further assays with appropriate positive and negative controls will need to be completed before definitive conclusions can be drawn from this data. As well as a comparison of the receptor activity in differentiated vs. Un-differentiated cells.

Stably transfected cell lines with ALS mutations express differences in membrane potential responses to a compound

Electrophysiological differences between mutations associated with ALS are of interest as studies with patient tissue have shown that there are differences associated with SOD1 mutations (Dong et al., 2014) however the systematic and controlled study of the possible effects of TDP-43 mutations on the electrophysiological properties is yet to be fully explored (Dong et al., 2014). The applicability of the real time membrane potential assay utilizing the Flex station is an attractive option to quickly assess differences across all of the TDP-43 mutations. NSC-34 cells do not synapse with other NSC-34 cells nonetheless they propagate action potentials (Cashman et al., 1992). NSC-34 cells depolarize and hyperpolarize via Na, K, Ca channels(Cashman et al., 1992) all of which can be explicitly targeted with the Flex station assay, and more in depth patch-clamp electrophysiological studies can be continued after the initial data collection.

The cells that were used in the assay were undifferentiated. When NCS-34 cells and Neuro2a cells are differentiated, there is a change in the amount of receptors present. This differentiation usually means that there will be an up-regulation compared to undifferentiated cells (Eggett et al., 2000).

The preliminary data indicates that the membrane potential assay is sensitive enough to identify small differences between cell expressing fusion proteins, further assays with appropriate positive and negative controls will need to be conducted before any conclusion is made. Osmolarity is an important control especially at the higher doses of KCl, this can affect the data severely. The compounds used should also be varied to help determine whether mutations affect the cell functioning on a whole or a specific receptors' activity.

Suitability of NSC-34 cells as a model of ALS

NSC-34 cells constitutively express a variety of characteristics, which are associated with primary motor neurons (Cashman et al., 1992). It is difficult to obtain and grow primary motor neuron cultures and established cell lines are easy, inexpensive and can be readily manipulated- transfections, infection or microinjection (Kaplan 1998). The closer we can

get our model to resemble the full characteristics of the in situ cell that we are investigating the more robust and applicable the findings/conclusions become

Future directions

More than 30 mutations have been identified in the *TARDBP* gene which encodes TDP-43 (Gitcho et al., 2008;Kabashi et al., 2008;Sreedharan et al., 2008;Van et al., 2008), but the roles of these mutations within the pathogenesis of ALS is not yet elucidated (Furukawa et al., 2011). The amount of genes and the associated mutations linked with ALS is now reaching the 100's (Al-Chalabi and Visscher, 2014;Benjamin Rix, 2014). Researchers are faced with the problem of "where to start" in elucidating the possible pathogenic mechanisms of the many ALS linked mutations, as a genotype does not always equal a phenotype. The traditional use of transgenic models is time consuming and expensive, highlighting the need for a cheap high throughput screening method.

The ultimate goals of this study was to develop two methods that could address the prion-like hypotheses of TDP-43 from two separate angles. The first being that of the single cellular electroporation of our fusion protein providing stable expression and the ability to map the movement of the TDP-43 protein within cultured neurons. The future direction of this aim was to then co-transfect single neurons within organotypic brain slices with our fusion protein and the c-terminal of the Tetanus toxin. The c-terminal of the tetanus toxin has a defined retrograded trans-synaptic movement. Co-localisation of the fusion protein and TTC would provide a map of the TDP-43's movement.

The second aim was to develop a stably transfected cell line that could assess the prion-like hypothesis in high throughput methods. Such as functional or bimolecular assays, to determine whether the creation of a cell line could become used for high throughput screening purposes.The third aim of the study which looked at the co-culture of the naïve cells with the double-transfected cell line had promising results, indicating the possible movement of the TDP-43 protein.

There is need to develop a tool or pipeline that can quickly assess mutations associated with ALS. Ultimately the results from this thesis demonstrated that the creation of an NSC-34 cell line that stably expresses our gene of interest could be used in different assays to look at functional or biomolecular properties successfully.

