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Thesis Title:

Preclinical research into amyotrophic lateral sclerosis. A comparison of established and novel techniques and models.

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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(Signature)

Declarations

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List of Abbreviations

2-AG	–	2-arachiodonoylglycerol
5-HTT	–	Serotonin transporter
AAV	–	Adeno-associated virus
ABR	–	Australian BioResources
AEA	–	Anandamide
ALS	–	Amyotrophic Lateral Sclerosis
ANOVA	–	Analysis of variance
ASR	–	Acoustic startle response
BDNF	–	brain derived neurotrophic factor
C9ORF72	–	*Chromosome 9 open reading frame 72*
CB1	–	Cannabinoid receptor 1
CB2	–	Cannabinoid receptor 2
CBD	–	Cannabidiol
CBN	–	Cannabinol
CREAE	–	Chronic relapsing experimental allergic encephalomyelitis
CS	–	Conditioned stimulus
DAG	–	Diacylglycerol
dB	–	Decibel
DMEM	–	Dulbecco's Modified Eagle Medium
EE	–	Environmental enrichment
EPM	–	Elevated plus maze
FAAH	–	Fatty acid amide hydrolase
fALS	–	Familial Amyotrophic Lateral Sclerosis
FC	–	Fear conditioning
FT	–	Filter top cage
FTD	–	Frontotemporal dementia

FUS	–	*Fused in sarcoma*
GDNF	–	Glial cell line-derived neurotrophic factor
GFP	–	Green Fluorescing Protein
Glut4	–	Glucose transporter isoform 4
GPR55	–	G-protein coupled receptor 55
IGF-1	–	Insulin-like growth factor 1
IGFBP2	–	Insulin-like growth factor-binding protein 2
IMDM	–	Iscove's modified Dulbecco's Medium
iTDP-43^{A315T}	–	*Tar DNA binding protein 43 (A315T)*
ITI	–	Inter trial interval
IVC	–	Individually ventilated cage
KO	–	Knock-out mouse model
MIA	–	Maternal immune activation
MS	–	Multiple sclerosis
NAPE-PLD	–	*N*-acyl-phosphatidylethanolamines-hydrolyzing phospholipase D
NFE2L2	–	Nuclear factor, erythroid 2 like 2
NMDA	–	N-methyl-D-aspartate
OF	–	Open field
PaGE	–	Paw grip endurance test
PBP	–	*Progressive bulbar palsy*
PGRN	–	Progranulin
PLS	–	*Primary lateral sclerosis*
PMA	–	*Progressive Muscular Atrophy*
PnC	–	Caudal pontine reticular nucleus
PND	–	Post-natal day
PPAR	–	Peroxisome proliferator activated receptors
PPAR-γ	–	Proliferator-activated receptor-γ

PPI	–	Prepulse inhibition
PVC	–	Polyvinyl chloride
RM ANOVA	–	Repeated measures analysis of variance
RNA	–	Ribonucleic acid
ROS	–	Reactive oxygen species
sALS	–	Sporadic Amyotrophic Lateral Sclerosis
SC	–	Superior colliculus
SEM	–	Standard error of the mean
SOD1	–	Superoxide dismutase 1
SOD1^{G93A}	–	*Superoxide dismutase 1 (G93A)*
SPT	–	Social preference test
TARDBP	–	*Tar DNA binding protein*
TDP-43	–	*Tar DNA binding protein 43*
THC	–	Δ^9– tetrahydrocannabinol
TNF	–	Tumor necrosis factor
TRAP1	–	TNF receptor associated protein 1
TRP	–	Transient receptor protein
UBQLN2	–	Ubiquilin-2
VEH	–	Vehicle
VPS29	–	*Vacuolar protein sorting-associated protein 29*
VPS35	–	*Vacuolar protein sorting-associated protein 35*
VR1	–	Vanilloid receptor type 1
VTA	–	Ventral tegmental area
WT	–	Wild-type like mouse

Abstract

Amyotrophic Lateral Sclerosis (ALS) is a rapidly progressing degenerative disease which leads to muscular atrophy via the degeneration of both upper and lower motor neurons with the majority of patient death resulting from respiratory failure. The majority (~90-95%) of ALS cases are non-inherited or sporadic ALS (sALS), with the remaining 5-10% inherited or familial ALS (fALS). ALS presents with a broad heterogeneity, as age of onset, symptoms and rate of disease progression are highly variable among patients, and this is further compounded by recent findings that ALS sits on a spectrum of diseases together with frontotemporal dementia (FTD) and being referred to as ALS-frontotemporal spectrum disorder. Only one drug is currently widely approved for the treatment of ALS (Riluzole) with many other drug candidates failing in clinical trials after seeing success in preclinical animal models for the disease.

One of the factors potentially contributing to the discrepancy between outcomes of preclinical testing and clinical trials for a new treatment candidate is the impact of the laboratory environment on ALS mouse models. Therefore, my initial aim was to assess environmental factors that impact on the behaviour of laboratory animals and what effect such factors may have on the validity of any particular mouse model for ALS. In a first experimental project, I assessed the effect of two techniques for handling mice on behavioural domains including anxiety, sensorimotor gating, and associative learning in non-transgenic C57BL/6J mice. Previous research has shown that handling mice by the base of the tail - as is commonly performed the majority of animal research facilities worldwide - is linked to increased anxiety behaviours in these mice. However, other behavioural domains including higher cognitive functioning have not been assessed to date. From these experiments, I found that less aversive tunnel handling reduces anxiety in a sex-dependent manner and that handling methods can impact on fear-associated memory and sensorimotor gating in mice. In a second experimental project, I assessed the effect of two different home caging systems

(individually ventilated versus filter top cage systems) on the development and progression of disease-relevant phenotypes in the *SOD1*^{G93A} transgenic mouse model for ALS. I found that the type of cage system did not alter the rapid degeneration of motor function characteristic of this model, however, housing in individually ventilated cages (IVCs; compared to filter top cage housing) improved sensorimotor gating in *SOD1*^{G93A} females and increased *freezing* of all mice in the cue trial of the fear conditioning test. Therefore, while IVC housing did not impact on the most commonly tested ALS-relevant phenotypes of transgenic mouse models, the choice of home cage holding system should be a key consideration when assessing relevance of these models to ALS-frontotemporal spectrum disorder phenotypes.

A recent gene-wide association study indicated a number of novel fALS mutations including one in the retromer subunit *VPS29* that is seen to co-localise with TDP-43 in the neurons of ALS patients. As such, to investigate possible gene-gene interactions, in a third experimental project, I evaluated the occurrence of gene-gene interactions in the iTDP-43^{A315T} mouse model for ALS/FTD. For this, I investigated motor function, exploratory behaviours and anxiety in iTDP-43^{A315T} transgenic mice when exposed to overexpression of human wild type or mutant VPS29 protein. Interestingly, only overexpression of human wild type VPS29 led to reduced weight gain in the iTDP-43^{A315T} mice compared to wild type controls. Neither protein led to an exacerbation of the ALS-relevant motor phenotype, however, iTDP-43^{A315T} mice overexpressing mutant VPS29 exhibited hyperlocomotion in the open field, and an anxiolytic-like phenotype in the elevated plus maze in a sex-dependent manner, indicating a *proof of concept* that this novel *VPS29* mutation may serve to promote an FTD-like phenotype in these mice.

Finally, in a fourth experimental project, I evaluated a new treatment candidate for ALS. The endocannabinoid system is implicated in the progression of ALS as the cannabinoid receptor 2 is

upregulated in spinal cord of end-stage ALS mouse models and patients. Particular cannabis constituents have been shown to delay the onset and slow progression of motor function decline. Thus, I evaluated the therapeutic potential of cannabidiol (CBD) for ALS in *SOD1*^{G93A} transgenic females. When started at symptom onset (15 weeks of age), 50mg/kg CBD administered via daily intraperitoneal injections was not seen to affect the progression of motor function deficits in these mice. These results imply that the age at which treatment is started in ALS models as well as dose and route of administration are also a key consideration in the translatability of prospective interventions.

In conclusion, the work presented in this thesis shows that mouse handling and mouse cage systems used for ALS transgenic mouse models can impact on anxiety and behaviours such as fear-associated learning and sensorimotor gating. These findings can have implications for the validity of these mouse models for research into human diseases as well as the particular experimental strategy chosen. Additionally, my work provides new insights into ALS-relevant gene-gene interactions that are capable of influencing behavioural phenotypes in established mouse models for the disease. Finally, I provide evidence that administration of 50mg/kg CBD as a novel treatment is not effective in female *SOD1*^{G93A} mice with the chosen parameters. Ultimately, laboratory environments / procedures should always be considered as potential test confounders and therefore be specifically selected for any preclinical research project. It is important to realise that these factors can impose on the validity of mouse models for ALS - even small alterations to the onset or progression of disease-relevant phenotypes can impact on experimental outcomes and thereby influence the translational potential of e.g. novel treatments in diseases with narrow treatment windows such as ALS.

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Chapter 1: Introduction

1.1 Amyotrophic Lateral Sclerosis (ALS)

1.1.1 Classification of ALS

Amyotrophic Lateral Sclerosis (ALS) is the most common category of motor neuron diseases (MND) and is a rapidly progressing degenerative disease which leads to muscular atrophy via the degeneration of both upper and lower motor neurons with the majority of patient death's resulting from respiratory failure. Whilst seen as a rare disease (median incidence rates of approximately 2.74 patients per 100,000 people [1,2]), the individual burden of patient costs on the healthcare system is high, with advanced disease averaged at AUD$1.13 million per person for care in 2015 [3]. The majority (~90-95%) of ALS cases are non-inherited or sporadic ALS (sALS) and are usually found to have onset at around 55-65 years of age. Making up the remaining 5-10% and showing an onset of symptomology on an average of 10 years earlier than that of sALS is inherited or familial ALS (fALS) [4,5].

ALS presents with a broad heterogeneity, as age of onset, symptoms and rate of disease progression are highly variable among patients. Spinal onset ALS is commonly defined as a presentation of limb weakness in either the upper or lower limbs and accounts for 65% of all ALS cases [6,7]. Patients may experience cramping and muscular fasciculations for extended periods of time reaching months to years prior to the onset of muscle weakness and atrophy [6]. Upper motor neuron or bulbar onset ALS patients show symptoms of dysphagia, the inability to swallow, or dysarthria, and slow or slurred speech. Muscle weakness in the limbs usually also develops at the same time or shortly after bulbar symptoms appear [6]. Bulbar onset ALS accounts for 30% of diagnosed ALS patients [7]. The remaining 5% of ALS cases present with respiratory difficulty (typically termed as 'respiratory onset') followed by progression of spinal motor neuron

degeneration soon after. Both spinal and bulbar onset ALS have a prognosis of approximately 3-5 years after disease presentation and diagnosis with respiratory onset ALS patients having an average lifespan of 2 years after diagnosis.

There are also phenotypical imitations of ALS, though they vary in the severity of motor neuron dysfunction and prognosis. However, these imitations, listed in the following, are also prone to develop into full ALS progression.

1. *Progressive bulbar palsy* (PBP), which initially impairs speech and the ability to swallow. Patients affected with this phenotype have a life expectancy of 2-3 years with aspiration pneumonia being the leading cause of death [8].
2. *Primary lateral sclerosis* (PLS), where patients initially present with only upper motor neuron symptoms such as muscle spasticity and weakness and symptoms can stabilize thereafter [9]. PLS yields an expected prognosis of greater than 20 years if there is no further progression. However, >75% of patients diagnosed with PLS go on to develop full ALS pathology [9-11].
3. *Progressive Muscular Atrophy* (PMA), only lower motor neurons are affected and a decrease in muscle tone and muscular atrophy is characteristic for this form. Patients with PMA face a better prognosis (≥20 years) compared to other motor neuron diseases, however, up to 30% of patients go on to develop full ALS pathology [12].

1.1.2 Pathogenesis of ALS

The development of ALS is presently believed to be resultant of an interplay of pathogenic cellular mechanisms such as oxidative stress [13], mitochondrial dysfunction [14,15], impairment of axonal transport [16], protein aggregation [17], abnormal RNA processing [18], and neuroinflammation [19]. Excitotoxicity as a result of excessive glutamate receptor activation on neurons appears to play a

major role as glutamate levels in the cerebrospinal fluid are increased in some patients with ALS [20] likely due to a reduction in glutamate reuptake as a result of reduced glutamate transporter expression in the synapses of ALS patients [21]. Genetic risk factors appear to play a significant role in the development of ALS pathogenesis and will be outlined in the following. Furthermore, the evidence for environmental risk factors is contradictory: a review of case-control studies has found that smoking [22] as well as highly physical, occupations such as military service [23], 'blue collar' occupations i.e. tradespeople [24] and professional full contact sports [25,26] may be associated with an increased risk of developing of ALS with other environmental factors such as alcohol consumption, physical activity and living in rural areas, playing little to no role [27]. Moreover, findings from the field have reported the opposite in that strenuous occupations and physical activity do not increase the risk of developing ALS and may provide protection against its development [28-31].

Recent advances in the field now indicate that many ALS-relevant polymorphisms sit on a spectrum of disorder with frontotemporal dementia (FTD) [34] with many ALS/FTD-relevant genes sharing the same pathological mechanisms as those mentioned above. However, the factors predicting which side of the ALS/FTD spectrum a given patient will fall on are still relatively unknown. The following list of genes are categorised along the spectrum from ALS to ALS/FTD. Notably the models listed are predominantly focus on familial mutations found in the patient population as the cause of sporadic disease is largely unknown and therefore difficult to model. However the models presented exhibit characteristics relevant for both familial and sporadic forms of the disease.

1.2 Genetic Risk Factors for ALS

1.2.1 Copper-Zinc superoxide dismutase (*SOD1*)

Initially discovered in 1993 [35], missense mutations in the *Copper-Zinc superoxide dismutase* (*SOD1*) gene are one of the leading genetic causes for ALS accounting for approximately 20% of familial ALS. However, of the 100+ mutations discovered during investigation into *SOD1*, no definite predilection or clustering on the gene has been observed [36]. Insights into *SOD1* biology and function underpin the majority of our knowledge regarding the mechanisms behind ALS pathogenesis. SOD1 is a ubiquitous cytoplasmic enzyme that catalyzes the breakdown of reactive oxygen species (ROS) thereby preventing harmful oxidative stress to cells and neurons [35]. Initial hypotheses posited that mutant *SOD1* caused ALS due to a loss of the dismutase activity [37] and consequential increase in ROS production. However, studies using *SOD1* knockout mice revealed that a loss of function was not the cause for ALS symptoms [37] as these mice did not show any loss in motor neurons nor exhibit an ALS-like phenotype by 6 months of age, however motor neuron loss was observed after axonal injury in these *SOD1* knockout mice indicating that *SOD1* is required following injury. Rather a toxic gain of enzymatic function in a mouse model for repeat copies of a missense *SOD1* point mutation of glycine 93 to alanine (G93A) was believed to cause increased formation of the strong ROS, peroxynitrite, and ultimately presented with motor neuron degeneration representative of human ALS [38]. Supporting this, increased mitochondrial ROS formation and ultimately dysfunction has recently been observed in response to $SOD1^{G93A}$ overexpression in the mouse [39].

The most commonly used mouse model for *SOD1* is the $SOD1^{G93A}$ transgenic mouse line which shows early disease onset at around 90-110 days of age with rapid degeneration of motor neurons with mice surviving until 4-5 months of age [40], mimicking what is seen in human fALS patients

with the same mutation in which disease onset is observed at 55-63 years of age and a rapid disease progression [41]. *SOD1* mouse models also develop many of the features of ALS such as progressive neuromuscular dysfunction, gliosis and the loss of motor neurons as well as rapid loss of body mass (for overview see Table 1.1). However, disease onset, rate of progression and maximum survival age are dependent on the specific *SOD1* mutation as well as gender and the level of *SOD1* transgene expression [42] (see Table 1.1).

utation	*Background*	*Sex*	*Onset*	*Survival*	*Phenotype*	*Ref*
G37R	C57BL/6J x C3H/HeJ	NR	4-6 months	13-14 months	Major motor deficits: - Reduced spontaneous movement - Hind limb clasping Poor grooming. Sensory motor deficits at 8 and 11 months of age.	[43]
H46R	C57BL/6 x DBA/2 F1	Male	5 months	6 months	Hindlimb weakness. Ubiquitin positive inclusions found in effected motor neurons. Degeneration found throughout the entire white matter. Degeneration in motor function tested by rotarod. Female mice not used.	[44]
D83G	C57BL/6J x C3H	Male & female	2 months	18-21 months	Loss in body weight from 4 weeks. Progressive loss of muscle strength. Motor deficits in accelorod.	[45]

utation	*Background*	*Sex*	*Onset*	*Survival*	*Phenotype*	*Ref*
					Female onset earlier than male.	
G85R	C57BL/6J x C3H/HeJ	NR	7.5 months	8 months	Rapid degeneration after onset resulting in near total paralysis 2 weeks after onset.	[46]
D90A	C57BL/6JBom	NR	12.5 months	14.5 months	Hind limb tremors. Slow developing ALS phenotype. Bladder distension as seen in the human disease.	[47]
G93A*	C57BL/6 x SJL	NR	3.5 months	4-5 months	Presents with inability to extend hind-limbs when held by the tail. Rapid motor function degeneration.	[48]
L126Z	C57BL/6J x C3/HeJ	NR	7-9 months	10-12 months	Eosinophilic inclusions. Motor neuron loss. Hind limb paralysis.	[49]
G127X	C57BL/6JBom	NR	9 months	9 months	Rapid disease progression *SOD1* ubiquitin inclusions.	[50]

Table 1.1: Overview of established SOD1 transgenic mouse models and their main phenotypic characteristics. * Mouse model used for this thesis project. NR: not reported

1.2.2 Tar DNA binding protein (TARDBP)

Encoded by the *Tar DNA binding protein* (*TARDBP*) gene, the TDP-43 protein was initially identified as a transcriptional repressor of the human immunodeficiency virus (HIV-1) disease. The protein's exact function is unknown but is seen to play key roles in important cellular mechanisms such as splicing and gene expression, neuronal development and embryogenesis [51,52]. In line with this, a homozygous knockout of *TARDBP* leads to early embryonic cell death [53]. Mutant TDP-43 has been found in ~5% of familial ALS cases. Whilst normally a nuclear protein, TDP-43 is uncharacteristically found in the cytosol and often as a constituent of protein inclusions in diseased motor neurons. While a number of missense *TARDBP* mutations have been discovered leading to pathogenic TDP-43 in the cytosol, the mechanisms leading to ALS pathogenesis are currently unknown, although studies have identified a number of cellular dysfunctions related to the mutant TDP-43 protein, such as abnormal neuron function [54], increased boutons at the neuromuscular junction [55] and proteasome dysfunction [56]. Furthermore, TDP-43 inclusions have also been linked to the development of frontotemporal dementia and cognitive decline in addition to ALS in some patients [57], initially paving the way for investigation into the now termed ALS-frontotemporal spectrum disorder [18].

Multiple rodent models of mutant TDP-43 have been generated, which exhibit large variability in phenotypical presentation, frontotemporal lobar degeneration related cognitive deficits such as impaired spatial working and long term memory and impaired associative memory [58] and extent of cytosolic *TDP-43* inclusions [59]. Much like other mouse models for ALS, symptom onset and severity are dependent on the promoter that is used and the level of *TDP-43* transgene expression [60] (see Table 1.2). However, unlike *SOD1* transgenic models, *TDP-43* mouse models develop

predominantly axonal changes with limited motor neuron degeneration [60] and do not progress to an 'end stage' phenotype, although overexpression of *TDP-43* (human wild type, human transgenic, or mouse transgenic) exhibits toxicity in a dose-dependent manner in these mice in which higher levels of transgene expression are linked to a more severe phenotypical presentation[60]. Though current belief is that any overexpression of *TDP-43* in mice inhibits normal function of the protein [61].

Mutation	*Background*	*Sex*	*Onset*	*Survival*	*Phenotype*	*Ref*
A315T*	C3H x C57BL/6	NR	7 months	No 'end stage'	Motor dysfunction starting at 8 months. Contextual learning deficits from 7 months.	[62]
Q331K	C57BL/6 x C3H	NR	3 months	No 'end stage'	Hindlimb tremors. Weaker hindlimb grip strength. Worse accelorod performance.	[63]
M337V	C57BL/6 x C3H	NR	10 months	No 'end stage'	Tremors at 10 months of age.	[63]
G348C	C3H x C57BL/6	NR	7 months	No 'end stage'	Motor dysfunction starting at 8 months. Contextual learning deficits from 7 months.	[62]
Human WT	C57BL/6 x C3H	NR	3 months	No 'end stage'	Development of tremor. No progressive degeneration.	[62]

Table 1.2: Overview about established TDP-43 transgenic mouse models and their phenotypic characteristics. * Mouse model used for this thesis project. NR: not reported

1.2.3 'Fused in sarcoma' (FUS)

Mutations in the '*Fused in sarcoma*' (*FUS*) gene account for approximately 5% of familial ALS cases [64,65] and are linked to abnormal RNA metabolism. FUS is normally located in the nucleus where it plays a role in DNA and RNA metabolism and it is also believed to contribute to tumorigenesis [66]. Mutated FUS is typically found in the cytosol and has been correlated with an earlier, juvenile onset (i.e. onset of under 25 years of age [67]) ALS compared to adult/middle age onset observed in the other major causative familial polymorphisms [68]. Mutant FUS has also been associated with a higher presentation of lower motor neuron symptoms and basophilic protein inclusion found in neurons located in the dorsal horn of the spinal cord [69].

Presently, there are four reported mouse models of *FUS,* two human *FUS* knock-out (KO) models show that FUS is vital for animal survival with homozygous KO mice failing to thrive [70,71]. However, models of overexpressed and abnormal human *FUS* present a distinct phenotypic disease presentation ranging from motor involvement with denervation of muscles [72,73], spinal motor neuron degeneration [72,73], to memory impairment and hippocampal degeneration (see overview in Table 1.3). Furthermore, *FUS* models are characterized by higher numbers of ubiquitin inclusions in affected motor neurons when compared to models of the other major polymorphisms, which lead to neuronal cell death [72]. The severity of symptoms and disease progression are dependent on the level of transgene expression and the promoter used to generate the model (see Table 1.3).

Mutation	*Background*	*Sex*	*Onset*	*Survival*	*Phenotype*	*Ref*
$KO^{FUS(+/-)}$	C57BL/6J	Male	12	No "end stage"	Homozygous KO mice failed to thrive. Home cage hyperactivity. Anxiolytic-like behaviour observed in elevated plus maze. Vacuolation in the hippocampus,	70
$KO^{FUS(-/-)}$	C57BL/6	NR			Death after 24 hours with chromosomal abnormalities.	71
$hFUS^{WT}$	C57BL/6 x SJL	NR	1 month	3 months	Failure to gain weight. Tremors. Paralysis at 10-12 weeks of age. Spinal MN loss was also seen.	74
R521G	C57BL/6	NR	2 months	1-3 months	Impaired motor function. Early mortality. Impaired bodyweight progression. Hind limb clasping. Impaired sociability in the resident intruder test.	75

Table 1.3: Overview about established FUS mouse models and their phenotypic characteristics. NR: not reported

1.2.4 Chromosome 9 open reading frame 72 (C9ORF72)

The GGGGCC hexanucleotide repeat expansion found in the non-coding region of *chromosome 9 open reading frame 72* (*C9ORF72)* gene has been found to be causative for 40-50% of all familial ALS cases and 2% of sporadic cases [76]. Though the function of the protein encoded by *C9ORF72* is still unknown, its role potentially lies in membrane trafficking. In 90% of healthy humans, the level of hexanucleotide repeats is low with less than 10 repeats [77]. However, in ALS patients, this number of repeats reaches into the hundreds or even thousands [78] although there is contention whether or not the number of repeats present is disease modifying with studies finding that repeat length may be positively correlated with age of ALS onset [79], and conversely that repeat length has no bearing on onset and progression [78]. These long repeat transcriptions are believed to interfere with normal RNA binding dependent on the number by creating nuclear foci that sequester RNA binding proteins [80]. Clinically, patients with significant repeat expansion are reported to have a higher incidence of bulbar onset ALS when compared to those without [81].

Whilst initially thought to be causative of ALS via a loss of function [82], *c9orf72* knockout mouse models have shown no degeneration of motor neurons. In contrast, human *C9ROF72* repeat expansion mouse models have been shown to develop behavioural abnormalities such as anxiety, hyperactivity and antisocial behaviours alongside the classical motor deficits present in other ALS animal models [83]. Mouse models with a high number of *hexanucleotide* repeats display repeat length-dependent motor phenotypes with mice carrying less than 100 repeats exhibiting no signs of limb weakness whereas mice carrying in excess of 500 repeats show limb weakness within 25 weeks of age (for overview see Table 1.4), in this regard as test mice are bred to express many repeat expansions, controls are often non-transgenic animals of the background strain.

No. Repeats	*Background*	*Sex*	*Onset*	*Survival*	*Phenotype*	*Ref*
66	C57BL/6	Male & Female	6 months	Not reported	RNA-foci found in brains at 6 months. Anxiety in the open field. Impaired sociability. lower latency to fall from the rotarod Female mice seen to have a repeat length-dependent decrease in body weight.	[83]
450	C57BL6/C3H	Male	12 months	No 'end stage'	RNA foci and loss of hippocampal neurons. Increased anxiety with age. Impaired spatial working and reference memory in Barnes maze and radial arm maze. Motor impairment not observed	[84]
500	FVB/NJ	Male & Female	1-1.5 years	No 'end stage'	Neuromuscular junction loss. Motor neuron degeneration with *TDP-43* inclusions. Anxiety & motor impairments in the open field test. 30-35% of female mice develop acute rapidly progressing disease at 20-40 weeks.	[85]

Table 1.4: Overview of established C9ORF72 mouse models and their phenotypic characteristics.