Conclusion

ALS has a large genetic component and considerable research interest; new mutations of current genes and new genes are rapidly being discovered. Having a library of assays that can be used to quickly interrogate possible electrophysiological, biochemical and molecular, and functional differences provides powerful data and a starting point of difference for more detailed acute investigation.

An immortal cell line though not an exact replication of motor neurons, provides a paired down, simple model for intricate questions to be asked. This thesis has demonstrated that the cell line can be an effective tool to act as an initial screening of mutations, before the more costly transgenic animal models are developed. This will hopefully help reduce the bottle neck of information regarding the numerous ALS linked mutations.

ALS is definitely a multi-process and multi-factorial disease, however the need to examine every possible component at a molecular level provides insight into the complex interactions not to mention possible therapeutic targets or biomarkers. To provide some hope and possible relief to the sufferers and their family members of the relentless disease that robs people of their lives.

Conflict of interest

The Author and the supervisory panel acknowledge that there were no conflicts of interest financial or otherwise

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Appendix I

Materials/ and solutions used

Luria-Bertani (LB) Broth
- 1% tryptone (w/v) 0.5% yeast extract (w/v) , 1% NaCl (w/v)

LB ampicillin agar plates
- 20g/L broth with 25 g/L agar added was autoclaved. After cooling 100mg/L Ampicillin (Sigma) was added in order to facilitate selection of recombinant bacterial colonies. 25ml was poured into a petri dish and allowed to set. After setting plates were stored upside down at 4^{o}C until use.

LB Kanamycin Agar plates
- The same protocol was followed as above, however instead of ampicillin 50mg/L of kanamycin was added to the LB agar mixture.

Agarose Gel electrophoresis
- For a 1% (w/v) agarose gel, 0.5 g of agarose was added to 50ml of 1x TBE buffer and heated in a microwave to dissolve the agarose. 5ul of SYBR safe (Invitrogen) was added once the agarose had dissolved. The cooled agarose was poured into a small casting tray with a comb inserted to firm wells. Once set 1x TAE buffer was added to and electrophoresis chamber (BioRad??) along with the gel tray. Samples along with 1x loadng dye (bioline) were loaded into wells along with 5ul easy ladder II (Bioline) in an adjacent/in the first) well. Gel electrophoresis was run using a 130 V current. DNA bands were visualized under UV illumination using a Gel documentation System (Biorad).

Tris-Borate -EDTA (TBE) buffer
- 90mM Tris base, 90mM boric acid, 2mM EDTA

DMEM (Sigma)
- 4500 mg glucose/L and 110 mg sodium pyruvate/L

10x Phosphate Buffered Saline (PBS)
- 0.8% NaCl, 0.02% KCl, 0.02 M PO4 (pH 7.3)

1x PBS
- 10 mL of 10 x PBS in 90 mL filter sterilised MilliQ Water

Paraformaldehyde (PFA)
- 4% (w/v) Paraformaldehye in 0.1 M Phosphate buffer (pH 7.4)

SOC medium
- 2% Tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10mM MgCl2, 10 mM MgSO4, 20 mM glucose. (pH 7.0)

Protinase K Solution
- 2ug/ul in10mM Tris-HCL (pH 7.5), 20 mM CaCl2, 50% glycerol

HBSS- Hanks Buffered Saline Solution low K
- NACl 145 mM, 22 mM HEPES, 0.388 mM Na2HPO4, 4.17 mM NaHCO3, 0.441 mM KH2PO4, 0.407 mM MgSo4, 0.493 mM MgCl2, 1.26 mM CaCl2, 5.56 mM glucose (pH 7.4 , Osmolarity 315 ± 5)

Supplementary Videos

Video 1

Singe cell electroporation of a NSC-34 cell with TMR Dextran

https://www.youtube.com/watch?v=h-XBs-wdar8

Video 2

Single cell electroporation of multiple NSC-34 cells with TMR Dextran

https://www.youtube.com/watch?v=1jHv4qn0N88

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