1.3 Gene-Gene interactions

The idea of 'gene by gene' interactions, known also as "epistasis" (the ability for one gene to interact with and/or silence another) is not a new idea and has been prevalent for over 100 years [86] and was originally used as a tool to help explain deviations from Mendelian genetics [87]. Along this line, it is no stretch to infer that gene-gene interaction can play a role in the formation of complex diseases, especially those with a heterogeneous symptomatology such as ALS. Genome-wide association studies have typically focused on single loci for causative polymorphisms [88], a limitation that is thought to be one of the contributing factors to the inability to replicate observed genetic mutations to phenotypes found in the human disease. Indeed, a comprehensive review of gene association studies focusing on common human disease found that of 166 commonly studied (reported three or more times) disease-associated polymorphisms, only six were consistently replicated [89]. These findings are indicative that interactions with disease-associated polymorphisms may prove more important for the cause of these diseases than single loci polymorphisms. Furthermore, whilst many single nucleotide polymorphisms have been identified as potentially causative for ALS, there is growing evidence for causative gene interactions [90,91] due to the heterogeneity of ALS presentation and onset. Thus, it is prudent to test these interactions to increase our understanding of the complex genetic contributors to ALS development. Moreover, recent work by Farrawell and colleagues has indicated that 3 of the common pathological protein inclusions observed in ALS, those being SOD1, TDP-43 and FUS, may also variably interact with each other to form combined protein aggregates, indicating that there is large scope for the investigation of these interactions [92]. Interestingly, this does not seem to be a result of interaction of gene expression and RNA but the propensity for mutant proteins to sequester wild-type proteins to further inhibit cellular function [92].

A genome-wide association study into rare causes of fALS identified a novel polymorphism in the gene encoding for VPS29. Moreover the mutant VPS29 protein was observed to co-aggregate with mutant TDP-43 in spinal cord protein inclusions of ALS patients (personal communication with Dr. Shu Yang from Macquarie University, lead researcher studying the role of VPS29 in ALS). This indicates that there may be an interplay or gene-gene interaction between these two polymorphisms and needs further investigation.

1.3.1 'Vacuolar protein sorting-associated protein 29' (*VPS29*)

Vacuolar protein sorting-associated protein 29 (VPS29) in combination with VPS35 and VPS26 forms what is known as the retromer complex [93]. Found on the endosomal membrane, the retromer plays a key role in recycling transmembrane receptors [94] and transport of proteins between the endoplasmic reticulum and the Golgi apparatus [94]. Whilst initially thought to be something of a 'bystander' protein, recent work indicates that VPS29 may potentially serve as a scaffold for the retromer complex and may modulate the sorting response of individual proteins [95]. Interestingly, exome sequencing on an Austrian family found a polymorphism in *VPS35* to be a rare cause of late onset Parkinson's disease [96]. Furthermore, knockout of *Vps35* and *Vps26a* for the purposes of animal modelling have proven to be embryonically fatal [97,98], with knockout of *Vps26b* leading to lower expression of both Vps29 and Vps35 due to the absence of the completed retromer complex [99] indicating that disruption of the retromer complex may have implications for both the motor system and viability as a whole.

1.4 The Validity of Modelling ALS using Laboratory Mice

ALS animal models that are used to understand the condition and guide treatment methodologies also exhibit a heterogeneity of disease relevant phenotypes similar to the human disease. However,

the modelling of these pathological genes in complex models is currently the best method we have at gaining a better understanding of the causative mechanisms. As patient databases and gene-wide association studies continue to unearth new and potentially causative polymorphisms, the use of *in vitro* and, more importantly, *in vivo* modelling is key to determine the biological function of genes identified and to clarify whether or not these polymorphisms mediate disease-relevant phenotypes and further, whether or not they provide a valid model for testing novel therapeutics [comprehensively reviewed in [100-102]].

In order for an animal model to be considered valid for research into human diseases, it must reflect the cause, presentation, underlying pathology and the outcome of treatments as is known for the human disease in question. An animal model of disease can be said to show construct validity if the underlying pathophysiological mechanisms are similar if not the same as the human disease. Subsequently a model can be described as showing face validity for a disease if the model mimics the symptoms and behaviours seen in the human disease. This is often limiting in the case of ALS models (especially since the recent prominence of research into ALS-frontotemporal spectrum disorder) as behavioural testing is often limited primarily to motor function with more complex cognitive behaviours rarely being assessed. However there is evidence that even in supposed 'pure' ALS models, such as the *SOD1*^{G93A} mouse model, cognitive impairments in learning and memory are present before the onset of ALS relevant phenotypes [103,104]. When discussing the outcome of interventions, a model can be determined to have predictive validity if the treatment, especially pre-existing medication for the disease is effective in the model [105,106].

Simple animal models such as *Caenorhabditis elegans*, *Drosophila melanogaster* and *Danio rerio* with their short life cycle, ease of transgenic model creation and relative commonality with the human genome, provide an excellent precursor to mouse models as a screening tool for

determining the construct validity of phenotypes. These models often present ALS-relevant pathologies, however, due to the simple nature of these models, more complex behaviours are difficult to discern [107]. In this regard, transgenic mouse models allow for the assessment of disease progression, associated behavioural symptoms beyond motor impairments (those being FTD relevant phenotypes such as anxiety and spatial and associative memory impairments seen in ALS-Frontotemporal spectrum disorder yet largely unstudied in assumed 'pure' ALS models) and the efficacy evaluation of new treatments (see Tables 1.1-4) in a complex system with efficient throughput and ease of transgenic modification.

1.4.1 Impact of laboratory environments on mouse model validity

The impact environmental factors such as housing cage systems, cage enrichment, and the handling of animals may have on the development and presentation of clinically relevant mouse model phenotypes is a largely underreported area of preclinical research. Many facilities have seen a change from the established open or filter-top (FT) cage system to the more recently developed individually ventilated cage (IVC) systems in an effort to provide a more stable microbiome [108] and reduce breeding stress [109,110], as well as reduce health and work risks for animal facility staff. However, this change has had unforeseen consequences on the validity of these strains. Behaviourally, inbred laboratory mice housed in IVC cages are prone to lower locomotor activity and exploration [111] as well as increased anxiety and sociability [112]. Physiologically, compared to FT cages, IVC systems have caused lower bodyweight (without changes in feeding habits) [111], induce cold stress [113] and increased dopamine and serotonin turnover in mice [111]. Furthering on from this, home cage enrichment, usually termed 'environmental enrichment (EE)', is the use of structures or 'toys' in the home cage of the animal to provide sensory and cognitive stimulation

that has been shown to increase brain weight [114], dendritic branching [115], improve plasticity [116], as well as improve cognitive functions in behavioural test such as the Morris water maze [117] and T-maze [118]. These diverse findings across standard inbred strains of laboratory mice suggests that transgenic models of human neurodegenerative disease are also likely to be impacted by these factors. Indeed, first studies evaluating the effects of IVCs on the validity of transgenic models found that individually ventilated cage systems diminished schizophrenia-relevant behaviours in *neuregulin 1* transmembrane domain mutant mice, an established genetic mouse model for preclinical research into schizophrenia [119]. Furthermore, IVC housing has confounded an established fear extinction impairment phenotype of serotonin transporter knockout rats [120], and prompted the re-establishment protocols for a widely used and well-established maternal immune activation (MIA) model for schizophrenia [121].

Mouse handling methods, may also present another confounding factor in behavioural testing as routine tail handling is reported to induce anxiety-like behaviours across test paradigms, aversive behaviours towards investigators [122-124], and induce anhedonia [125] when compared to less aversive handling (e.g. use of a tunnel to pick up mice from the home cage). On a larger scale, past research has also shown evidence that testing facility affects established behavioural differences between common inbred and outbred laboratory strains. Differences in anxiety and locomotion between strains were observed in some but not all facilities where identical test protocols were used [126,127]. Notably, the effect of different handling approaches on the phenotype of ALS mice has yet to be investigated.

Previously studies in to environmental effects in ALS modes have found that EE in the *SOD1*^{G93A} model found that female *SOD1*^{G93A} mice in enriched housing conditions reached and "end-stage" phenotype sooner than their standard-housed littermates. [128]. Furthermore, in a recent study in the

SOD1^{G93A} mouse model, mice exposed to a disrupted day/night cycle in the holding room to emulate circadian rhythm disfunction were found to have earlier onset of symptoms, more rapid degeneration of motor function, and increased number of inflammatory markers when compared to their normal light/dark cycle counterparts [129]. These studies indicate that environmental factors can indeed affect the development and progression of ALS-relevant mouse phenotypes and therefore need careful consideration when planning preclinical research into ALS. However, there is a lack of studies into the effects of many more environmental factors inherent to animal facilities.

1.5 Current treatments available for ALS and novel candidates

Presently, Riluzole is the only widely available disease-modifying drug that has been shown to prolong life in ALS patients, though only for 2-3 months [130]. However, it has presented conflicting preclinical results as both increases in life expectancy in *SOD1*^{G93A} mice [131], as well as no changes in the lifespan of not only the *SOD1*^{G93A} mouse model, but *TDP-43*^{A315T} and *FUS(1-359)* models [132] without effecting phenotype onset in either case, indicating the pressing need for more effective treatments. Riluzole preferentially acts on tetrodotoxin-sensitive sodium channels implicated in damaged neurons [133]. There is also some evidence that it is able to act as an N-methyl D-aspartate (NMDA) pathway antagonist as it interferes with NMDA receptors, increases the reuptake of NMDA, and inhibits its release from vesicles [134]. Whilst the clinical effect of Riluzole is largely unknown, it's mechanism of action and ability to inhibit glutamate signaling [135] may indicate that it is beneficial in partially ameliorating the excitotoxicity seen in ALS. Recently gaining approval in both Japan and America is the free radical scavenging drug Edaravone [136,137]. Initially developed for the treatment of ischemic stroke, Edaravone has shown promise by significantly slowing the degeneration of motor function in a subset of Japanese ALS patients, however, the mechanism of action of this drug with regard to ALS and how it may be beneficial is presently unknown [136,137].

A significant number of other neuroprotective and immune-modifying drugs have been tested preclinically using *in vitro* and *in vivo* models, with the most common *in vivo* models being genetically modified mice [138]. Whilst many of these novel treatment candidates have shown promising results as single therapy primarily in the *SOD1*^{G93A} mouse model, for example dexpramipexole [139], minocycline [140] and thalidomide [141,142], there have been no drug candidates thus far that have been successful in phase II or III clinical trials [this is further reviewed in [138]]. In the case of the antibiotic minocycline, while effective in extending life in preclinical models, an extended treatment paradigm hastened disease progression in human phase III clinical trials, there have been no drug candidates thus far that have been successful in phase II or III clinical trials[143]. Gene therapies are another treatment methodology that are presently being assessed in ALS, having already been shown to enhance patient survival in other neurodegenerative diseases such as Alzheimer's [144] and Parkinson's disease [145]. Though presently limited to preclinical models, adeno-associated viral (AAV) delivery systems for insulin-like growth factor 1 (IGF-1) and glial cell line-derived neurotrophic factor (GDNF) have shown promise in extending the lifespan of *SOD1*^{G93A} transgenic mice due to their role in the proliferation, differentiation and survival of spinal neurons affected in ALS [146,147]. On the other hand, stem cell therapy where multiple types of stem cells were transplanted to replace atrophied motor neurons *in vivo* showed a delay in the onset of symptoms [148] and an increase in lifespan of *SOD1*^{G93A} transgenic mice [149-152]. However, other than the inherent risk of teratoma formation that stem cell therapy carries with it, there are doubts as to whether a single treatment with stem cells will be effective due to the progressive nature of ALS [152].

In the wake of poor drug translation and limitations of current stem cell therapy approaches, new strategies for the treatment of ALS are needed. Due to their anti-excitotoxic and neuroprotective

effects, cannabinoids have been identified as a treatment option for diseases similar in nature to ALS, such as multiple sclerosis (MS). For example, *Sativex* is a cannabis-derived therapy, which contains a close to equal mix of the phytocannabinoids Δ^9-tetrahydrocannabinol (THC) (~27 mg/ml) and cannabidiol (CBD) (~25 mg/ml) and has shown efficacy in the management of neuropathic pain and muscle spasticity in MS [153,154]. The following chapter will outline basics of the endocannabinoid system and studies exploring whether cannabinoids are potentially valuable candidates for comprehensive, in-depth discovery research into the treatment of ALS.

1.5.1 The Endocannabinoid system

Endocannabinoids are synthesised and released into the synapse when needed for the regulation of dopaminergic activity and CNS activation [155] and is rapidly transported via carrier-mediated uptake into nerve endings and glia [156] where they are metabolised once again within minutes [157]. In addition to the modulation of neurotransmitter release [158], endocannabinoids also act to prevent the release of pro-inflammatory mediators such as interleukins and tumor necrosis factors [159]. The two main endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) and both have been found to be elevated in the spinal cord of *SOD1* and *TDP-43* transgenic mice [160,161]. However, this finding has yet to be confirmed in human ALS patients. Endocannabinoid activity is primarily mediated by the CB_1 and CB_2 G-protein coupled receptors [162], although endocannabinoids also have an affinity for, among others, peroxisome proliferator activated receptors (PPAR) [163,164] and transient receptor protein (TRP) channels, namely TRPV1 [165,166], and G-protein coupled receptor 55 (GPR55) [167].

The synthesis of anandamide at the nerve terminal occurs via the hydrolysis of N-arachidonoyl phosphatidylethanolamide (NAPE) by a NAPE-specific phospholipase D enzyme [167]. Anandamide is an agonist of CB_1 receptors and whilst evidence shows potential CB_2 receptor binding, it does

so with a low affinity and may instead act as an antagonist [168]. 2-AG is synthesized via the action of diacylglycerol (DAG) lipase from DAG [169]. 2-AG primarily elicits effects thought the CB_1 receptor where it has roles in the management of appetite [170], pain management [171] and is also believed to play a role in CB_1 receptor mediated neuronal plasticity in the hippocampus [172].

1.5.2 Phytocannabinoids and Synthetic Cannabinoids with Treatment Potential for ALS Mouse Models

Exogenous cannabinoids extracted from the *cannabis sativa* plant (i.e. phytocannabinoids) and synthetic cannabinoids have been found to have potential as disease-modifying therapeutic agents in ALS mouse models. In the *SOD1*^{G93A} mouse model, THC has not only been shown to delay disease progression, but treatment with 10/20 mg/kg also led to a significant increase in the survival of these mice by an average of 4.9 and 6.1days respectively compared to control treated mice[173]. Cannabinol (CBN) has been shown to delay the onset of disease progression in the same model without effecting survival, this was thought to be either as a result of CBN acting as an antispasmodic thereby masking early symptoms, or a potential acute toxicity [174] (which has since been observed in zebrafish [175]) and the synthetic CB_2 receptor-specific agonist AM1214 prolonged survival of *SOD1*^{G93A} mice when treatment was started at symptom onset [176].

CBD is a main constituent of the *cannabis sativa* plant that has been seen to elicit anti-convulsive [177], anxiolytic-like [178], anti-nausea [179] and anti-inflammatory actions [180] without possessing psychoactive-like properties seen in THC (i.e. CBD is not known to be intoxicating). CBD has also been found to have weak binding activities at both CB_1 and CB_2 receptors when compared to other cannabinoids such as WIN-55,212-2 and Δ^9-THC [181]. However, CBD also functions as an agonist on the GPR55 and the vanilloid receptor type 1 (VR1), indicating a potential anti-inflammatory effect usually elicited by capsaicin at these receptors [182]. Interestingly, CBD also

appears to protect against NMDA receptor-mediated neurotoxicity when assessed in the brain tissue of Sprague-Dawley rats [183]. When tested in *in vitro* ALS model systems using mesenchymal stromal cells, exposure to CBD triggered changes in the expression of ALS-relevant genes and up-regulation of tumor necrosis factor (TNF)-related proteins, TNF receptor associated protein 1 (TRAP1) and nuclear factor, erythroid 2 like 2 (NFE2L2), both of which are known to inhibit excitotoxicity related cell death [184]. Importantly, increased CB_2 receptor expression in the spinal cords of male and female $SOD1^{G93A}$ mice, as well as an increase in the N-acetylphosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) enzyme in male mice has been observed [185]. Treatment with a "*Sativex*-like" formulation (containing a combination of THC and CBD) in this model reduced symptom progression and extended the lifespan of these mice indicating that the cannabinoid system potentially provides a valid novel treatment target. Importantly, CBD alone has not yet been tested in animal models for ALS.

1.5.3 Clinical trials evaluating cannabinoid therapy

Whilst there has been limited testing of cannabinoids in human clinical trial, the focus has been on the management of pain, cramping and muscle spasticity in ALS patients [186]. One trial assessing the effects of chronic oral THC (dronabinol) administration (i.e. 5 mg twice daily) found no beneficial effects of cannabinoid treatment on these symptoms [187]. However, when assessing a ~1:1 ratio combination of CBD and THC (nabiximols) administered intranasally, patients reported an improvement in pain and spasticity (though improvements in spasticity were not confirmed via objective methodology, clinical trial ID: NCT01776970) [188], indicating that both of these cannabinoids may be necessary for the symptomatic improvement of pain and spasticity. Interestingly, in a survey conducted on an ALS community website investigating the personal use of *cannabis sativa* for the treatment of muscle spasticity, pain and appetite, cannabis was reported

to be mildly effective in the management of these symptoms. However, it must be noted that the sample size of the survey was limited as from 131 participants only 13 reported personal use of marijuana within the last 12 months [189]. Thus, the potential for cannabinoids as a single or adjunct therapy to influence the disease progression of ALS when initiated at symptom onset has yet to be explored.

1.6 Aims

In this thesis, I will evaluate i) how two different handling methods effect mouse behavioural domains and ii) whether IVC or FT cage systems impact on disease-relevant phenotypes of the *SOD1*^{G93A} transgenic mouse model. These initial steps are necessary as the Karl laboratory has only recently started testing ALS transgenic mouse models in their new phenotyping test facility at Western Sydney University. Following on from this initial work, I will have the opportunity to investigate a new genetic risk factor for ALS (i.e. ALS-associated polymorphism) and its potentially disease-modifying impact on an established genetic mouse model for ALS, i.e. when co-expressed in the established *TDP-43* transgenic mouse model of ALS. Finally, I will also determine the therapeutic properties of CBD in the *SOD1*^{G93A} mouse model of ALS

Aim 1: To evaluate if the choice of handling method, i.e. tail handling compared to tunnel handling, affect baseline behaviours of laboratory mice in a newly established mouse testing facility

Hypothesis 1: I hypothesise that the use of less aversive handling methods will positively alter the behaviour of mice when exposed to a test paradigm assessing exploration and anxiety-like behaviours.

Aim 2: To determine if individually ventilated cages (IVC) compared to filter top (FT) cages, effect the development and progression of the behavioural phenotype of the established *SOD1*^{G93A} transgenic mouse model

Hypothesis 2: That the use of the newer 'IVC' cages will impact on the progression of the ALS-like phenotype seen in the *SOD1*^{G93A} mouse model.

Aim 3: To determine the disease-modifying potential of a novel *VPS29* polymorphism in the *TDP-43* transgenic mouse model of ALS and FTD

Hypothesis 3: That the overexpression of human mutant VPS29 in the *TDP-43*^{A315T} mouse model for ALS will accelerate the development of disease-relevant motor phenotypes in this mouse.

Aim 4: To determine if cannabidiol (CBD) is an effective intervention in the progression of motor degeneration of SOD1 G93A mice

Hypothesis 4: That the administration of CBD in *SOD1*^{G93A} mice at symptom onset will delay the progression of motor function decline and possibly extend lifespan

1.7 Research methods

Aim 1: To evaluate if the choice of handling method, i.e. tail handling compared to tunnel handling, affect baseline behaviours of laboratory mice in a newly established testing facility

Prior to the start of testing in transgenic animals we will assess if the method by which animals are handled impacts (and to what extent) on several complex behavioural domains [103]. C57BL/6J wild type-like mice of both sexes will be assigned to either tail (Fig. 1) or tunnel (Fig, 2) handling

methods. Mice will be housed in groups of 2-3 provided with nesting material and water and lab chow *ad libitum*.

Behavioural assessment: I have screened cohorts of male and female C57BL/6J mice (n=12 per experimental factor) in tests for locomotion, exploration and anxiety behaviours (open field and elevated plus maze paradigms) in addition to the assessment of sensorimotor gating (prepulse inhibition test), fear-associated memory (fear conditioning test), sociability, and social recognition memory (social novelty/preference test). Detailed methodology and experimental timelines provided in section 2.2 of this thesis titled: Materials and methods.

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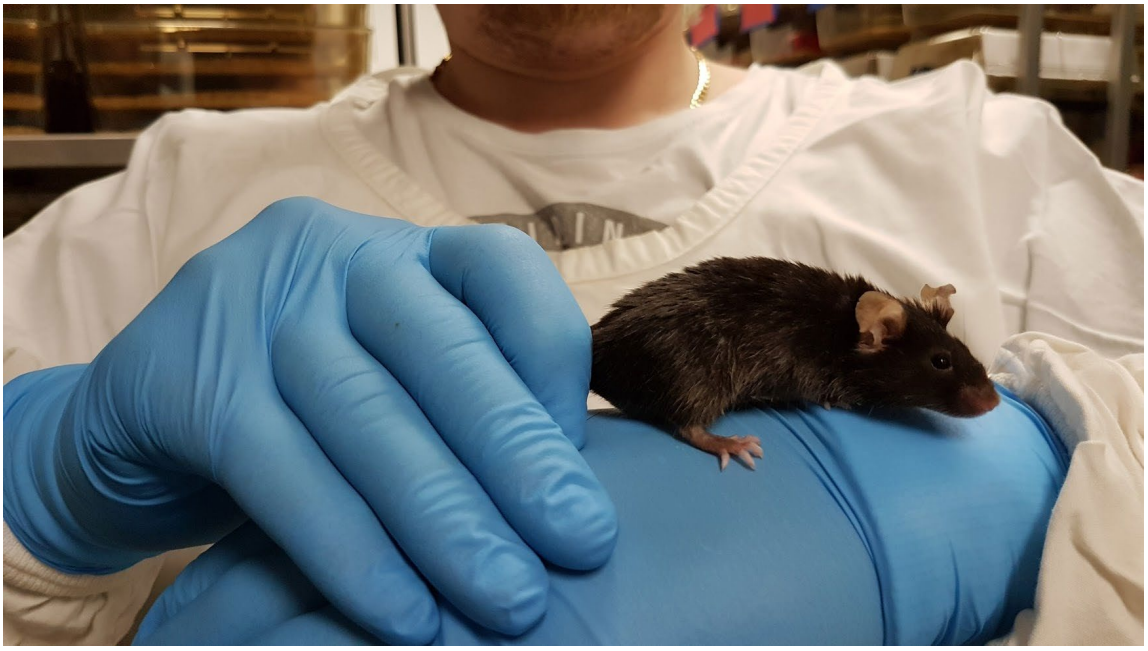

Figure 1.1 Supported tail handling

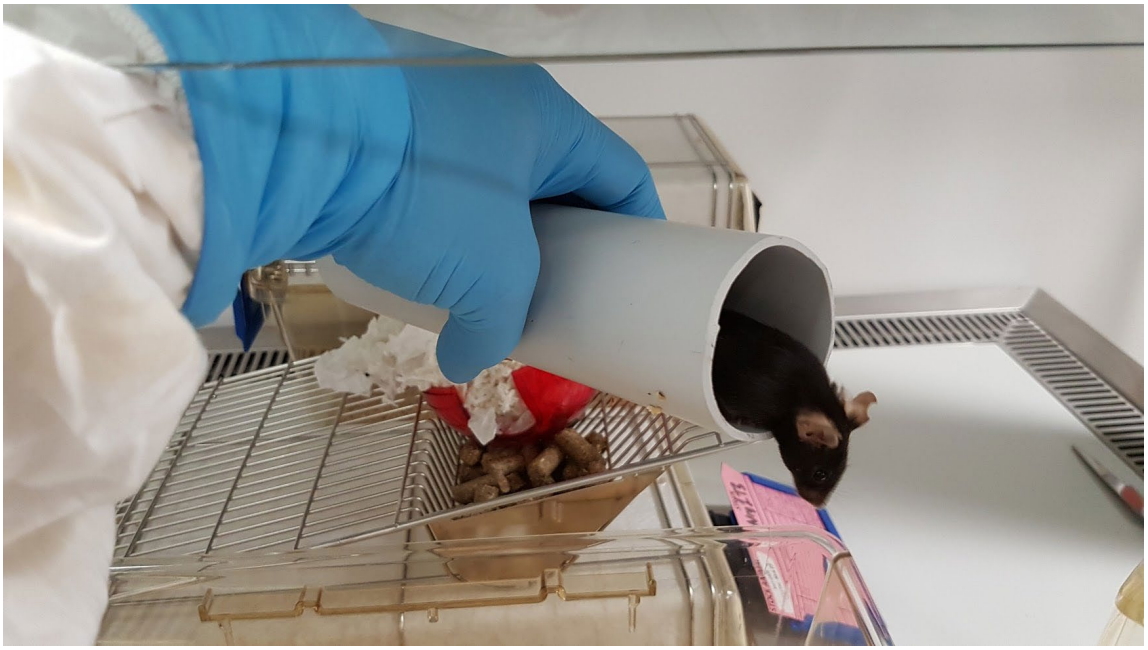

Figure 1.2 Tunnel handling.

Aim 2: To determine if housing conditions, i.e. individually ventilated cages (IVC) compared to filter top (FT) cages, effect the development and progression of the established *SOD1*^{G93A} phenotype.

Subsequent to the establishment of *SOD1*^{G93A} breeding pairs at Western Sydney University, pups will be assigned to one of either IVC (Fig. 3) or FT (Fig. 4) housing conditions post weaning and genotyping (PND 30±5). Both males and females will be housed in groups of 2-3 provided with nesting material and water and lab chow *ad libitum*. All mice will be weighed weekly to assess bodyweight development. Disease-relevant behavioural testing will begin at 15 weeks of age (in line with Kreilaus, F. et Al. [87]).

Behavioural assessment: I will assess the motor function of four cohorts of male and female *SOD1*^{G93A} transgenic mice (n=12 per experimental condition) weekly from 15 weeks of age using tests of motor co-ordination and endurance (accelerod and pole test paradigms) as well as the assessment of sensorimotor gating (prepulse inhibition test), fear associated memory (fear conditioning test), sociability, and social recognition memory (social novelty/preference test). Detailed methodology and experimental timelines provided in section 3.2 of this thesis titled: Materials and methods.

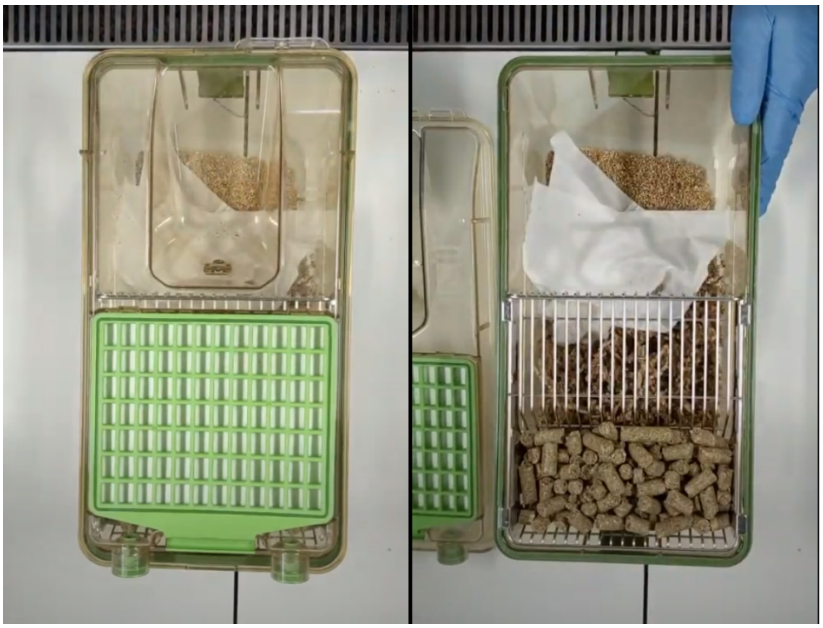

Figure 1.3 Individually Ventilated Cage (IVC).

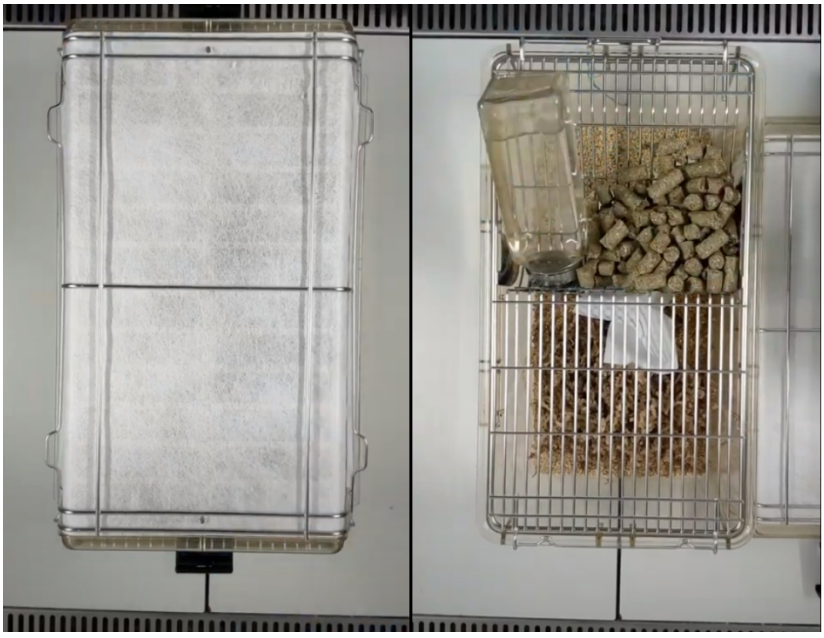

Figure 1.4 Filter Top (FT) cage.

Aim 3: To determine the validity of a novel *VPS29* mutation in the TDP-43 mouse model of ALS and FTD.

To achieve this aim, we will work in collaboration with colleagues at Macquarie university to determine the face validity of a novel potentially disease modifying mutation of *VPS29* in the *TDP-43* transgenic mouse model of ALS. Separate cohorts of these mice containing both WT and transgenic *VPS29* and Green Fluorescent Protein (GFP) controls will be injected with their respective virus at PND 0-1 and then tested in from 1-week post weaning (age 4 weeks).

Behavioural assessment: We will systematically screen cohorts of male and female WT and *TDP-43* transgenic mice expressing WT or transgenic *VPS29* or GFP as control (n = 12) in fortnightly tests of motor co-ordination, endurance (accelerod and pole test) and a singular test of locomotion and anxiety (elevated plus maze and open field paradigms). Detailed methodology and experimental timelines provided in section 4.2 of this thesis titled: Materials and methods.

Aim 4: To determine if cannabidiol (CBD) is an effective intervention in the progression of motor degeneration of *SOD1* ^{G93A} mice

I will carry out a pilot study in female *SOD1*^{G93A} mice to evaluate the therapeutic properties of chronic treatment with 50 mg/kg CBD [due to the increased cannabinoid receptor proliferation at this dose [195] and effectiveness in Alzheimer's models [196], as well as the lack of toxicity seen at this dose] prior to the onset of ALS-relevant phenotypes and disease progression. ALS transgenic mice will be treated daily for three weeks prior to testing and during the testing period with vehicle or CBD via i.p. injections starting at PND100. The effect of CBD will be characterized across behavioural domains. All mice will be assessed in ALS-relevant behavioural tests for motor deficits.

Behavioural Assessment:

We will assess the motor function and other behavioural domains in female *SOD1*^{G93A} transgenic females (n = 6-7 per condition) weekly from 100 days of age using tests of motor co-ordination and endurance (accelorod, grip endurance and pole test paradigms). Brains, spinal cords and whole blood will be collected post testing for potential analysis of CBD levels, cannabinoid receptor levels and SOD1 inclusion pathologies in case CBD shows therapeutic effects on behavioural deficits. Detailed methodology and experimental timelines provided in section 5.2 of this thesis titled: Materials and methods.

1.8 References

1. Chio A, Logroscino G, Traynor B, et al. Global epidemiology of amyotrophic lateral sclerosis: a systematic review of the published literature. *Neuroepidemiology.* 2013;41(2):118-130.
2. McCann EP, Williams KL, Fifita JA, et al. The genotype-phenotype landscape of familial amyotrophic lateral sclerosis in Australia. *Clin Genet.* 2017;92(3):259-266.
3. Deloitte Access Economics 2015, Economic analysis of motor neurone disease in Australia, report for Motor Neurone Disease Australia. 2015; http://www.mndaust.asn.au/Influencing-policy/Economic-analysis-of-MND-(1)/Economic-analysis-of-MND-in-Australia.aspx. Accessed 14 July, 2021.
4. Cudkowicz M, McKenna-Yasek D, Sapp P, et al. Epidemiology of mutations in superoxide dismutase in amyotrophic lateal sclerosis. *Annals of neurology.* 1997;41(2):210-221.
5. Testa D, Lovati R, Ferrarini M, Salmoiraghi F, Filippini G. Survival of 793 patients with amyotrophic lateral sclerosis diagnosed over a 28-year period. *Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases.* 2004;5(4):208-212.
6. Wijesekera LC, Leigh PN. Amyotrophic lateral sclerosis. *Orphanet journal of rare diseases.* 2009;4(1):3.
7. Logroscino G, Traynor BJ, Hardiman O, et al. Incidence of amyotrophic lateral sclerosis in Europe. *Journal of neurology, neurosurgery, and psychiatry.* 2010;81(4):385-390.
8. Ferguson TA, Elman LB. Clinical presentation and diagnosis of amyotrophic lateral sclerosis. *NeuroRehabilitation.* 2007;22(6):409-416.

9. Brugman F, Veldink JH, Franssen H, et al. Differentiation of hereditary spastic paraparesis from primary lateral sclerosis in sporadic adult-onset upper motor neuron syndromes. *Archives of neurology.* 2009;66(4):509-514.

10. Gordon P, Cheng B, Katz I, et al. The natural history of primary lateral sclerosis. *Neurology.* 2006;66(5):647-653.

11. Brugman F, Wokke J, de Jong JV, Franssen H, Faber C, Van den Berg L. Primary lateral sclerosis as a phenotypic manifestation of familial ALS. *Neurology.* 2005;64(10):1778-1779.

12. Visser J, van den Berg-Vos RM, Franssen H, et al. Disease course and prognostic factors of progressive muscular atrophy. *Archives of neurology.* 2007;64(4):522-528.

13. Ferrante RJ, Browne SE, Shinobu LA, et al. Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *Journal of neurochemistry.* 1997;69(5):2064-2074.

14. Kong J, Xu Z. Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD1. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 1998;18(9):3241-3250.

15. Krasnianski A, Deschauer M, Neudecker S, et al. Mitochondrial changes in skeletal muscle in amyotrophic lateral sclerosis and other neurogenic atrophies. *Brain : a journal of neurology.* 2005;128(8):1870-1876.

16. De Vos KJ, Grierson AJ, Ackerley S, Miller CC. Role of axonal transport in neurodegenerative diseases. *Annual review of neuroscience.* 2008;31:151-173.

17. Ince PG, Wharton SB. Cytopathology of the motor neuron. *Handbook of clinical neurology.* 2007;82:89-119.

18. Neumann M, Sampathu DM, Kwong LK, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science.* 2006;314(5796):130-133.

19. Kawamata T, Akiyama H, Yamada T, McGeer P. Immunologic reactions in amyotrophic lateral sclerosis brain and spinal cord tissue. *The American journal of pathology.* 1992;140(3):691.

20. Rothstein JD, Tsai G, Kuncl RW, et al. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. *Annals of neurology.* 1990;28(1):18-25.

21. Bristol LA, Rothstein JD. Glutamate transporter gene expression in amyotrophic lateral sclerosis motor cortex. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society.* 1996;39(5):676-679.

22. Armon C. Smoking may be considered an established risk factor for sporadic ALS. *Neurology.* 2009;73(20):1693-1698.

23. Weisskopf M, O'reilly E, McCullough M, et al. Prospective study of military service and mortality from ALS. *Neurology.* 2005;64(1):32-37.

24. Farrugia Wismayer M, Borg R, Farrugia Wismayer A, et al. Occupation and amyotrophic lateral sclerosis risk: a case-control study in the isolated island population of Malta. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration.* 2021:1-7.

25. Abel EL. Football increases the risk for Lou Gehrig's disease, amyotrophic lateral sclerosis. *Perceptual and motor skills.* 2007;104(3_suppl):1251-1254.

26. Chio A, Benzi G, Dossena M, Mutani R, Mora G. Severely increased risk of amyotrophic lateral sclerosis among Italian professional football players. *Brain : a journal of neurology.* 2005;128(3):472-476.

27. Armon C. An evidence-based medicine approach to the evaluation of the role of exogenous risk factors in sporadic amyotrophic lateral sclerosis. *Neuroepidemiology.* 2003;22(4):217-228.

28. Pupillo E, Messina P, Giussani G, et al. Physical activity and amyotrophic lateral sclerosis: a European population-based case-control study. *Ann Neurol.* 2014;75(5):708-716.

29. Huisman MH, Seelen M, de Jong SW, et al. Lifetime physical activity and the risk of amyotrophic lateral sclerosis. *Journal of neurology, neurosurgery, and psychiatry.* 2013;84(9):976-981.

30. Longstreth W, McGuire V, Koepsell T, Wang Y, Van Belle G. Risk of amyotrophic lateral sclerosis and history of physical activity: a population-based case-control study. *Archives of neurology.* 1998;55(2):201-206.

31. Gallo V, Vanacore N, Bueno-de-Mesquita HB, et al. Physical activity and risk of amyotrophic lateral sclerosis in a prospective cohort study. *European journal of epidemiology.* 2016;31(3):255-266.

32. O'Toole O, Traynor BJ, Brennan P, et al. Epidemiology and clinical features of amyotrophic lateral sclerosis in Ireland between 1995 and 2004. *Journal of neurology, neurosurgery, and psychiatry.* 2007.

33. Sutedja NA, Fischer K, Veldink JH, et al. What we truly know about occupation as a risk factor for ALS: a critical and systematic review. *Amyotrophic Lateral Sclerosis.* 2009;10(5-6):295-301.

34. Ling S-C, Polymenidou M, Cleveland DW. Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis. *Neuron.* 2013;79(3):416-438.

35. Rosen DR. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature.* 1993 Mar 4;362(6415):59-62.

36. Andersen PM, Sims KB, Xin WW, et al. Sixteen novel mutations in the Cu/Zn superoxide dismutase gene in amyotrophic lateral sclerosis: a decade of discoveries, defects and disputes. *Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases.* 2003;4(2):62-73.

37. Reaume AG, Elliottº JL, Hoffman EK, et al. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop. *Nature genetics.* 1996;13(1):43-47.

38. Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science.* 1994;264(5166):1772-1775.

39. Xiao Y, Karam C, Yi J, et al. ROS-related mitochondrial dysfunction in skeletal muscle of an ALS mouse model during the disease progression. *Pharmacological research.* 2018;138:25-36.

40. Zang DW, Cheema SS. Degeneration of corticospinal and bulbospinal systems in the superoxide dismutase 1(G93A G1H) transgenic mouse model of familial amyotrophic lateral sclerosis. *Neurosci Lett.* 2002;332(2):99-102.

41. Synofzik M, Fernández-Santiago R, Maetzler W, Schöls L, Andersen PM. The human G93A SOD1 phenotype closely resembles sporadic amyotrophic lateral sclerosis. *Journal of neurology, neurosurgery, and psychiatry.* 2010;81(7):764-767.

42. Alexander GM, Erwin KL, Byers N, et al. Effect of transgene copy number on survival in the G93A SOD1 transgenic mouse model of ALS. *Brain research Molecular brain research.* 2004;130(1):7-15.

43. Wong PC, Pardo CA, Borchelt DR, et al. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron.* 1995;14(6):1105-1116.

44. Chang-Hong R, Wada M, Koyama S, et al. Neuroprotective effect of oxidized galectin-1 in a transgenic mouse model of amyotrophic lateral sclerosis. *Exp Neurol.* 2005;194(1):203-211.

45. Joyce PI, Mcgoldrick P, Saccon RA, et al. A novel SOD1-ALS mutation separates central and peripheral effects of mutant SOD1 toxicity. *Human molecular genetics.* 2014;24(7):1883-1897.

46. Bruijn LI, Becher MW, Lee MK, et al. ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron.* 1997;18(2):327-338.

47. Jonsson PA, Graffmo KS, Brannstrom T, Nilsson P, Andersen PM, Marklund SL. Motor neuron disease in mice expressing the wild type-like D90A mutant superoxide dismutase-1. *J Neuropathol Exp Neurol.* 2006;65(12):1126-1136.

48. Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science.* 1994:1772-1772.

49. Wang J, Xu G, Li H, et al. Somatodendritic accumulation of misfolded SOD1-L126Z in motor neurons mediates degeneration: alphaB-crystallin modulates aggregation. *Hum Mol Genet.* 2005;14(16):2335-2347.

50. Jonsson PA, Ernhill K, Andersen PM, et al. Minute quantities of misfolded mutant superoxide dismutase-1 cause amyotrophic lateral sclerosis. *Brain : a journal of neurology.* 2004;127(1):73-88.

51. Buratti E, Baralle FE. Multiple roles of TDP-43 in gene expression, splicing regulation, and human disease. *Frontiers in bioscience : a journal and virtual library.* 2008;13(867):78.

52. Sephton CF, Good SK, Atkin S, et al. TDP-43 is a developmentally regulated protein essential for early embryonic development. *Journal of Biological Chemistry.* 2010;285(9):6826-6834.

53. Wu LS, Cheng WC, Hou SC, Yan YT, Jiang ST, Shen CKJ. TDP-43, a neuro-pathosignature factor, is essential for early mouse embryogenesis. *Genesis.* 2010;48(1):56-62.

54. Godena VK, Romano G, Romano M, et al. TDP-43 regulates Drosophila neuromuscular junctions growth by modulating Futsch/MAP1B levels and synaptic microtubules organization. *PloS one.* 2011;6(3):e17808.

55. Lin M-J, Cheng C-W, Shen C-KJ. Neuronal function and dysfunction of Drosophila dTDP. *PloS one.* 2011;6(6):e20371.

56. Estes PS, Boehringer A, Zwick R, Tang JE, Grigsby B, Zarnescu DC. Wild-type and A315T mutant TDP-43 exert differential neurotoxicity in a Drosophila model of ALS. *Human molecular genetics.* 2011;20(12):2308-2321.

57. Mackenzie IR, Rademakers R, Neumann M. TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia. *Lancet Neurol.* 2010;9(10):995-1007.

58. Wang W, Arakawa H, Wang L, et al. Motor-coordinative and cognitive dysfunction caused by mutant TDP-43 could be reversed by inhibiting its mitochondrial localization. *Molecular Therapy.* 2017;25(1):127-139.

59. Tan RH, Yang Y, Kim WS, et al. Distinct TDP-43 inclusion morphologies in frontotemporal lobar degeneration with and without amyotrophic lateral sclerosis. *Acta neuropathologica communications.* 2017;5(1):76.

60. Tsao W, Jeong YH, Lin S, et al. Rodent models of TDP-43: recent advances. *Brain research.* 2012;1462:26-39.

61. Tsai K-J, Yang C-H, Fang Y-H, et al. Elevated expression of TDP-43 in the forebrain of mice is sufficient to cause neurological and pathological phenotypes mimicking FTLD-U. *Journal of Experimental Medicine.* 2010;207(8):1661-1673.

62. Swarup V, Phaneuf D, Bareil C, et al. Pathological hallmarks of amyotrophic lateral sclerosis/frontotemporal lobar degeneration in transgenic mice produced with TDP-43 genomic fragments. *Brain : a journal of neurology.* 2011;134(9):2610-2626.

63. Arnold ES, Ling S-C, Huelga SC, et al. ALS-linked TDP-43 mutations produce aberrant RNA splicing and adult-onset motor neuron disease without aggregation or loss of nuclear TDP-43. *Proceedings of the National Academy of Sciences.* 2013;110(8):E736-E745.

64. Kwiatkowski TJ, Bosco D, Leclerc A, et al. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science.* 2009;323(5918):1205-1208.

65. Vance C, Rogelj B, Hortobágyi T, et al. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science.* 2009;323(5918):1208-1211.

66. Rabbitts T, Forster A, Larson R, Nathan P. Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t (12; 16) in malignant liposarcoma. *Nature genetics.* 1993;4(2):175-180.

67. Conte A, Lattante S, Zollino M, et al. P525L FUS mutation is consistently associated with a severe form of juvenile amyotrophic lateral sclerosis. *Neuromuscular Disorders.* 2012;22(1):73-75.

68. Waibel S, Neumann M, Rosenbohm A, et al. Truncating mutations in FUS/TLS give rise to a more aggressive ALS-phenotype than missense mutations: a clinico-genetic study in Germany. *European journal of neurology.* 2013;20(3):540-546.

69. King A, Troakes C, Smith B, et al. ALS-FUS pathology revisited: singleton FUS mutations and an unusual case with both a FUS and TARDBP mutation. *Acta neuropathologica communications.* 2015;3(1):62.

70. Kino Y, Washizu C, Kurosawa M, et al. FUS/TLS deficiency causes behavioral and pathological abnormalities distinct from amyotrophic lateral sclerosis. *Acta neuropathologica communications.* 2015;3(1):24.

71. Hicks GG, Singh N, Nashabi A, et al. Fus deficiency in mice results in defective B-lymphocyte development and activation, high levels of chromosomal instability and perinatal death. *Nature genetics.* 2000;24(2):175-179.

72. Mitchell JC, McGoldrick P, Vance C, et al. Overexpression of human wild-type FUS causes progressive motor neuron degeneration in an age-and dose-dependent fashion. *Acta neuropathologica.* 2013;125(2):1-16.

73. Sephton CF, Tang AA, Kulkarni A, et al. Activity-dependent FUS dysregulation disrupts synaptic homeostasis. *Proceedings of the National Academy of Sciences.* 2014;111(44):E4769-E4778.

74. Mitchell JC, McGoldrick P, Vance C, et al. Overexpression of human wild-type FUS causes progressive motor neuron degeneration in an age-and dose-dependent fashion. *Acta neuropathologica.* 2013;125(2):273-288.

75. Qiu H, Lee S, Shang Y, et al. ALS-associated mutation FUS-R521C causes DNA damage and RNA splicing defects. *The Journal of clinical investigation.* 2014;124(3):981-999.

76. Renton AE, Majounie E, Waite A, et al. A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. *Neuron.* 2011;72(2):257-268.

77. DeJesus-Hernandez M, Mackenzie IR, Boeve BF, et al. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron.* 2011;72(2):245-256.

78. Suh E, Lee EB, Neal D, et al. Semi-automated quantification of C9orf72 expansion size reveals inverse correlation between hexanucleotide repeat number and disease duration in frontotemporal degeneration. *Acta neuropathologica.* 2015;130(3):363-372.

79. Hubers A, Marroquin N, Schmoll B, et al. Polymerase chain reaction and Southern blot-based analysis of the C9orf72 hexanucleotide repeat in different motor neuron diseases. *Neurobiol Aging.* 2014;35(5):1214 e1211-1216.

80. Wojciechowska M, Krzyzosiak WJ. Cellular toxicity of expanded RNA repeats: focus on RNA foci. *Human molecular genetics.* 2011;20(19):3811-3821.

81. Byrne S, Elamin M, Bede P, et al. Cognitive and clinical characteristics of patients with amyotrophic lateral sclerosis carrying a C9orf72 repeat expansion: a population-based cohort study. *The Lancet Neurology.* 2012;11(3):232-240.

82. La Spada AR, Taylor JP. Repeat expansion disease: progress and puzzles in disease pathogenesis. *Nature reviews genetics.* 2010;11(4):247-258.

83. Chew J, Gendron TF, Prudencio M, et al. C9ORF72 repeat expansions in mice cause TDP-43 pathology, neuronal loss, and behavioral deficits. *Science.* 2015;348(6239):1151-1154.

84. Jiang J, Zhu Q, Gendron TF, et al. Gain of toxicity from ALS/FTD-linked repeat expansions in C9ORF72 is alleviated by antisense oligonucleotides targeting GGGGCC-containing RNAs. *Neuron.* 2016;90(3):535-550.

85. Liu Y, Pattamatta A, Zu T, et al. C9orf72 BAC mouse model with motor deficits and neurodegenerative features of ALS/FTD. *Neuron.* 2016;90(3):521-534.

86. Bateson W. Mendel's Principles of Heredity: Cambridge University Press. *März 1909; 2nd Impr.* 1909;3:1913.

87. Phillips PC. Epistasis--the essential role of gene interactions in the structure and evolution of genetic systems. *Nat Rev Genet.* 2008;9(11):855-867.

88. Weiss KM, Terwilliger JD. How many diseases does it take to map a gene with SNPs? *Nature genetics.* 2000;26(2):151-157.

89. Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K. A comprehensive review of genetic association studies. *Genetics in medicine.* 2002;4(2):45-61.

90. Kim NC, Andrews PC, Asselbergs FW, et al. Gene ontology analysis of pairwise genetic associations in two genome-wide studies of sporadic ALS. *BioData mining.* 2012;5(1):1-6.

91. Majoor-Krakauer D, Willems P, Hofman A. Genetic epidemiology of amyotrophic lateral sclerosis. *Clinical genetics.* 2003;63(2):83-101.

92. Farrawell NE, Lambert-Smith IA, Warraich ST, et al. Distinct partitioning of ALS associated TDP-43, FUS and SOD1 mutants into cellular inclusions. *Scientific reports.* 2015;5(1):1-14.

93. Seaman MN. The retromer complex–endosomal protein recycling and beyond. *Journal of cell science.* 2012;125(20):4693-4702.

94. Bonifacino JS, Rojas R. Retrograde transport from endosomes to the trans-Golgi network. *Nature reviews Molecular cell biology.* 2006;7(8):568-579.

95. Banos-Mateos S, Rojas AL, Hierro A. VPS29, a tweak tool of endosomal recycling. *Curr Opin Cell Biol.* 2019;59:81-87.

96. Zimprich A, Benet-Pagès A, Struhal W, et al. A mutation in VPS35, encoding a subunit of the retromer complex, causes late-onset Parkinson disease. *The American Journal of Human Genetics.* 2011;89(1):168-175.

97. Lee JJ, Radice G, Perkins CP, Costantini F. Identification and characterization of a novel, evolutionarily conserved gene disrupted by the murine H beta 58 embryonic lethal transgene insertion. *Development.* 1992;115(1):277-288.

98. Wen L, Tang FL, Hong Y, et al. VPS35 haploinsufficiency increases Alzheimer's disease neuropathology. *J Cell Biol.* 2011;195(5):765-779.

99. Kim E, Lee Y, Lee HJ, et al. Implication of mouse Vps26b-Vps29-Vps35 retromer complex in sortilin trafficking. *Biochem Biophys Res Commun.* 2010;403(2):167-171.

100. McGoldrick P, Joyce PI, Fisher EM, Greensmith L. Rodent models of amyotrophic lateral sclerosis. *Biochimica et biophysica acta.* 2013;1832(9):1421-1436.

101. Ittner LM, Halliday GM, Kril JJ, Götz J, Hodges JR, Kiernan MC. FTD and ALS [mdash] translating mouse studies into clinical trials. *Nature reviews neurology.* 2015;11(6):360-366.

102. Mancuso R, Navarro X. Amyotrophic lateral sclerosis: Current perspectives from basic research to the clinic. *Progress in neurobiology.* 2015;133:1-26.

103. Kreilaus F, Guerra S, Masanetz R, Menne V, Yerbury J, Karl T. Novel behavioural characteristics of the superoxide dismutase 1 G93A (SOD1G93A) mouse model of amyotrophic lateral sclerosis include sex-dependent phenotypes. *Genes, Brain and Behavior.* 2019:e12604.

104. Sgobio C, Trabalza A, Spalloni A, et al. Abnormal medial prefrontal cortex connectivity and defective fear extinction in the presymptomatic G93A SOD1 mouse model of ALS. *Genes, Brain and Behavior.* 2008;7(4):427-434.

105. Takao K, Yamasaki N, Miyakawa T. Impact of brain-behavior phenotypying of genetically-engineered mice on research of neuropsychiatric disorders. *Neuroscience research.* 2007;58(2):124-132.

106. Van Den Buuse M, Garner B, Gogos A, Kusljic S. Importance of animal models in schizophrenia research. *Australian and New Zealand Journal of Psychiatry.* 2005;39(7):550-557.

107. Patten SA, Parker JA, Wen X-Y, Drapeau P. Simple animal models for amyotrophic lateral sclerosis drug discovery. *Expert opinion on drug discovery.* 2016;11(8):797-804.

108. Borrello P, D'Amore E, Panzini G, Valeri M, Lorenzini RN. Individually ventilated cages–Microbiological containment testing. *Scandinavian Journal of Laboratory Animal Sciences.* 2000;27(3):142-152.

109. Spangenberg E, Wallenbeck A, Eklöf A, Carlstedt-Duke J, Tjäder S. Housing breeding mice in three different IVC systems: maternal performance and pup development. *Laboratory animals.* 2014;48(3):193-206.

110. Tsai P-P, Oppermann D, Stelzer H, Mähler M, Hackbarth H. The effects of different rack systems on the breeding performance of DBA/2 mice. *Laboratory animals.* 2003;37(1):44-53.

111. Pasquarelli N, Voehringer P, Henke J, Ferger B. Effect of a change in housing conditions on body weight, behavior and brain neurotransmitters in male C57BL/6J mice. *Behavioural brain research.* 2017;333:35-42.

112. Logge W, Kingham J, Karl T. Behavioural consequences of IVC cages on male and female C57BL/6J mice. *Neuroscience.* 2013;237:285-293.

113. David JM, Knowles S, Lamkin DM, Stout DB. Individually ventilated cages impose cold stress on laboratory mice: a source of systemic experimental variability. *Journal of the American Association for Laboratory Animal Science.* 2013;52(6):738-744.

114. Bennett EL, Rosenzweig MR, Diamond MC. Rat brain: effects of environmental enrichment on wet and dry weights. *Science.* 1969;163(3869):825-826.

115. Diamond MC. *Enriching heredity: the impact of the environment on the anatomy of the brain.* Free Press; 1988.

116. Nithianantharajah J, Hannan AJ. Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nature Reviews Neuroscience.* 2006;7(9):697-709.

117. Nilsson M, Perfilieva E, Johansson U, Orwar O, Eriksson PS. Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *Journal of neurobiology.* 1999;39(4):569-578.

118. Pacteau C, Einon D, Sinden J. Early rearing environment and dorsal hippocampal ibotenic acid lesions: long-term influences on spatial learning and altenation in the rat. *Behavioural brain research.* 1989;34(1-2):79-96.

119. Logge W, Kingham J, Karl T. Do individually ventilated cage systems generate a problem for genetic mouse model research? *Genes, Brain and Behavior.* 2014;13(7):713-720.

120. Shan L, Schipper P, Nonkes LJ, Homberg JR. Impaired fear extinction as displayed by serotonin transporter knockout rats housed in open cages is disrupted by IVC cage housing. *PloS one.* 2014;9(3):e91472.

121. Mueller FS, Polesel M, Richetto J, Meyer U, Weber-Stadlbauer U. Mouse models of maternal immune activation: Mind your caging system! *Brain, behavior, and immunity.* 2018;73:643-660.

122. Gouveia K, Hurst JL. Reducing mouse anxiety during handling: effect of experience with handling tunnels. *PloS one.* 2013;8(6):e66401.

123. Gouveia K, Hurst JL. Optimising reliability of mouse performance in behavioural testing: the major role of non-aversive handling. *Scientific reports.* 2017;7:44999.

124. Hurst JL, West RS. Taming anxiety in laboratory mice. *Nature methods.* 2010;7(10):825-826.

125. Clarkson JM, Dwyer DM, Flecknell PA, Leach MC, Rowe C. Handling method alters the hedonic value of reward in laboratory mice. *Scientific reports.* 2018;8(1):2448.

126. Wahlsten D, Bachmanov A, Finn DA, Crabbe JC. Stability of inbred mouse strain differences in behavior and brain size between laboratories and across decades. *Proceedings of the national academy of sciences.* 2006;103(44):16364-16369.

127. Crabbe JC, Wahlsten D, Dudek BC. Genetics of mouse behavior: interactions with laboratory environment. *Science.* 1999;284(5420):1670-1672.

128. Stam NC, Nithianantharajah J, Howard ML, Atkin JD, Cheema SS, Hannan AJ. Sex-specific behavioural effects of environmental enrichment in a transgenic mouse model of amyotrophic lateral sclerosis. *European Journal of Neuroscience.* 2008;28(4):717-723.

129. Huang Z, Liu Q, Peng Y, et al. Circadian rhythm dysfunction accelerates disease progression in a mouse model with amyotrophic lateral sclerosis. *Frontiers in neurology.* 2018;9:218.

130. Doble A. The pharmacology and mechanism of action of riluzole. *Neurology.* 1996;47(6 Suppl 4):233S-241S.

131. Gurney ME, Cutting FB, Zhai P, et al. Benefit of vitamin E, riluzole, and gababapentin in a transgenic model of familial amyotrophic lateral sclerosis. *Annals of neurology.* 1996;39(2):147-157.

132. Hogg MC, Halang L, Woods I, Coughlan KS, Prehn JH. Riluzole does not improve lifespan or motor function in three ALS mouse models. *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration.* 2018;19(5-6):438-445.

133. Song J-H, Huang C-S, Nagata K, Yeh JZ, Narahashi T. Differential action of riluzole on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. *Journal of Pharmacology and Experimental Therapeutics.* 1997;282(2):707-714.

134. Distad BJ, Meekins GD, Liou LL, Weiss MD, Carter GT, Miller RG. Drug therapy in amyotrophic lateral sclerosis. *Physical medicine and rehabilitation clinics of North America.* 2008;19(3):633-651.

135. Bellingham MC. A review of the neural mechanisms of action and clinical efficiency of riluzole in treating amyotrophic lateral sclerosis: what have we learned in the last decade? *CNS neuroscience & therapeutics.* 2011;17(1):4-31.

136. Hardiman O, van den Berg LH. Edaravone: a new treatment for ALS on the horizon? *Lancet Neurol.* 2017;16(7):490-491.

137. Watanabe T, Tahara M, Todo S. The novel antioxidant edaravone: from bench to bedside. *Cardiovasc Ther.* 2008;26(2):101-114.

138. Zhu Y, Fotinos A, Mao LL, et al. Neuroprotective agents target molecular mechanisms of disease in ALS. *Drug discovery today.* 2015;20(1):65-75.

139. Gribkoff VK, Bozik ME. KNS-760704 [(6R)-4, 5, 6, 7-tetrahydro-N6-propyl-2, 6-benzothiazole-diamine dihydrochloride monohydrate] for the Treatment of Amyotrophic Lateral Sclerosis. *CNS neuroscience & therapeutics.* 2008;14(3):215-226.

140. Keller AF, Gravel M, Kriz J. Treatment with minocycline after disease onset alters astrocyte reactivity and increases microgliosis in SOD1 mutant mice. *Experimental neurology.* 2011;228(1):69-79.

141. Stommel EW, Cohen JA, Fadul CE, et al. Efficacy of thalidomide for the treatment of amyotrophic lateral sclerosis: a phase II open label clinical trial. *Amyotrophic lateral sclerosis.* 2009;10(5-6):393-404.

142. Kiaei M, Petri S, Kipiani K, et al. Thalidomide and lenalidomide extend survival in a transgenic mouse model of amyotrophic lateral sclerosis. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2006;26(9):2467-2473.

143. Gordon PH, Moore DH, Miller RG, et al. Efficacy of minocycline in patients with amyotrophic lateral sclerosis: a phase III randomised trial. *The Lancet Neurology.* 2007;6(12):1045-1053.

144. Tuszynski MH, Blesch A. Nerve growth factor: from animal models of cholinergic neuronal degeneration to gene therapy in Alzheimer's disease. *Progress in brain research.* 2004;146:439-449.

145. Luo J, Kaplitt MG, Fitzsimons HL, et al. Subthalamic GAD gene therapy in a Parkinson's disease rat model. *Science.* 2002;298(5592):425-429.

146. Kaspar BK, Lladó J, Sherkat N, Rothstein JD, Gage FH. Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science.* 2003;301(5634):839-842.

147. Wang L-J, Lu Y-Y, Muramatsu S-i, et al. Neuroprotective effects of glial cell line-derived neurotrophic factor mediated by an adeno-associated virus vector in a transgenic animal model of amyotrophic lateral sclerosis. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2002;22(16):6920-6928.

148. Zhao C-P, Zhang C, Zhou S-N, et al. Human mesenchymal stromal cells ameliorate the phenotype of SOD1-G93A ALS mice. *Cytotherapy.* 2007;9(5):414-426.

149. Giordano A, Galderisi U, Marino IR. From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells. *Journal of cellular physiology.* 2007;211(1):27-35.

150. Kim SU, De Vellis J. Stem cell-based cell therapy in neurological diseases: a review. *Journal of neuroscience research.* 2009;87(10):2183-2200.

151. Meamar R, Nasr-Esfahani MH, Mousavi SA, Basiri K. Stem cell therapy in amyotrophic lateral sclerosis. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia.* 2013;20(12):1659-1663.

152. Thomsen GM, Gowing G, Svendsen S, Svendsen CN. The past, present and future of stem cell clinical trials for ALS. *Experimental neurology.* 2014;262:127-137.

153. Novotna A, Mares J, Ratcliffe S, et al. A randomized, double-blind, placebo-controlled, parallel-group, enriched-design study of nabiximols (Sativex®), as add-on therapy, in subjects with refractory spasticity caused by multiple sclerosis. *European Journal of Neurology.* 2011;18(9):1122-1131.

154. Barnes MP. Sativex®: clinical efficacy and tolerability in the treatment of symptoms of multiple sclerosis and neuropathic pain. *Expert opinion on pharmacotherapy.* 2006;7(5):607-615.

155. Giuffrida A, Piomelli D. The endocannabinoid system: a physiological perspective on its role in psychomotor control. *Chemistry and physics of lipids.* 2000;108(1-2):151-158.

156. Freund TF, Katona I, Piomelli D. Role of endogenous cannabinoids in synaptic signaling. *Physiological reviews.* 2003.

157. Di Marzo V, Fontana A, Cadas H, Schinelli S. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature.* 1994;372(6507):686.

158. Howlett A, Barth F, Bonner T, et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacological reviews.* 2002;54(2):161-202.

159. Carrier EJ, Auchampach JA, Hillard CJ. Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. *Proceedings of the National Academy of Sciences.* 2006;103(20):7895-7900.

160. Bilsland LG, Dick JR, Pryce G, et al. Increasing cannabinoid levels by pharmacological and genetic manipulation delay disease progression in SOD1 mice. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2006;20(7):1003-1005.

161. Witting A, Weydt P, Hong S, Kliot M, Mőller T, Stella N. Endocannabinoids accumulate in spinal cord of SOD1G93A transgenic mice. *Journal of neurochemistry.* 2004;89(6):1555-1557.

162. Mechoulam R, Parker LA. The endocannabinoid system and the brain. *Annual review of psychology.* 2013;64:21-47.

163. Sun Y, Alexander S, Kendall D, Bennett A. Cannabinoids and PPARα signalling. Portland Press Limited; 2006.

164. Burstein S. PPAR-γ: a nuclear receptor with affinity for cannabinoids. *Life sciences.* 2005;77(14):1674-1684.

165. Smart D, Gunthorpe M, Jerman J, et al. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *British journal of pharmacology.* 2000;129(2):227-230.

166. Zygmunt PM, Petersson J, Andersson DA, et al. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature.* 1999;400(6743):452-457.

167. Drmota T, Greasley P, Groblewski T. Screening assays for cannabinoid-ligand-type modulators of GPR55. Google Patents; 2004.

168. Gonsiorek W, Lunn C, Fan X, Narula S, Lundell D, Hipkin RW. Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide. *Molecular pharmacology.* 2000;57(5):1045-1050.

169. Sugiura T, Kobayashi Y, Oka S, Waku K. Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA).* 2002;66(2-3):173-192.

170. Di Marzo V, Matias I. Endocannabinoid control of food intake and energy balance. *Nature neuroscience.* 2005;8(5):585-589.

171. Khasabova IA, Chandiramani A, Harding-Rose C, Simone DA, Seybold VS. Increasing 2-arachidonoyl glycerol signaling in the periphery attenuates mechanical hyperalgesia in a model of bone cancer pain. *Pharmacological research.* 2011;64(1):60-67.

172. Katona I, Urbán GM, Wallace M, et al. Molecular composition of the endocannabinoid system at glutamatergic synapses. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2006;26(21):5628-5637.

173. Raman C, McAllister SD, Rizvi G, Patel SG, Moore DH, Abood ME. Amyotrophic lateral sclerosis: delayed disease progression in mice by treatment with a cannabinoid. *Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases.* 2004;5(1):33-39.

174. Weydt P, Hong S, Witting A, Möller T, Stella N, Kliot M. Cannabinol delays symptom onset in SOD1 (G93A) transgenic mice without affecting survival. *Amyotrophic Lateral Sclerosis.* 2005;6(3):182-184.

175. Chousidis I, Chatzimitakos T, Leonardos D, Filiou MD, Stalikas CD, Leonardos ID. Cannabinol in the spotlight: Toxicometabolomic study and behavioral analysis of zebrafish embryos exposed to the unknown cannabinoid. *Chemosphere.* 2020;252:126417.

176. Shoemaker JL, Seely KA, Reed RL, Crow JP, Prather PL. The CB2 cannabinoid agonist AM-1241 prolongs survival in a transgenic mouse model of amyotrophic lateral sclerosis when initiated at symptom onset. *Journal of neurochemistry.* 2007;101(1):87-98.

177. Carlini EA, Cunha JM. Hypnotic and antiepileptic effects of cannabidiol. *Journal of clinical pharmacology.* 1981;21(S1):417S-427S.

178. Zuardi A, Guimares F. Cannabidiol as an Anxiolytic and Antipsychotic in Cannabis: The Medicine Plant McFarland & Co. London; 1991.

179. Parker LA, Mechoulam R, Schlievert C. Cannabidiol, a non-psychoactive component of cannabis and its synthetic dimethylheptyl homolog suppress nausea in an experimental model with rats. *Neuroreport.* 2002;13(5):567-570.

180. Hampson AJ, Grimaldi M, Axelrod J, Wink D. Cannabidiol and (-)Delta9-tetrahydrocannabinol are neuroprotective antioxidants. *Proceedings of the National Academy of Sciences of the United States of America.* 1998;95(14):8268-8273.

181. Thomas BF, Gilliam AF, Burch DF, Roche MJ, Seltzman HH. Comparative receptor binding analyses of cannabinoid agonists and antagonists. *Journal of Pharmacology and Experimental Therapeutics.* 1998;285(1):285-292.

182. Bisogno T, Hanuš L, De Petrocellis L, et al. Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *British journal of pharmacology.* 2001;134(4):845-852.

183. El-Remessy AB, Khalil IE, Matragoon S, et al. Neuroprotective effect of (−) Δ 9-tetrahydrocannabinol and cannabidiol in N-methyl-D-aspartate-induced retinal neurotoxicity: involvement of peroxynitrite. *The American journal of pathology.* 2003;163(5):1997-2008.

184. Rajan TS, Scionti D, Diomede F, et al. Gingival stromal cells as an in vitro model: Cannabidiol modulates genes linked with amyotrophic lateral sclerosis. *Journal of cellular biochemistry.* 2017;118(4):819-828.

185. Moreno-Martet M, Espejo-Porras F, Fernández-Ruiz J, Lago E. Changes in Endocannabinoid Receptors and Enzymes in the Spinal Cord of SOD1G93A Transgenic Mice and Evaluation of a Sativex®-like Combination of Phytocannabinoids: Interest for Future Therapies in Amyotrophic Lateral Sclerosis. *Cns neuroscience & therapeutics.* 2014;20(9):809-815.

186. Weber M, Goldman B, Truniger S. Tetrahydrocannabinol (THC) for cramps in amyotrophic lateral sclerosis: a randomised, double-blind crossover trial. *Journal of neurology, neurosurgery, and psychiatry.* 2010;81(10):jnnp. 2009.200642.

187. Weber M, Goldman B, Truniger S. Tetrahydrocannabinol (THC) for cramps in amyotrophic lateral sclerosis: a randomised, double-blind crossover trial. *Journal of neurology, neurosurgery, and psychiatry.* 2010;81(10):1135-1140.

188. Riva N, Mora G, Sorarù G, et al. Safety and efficacy of nabiximols on spasticity symptoms in patients with motor neuron disease (CANALS): a multicentre, double-blind, randomised, placebo-controlled, phase 2 trial. *The Lancet Neurology.* 2019;18(2):155-164.

189. Amtmann D, Weydt P, Johnson KL, Jensen MP, Carter GT. Survey of cannabis use in patients with amyotrophic lateral sclerosis. *American Journal of Hospice and Palliative Medicine.* 2004;21(2):95-104.

190. Deng H-X, Shi Y, Furukawa Y, et al. Conversion to the amyotrophic lateral sclerosis phenotype is associated with intermolecular linked insoluble aggregates of SOD1 in

mitochondria. *Proceedings of the National Academy of Sciences.* 2006;103(18):7142-7147.

191. Filali M, Lalonde R, Rivest S. Sensorimotor and cognitive functions in a SOD1 G37R transgenic mouse model of amyotrophic lateral sclerosis. *Behavioural brain research.* 2011;225(1):215-221.

192. Chen W, Zhai P, Sufit RL, Siddique T. Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science.* 1994;264:17.

193. Wegorzewska I, Bell S, Cairns NJ, Miller TM, Baloh RH. TDP-43 mutant transgenic mice develop features of ALS and frontotemporal lobar degeneration. *Proceedings of the National Academy of Sciences.* 2009;106(44):18809-18814.

194. Xu Y-F, Zhang Y-J, Lin W-L, et al. Expression of mutant TDP-43 induces neuronal dysfunction in transgenic mice. *Molecular neurodegeneration.* 2011;6(1):73.

195. Hayakawa K, Mishima K, Hazekawa M, et al. Cannabidiol potentiates pharmacological effects of Δ9-tetrahydrocannabinol via CB1 receptor-dependent mechanism. *Brain research.* 2008;1188:157-164.

196. Watt G, Shang K, Zieba J, et al. Chronic Treatment with 50 mg/kg Cannabidiol Improves Cognition and Moderately Reduces Aβ 42 Levels in 12-Month-Old Male AβPP swe/PS1ΔE9 Transgenic Mice. *Journal of Alzheimer's Disease.* 2020(Preprint):1-14.

Note: The following chapter is an excerpt from work titled:

Effects of handling on the behavioural phenotype of the *neuregulin 1 type III* transgenic mouse model for schizophrenia

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This was an opportunistic study as ALS mouse models were not yet ready for study due complications at the animal breeding facility (detailed in Chapter 6. Limitations). Thus, to not confuse the message of this thesis by introducing the topic of Schizophrenia only the Wild-type animals have been analysed.

Declaration

I certify that this publication was a direct result of my research towards this PhD and that reproduction in this thesis does not breach copyright regulations.

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Stefan Guerra

Chapter 2: Evaluation of the effect of handling method on the baseline behaviours of laboratory mice in a newly established testing facility.

2.1 Introduction

An often-neglected area in mouse research is the impact laboratory housing conditions, such as holding cage systems and cage enrichment structures, can have on the neuro-behavioural phenotype of laboratory mice [1-3], and in particular on genetic mouse models for human diseases [4-6]. Most recently, the method of handling laboratory mice for general husbandry or experimental purposes has been identified as another environmental factor which could potentially confound experimental test outcomes. Hurst and colleagues found that 'tail handling', which involves the picking up and restraining of laboratory mice by the base of the tail, elicits an aversive response from the test mouse and this aversion actually increased in severity over the course of a testing period [7]. The team found that, once habituated, using plastic tunnels or cupped hands to pick up laboratory mice increased the voluntary interaction of test mice with the researcher in the home cage, decreased anxiety in the elevated plus maze (EPM), and increased exploration of a dishabituated stimulus in an open arena [8] when compared to standard handling procedures [7,9]. This effect of non-aversive handling was investigated further by other groups and, in addition to the behaviours previously mentioned, tunnel-handled mice showed a prohedonic response to a sucrose reward compared to tail-handled mice, indicating a potential depressive-like effect of tail handling compared to other handling alternatives [10]. Furthermore, handling using the 'cupped hand' method has decreased anxiety measures in the EPM, improved glucose tolerance, and lowered serum corticosterone levels [8,11]. Importantly, a study performed by Gouveia and co-workers reported that

tunnel handling reduces variation in test results across mice within individual behavioural tests in the ICR mouse strain when compared to tail handling [12]. Thus, prior to the commencement of testing with transgenic animals of human disease, it was prudent to establish what effects, if any, less aversive handling would have in our newly established facility. As such we assessed the effect of tail handling versus the less aversive tunnel handling (handling via the use of cupped hands was not assessed due to the potential for inter-investigator variability in the procedure used and the potential for olfactory confounds) on C57BL/6Tac mice in a standard battery of behavioural test. We also evaluated potential sex differences in the response to handling as this perspective had not been a factor in prior research performed in this domain.

2.2 Materials and methods

2.2.1 Animals

Experimental animals were male and female C57BL/6Tac mice bred at the Australian BioResources [ABR Moss Vale, Australia. Breeding colonies at ABR were housed in individually ventilated cages (Type Mouse Version 1; Airlaw, Smithfield, Australia; air change: 90-120 times per hour averaged; passive exhaust ventilation system)]. The test mice were transported to the animal facility at Western Sydney University (School of Medicine, Campbelltown, Australia) at 3 months of age. Mice from the same litter were group-housed (2-3 per cage) in filter top cages (1144B: Techniplast, Rydalmere Australia) with corn cob bedding (PuraCob premium: Able Scientific, Perth Australia), tissues for nesting material and a red igloo (Bioserv, Frenchtown, NJ, USA). Mice were kept on a 12h light/dark cycle (9am – 9pm/9pm – 9pm respectively and tested during the first 5 hours of the light phase) and food and water were provided *ad libitum*. Prior to the start of the experiments, mice were only handled during fortnightly cage changes using the tail handling method. For the social preference test, sex-matched, adult A/JArc mice from the Animal Resources Centre (ARC: Cunning Vale, Australia) were used as social conspecifics.

All research and animal care procedures were approved by the Western Sydney University Animal Care and Ethics Committee (#A11746) and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2.2 Handling habituation

The two different handling procedures were started when mice were 25.5 ± 2.5 weeks of age. Mice were assigned a handling method in a quasi-randomised fashion per cage and handled by their designated handling method for the entire experimental period. Prior to each handling session,

nesting material and environmental enrichment were removed from the cage. Handling techniques used were adapted from the research performed by Hurst and coworkers [7-9]. Tail handling 'training' consisted of grasping the mouse at the base of the tail between the thumb and forefinger, the mouse was then supported on the back of the opposite hand and held for 30 s before being released back into the cage. Tunnel handling 'training' consisted of guiding the mouse into a cage specific tunnel (used for the duration of the testing period and cleaned at the end of each session; opaque grey PVC tunnels: 200 mm x 40 mm) which was then held above the cage for 30 seconds before releasing the animal back into the cage. Handling training was carried out twice a day, with a 60 seconds inter-trial interval, for a total of 10 days before any behavioural testing commenced. From the start of handling habituation, all cages were changed twice weekly by the investigator using the assigned handling method.

2.2.3 Behavioural testing

After 10 days of handling training, mice (n = 10-14 per experimental condition, see Table 1) were tested in a standard battery of behavioural domains. During all behavioural testing, mice were placed into and retrieved from the apparatus via their assigned handling method. On non-testing days, mice were handled in the same way as during their handling training. Test order, sample sizes, and test ages are outlined in Table 1.

2.2.3.1 Open Field (OF)

To assess locomotion and exploration [13] in response to handling, mice were placed into infrared photobeam controlled OF test chambers (MED Associates Inc., St Albans, USA) for 30 minutes. The test area (43.2 cm x 43.2 cm) was divided into a central and peripheral zone (MED software coordinates for central zone: 3/3, 3/13, 13/3, 13/13); total distance travelled, and vertical activity

were automatically measured (locomotion defined as two infrared beam breaks in a 100ms window). In addition, the ratio of distance travelled, time spent in the central zone and velocity over the test duration were used to analyse anxiety-related behaviours. All measures were analysed at both 10 and 30 minutes

2.2.3.2 Elevated Plus Maze (EPM)

The EPM measures anxiety-related behaviours as well as exploration and locomotion [14]. Mice were placed in the centre of the apparatus (Elevated Plus Maze, No. 7001-0316; San Diego Instruments, San Diego, USA) facing the closed arm and allowed to explore for a total of 5 minutes. The number of entries, total time and total distance spent in the open arms and closed arms was automatically scored using Any-Maze™ tracking software (Stoelting Co.; Illinois, USA). Manual scoring was used for exploratory behaviours such as *rearing* and risk assessment behaviour (i.e. stretch-attend postures). Time spent on open arms as well as percentage locomotion on open arms (as a percentage of total time spent on open arms / percentage total distance travelled on arms) were used to assess anxiety-like behaviours.

2.2.3.3 Social Preference Test (SPT)

The SPT was used to measure if handling impacted on social approach behaviour and social recognition memory. The apparatus consists of three connected chambers: a central chamber (length: 10 cm, width: 19 cm and height: 20 cm) and two outer chambers (17 cm x 19 cm x 20 cm) with clear Plexiglas dividing walls with square passages (height: 4 cm and width: 4 cm). One circular enclosure (height: 15 cm, diameter: 7 cm; bars spaced 0.5 cm apart) was placed into each outer chamber to allow contact between mice but prevent fighting. Fresh bedding was added to all chambers prior to each test trial. Test animals were isolated for 1 hour prior to testing in a new cage with fresh nesting material. Test mice were then allowed to habituate to the apparatus for a period 5 minute before being removed. For the test of social novelty, an unfamiliar social

conspecific (i.e. a sex-matched, adult A/J mouse), was placed in one of the two opponent outer chambers in a quasi-randomised manner before the test mouse was returned to the central chamber and allowed to explore freely for 10 minutes. Finally, test animals were observed in a 10 minutes social recognition test trial in which a second unfamiliar opponent A/J mouse was placed in the chamber that was previously empty during the social preference trial so that the test mice could explore either the familiar mouse (from the previous trial) or the novel, unfamiliar mouse. Any-MazeTM tracking software was used to determine the time the test mice spent in the different chambers during the trial.

2.2.3.4 Fear Conditioning (FC)

FC measures the association of a previously neutral stimulus (e.g. a tone or a context) with an aversive stimulus (e.g. an electric foot shock). The FC task occurred over three days [methods adapted from previous work in our lab [14]]. On the first day (i.e. conditioning day), mice were placed in a test chamber (Med Associates product: MED-VFC-SCT-M) with a vanilla scent cue for 120 seconds, with no sound (i.e. baseline period), before an 80 dB conditioned stimulus (CS) was presented for 30 s co-terminating with a 0.4 mA 2 second foot shock (unconditioned stimulus). This procedure was repeated with an inter-stimulus interval of 120 s with the test concluding 120 seconds later. On the second day of testing (context test), mice were returned to the apparatus for 7 minutes with vanilla scent cue and no sound. On the third day of testing (cue test), mice were placed in an altered environment (i.e. a triangular insert added to the chamber to change its overall shape with no scent cue present) for 9 minutes. After 120 seconds (pre-CS/baseline), the CS was continuously presented for 5 minutes with the test concluding 120 seconds afterwards (i.e. no cue presentation in the last 2 minutes). Time spent *freezing* per minute (and as a total per trial) was measured in all three trials using the video-freeze^{TM} software (freezing threshold of 15 frames). In

addition, the *freezing* response to the cue was also assessed by comparing average *freezing* for 2 minutes prior to cue presentation to the average *freezing* for the 5 minutes during cue presentation.

2.2.3.5 Sensorimotor gating - Prepulse inhibition (PPI)

Sensorimotor gating, as indicated by the attenuation of the startle response by a non-startling stimulus [15], was measured using SR-LAB startle chambers (San Diego Instruments, San Diego, USA). Mice were initially habituated to the apparatus for three days prior to the test by being placed into the test chamber and exposed to 70 decibel (dB) white background noise for a period of 5 minutes. The PPI test consisted of an initial habituation period with the presentation of 70 dB white background noise for 5 minutes. Following this, 97 trials presented in a pseudorandom order: 5 x 70 dB trials (background); 5 x 100 dB trials; 15 x 120 dB trials [startle; separated into blocks of 5 startle trials each (first, middle and last startle block)] and 4 sets of 12 trials including a prepulse of either 74, 82 or 86 dB presented at 32, 64, 128 or 254 milliseconds (variable interstimulus interval: ISI) prior to a startling pulse of 120 dB (PPI response). The interval between trials differed randomly between 10-20 seconds. Acoustic startle response was calculated as the mean amplitude to all startle trials. Percentage of PPI (%PPI) was calculated as: [mean startle response (120 dB) - PPI response / mean startle response (120 dB)] x 100. PPI was averaged across ISIs to produce a mean % PPI for each prepulse intensity.

2.2.5 Statistical analysis

Data were initially analysed using two-way analysis of variance (ANOVA) for the main test parameters ‘Sex’ and ‘Handling’. Three-way repeated measures (RM) ANOVAs were also carried out for within factors ‘chamber’ (SPT), ‘time’ and ‘cue’ (FC), as well as ‘pulse’ and ‘prepulse’ (PPI). When significant interactions were detected, we split by the corresponding factor. Data are

shown as means ± standard error of the mean (SEM) and only presented separately for males and females in cases where we found significant interactions of 'Sex' and 'Handling'. F-values and degrees of freedom are presented for ANOVAs and significant handling effects are shown in figures and tables as '*' (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). Sex effects are indicated using '#' ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$) and interactions between 'Sex' and 'Handling' are shown as '+' ($^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$). Sociability testing was analysed via independent samples t-test *versus* a chance level of 50%. Significant effects versus 50% 'chance' are shown in figures as '^' (^$p < 0.05$, ^^$p < 0.01$ and ^^^$p < 0.001$). All analyses were performed using IBM SPSS Statistics v24 and were run discreetly for this reported cohort.

2.3. Results

2.3.1 Locomotion and exploration:

Two-way ANOVA found effects of 'Sex' on total distance travelled in the OF with male mice travelling less than female mice [trend at 10 mins: $F(1,44) = 3.91$, $p = 0.054$ - significant at 30 mins: $F(1,44) = 4.44$, $p = 0.041$] (Fig. 2.1A-B). Exploration (i.e. vertical activity) was significantly higher in male mice compared to female mice after 10 minutes [$F(1,44) = 10.98$, $p = 0.002$] as well as 30 minutes of testing [$F(1,44) = 7.921$, $p = 0.007$] (Fig. 2.1C-D). Overall, tunnel-handled mice had higher levels of exploration compared to tail-handled mice in the first 10 minutes of testing [$F(1,44) = 7.77$, $p = 0.008$, Fig. 2.1C], however, no significant impact of Handling on exploration was found after 30 minutes [$F(1,44) = 3.26$, $p = 0.08$] (no 'Sex' by 'Handling' interactions, *p's* > 0.28). Tunnel handling also led to an increase in average locomotion speed in the first 10 minutes of testing [$F(1,44) = 4.64$, $p = 0.037$; Table 2.2] compared to the control condition (not evident when assessing full 30 min test period, $p = 0.25$). No 'Sex' effects (all $p > 0.198$) or 'Sex' by 'Handling' interactions (all $p > 0.168$) were observed for locomotion speed at either time point.

In the EPM, no main effects or interactions of 'Sex' and 'Handling' were seen for total entries into an arm (all *p's* > 0.05; Table 2.2). A trend for 'Handling' on explorative behaviours was evident, as tunnel-handled animals tended to spend more overall time *head dipping* [$F(1,44) = 3.57$, $p = 0.065$; Fig. 2.1E] compared to tail-handled animals. Additionally, an significant 'Sex' by 'Handling' interaction for total *rearing* time [$F(1,44) = 4.87$, $p = 0.032$; Fig. 2.1F] was present with male tunnel-handled animals showing higher levels of exploration than tail-handled males ['Handling' effect when split by Sex: $F(1,25) = 6.15$, $p = 0.02$]. This handling effect was absent in female mice [$F(1,19) = 0.6$, $p = 0.45$].

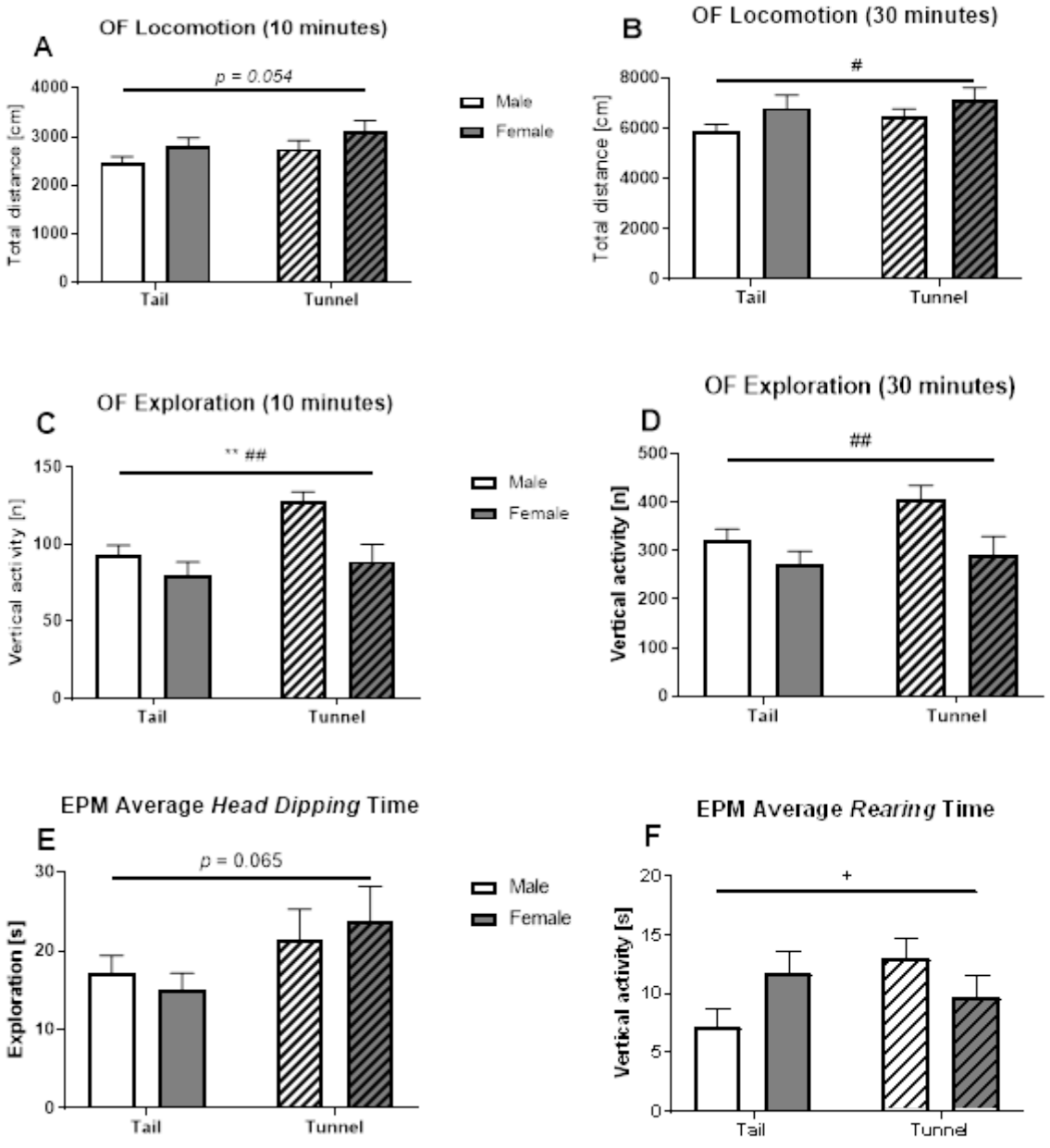

Figure 2.1A-F: Locomotion and exploration in the open field (OF) and elevated plus maze (EPM): Total distance travelled [cm] (**A/C**) and total vertical activity (i.e. rearing frequency) [n] (**B/D**) after 10 minutes **(A/C)** and **(B/D)** 30 minutes of OF testing as well as average time spent head dipping **(E)** and rearing **(F)** on the EPM. Data are shown for tail-handled (tail) and tunnel-handled (tunnel) C57BL/6Tac mice (split for sex as main effects of 'Sex' were evident). Data are presented as mean ± SEM. Main handling effects are shown as '*' ($^{**}p < 0.01$) and main sex effects are shown as '#' ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$). A trend for a main effect of 'Sex' for locomotion during the first 10 minutes has been indicated as well ($p = 0.054$) (**Fig 1A**). A trend for 'Handling' in average time head dipping in the EPM is indicated by $p = 0.065$ (**Fig. 3B**). Interaction of 'Sex' and 'Handling' for rearing in the EPM indicated by '+' ($^{+}p < 0.05$).

2.3.2 Anxiety-related behaviours:

Two-way ANOVA revealed a trend for a 'Sex' effect [$F(1,44) = 3.66$, $p = 0.062$] after 10 minutes of OF testing as male mice travelled a higher percentage in the centre zone than female mice [not evident at 30 minutes; $F(1,44) = 2.95$, $p > 0.09$] (Fig. 2.2A-B). The method of Handling did not have an effect on percentage centre distance travelled ($F(1,44) = 1.92$, $p = 0.17$; Fig. 2.2A) or percentage centre time ($F(1,44) = 0.89$, $p = 0.35$; Fig. 2.2C) after 10 minutes. However, after 30 minutes, a main effect of 'Handling' was evident for both measures [i.e. % centre distance: $F(1,44) = 8.39$, $p = 0.006$, Fig. 2.2B - % centre time: $F(1,44) = 5.5$, $p = 0.024$, Fig. 2.2D] indicating that tunnel-handled animals displayed reduced aversion of the centre zone compared to tail-handled mice. Supporting these findings, in the EPM, two-way ANOVA found that tunnel-handled animals travelled a higher percentage [$F(1,44) = 8.86$, $p = 0.005$; Fig. 2.2E] and spent a higher percentage of time [$F(1,44) = 6.96$, $p = 0.01$; Fig. 2.2F] on the open arms compared to tail-handled mice overall. Sex did not affect these EPM behaviours (no overall effect of 'Sex' and no 'Sex' by 'Handling' interactions: all p'$s \geq 0.146$).

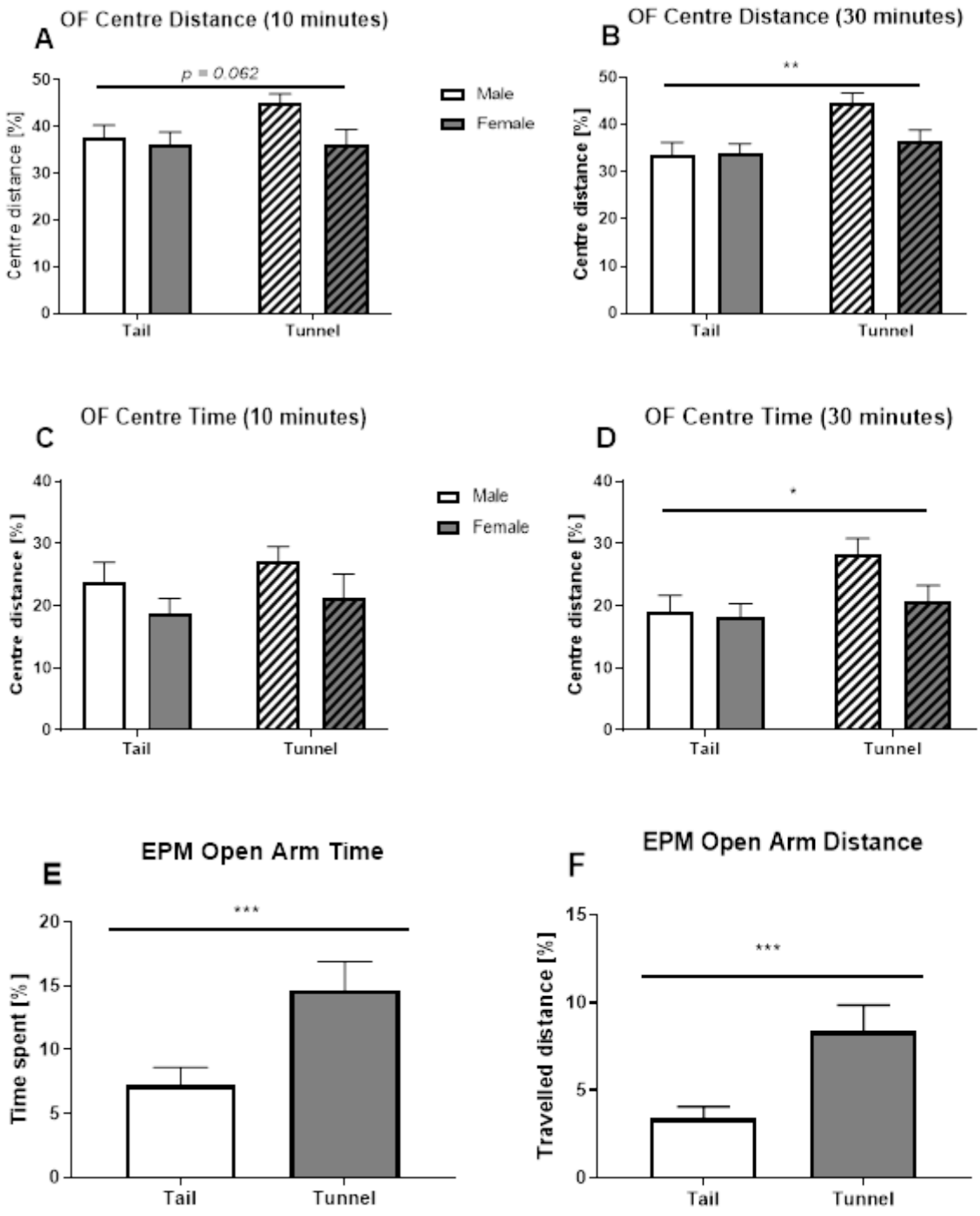

Figure 2.2A-F: Anxiety behaviours in the open field (OF) and the elevated plus maze (EPM): Percentage distance travelled for male and female mice as well as percentage time spent [%] in the OF centre at **(A/C)** 10 minutes and **(B/D)** 30 minutes, percentage distance travelled on the open arms [%] (combined for males and females as no 'Sex' by 'Handling' interactions were detected) at **(E)** 10 minutes and **(F)** 30 minutes. Data are shown for tail-handled (tail) and tunnel-handled (tunnel) C57BL/6Tac mice as mean ± SEM. Main handling effects are shown as '*' (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

2.3.3 Sociability testing:

Sociability test:

All mice spent more time in a chamber with a mouse compared to an empty chamber [three-way RM ANOVA: $F(1,44) = 52.7$, $p < 0.001$]. There were no main effects of 'Sex' or 'Handling' for total time in mouse chamber and no interactions with 'Chamber' (all $p's > 0.291$). In line with this, two-way ANOVA revealed no main effects or interactions of 'Sex' or 'Handling' for percentage time spent in the mouse chamber (all $p's > 0.237$ Fig. 2.3A). Finally, single sample t-tests against chance levels for percentage time in the mouse chamber confirmed that all mice developed a preference for a mouse over an empty chamber [Male: Tail: $t(13) = 3.87$; $p = 0.02$ - Tunnel: $t(14) = 5.6$; $p < 0.001$; Female: Tail: $t(10) = 2.62$; $p = 0.03$ - Tunnel: $t(10) = 3.822$; $p = 0.004$, Fig. 2.3A].

Social recognition memory:

All mice spent more time *nosing* the novel mouse [$F(1,44) = 25.1$, $p < 0.001$]. No main effects of 'Sex' or 'Handling' or interactions with 'Chamber' were detected for total time *nosing* the novel mouse (all $p's > 0.187$, Fig. 2.3B). Two-way ANOVA revealed no main effects of 'Sex' or 'Handling' nor an interaction for percentage time *nosing* a novel mouse either (all $p's > 0.27$). Interestingly, single sample t-tests against chance levels for percentage time *nosing* the novel mouse revealed that all male mice developed a preference for the novel mouse [Tail: $t(13) = 3.05$; $p = 0.01$ - Tunnel: $t(14) = 3.1$; $p = 0.009$; Fig. 2.3B] whereas in females, only tail-handled mice tended to develop a preference for the novel mouse [Tail: $t(9) = 2.1$; $p = 0.07$ - Tunnel: $t(10) = 0.83$; $p = 0.42$; Fig. 2.3B].

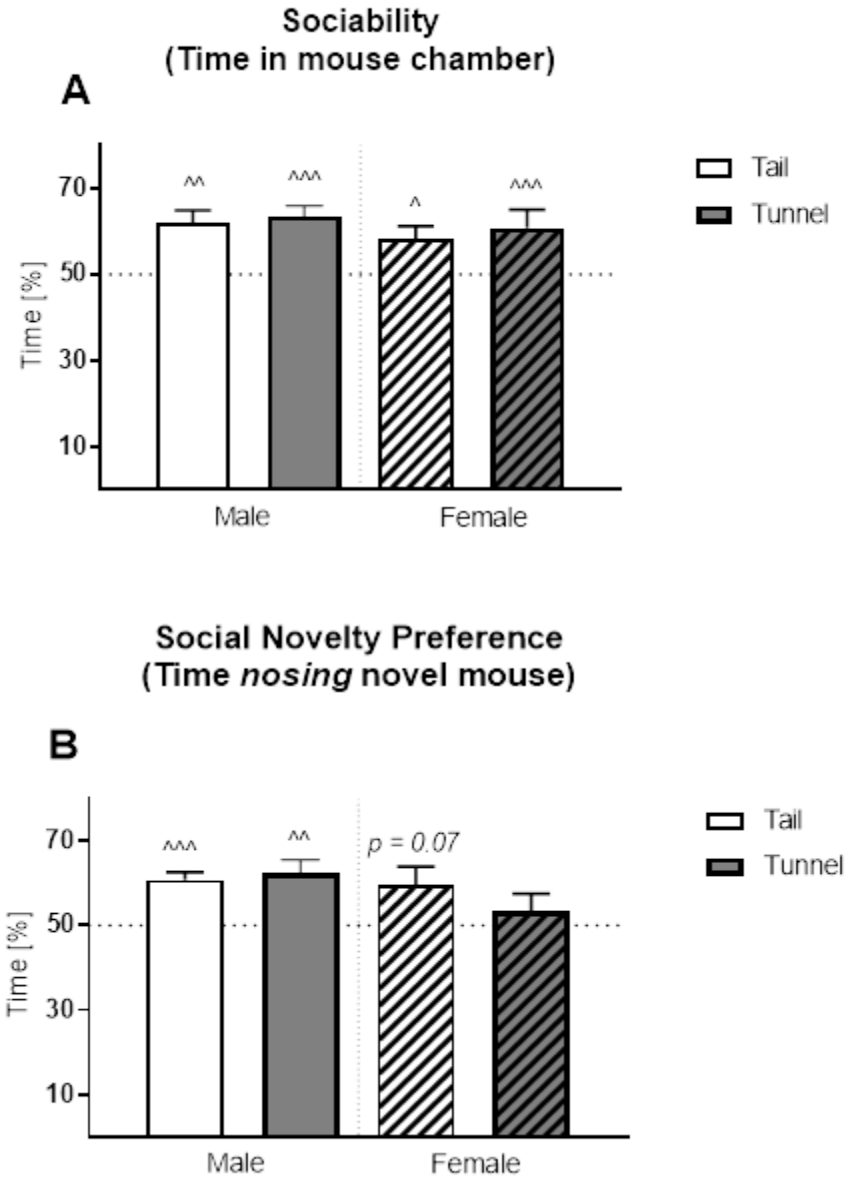

Figure 2.3A-B: Sociability and social recognition memory in the Social Preference Test (SPT): (A) Percentage time [%] spent with a mouse over empty chamber (i.e. sociability) and **(B)** percentage time [%] nosing a novel mouse over a familiar mouse (i.e. social novelty preference/memory). Data are shown for tail-handled (tail) and tunnel-handled (tunnel) C57BL/6Tac mice separated by sex as sex effects are often evident in this test paradigm [16]. Single sample t-test results versus 50% chance levels are shown as '^' (^^p < 0.01, ^^^p <0.001). A weak trend for social novelty preference is indicated by p = 0.07 (**Fig. 3B**).

2.3.4 Fear-associated memory:

Two-way ANOVA found no differences in baseline levels of *freezing* in the first 2 min of the conditioning session between sex or handling methods used (all p's > 0.08; Table 2.3). Additionally, three-way RM ANOVA across 1-min blocks during conditioning showed an increased *freezing* response to consecutive foot shocks in all mice ['Time': $F(6,264) = 55.8$, $p < 0.001$] and no effects or interactions of 'Sex' or 'Handling' with 'time' were detected (all p's > 0.187; data not shown).

In the context test, three-way RM ANOVA across 1-min blocks revealed no differences in contextual *freezing* between sex or handling methods (no main effects) and no interactions thereof or with 'Time' were detected either (all p's > 0.251; Fig. 2.4A). In the cue test, three-way RM ANOVA revealed that male mice exhibited lower cue *freezing* across the test period compared to females [main 'Sex' effect: $F(1,44) = 4.12$, $p = 0.049$; Fig. 2.4B/C]. In addition, a strong trend for a 'time' by 'Sex' by 'Handling' interaction for time spent *freezing* across 1-min blocks was observed [$F(8,352) = 2.26$, $p = 0.05$]. Thus, data were split by sex, and a trend for a 'Time' by 'Handling' interaction [$F(8,200) = 2.22$, $p = 0.068$] was evident in male mice (Fig. 4B). *Freezing* at cue presentation appeared to be initially higher in tunnel-handled males and then diminished more rapidly once the cue presentation had finished compared to tail-handled littermates (Fig. 2.4C).

Analysing the *freezing* response to the cue (i.e. comparing average *freezing* pre-cue with average *freezing* during cue presentation) revealed that all mice showed a significant response to the cue regardless of experimental condition [$F(1,44) = 256.87$, $p < 0.001$; Fig. 2.4D]. No main effects of 'Sex' or 'Handling' or interactions with 'Cue' were detected (all p's > 0.094).

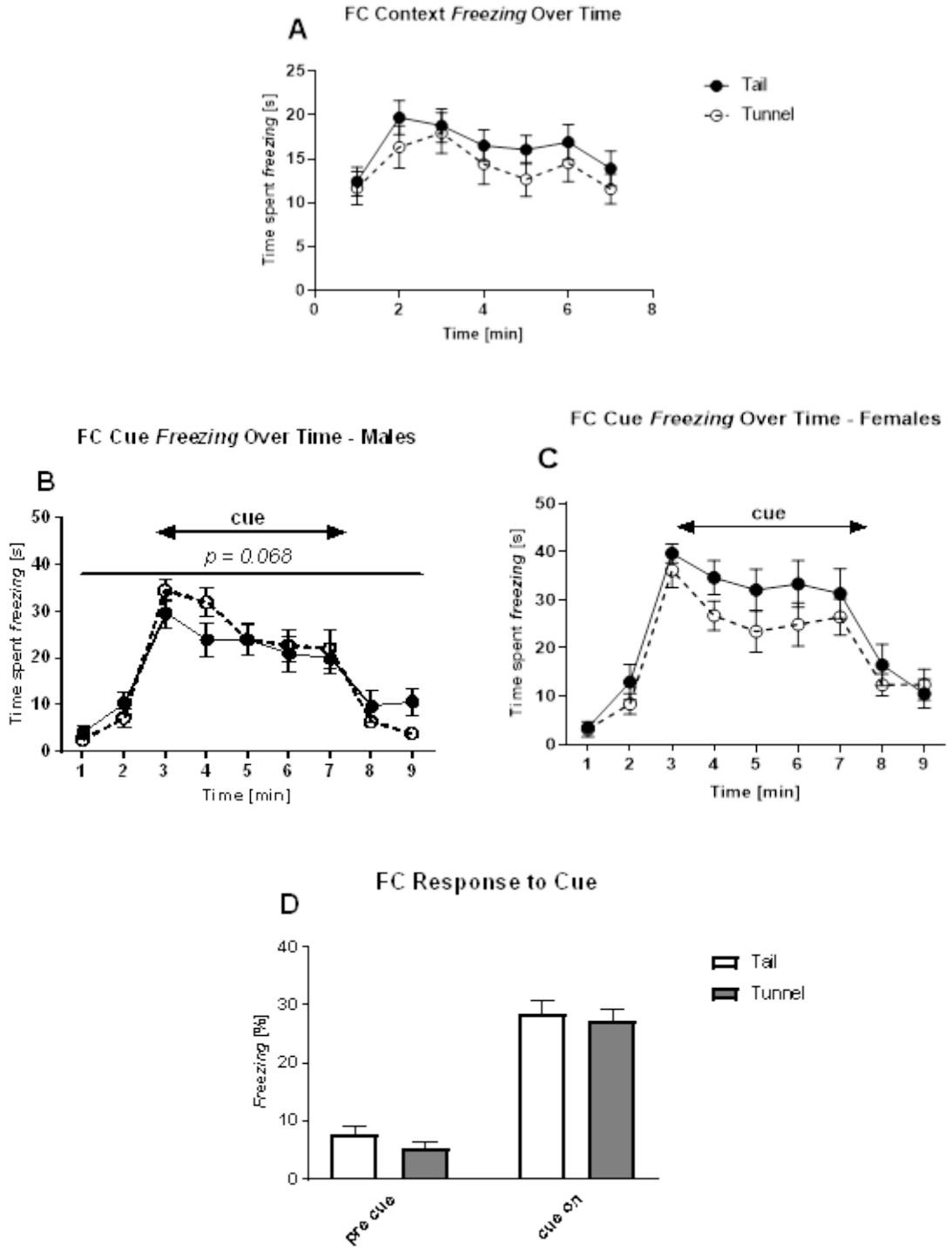

Figure 2. 4A-F: Fear-associated memory in the fear conditioning (FC) test: (A) Time spent freezing [s] per 1-min block during context testing, **(B,C)** Time spent freezing [s] per 1-min block during cue testing (data separated for male **(B)** and female **(C)** mice], and **(D)** average freezing prior to and during cue presentation. Data are shown for tail-handled (tail) and tunnel-handled (tunnel) female C57BL/6Tac mice. Data are presented as mean ± SEM. A strong trend for interaction of 'Time' 'Sex' and 'Handling' ($p = 0.05$) for time spent freezing across 1-min blocks in the cue was also detected. A trend for interaction of 'time' and 'Handling' ($p = 0.068$) for time spent freezing across 1-min blocks in the cue was seen for male mice

2.3.5 Sensorimotor gating

Acoustic startle response (ASR): All mice responded to increasing acoustic startle pulse intensities with increasing ASRs [three-way RM ANOVA 'pulse': [$F(2,88) = 78.03$, $p < 0.001$; Fig. 2.5A]. An interaction of 'Pulse' and 'Handling' [$F(2,88) = 6.37$, $p = 0.007$] suggested that tunnel-handled mice had a reduced response to increasing startle pulse intensities compared to tail-handled mice. Split by 'Pulse', this handling-dependent reduction was most evident at 120dB [$F(1,44) = 5.50$, $p = 0.024$. Split by handling, two-way RM ANOVA revealed a main effect of 'Sex' [$F(1,23) = 6.6$, $p = 0.017$] in tunnel-handled mice as male mice exhibited an overall stronger startle response compared to female mice. This sex effect was not present in tail-handled animals [$F(2,42) = 0.34$, $p > 0.621$]. However, both tail- [$F(2,42) = 35.54$, $p < 0.001$] and tunnel-handled [$F(2,46) = 75.9$, $p < 0.001$] groups exhibited increasing startle response with increasing ASR dB levels indicating intact startle response in all mice (no 'Pulse' by 'Sex' interaction for either handling condition, $p > .05$).

Prepulse inhibition (PPI): Three-way RM ANOVA revealed that all mice exhibited increasing PPI with increasing prepulse intensities ['prepulse': $F(2,88) = 364.6$, $p < 0.001$]. Furthermore, an interaction of 'Prepulse' and 'Sex' [$F(2,88) = 3.82$, $p = 0.04$] was seen as male mice exhibited a larger increase in PPI across increasing prepulse intensities compared to female mice (Fig. 5B). In addition, a trend for a 'Prepulse' by 'Handling' interaction was also observed [$F(2,88) = 3.1$, $p = 0.07$] as tunnel-handled animals tended to have a lower increase in PPI compared to tail-handled animals (Fig. 5B). Split by 'Sex', both male ['Prepulse': $F(2,50) = 264.8$, $p < 0.001$] and female ['Prepulse': $F(2,38) = 123.5$, $p < 0.001$] animals showed an increase in %PPI with increasing prepulse intensities. No handling effects or interactions with 'Prepulse' were observed in male mice (all p's > 0.43). Tunnel-handled females tended to exhibit a lower increase in PPI with

elevating prepulse intensities compared to tail-handled littermates [trend for 'Prepulse' by 'Handling' interaction: $F(2,38) = 3.6$, $p = 0.056$]. No effects or interactions were seen when assessing PPI averaged across prepulse intensities (all p's > 0.311, Fig. 2.5B).

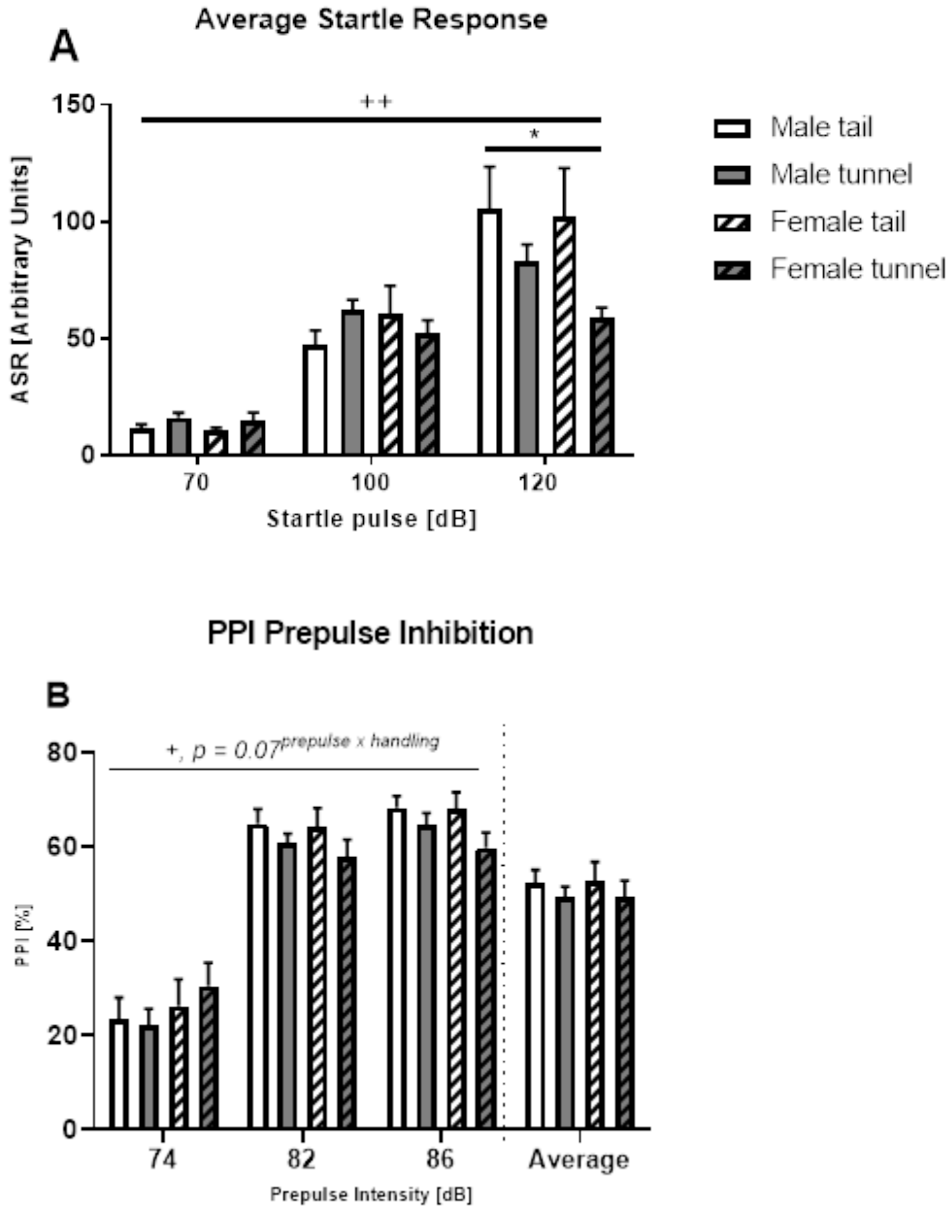

Figure 2. 5A-B: Acoustic startle response (ASR) and sensorimotor gating behaviours in the prepulse inhibition test (PPI): (A) Average startle response across startle intensities, and **(B)** prepulse inhibition per prepulse intensity and averaged across intensities. Data are shown for tail-handled (tail) and tunnel-handled (tunnel) C57BL/6Tac mice. Data are presented as mean ± SEM (PPI data separated for sex). A significant interaction of 'pulse' and 'Handling' for startle response across intensities is indicated by '+' ($^{++}p < 0.01$) as tunnel-handled animals had a lower increase in startle response compared to tail-handled animals. A main effect of 'Sex' was also evident in tunnel handled animals as male mice had increased startle response compared to female mice ($p = 0.017$). An interaction of 'Prepulse' and 'Sex' ($p = 0.04$) for %PPI over prepulse intensities is indicated by '+' ($^{+}p < 0.05$). A trend for 'Prepulse' by 'Handling' interaction is indicated by $p = 0.07$ as tunnel-handled mice tended to show a lower increase in PPI over prepulse intensities. Additionally a trend for 'prepulse' by 'Handling' interaction in female mice ($p = 0.056$) was seen as tunnel-handled mice tended to show a lower increase in PPI over prepulse intensities.

2.4. Discussion

This experiment investigated the impact of two different handling methods on the behaviour of C57BL/6 mice whilst also considering sex differences in our new mouse phenotyping facility at Western Sydney University. Tunnel handling increased locomotion speed (first 10 minutes of OF testing), explorative behaviours more generally, and also decreased spatio-temporal anxiety behaviours. Furthermore, tunnel handling exhibited a moderate effect on the time course of cue *freezing* in male mice attenuated the startle response to increasing startle pulse intensities. Finally, tunnel-handled females tended to show a reduced PPI response to increasing prepulse intensities compared to tail-handled animals. Compared to female mice, males regardless of handling condition exhibiting reduced OF locomotion, increased OF exploration, increased locomotion in the more aversive OF centre, lower levels of *cue* freezing as well as a more robust social novelty preference and a stronger PPI response to increasing prepulse intensities.

Tunnel handling had no effect on total locomotive distance but increased exploratory behaviours and locomotion speed. Previous studies also found an increase in exploratory behaviours in the habituation-dishabituation test of odour discrimination whereas tail-handled mice showed little motivation to explore the odour stimuli [8]. However, it should be noted here that the explorative parameter investigated in the earlier study was '*sniffing*'. Effects of handling on *rearing* behaviour have not been considered in any detail in previous work in the field, indicating that the findings in this study are novel. The observed sexually dimorphic effect of tunnel handling on *rearing* in the EPM may be a result of how male and female rodents perceive the respective test paradigm. For example, activity is the most important factor for female Wistar rats when being tested in the EPM, whereas males appear predominantly driven by their anxiety response during EPM testing [17]. Thus, tunnel handling may have reduced anxiety and thereby stimulated exploration in male C57BL/6J mice whereas this anti-anxiety effect may be less important for explorative tendencies of female

mice. Tunnel handling has previously been seen to increase locomotor activity in male C57BL/6 mice compared to tail-handled controls [10]. This increase was not seen in the current study, although locomotor velocity was increased by tunnel handling in both the current as well as the aforementioned study. Females had not been tested in this earlier work, prompting our investigation into potential sex effects.

The finding of this study that tunnel handling reduced anxiety-like responses across behavioural paradigms and regardless of sex confirms the initial work by Hurst and colleagues [7-9,11]. The results are in line with the idea that mice perceive tail handling as a more stressful, aversive handling method – potentially somewhat similar to a predatory attack [7]. Supporting this idea, hyperthermia [18], increased corticosterone levels [19,20], and immunosuppression [21] are often seen after routine laboratory procedures [22] including tail handling or moving the home cage [23,24]. Furthermore, tail handling (compared to tunnel handling) has been found to elicit anhedonia in male C57BL/6J mice [10], a behavioural response similar to the one of mice which are exposed to unexpected chronic mild stress [25,26] or restraint stress [27].

In the social preference test, all mice exhibited intact sociability and male mice regardless of handling method also developed a preference for a novel mouse. However, tail-handled females only tended to show a preference for the novel mouse which was not present in female handled animals. Interestingly, two different stress paradigms, those being chronic variable stress and unexpected chronic mild stress, have shown an increase in sociability in stressed compared to unstressed female C57BL/6NCrl [28] and CD1 [29] mice respectively and is potentially due to the effect of 'social buffering' in which socialisation reduces anxiety and lowers activation of the hypothalamic–pituitary–adrenocortical axis [30]. Furthermore, Sex-specific differences in the social preference test have been found in previous studies [31] and are likely due to social hierarchies being

more competitive in male mice but based on unknown intrinsic qualities in female mice, indicating that females may not spend more time with animals simply because they are novel [22,32,33].

Tunnel handling did not impact on contextual fear-associated memory. However, this is an interesting finding as lower levels of corticosterone, which have been observed in tunnel-handled animals, in adrenalectomized Long-Evans hooded rats led to impaired contextual fear memory [34]. However, this is speculative as further investigation into corticosterone levels in tunnel handled mice pre and post fear conditioning should be investigated. With regard to this finding, the current test protocol included an olfactory cue during conditioning and context. Importantly, the rodent olfactory system is directly linked to the cortico-amygdaloid group [35] and odors used as an additional conditioning stimulus results in highly salient fear-associated memory in mice [36]. Thus, the vanilla scent present in the current protocol may have acted as a secondary conditioned stimulus and thereby interfered with the test outcomes in the context test.

Tunnel handling modulated the cue association in male mice with tunnel-handled males displaying an initially higher response to the cue presentation which diminished more rapidly than their tail-handled counterparts post cue presentation. This novel finding is likely due to lower levels of stress and stress hormones seen in the tunnel-handled animals [24] as it has previously been shown that stressful experiences (i.e. 30 min acute restraint stress) following the conditioning of fear can increase the consolidation of fear memory and dendritic spine density in the amygdala [37]. In addition, brief forced swim stress 24 hours prior to testing impaired fear extinction in male C57BL/6J mice compared to non-stressed controls [38]. However, it is important to mention there that assessment of the average response to the cue (i.e. percentage *freezing* prior and during cue presentation) suggests that the initial association with the cue is intact in all experimental test groups.

All mice responded to increasing startle stimulus intensities with increasing ASR but this response was attenuated in tunnel-handled mice. Interestingly, there is a positive correlation between stress hormone levels such as corticotropin and elevated ASR [39] indicating that less stressful pre-test handling lowered the startle response in tunnel handled mice due to lower levels of stress hormone release [23]. Tunnel-handled male mice showed a higher overall startle response compared to tunnel-handled female mice averaged across the startle intensities.

All mice exhibited intact PPI, however, tunnel-handled mice and in particular tunnel-handled females showed a reduced elevation in PPI across increasing prepulse intensities. This is a novel finding as the effect of handling on sensorimotor gating has not previously been assessed. Interestingly, another environmental modification, home cage environmental enrichment has also been seen to reduce the PPI response in both male and female Sprague-Dawley rats [40] as well as C57BL/6J mice [41]. However, this effect was thought to be mediated by a combination of long exposure to the enriched environment (12 weeks) post-weaning when compared to similar studies in which rats were only exposed to EE for 2 [42] or 8 [43] weeks, the age at which animals were first exposed, and the age at which they were tested. Notably, dopamine is seen to be a key modulator of sensorimotor gating in the brain [44] and disruption of which interferes with prepulse inhibition. Moreover, previous studies have seen reductions in dopaminergic neurons and thereby lower expression of dopamine transporters in the striatum of environmentally-enriched animals [45-47] indicating that laboratory conditions can induce PPI-relevant physiological changes which may ultimately lead to disrupted prepulse inhibition.

Finally, it was found in this experiment that, irrespective of the method of handling used, male mice showed increased exploratory behaviours and lower levels of anxiety in the open field compared to female mice. Similar sex differences in open field anxiety have previously been found

for C57BL/6 mice [48]. Furthermore, sexually dimorphic responses to fear conditioning paradigms are evident in both rats [49] and mice [50] with males exhibiting higher levels of *freezing* than their female counterparts, similar to what has been observed in this study. The study also detected a stronger PPI response to increasing prepulse intensities in male mice, a phenomenon attributed to the effects of sex hormones and the stage of the estrous cycle at which female animals are tested [51]. The finding is important as some previous work confirms this sex effect in Wistar rats [52] whereas other studies, for example in Sprague Dawley rats, did not observe such a difference [53].

Overall, handling method appears to constitute an important environmental factor which can modulate a variety of behavioural domains including locomotion, exploration, anxiety, socability and the acoustic startle response in a sex-dependent manner. Presently, one of the main detractions from the implementation of less aversive handling methods, including tunnel handling, is the time necessary to training animal staff and the animals themselves. However, in a recent paper by Gouveia et al., it has been reported that a 2-second-exposure is sufficient to habituate mice to tunnel handling [54]. Therefore, when assessing anxiety and stress-related behaviours, it may be prudent to use less aversive handling methods to avoid confounding any e.g. stress-related responses of animals, which include behavioural but also physiological or immunological parameters. The data also suggest that handling methods can have implications for the validity of mouse models which are characterized by small effect sizes.

2.5. References

1. Wurbel H. Ideal homes? Housing effects on rodent brain and behaviour. *Trends Neurosci.* 2001;24(4):207-211.
2. Tsai P-P, Pachowsky U, Stelzer H, Hackbarth H. Impact of environmental enrichment in mice. 1: effect of housing conditions on body weight, organ weights and haematology in different strains. *Laboratory animals.* 2002;36(4):411-419.
3. Wahlsten D, Bachmanov A, Finn DA, Crabbe JC. Stability of inbred mouse strain differences in behavior and brain size between laboratories and across decades. *Proceedings of the national academy of sciences.* 2006;103(44):16364-16369.
4. Laviola G, Hannan AJ, Macri S, Solinas M, Jaber M. Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. *Neurobiol Dis.* 2008;31(2):159-168.
5. Burrows EL, McOmish CE, Hannan AJ. Gene-environment interactions and construct validity in preclinical models of psychiatric disorders. *Prog Neuropsychopharmacol Biol Psychiatry.* 2011;35(6):1376-1382.
6. Logge W, Kingham J, Karl T. Do individually ventilated cage systems generate a problem for genetic mouse model research? *Genes, Brain and Behavior.* 2014;13(7):713-720.
7. Hurst JL, West RS. Taming anxiety in laboratory mice. *Nature methods.* 2010;7(10):825-826.
8. Gouveia K, Hurst JL. Optimising reliability of mouse performance in behavioural testing: the major role of non-aversive handling. *Scientific reports.* 2017;7:44999.
9. Gouveia K, Hurst JL. Reducing mouse anxiety during handling: effect of experience with handling tunnels. *PloS one.* 2013;8(6):e66401.

10. Clarkson JM, Dwyer DM, Flecknell PA, Leach MC, Rowe C. Handling method alters the hedonic value of reward in laboratory mice. *Scientific reports.* 2018;8(1):2448.

11. Ghosal S, Nunley A, Mahbod P, et al. Mouse handling limits the impact of stress on metabolic endpoints. *Physiol Behav.* 2015;150:31-37.

12. NAKAMURA Y, SUZUKI K. Tunnel use facilitates handling of ICR mice and decreases experimental variation. *Journal of Veterinary Medical Science.* 2018;80(6):886-892.

13. Olaya JC, Heusner CL, Matsumoto M, et al. Overexpression of Neuregulin 1 Type III Confers Hippocampal mRNA Alterations and Schizophrenia-Like Behaviors in Mice. *Schizophrenia Bulletin.* 2017.

14. Olaya JC, Heusner CL, Matsumoto M, Shannon Weickert C, Karl T. Schizophrenia-relevant behaviours of female mice overexpressing neuregulin 1 type III. *Behav Brain Res.* 2018;353:227-235.

15. Karl T, Burne TH, Van den Buuse M, Chesworth R. Do transmembrane domain neuregulin 1 mutant mice exhibit a reliable sensorimotor gating deficit? *Behav Brain Res.* 2011;223(2):336-341.

16. Cheng D, Low JK, Logge W, Garner B, Karl T. Novel behavioural characteristics of female APPSwe/PS1DeltaE9 double transgenic mice. *Behav Brain Res.* 2014;260:111-118.

17. Fernandes C, Gonzalez M, Wilson C, File SE. Factor analysis shows that female rat behaviour is characterized primarily by activity, male rats are driven by sex and anxiety. *Pharmacology Biochemistry and Behavior.* 1999;64(4):731-736.

18. Zethof TJ, Van der Heyden JA, Tolboom JT, Olivier B. Stress-induced hyperthermia in mice: a methodological study. *Physiol Behav.* 1994;55(1):109-115.

19. Bowers SL, Bilbo SD, Dhabhar FS, Nelson RJ. Stressor-specific alterations in corticosterone and immune responses in mice. *Brain Behav Immun.* 2008;22(1):105-113.

20. Tabata H, Kitamura T, Nagamatsu N. Comparison of effects of restraint, cage transportation, anaesthesia and repeated bleeding on plasma glucose levels between mice and rats. *Laboratory animals.* 1998;32(2):143-148.

21. Moynihan J, Brenner G, Koota D, Breneman S, Conen N, Ader R. The effects of handling on antibody production, mitogen responses, spleen cell number, and lymphocyte subpopulations. *Life Sci.* 1990;46(26):1937-1944.

22. van den Berg WE, Lamballais S, Kushner SA. Sex-specific mechanism of social hierarchy in mice. *Neuropsychopharmacology.* 2015;40(6):1364-1372.

23. Balcombe JP, Barnard ND, Sandusky C. Laboratory routines cause animal stress. *Contemp Top Lab Anim Sci.* 2004;43(6):42-51.

24. Ono M, Sasaki H, Nagasaki K, et al. Does the routine handling affect the phenotype of disease model mice? *Japanese Journal of Veterinary Research.* 2016;64(4):265-271.

25. Willner P, Muscat R, Papp M. Chronic mild stress-induced anhedonia: a realistic animal model of depression. *Neurosci Biobehav Rev.* 1992;16(4):525-534.

26. Muscat R, Willner P. Suppression of sucrose drinking by chronic mild unpredictable stress: a methodological analysis. *Neurosci Biobehav Rev.* 1992;16(4):507-517.

27. Chiba S, Numakawa T, Ninomiya M, Richards MC, Wakabayashi C, Kunugi H. Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Prog Neuropsychopharmacol Biol Psychiatry.* 2012;39(1):112-119.

28. Borrow AP, Bales NJ, Stover SA, Handa RJ. Chronic Variable Stress Induces Sex-Specific Alterations in Social Behavior and Neuropeptide Expression in the Mouse. *Endocrinology.* 2018;159(7):2803-2814.

29. Dadomo H, Gioiosa L, Cigalotti J, Ceresini G, Parmigiani S, Palanza P. What is stressful for females? Differential effects of unpredictable environmental or social stress in CD1 female mice. *Hormones and behavior.* 2018;98:22-32.

30. Hostinar CE, Sullivan RM, Gunnar MR. Psychobiological mechanisms underlying the social buffering of the hypothalamic-pituitary-adrenocortical axis: a review of animal models and human studies across development. *Psychol Bull.* 2014;140(1):256-282.

31. Olaya JC, Heusner CL, Matsumoto M, Weickert CS, Karl T. Schizophrenia-relevant behaviours of female mice overexpressing neuregulin 1 type III. *Behavioural brain research.* 2018.

32. Choleris E, Kavaliers M. Social learning in animals: sex differences and neurobiological analysis. *Pharmacol Biochem Behav.* 1999;64(4):767-776.

33. Haller J, Fuchs E, Halász J, Makara GB. Defeat is a major stressor in males while social instability is stressful mainly in females: towards the development of a social stress model in female rats. *Brain research bulletin.* 1999;50(1):33-39.

34. Pugh CR, Tremblay D, Fleshner M, Rudy JW. A selective role for corticosterone in contextual-fear conditioning. *Behav Neurosci.* 1997;111(3):503-511.

35. Price JL. An autoradiographic study of complementary laminar patterns of termination of afferent fibers to the olfactory cortex. *J Comp Neurol.* 1973;150(1):87-108.

36. Jones SV, Heldt SA, Davis M, Ressler KJ. Olfactory-mediated fear conditioning in mice: simultaneous measurements of fear-potentiated startle and freezing. *Behav Neurosci.* 2005;119(1):329-335.

37. Giachero M, Calfa GD, Molina VA. Hippocampal structural plasticity accompanies the resulting contextual fear memory following stress and fear conditioning. *Learn Mem.* 2013;20(11):611-616.

38. Izquierdo A, Wellman CL, Holmes A. Brief uncontrollable stress causes dendritic retraction in infralimbic cortex and resistance to fear extinction in mice. *J Neurosci.* 2006;26(21):5733-5738.

39. Lee Y, Schulkin J, Davis M. Effect of corticosterone on the enhancement of the acoustic startle reflex by corticotropin releasing factor (CRF). *Brain Res.* 1994;666(1):93-98.

40. Pena Y, Prunell M, Rotllant D, Armario A, Escorihuela RM. Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats. *Psychoneuroendocrinology.* 2009;34(9):1390-1404.

41. Hendershott TR, Cronin ME, Langella S, McGuinness PS, Basu AC. Effects of environmental enrichment on anxiety-like behavior, sociability, sensory gating, and spatial learning in male and female C57BL/6J mice. *Behav Brain Res.* 2016;314:215-225.

42. Varty GB, Paulus MP, Braff DL, Geyer MA. Environmental enrichment and isolation rearing in the rat: effects on locomotor behavior and startle response plasticity. *Biological psychiatry.* 2000;47(10):864-873.

43. Schneider T, Turczak J, Przewłocki R. Environmental enrichment reverses behavioral alterations in rats prenatally exposed to valproic acid: issues for a therapeutic approach in autism. *Neuropsychopharmacology.* 2006;31(1):36-46.

44. Zhang J, Forkstam C, Engel JA, Svensson L. Role of dopamine in prepulse inhibition of acoustic startle. *Psychopharmacology.* 2000;149(2):181-188.

45. Del Arco A, Segovia G, Canales JJ, et al. Environmental enrichment reduces the function of D1 dopamine receptors in the prefrontal cortex of the rat. *J Neural Transm (Vienna).* 2007;114(1):43-48.

46. Segovia G, Del Arco A, de Blas M, Garrido P, Mora F. Effects of an enriched environment on the release of dopamine in the prefrontal cortex produced by stress and on working memory during aging in the awake rat. *Behav Brain Res.* 2008;187(2):304-311.

47. Bezard E, Dovero S, Belin D, et al. Enriched environment confers resistance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and cocaine: involvement of dopamine transporter and trophic factors. *J Neurosci.* 2003;23(35):10999-11007.

48. An XL, Zou JX, Wu RY, et al. Strain and sex differences in anxiety-like and social behaviors in C57BL/6J and BALB/cJ mice. *Exp Anim.* 2011;60(2):111-123.

49. Fanselow MS, Landeira-Fernandez J, DeCola JP, Kim JJ. The immediate-shock deficit and postshock analgesia: Implications for the relationship between the analgesic CR and UR. *Animal Learning & Behavior.* 1994;22(1):72-76.

50. Wiltgen BJ, Sanders MJ, Behne NS, Fanselow MS. Sex differences, context preexposure, and the immediate shock deficit in Pavlovian context conditioning with mice. *Behav Neurosci.* 2001;115(1):26-32.

51. Koch M. Sensorimotor gating changes across the estrous cycle in female rats. *Physiol Behav.* 1998;64(5):625-628.

52. Lehmann J, Pryce CR, Feldon J. Sex differences in the acoustic startle response and prepulse inhibition in Wistar rats. *Behav Brain Res.* 1999;104(1-2):113-117.

53. Swerdlow NR, Auerbach P, Monroe SM, Hartston H, Geyer MA, Braff DL. Men are more inhibited than women by weak prepulses. *Biological psychiatry.* 1993;34(4):253-260.

54. Gouveia K, Hurst JL. Improving the practicality of using non-aversive handling methods to reduce background stress and anxiety in laboratory mice. *Scientific Reports.* 2019;9(1):1-19.

2.8. Tables

Handling	Tail		Tunnel	
	Male (n = 13)	Female (n = 10)	Male (n = 14)	Female (n = 11)
Start Handling	27 ± 11	25 ± 10	27 ± 11	27 ± 8
Open Field	28 ± 11	27 ± 10	29 ± 11	29 ± 8
EPM	28 ± 11	27 ± 10	29 ± 11	29 ± 8
Social Preference	29 ± 11	28 ± 10	29 ± 11	29 ± 8
Fear Conditioning	29 ± 11	28 ± 10	30 ± 11	30 ± 8
Prepulse Inhibition	30 ± 11	29 ± 10	31 ± 11	30 ± 8

Table 2.1: Test biography and test age [weeks] of male and female C57BL/6Tac animals. Ages are presented as average weeks ± days. Sample sizes are indicated.

Handling	Tail	Tunnel
OF		
Velocity [cm/s]		
10 minutes	15.42± 0.18	16.34 ± 0.34*
30 minutes	15.94 ± 0.20	16.47 ± 0.32
EPM		
Total arm entries [n]	20.67 ± 1.12	22.91 ± 1.1

Table 2.2: Locomotion in the open field (OF) and elevated plus maze (EPM). Data are presented as mean ± SEM for C57BL/6Tac mice. Significant main effects of tunnel handling compared to tail handling are shown by '*' *($^{*}p < 0.05$)*

Handling	Tail		Tunnel	
	Male	Female	Male	Female
Fear Conditioning				
Baseline *freezing [s]*	0.92 ± 0.48	0.4 ± 0.27	0.724 ± 0.32	0 ± 0

Table 2.3: Average baseline freezing during first two minutes of FC conditioning trial for all mice. Data are presented as mean ± SEM for C57BL/6Tac mice.

Note: The following chapter is identical to the work titled:

Behavioural effects of cage systems on the *G93A Superoxide Dismutase 1* transgenic mouse model for Amyotrophic Lateral Sclerosis

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Declaration

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Chapter 3: Behavioural effects of cage systems on the *G93A Superoxide Dismutase 1* transgenic mouse model for Amyotrophic Lateral Sclerosis

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Abstract

Environmental factors inherent to animal facilities can impact on the neuro-behavioural phenotype of laboratory mice and genetic mouse models for human diseases. Many facilities have upgraded from traditional 'open filter top' cages (FT) to individually ventilated cage (IVC) systems, which have been shown to modify various behavioural responses of laboratory mice. Importantly, the impact of IVC housing on the *G93A superoxide dismutase 1* mouse model of amyotrophic lateral sclerosis (ALS) is currently unknown. Male and female wild type-like (WT) and heterozygous *SOD1*^{G93A} mice were group-housed in FT or IVC systems from PND 30±5 onwards. Body weight and motor function were assessed weekly from 15 weeks onward. Mice were also tested for cognitive abilities (i.e. fear conditioning and social recognition memory) and sensorimotor gating (i.e. prepulse inhibition: PPI). *SOD1*^{G93A} mice lost body weight, and their motor function degenerated over time compared to control littermates. Motor impairments developed faster when *SOD1*^{G93A} females were housed in IVCs. Context and cue *freezing* were increased in *SOD1*^{G93A} females compared to controls, whereas all *SOD1*^{G93A} mice exhibited lower acoustic startle and PPI than WT mice. IVC housing led to an increase in cue *freezing* in males and reduced the severity of PPI deficits in *SOD1*^{G93A} females. Overall, IVC housing impacted moderately on the *SOD1*^{G93A} phenotype. Still, central behavioural deficits were evident across housing conditions. Nonetheless, our findings indicate the importance of assessing the effect of cage system in genetic mouse models as these systems can modulate the magnitude and onset of genotypic differences.

3.1 Introduction

In the past few decades, there has been increasing interest in understanding the effects of environmental factors prevalent in animal facilities on the validity of genetic mouse models of human disease. We know that environmental enrichment [1], the gender of the investigator carrying out experiments [2] and standard procedures like routine handling [3-5] may impact on the neuro-behavioural phenotype of laboratory mice tested within one research facility, not to mention phenotypic differences observed when the same mouse model is evaluated across multiple test facilities [6].

Many facilities have upgraded facilities from traditional 'open top' or 'open filter top' cage systems (FT) to individually ventilated cage (IVC) systems. IVC housing has been introduced to reduce the costs of mouse model research, increase breeding productivity, reduce microbiological monitoring requirements, and increase work health and safety of staff in those holding facilities. Indeed, IVC cage systems allow for increased cage density of mice (i.e. more IVC holding cages fit into the same space compared to FT systems) [7] and time between cage changes (i.e. due to measurably lower levels of intra-cage ammonia) [8], reduce the chance of airborne infection transmission, and lower allergen levels in holding rooms [9,10]. However, the caveat to these improvements is the fact that IVCs provide a different housing environment than FT cage systems. IVC systems using active ventilation systems have increased airflow, which may lead to cold stress in animals [11], and can generate vibrations in the rack with presently unknown effects on mice [12,13]. Importantly, increased caged density in IVCs could potentially also lead to low-grade hypoxia [14]. Additionally, compared to FT systems, IVCs (both passive and active airflow systems) may decrease climbing opportunities as some systems use Perspex lids rather than wire lids [15] and

present a more stimulation-deprived environment (i.e. a form of isolation) as intercage exchange of olfactory and auditory cues is very limited or completely absent in IVCs [16].

Mice are born, raised, and held for their full life cycle in these cage systems so it is no stretch to hypothesize that these environmental differences may impact on the phenotype of mice held in these cages. Indeed, IVC housing can induce anxiety-like phenotypes, reduce body weight, impact on body temperature [17], exploration and social behaviours [13,15,17,18] and increase the sensitivity of mice to the locomotion-stimulating effects of the *N*-methyl-D-aspartate (NMDA) antagonist MK-801 [19]. Most important for preclinical research into human diseases is the finding that IVC systems can compromise well-characterized and established disease-relevant behavioural deficits in genetic rodent models, e.g. for the schizophrenia risk gene *neuregulin 1* [20] and the serotonin transporter (5-HTT) [21], as well as neurodevelopmental mouse model of schizophrenia [i.e. maternal immune activation (MIA) using polyI:C] [22].

Amyotrophic lateral sclerosis (ALS) is a rapidly progressing neurodegenerative disease [23]. The majority of ALS cases are sporadic (90-95%), the remaining 5-10% of ALS cases are inherited or familial (fALS). Accounting for approximately 20% of fALS, mutations in *copper-zinc superoxide dismutase 1* (*SOD1*) are one of the primary genetic causes of fALS (Parton et al [24], Turner et al [25]). Recent promising human clinical trials for the use of antisense oligonucleotide [26] and adenosine associated viral vector [27] therapies in *SOD1*-mutant ALS patients confirm the continuing relevance of the well-established $SOD1^{G93A}$ transgenic mouse model for research into ALS therapies. Unfortunately, inconsistent reporting (and consideration) of sex effects [6], age of onset/death [28], and a lack of studies evaluating the effect of holding facility conditions on this model has been evident. Importantly, as discussed above, these factors can confound experimental outcomes and thereby the validity of preclinical research models. This gap in knowledge is surprising as

environmental factors such as home cage enrichment [29] are known to accelerate the progression of disease-relevant parameters in *SOD1*^{G93A} transgenic mice [30] showing the sensitivity of this mouse model to environmental factors. Thus, we systematically compared the onset and severity of the behavioural phenotype of *SOD1*^{G93A} transgenic mice of both sexes kept in either FT or IVC housing from weaning onwards. Recent genetic evidence suggests that ALS and frontotemporal dementia overlap [31] and form an ALS- frontotemporal spectrum disorder, which includes motor and cognitive symptoms [31]. Indeed, our previous work discovered sex-specific sensory and spatial memory deficits in *SOD1*^{G93A} transgenic mice [31]. Thus, our behavioural evaluation utilised a more comprehensive behavioural evaluation strategy compared to most previous ALS mouse model studies.

3.2 Materials and Methods

3.2.1 Animals

Test animals were male and female heterozygous *G93A superoxide dismutase 1* transgenic mice [*SOD1*^{G93A}: B6-Tg(SOD1-G93A)1GUr/j; The Jackson Laboratory Stock Number: 004435] as well as wild type-like control littermates (n = 11-16). Mice were bred at the animal facility of the School of Medicine, Western Sydney University (Campbelltown, New South Wales, Australia). Breeding pairs were housed in IVC cages in a temperature (22-24°C), and humidity (50-70%) controlled room in the facility. Pups born were weaned at around postnatal days 18-21 and remained in IVC housing in the breeding room until the completion of genotyping, at which point they were moved to their dedicated cage system in the test animal holding room (further info under *2.2 Housing*). Adult A/JArc mice were purchased from the Animal Resources Centre (ARC: Cunning Vale, Australia) as age- and sex-matched conspecifics for the social preference test.

3.2.2 Housing

Once genotypes were confirmed (30±5 days of age), control and *SOD1*^{G93A} transgenic mice were group-housed (2-3 mice per cage) in a quasi-randomized manner in either FT (1144B: Techniplast, Rydalmere Australia) or IVC cage systems (GM500 Green, Techniplast Australia Pty Ltd, Rydalmere, Australia; system uses active airflow of 75 air exchanges per hour). The cage environment for both methods of housing included corn cob bedding (PuraCob Premium: Able Scientific, Perth, Australia) with tissues and 'crinkle nest' as nesting materials but no further enriching structures were provided. Food and water were provided *ad-libitum*. Mice were housed in their respective cage system within the same holding room until the end of the experiment.

3.2.3 Behavioural phenotyping

Mice were tested during the first five hours of the light phase: motor function testing started at 15 weeks of age, prepulse inhibition (PPI) and fear conditioning (FC) were carried out at 17 weeks of age and social preference testing (SPT) at 19 weeks of age (inter-test interval of at least 24 h). Animals were examined using a number of behavioural tests with relevance to ALS and FTD in line with a previous report from our lab [31]. Body weights were recorded weekly. At the completion of testing all test devices were thoroughly cleaned with 80% ethanol. A minimum inter-test-interval of 24 hours was used when mice were tested in different paradigms within one week, such as in the case of accelerod and pole test. Sample sizes for all test paradigms are as follows: male FT [WT(13), *SOD1*^{G93A}(11)], male IVC [WT(16), *SOD1*^{G93A}(14)], female FT [WT(14), *SOD1*^{G93A}(13)], female IVC [WT(11), *SOD1*^{G93A}(12)].

3.2.3.1 Accelerod

To assess motor coordination and across time in the ALS phenotype, mice were first trained on the rotarod apparatus (ENV-574M, MED Associates Inc., St Albans, VT, USA) at a fixed speed of 12 rotations per minute (RPM) for two minutes. This training was repeated a total of three times one day prior to the first test to ensure that the mice understood the task. On the test day, mice were placed on the rotarod apparatus at a starting speed of 4 RPM, after which an acceleration program of 4-40 RPM ran for 300 seconds (i.e. reaching the top speed at 270 seconds). The latency for mice to fall from the rod was recorded. The test was performed twice with a 1-hour inter-trial interval (ITI); the average run time from both trials was analyzed. The accelerod was run weekly from 15-21 weeks of age.

3.2.3.2 Pole test

The pole test was used to assess the degeneration of gross motor co-ordination, balance and motor function in line with our previous paper [32]. Mice were placed (facing upwards) on a vertical pole (diameter: 1 cm; length: 51 cm) and the latency to reach the platform was recorded. Mice were allowed 60 s to complete the test; animals not completing the test (i.e. were unable to turn or climb down or fell from the pole) were awarded the maximum test time. The test was performed twice with a 30-minute ITI; the average latency to reach the platform was analyzed. The pole test was run weekly from 15-21 weeks of age.

3.2.3.3 Sensorimotor gating – Prepulse inhibition (PPI)

Acoustic startle response (ASR) and sensorimotor gating, as indicated by the attenuation of the startle response by a non-startling stimulus [33], was carried out as follows: test mice were placed in Plexiglas mouse enclosures of the startle chambers (SR-Lab: San Diego Instruments, San Diego, USA) and allowed to habituate to the enclosure and test apparatus for 5 min over three consecutive days prior to PPI testing with a consistent background noise of 70 dB. The 30 min PPI test session consisted of a 5 min acclimation period to 70 dB background noise, followed by 97 trials presented in a pseudorandomised order: 5 × 70 dB trials; 5 × 100 dB trials; 15 × 120 dB trials to measure the acoustic startle response (ASR) and 15 sets of 5 trials comprising of a prepulse of either 74, 82 or 86 dB presented 32, 64, 128, or 256 ms (variable interstimulus interval: ISI) prior to a startle pulse of 120 dB to measure the PPI response. The intertrial interval (ITI) varied randomly from 10 to 20 s. Responses to each trial were calculated as the average mean amplitude detected by a piezoelectric accelerometer on the base of the enclosure. ASR was calculated as the mean amplitude to all startle trials. For ASR habituation, blocks (i.e. averaged across 5 trials) of the ASR

to 120 dB startle pulses presented at the beginning, in the middle and at the end of the PPI protocol were used to determine the effect of 'startle block'. Percentage PPI (%PPI) was calculated as [(mean startle response (120 dB) − PPI response)/mean startle response (120 dB)] × 100. %PPI was averaged across ISIs to produce a mean %PPI for each prepulse intensity. We analysed both the %PPI for the mean startle response across all 120 dB startle trials (i.e. 15 trials) as well as %PPI for the mean startle response across only the middle 120 dB startle trials (i.e. 5 trials).

3.2.3.4 Fear Conditioning (FC)

Fear conditioning was used to assess the impact of housing condition on the decline in associative memory in the *SOD1*^{G93A} model previously seen in our lab [32]. FC measures the association of a previously neutral stimulus (e.g. a tone or a context) with an aversive stimulus (e.g. an electric foot shock). The FC task occurred over three days [methods adapted from previous work in our lab [34]]. On the first day (i.e. conditioning day), mice were placed in a test chamber (Med Associates product: MED-VFC-SCT-M) which contained a vanilla scent cue for 120 seconds, with no sound (i.e. baseline period), before an 80 dB conditioned stimulus (CS) was presented for 30s co-terminating with a 0.4 mA 2-s foot shock (unconditioned stimulus). This procedure was repeated once with an inter-stimulus interval of 120 s and the conditioning test concluding 120 s later. On the second day of testing (context test), mice were returned to the apparatus for 7 minutes still containing the vanilla scent cue, but no sound was played. On the third day of testing (cue test), mice were placed in an altered environment (i.e. same chamber but including a triangular insert and no scent cue present) for 9 minutes. After 120 s (pre-CS/baseline), the CS was continuously presented for 5 minutes with the test concluding 120 s afterwards (i.e. no cue presentation in the last 2 minutes). Time spent *freezing* per minute (and as a total per trial) was measured in all three

trials using the video-freeze^{TM} software (freezing threshold of 15 frames). In addition, the *freezing* response to the cue was also assessed by comparing average *freezing* for 2 minutes prior to cue presentation to the average *freezing* for the 5 minutes during cue presentation.

3.2.3.5 Social Preference Test (SPT)

The SPT was used to measure social approach behaviour and social recognition memory. The three-chamber apparatus was similar to previous studies [31]. Test animals were isolated for 1 hour prior to testing in a new cage with fresh nesting material. They were then allowed to habituate to the test apparatus for a period 5 min before being removed. For the test of sociability, an unfamiliar social conspecific (i.e. a sex-matched, adult A/J mouse), was placed in one of two circular enclosures (allowing *sniffing* contact between mice but prevented fighting) in a quasi-randomized manner before the test mouse was returned to the central chamber and allowed to explore freely for 10 minutes. Finally, test animals were observed in a 10 minute social recognition memory (i.e. novelty preference) test trial in which a second unfamiliar opponent A/J mouse was placed in the previously empty enclosure so that the test mice could explore either the familiar mouse (from the previous trial) or the novel, unfamiliar mouse. An inter-trial interval of 5 minutes was used between each stage of the test. Any-MazeTM tracking software was used to determine the time the test mice spent in the different chambers during the test trials and assisted in the analysis of the more relevant time spent *nosing* the social conspecific (i.e. for social novelty preference testing).

3.2.4 Statistical analysis

Data were initially analyzed using three-way analysis of variance (ANOVA) for the main between test parameters ‘Sex’, ‘Genotype’ and ‘Housing’ as well as four-way repeated measures (RM)

ANOVA for the within measures: 'week' (body weight development and motor function tests across weekly testing), 'pulse' and 'prepulse' (PPI), and '1-min block' and 'cue' (FC). Where significant interactions were found data were split for corresponding factors and further analyses carried out (e.g. for main three-way ANOVA effect of 'sex': data are split by 'sex' and then a two-way ANOVA for 'genotyping' and 'housing' is carried out). In line with Rothman [35] and Perneger [36] the data were not adjusted for multiple comparisons and were interpreted as such in the discussion. The social preference test (SPT) was analyzed using single sample t-tests *versus* a chance level of 50%. Data are shown as means ± standard error of the mean (SEM) and data were deemed significant if $p < 0.05$. F-values and degrees of freedom are presented for ANOVAs and significant genotype effects *versus* corresponding WT are shown in figures and tables as '*' (*$p <$ 0.05, **$p < 0.01$ and ***$p < 0.001$). Housing effects are indicated using '#' ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$) and interactions between 'genotype' and 'housing' are shown as '+' ($^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$). For the SPT testing, significant t-test effects versus 50% 'chance' are shown in figures as '^' (*$p < 0.05$, ^^$p < 0.01$ and ^^^$p < 0.001$). All analyses were performed in IBM SPSS Statistics v24.

3.3 Results

3.3.1 Body weight development

3.1 Body weight development

Male mice were overall heavier than females [three-way ANOVA for 'Sex': $F(1,94) = 577.2$, $p < 0.001$] and *SOD1*^{G93A} transgenic mice were lighter than control littermates ['Genotype': $F(1,94) = 112.7$, $p < 0.001$] (Fig. 1A-B). When body weight was analysed across weeks using four-way RM ANOVA, *SOD1*-related body weight differences were more pronounced in male than female mice ['Sex' by 'Genotype' interaction: $F(1,94) = 5.3$, $p = 0.023$]. When split by 'Sex', both male ['Genotype' by 'Week' interaction: $F(6,288) = 19.413$, $p < 0.001$] as well as female [$F(6,276) = 10.7$, $p < 0.001$] *SOD1*^{G93A} transgenic mice lost body weight over the testing period regardless of housing condition whereas control mice gained weight (Fig. 1A-B). This difference was not affected by housing condition (no 'Genotype' by 'Week' by 'Housing' interactions for either sex, *p's* > 0.08). Interestingly, a 'Week' by 'Housing' interaction was detected in male mice [$F(6,288) = 2.2$, $p = 0.047$] indicating that IVC housing impacted on body weight development in males (Fig. 1A).

To clarify these findings further, the percentage change in body weight across weeks was analysed. Similar to overall body weight development, male ['Genotype' by 'Week' interaction: $F(6,288) = 13.44$, $p < 0.001$] and female [$F(6,276) = 4.65$, $p < 0.001$] *SOD1*^{G93A} transgenic mice lost body weight over the testing period regardless of housing condition (no 'Genotype' by 'Week' by 'Housing' interactions for either sex, *p's* > 0.05) (Fig. S1A-B). Again, an interaction of 'Week' by 'Housing' was detected in male mice [$F(6,288) = 2.5$, $p = 0.049$] as IVC-housed males exhibited a moderately slower change in body weight across weeks compared to FT-housed males (Fig. S1A).

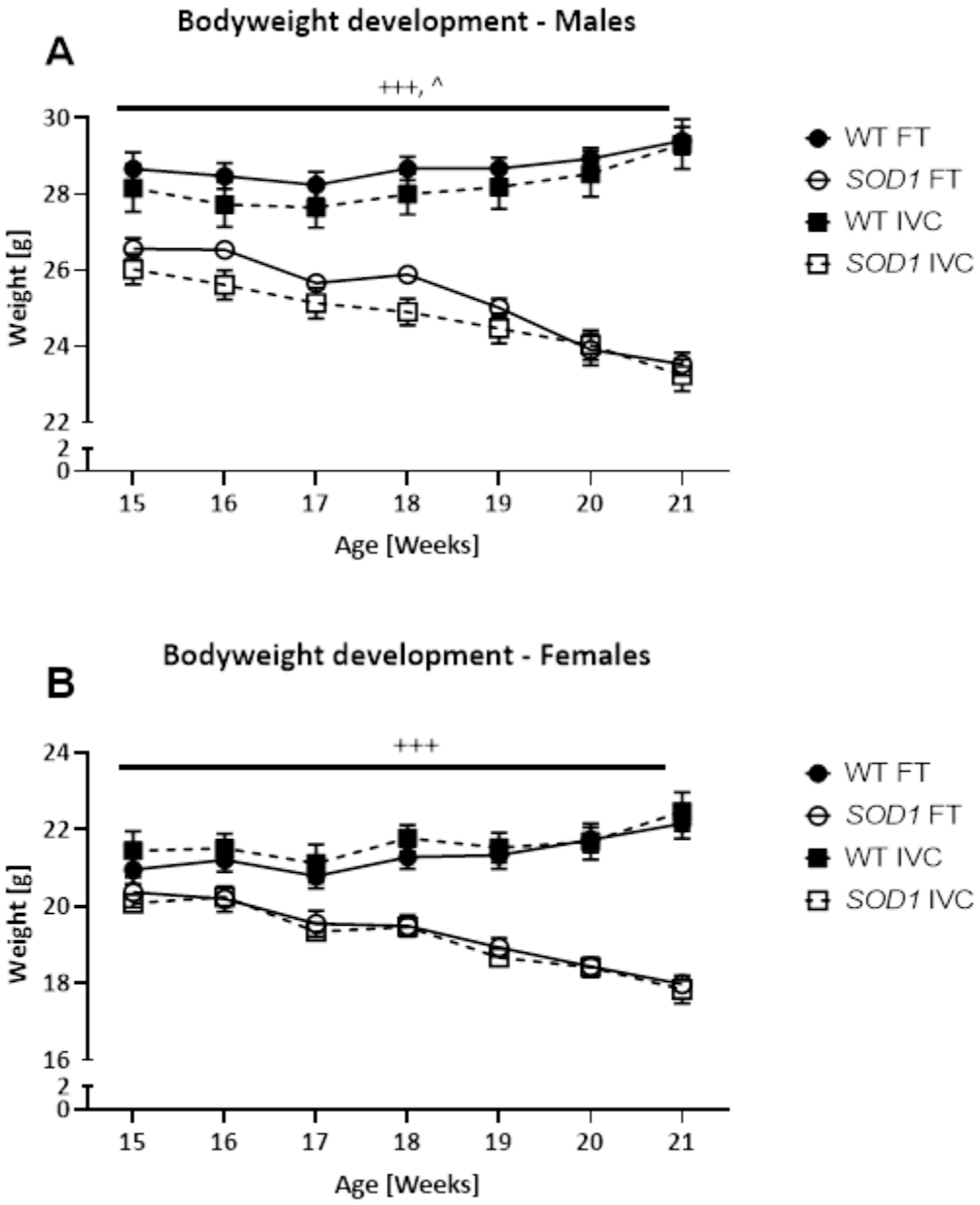

Figure 3. 1A-B Body weight progression over the testing period: Body weight per week [g] is shown for male **(A)** and female **(B)** G93A superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates kept in filter top (FT) or individually ventilated cage systems (IVC). Data are presented as mean ± SEM. 'Week' by 'Genotype' interactions are indicated by $^{+++}p < 0.001$ and a

'week' by 'Housing' interaction is shown by ^p < 0.05. Overall 'Sex' (p < 0.001) and 'Sex' by 'Genotype' interactions (p = 0.023) were observed for body weight

3.3.2 Motor function

Accelerod: Four-way RM ANOVA detected a 'Week' by 'Sex' by 'Genotype' by 'Housing' interaction [F(5,480) = 2.6, p = 0.031] (Fig. 2A-B). To clarify this interaction, we split data by 'Sex'. *SOD1*^{G93A} animals of both sexes exhibited progressively worsening motor performance (i.e. lower latencies to fall) compared to WT counterparts ['Week' by 'Genotype' interaction; males: F(5,250) = 33.0, p < 0.001 – females: [F(5,230) = 27.8, p < 0.001]. Importantly, a 'Week' by 'Genotype' by 'Housing' interaction was detected in female mice [F(5,230) = 2.6, p = 0.025 - p > 0.08 in males] indicating that this progressive impairment was more pronounced in IVC-housed females (Fig. 2B). However, split by 'Housing', a significant 'Week' by 'Genotype' interaction was still evident for both IVC [F(5,105) = 24.4, p < 0.001] and FT housing conditions [F(5,125) = 9.3, p < 0.001]. Interestingly, when splitting the female data by 'Genotype', a significant 'Week' by 'Housing' interaction was seen in *SOD1*^{G93A} females [F(5,115) = 2.4, p = 0.042] whereas no significant interaction was evident in WT mice [F(5,115) = 2.3, p = 0.053]. IVC-housed *SOD1*^{G93A} females had a moderately accelerated motor degeneration compared to transgenic mice in FT housing (Fig. 2B).

Pole test: Total time to reach the platform revealed that *SOD1*^{G93A} mice took significantly longer to complete the test compared to WT littermates ['Genotype' effect: F(1,96) = 123.5, p < 0.001] which progressively worsened over time ['Week' by 'Genotype': F(5,480) = 39.2, p < 0.001] (Fig. 2C-D). In addition, a 'Week' by 'Sex' by 'Genotype' interaction [F(5,480) = 3.8, p = 0.004] suggests that this genotype difference was influenced by sex. Indeed, split by 'Sex' and 'Week', *SOD1*^{G93A} transgenic males began to show motor deficits at 16 weeks of age whereas female *SOD1*^{G93A} mice exhibited motor impairments from 18 weeks of age onwards when compared to

WT littermates (Fig. 2C-D). In addition, over the testing period, an ever-increasing percentage of *SOD1*^{G93A} animals fell from the pole and were unable to complete the test (Table 1).

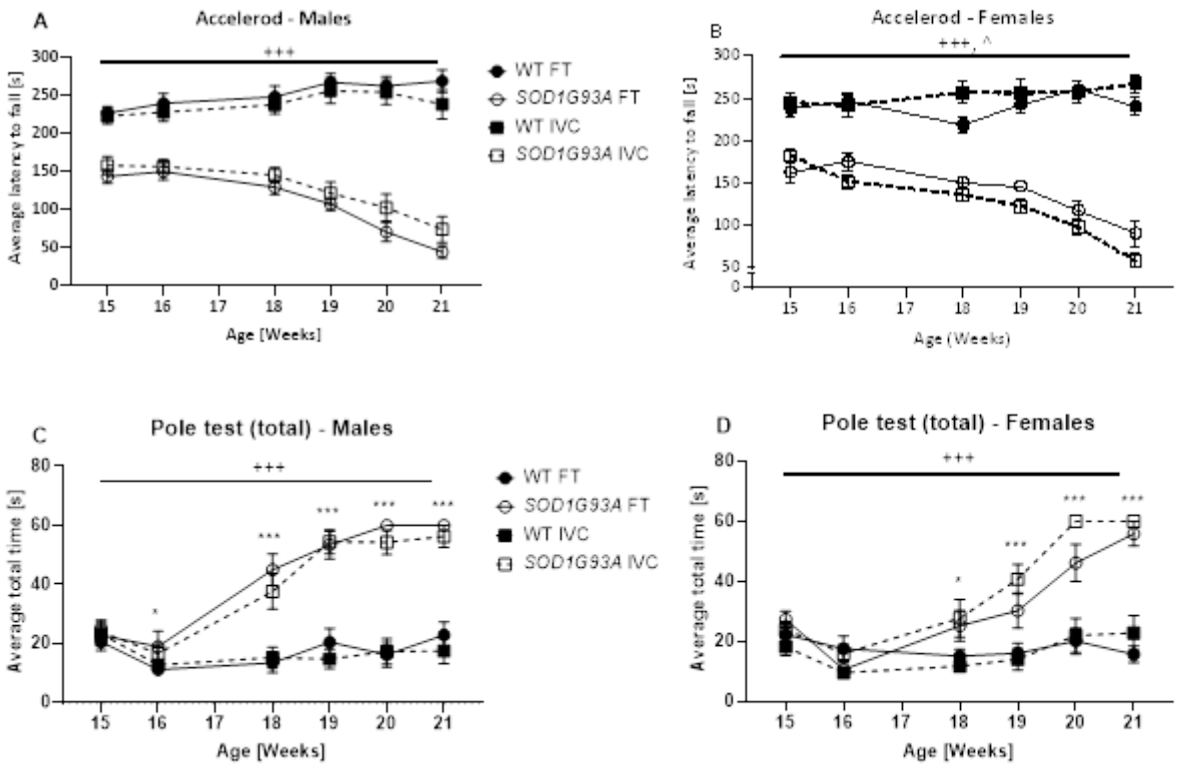

Figure 3.2A-D Motor function assessment over the testing period: (A/B) Average latency to fall from the accelerod [s] and **(C/D)** average total test time in the pole test [s] are shown as mean ± SEM for male **(A/C)** and female **(B/D)** G93A superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates kept in filter top (FT) or individually ventilated cage systems (IVC). 'Week' by 'Genotype' interactions are indicated by $^{+++}p < 0.001$ (main effects of Genotype across Housing conditions are indicated by $^{*}p < 0.05$, $^{***}p < 0.001$) and a 'week' by 'Genotype' by 'Housing' interaction is shown by $^{\wedge}p < 0.05$. A 'week' by 'Sex' by 'Genotype' by 'Housing' ($p = 0.031$) interaction for accelerod **(A/B)** and a 'week' by 'Sex' by 'Genotype' interaction ($p = 0.004$) for pole test were observed **(C/D)**.

3.3.3 Acoustic startle response and sensorimotor gating

Acoustic startle response: Four-way RM ANOVA revealed a main effect of 'Intensity' [$F(2,196) = 180.1$, $p < 0.001$] as all animals responded to increasing dB levels of startle stimuli with male mice exhibiting a more pronounced startle response than female mice ['Intensity' by 'Sex': $F(2,196) = 13.9$, $p < 0.001$] (Fig. 3A/B). All *SOD1*^{G93A} exhibited a significantly reduced increase in startle response across intensities compared to WT littermates ['Intensity' by 'Genotype': $F(2,196) = 75.5$, $p < 0.001$] but this genotype effect was affected by sex as it appeared less prominent in females ['Intensity' by 'Sex' by 'Genotype' interaction: $F(2,196) = 3.4$, $p = 0.036$]. Split by 'Sex' and 'Intensity' there was no difference seen in any mice of either sex at 70dB baseline startle (all $p > 0.08$), whereas at 100dB, while female *SOD1*^{G93A} did indeed show significantly reduced startle compared to WT ($p = 0.046$), this was not to the same magnitude as seen in male mice ($p < 0.0001$), at 120dB the startle response for both male and female *SOD1*^{G93A} was drastically lower than WT littermates (both *p's* < 0.0001).

Sensorimotor gating: Four-way RM ANOVA revealed a main effect of 'Prepulse' [$F(2,196) = 359.8$, $p < 0.001$] as %PPI increased with increasing prepulse intensities in all animals indicating that the protocol was effective regardless of experimental test condition (no interactions of 'Sex', 'Genotype' or 'Housing' with 'Prepulse': all *p's* > 0.08; data not shown). When analyzing %PPI averaged across prepulse intensities, a main effect of 'Genotype' [$F(1,98) = 59.1$, $p < 0.001$] was found with *SOD1*^{G93A} transgenic mice exhibiting lower PPI than WT animals regardless of sex (no 'Genotype' by 'Sex' interaction, $p > 0.08$; Fig. 3C-D). Importantly, a 'Genotype' by 'Housing' interaction [$F(1,98) = 6.3$, $p = 0.014$] indicated that *SOD1*^{G93A} genotype differences were less severe (but still evident) in IVC-housed females compared to females in FT housing – this was not evident in male mice (no 'Genotype' by 'Housing' by 'Sex' interaction, $p > 0.08$, Fig. 3A-B).

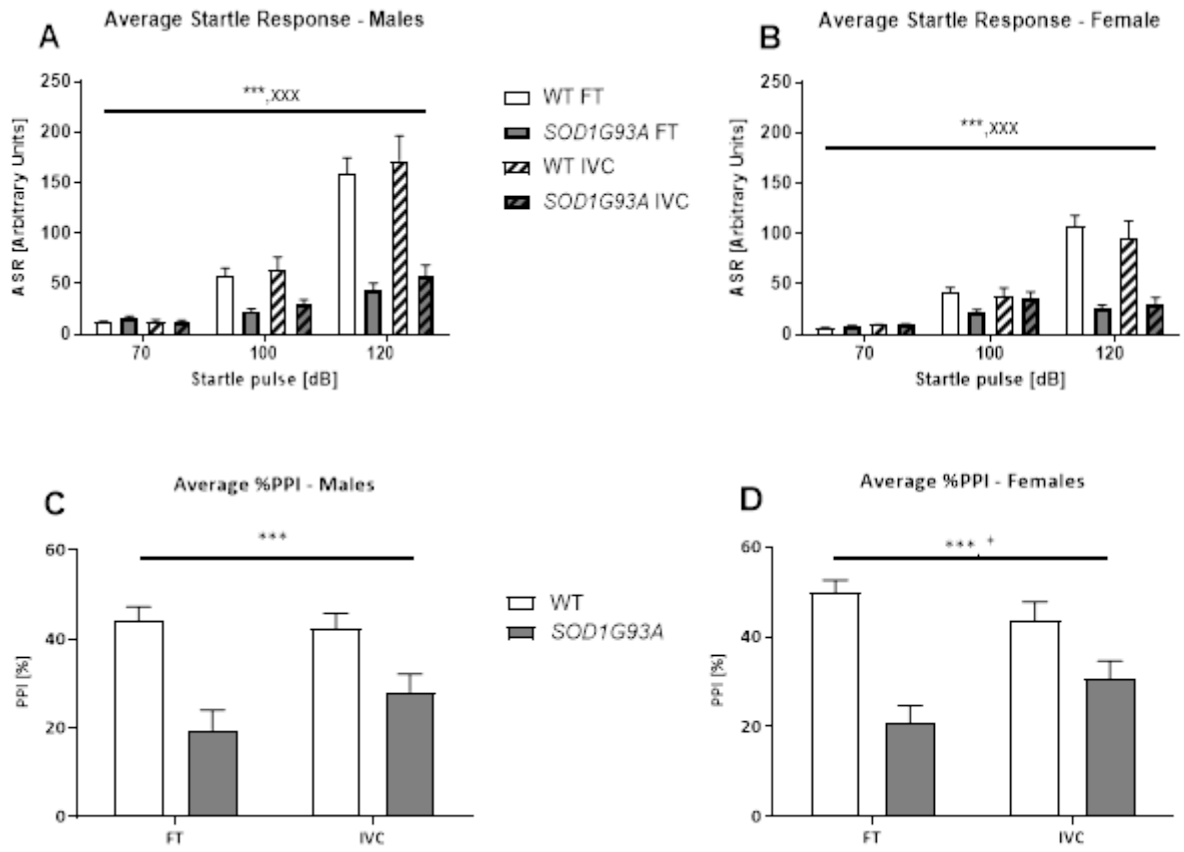

Figure 3. 3A-D Acoustic startle response (ASR) and sensorimotor gating (i.e. prepulse inhibition, PPI): (A/B) Average ASR across startle intensities [arbitrary units] and **(C/D)** percentage PPI [%] averaged across prepulse intensities [%PPI] are shown as mean ± SEM for male **(A/C)** and female **(B/D)** G93A superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates kept in filter top (FT) or individually ventilated cage systems (IVC). Main Genotype effects across housing conditions versus WT are shown as ***$p < 0.001$ **(A-D)**. 'Intensity' by 'Genotype' interactions are indicated by $^{xxx}p < 0.001$ **(A/B)** and a 'Genotype' by 'Housing' interaction is indicated by $^{+}p < 0.05$ **(D)**. The ASR response of mice was sex-dependent, as evidenced by 'intensity' by 'Sex' ($p < 0.001$) and 'intensity' by 'Sex' by 'Genotype' ($p = 0.036$) interactions **(A/B)**.

3.3.4 Fear-associated memory

Three-way ANOVA found no differences in baseline levels of *freezing* in the first 2 min of the conditioning session across sex, genotypes or housing methods (all *p's* > 0.08; data not shown).

<u>*Contextual fear conditioning:*</u> Main effects of 'Sex' [F(1,98) = 11.7, $p = 0.001$] and 'Genotype' [F(1,98) = 12.547, $p = 0.001$] were seen for total *freezing* during context testing with male mice exhibiting lower contextual *freezing* compared to female mice and $SOD1^{G93A}$ mice showing increased contextual *freezing* compared to WT mice (Table 2). However, a 'Sex' by 'Genotype' interaction clarified [F(1,98) = 12.5, $p = 0.001$] that only female $SOD1^{G93A}$ mice exhibited increased *freezing* to the context compared to control females [F(1,46) = 19.9, $p < 0.001$]. Indeed, split by sex, no main effects or interactions were seen in male mice during the context test (all *p's* > 0.08), whereas a main effect of 'Genotype' was evident in female mice [F(1,46) = 19.9, $p < 0.001$] with *SOD1* females showing increased total contextual *freezing* compared to WT littermates (Table 1).

<u>*Cue fear conditioning:*</u> Looking at total *freezing* during the 9-min cue test session, female mice *froze* overall more than males [F(1,98) = 12.35, $p = 0.001$] (Table 1). Split by 'Sex', no significant interaction for 'Genotype' by 'Housing' [F(1,52) = 3.5, $p = 0.069$] was seen in male mice although IVC-housed $SOD1^{G93A}$ males appeared to *freeze* more than their IVC-housed WT littermates, whereas this phenomenon was not evident in FT housing (i.e. total *freezing*: Table 1). Female $SOD1^{G93A}$ mice exhibited increased total cue *freezing* compared to control females [F(1,46) = 17.3, $p < 0.001$; Table 1]; no effects of housing were evident (all *p's* > 0.08).

Analysing *freezing* per 1-min block over the test period, a '1-min block' by 'Sex' by 'Genotype' interaction was detected [F (8,784) = 2.2, $p = 0.044$] as genotype differences in cue *freezing* across time appeared only evident in female mice (Fig. 4A-B). Indeed, analysing cue *freezing* across time

for each sex, a '1-min block' by 'Genotype' interaction was evident in female mice [three-way RM ANOVA: $F(8,368) = 4.2$, $p < 0.001$ - $p > 0.08$ in male mice] (Fig. 4A-B). Two-way ANOVAs split by '1-min block' confirmed that *SOD1*^{G93A} transgenic females *froze* more than WT females in the first two 1-min blocks of cue testing as well as from minute 6 onwards (Fig. 4B). Housing had no effects in female mice (all p's > 0.08). In male mice, a '1-min block' by 'Housing' interaction was found [$F(8,368) = 4.2$, $p < 0.001$]. IVC-housed males developed higher levels of *freezing* over the test period when compared to FT-housed males, and this was most evident in the first two 1-min blocks of cue testing (Fig. 4A).

Looking at average *freezing* prior to cue presentation compared to the average *freezing* during cue presentation, a 'Cue' by 'Housing' interaction was detected in male mice [$F(1,52) = 6.4$, $p = 0.014$ - $p > 0.08$ in female mice] (Fig. 4C-D). IVC-housed males regardless of genotype (no significant 'Cue' by 'Housing' by 'Genotype' interaction, $p > 0.08$) showed a weaker response to the cue than males kept in FT. This appeared to be driven by the increased *freezing* of IVC males in the first 2 min of cue testing (Fig. 4C). Indeed, when assessing average *freezing* prior to cue presentation separately, a main effect of 'Housing' was observed [$F(1,53) = 7.25$, $p = 0.01$] with IVC-housed males *freezing* more than their FT-housed littermates.

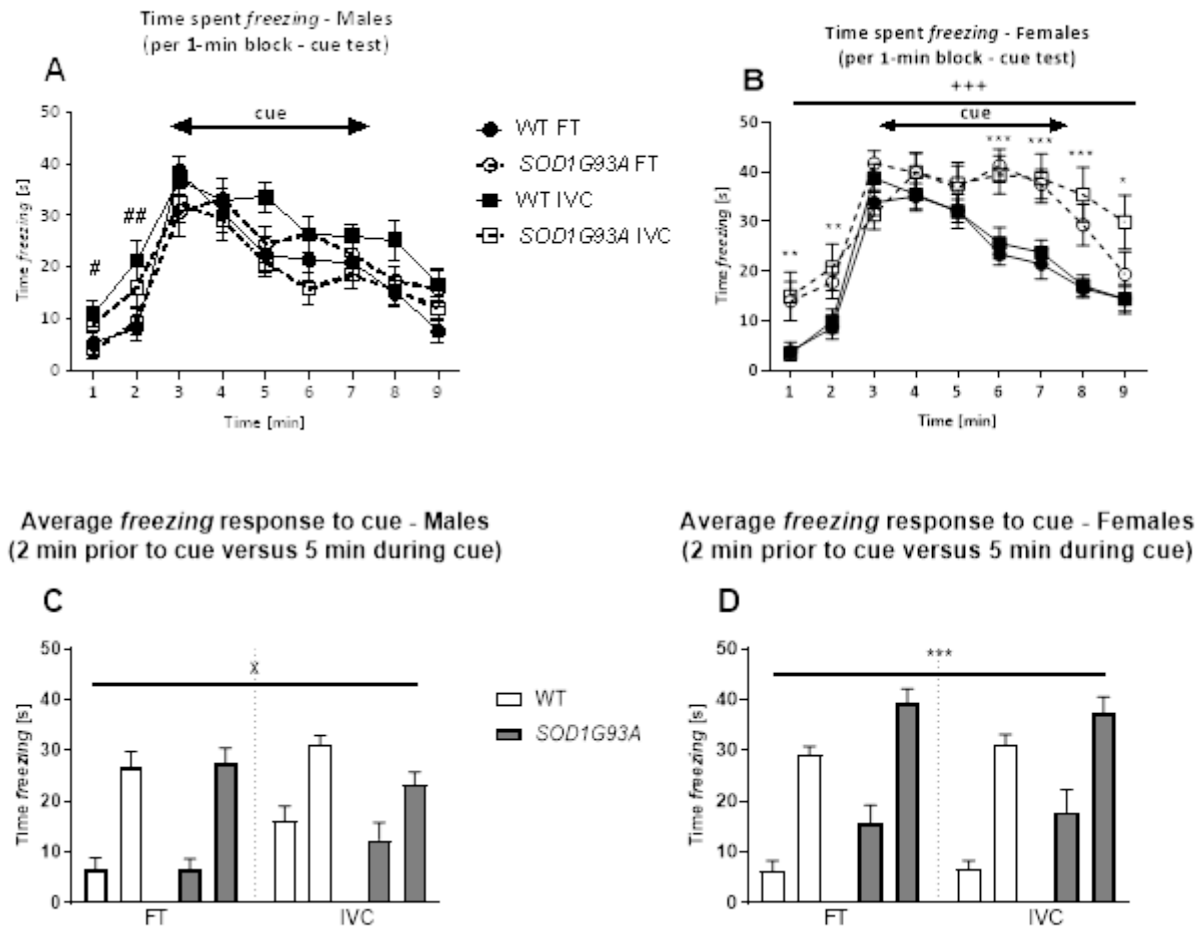

Figure 3. 4A-D Fear-associated memory: (A/B) Time spent freezing [s] in the cue test across 1-min blocks (total of 9 min test session) and **(C/D)** average freezing [s/min] in the 2 mins prior to cue presentation compared to average freezing during the 5 minutes of cue presentation. Data are shown as mean ± SEM for male **(A/C)** and female **(B/D)** G93A superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates kept in filter top (FT) or individually ventilated cage systems (IVC). Main Genotype effects across housing condition versus WT (total or per 1-min block) are shown as '*' (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Housing effects across Genotype versus FT (per 1-min block) are shown as '$^{\#}$' ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$). A '1-min block' by 'Sex' by 'Genotype' interaction ($p = 0.044$) was evident for cue freezing across time **(A/B)**. There was also a 'Time' by 'Genotype' interaction for cue freezing across 1-min blocks in females ($^{+++}p < 0.001$) **(B)** and a 'Time' by 'Housing' interaction for the cue response for males ($^{x}p < 0.05$) **(C)**.

3.3.5 Social preference test:

The social preference test was analyzed split by sex as our previous research has found that the response to the test protocol is highly sex-specific [38-40].

Sociability: Single sample t-tests against 50% chance levels revealed that all male mice regardless of experimental condition showed intact sociability, i.e. all males except for IVC-housed WT males exhibited a preference for a mouse over an empty chamber [WT FT: $t(12) = 4.4$; $p < 0.001$; *SOD1*^{G93A} FT: $t(11) = 2.6$; $p = 0.03$; WT IVC: $t(15) = 1.9$, $p = 0.075$; *SOD1*^{G93A} IVC: $t(13) = 5.4$; $p < 0.001$; Figure 5A]. In females, all experimental groups except FT-housed WT females [$t(13) = 1.2$, $p = 0.12$] displayed intact sociability [*SOD1*^{G93A}FT: $t(12) = 3.1$; $p = 0.01$; WT IVC: $t(10) = 2.3$; $p = 0.04$; *SOD1*^{G93A} IVC: $t(10) = 2.3$; $p = 0.046$; Fig. 5B].

Social novelty preference: All male mice exhibited intact social recognition memory regardless of the experimental condition [i.e. all mice showed an above chance level preference (i.e. time spent *nosing*) for the novel mouse: WT FT: $t(12) = 2.8$; $p = 0.016$; *SOD1*^{G93A} FT: $t(11) = 2.3$; $p = 0.041$; WT IVC: $t(15) = 2.3$, $p = 0.006$; *SOD1*^{G93A} IVC: $t(13) = 5.2$; $p < 0.001$; Fig. 5C]. Interestingly, in females, only FT-housed *SOD1*^{G93A} transgenic females showed intact social recognition memory [$t(12) = 3.3$, $p = 0.006$] whereas all remaining experimental groups failed to show a preference for the novel mouse (all *p's* > 0.08) (Fig. 5D). Data for the less relevant chamber time are presented as supplementary info (Supplementary Figure 2A-B).

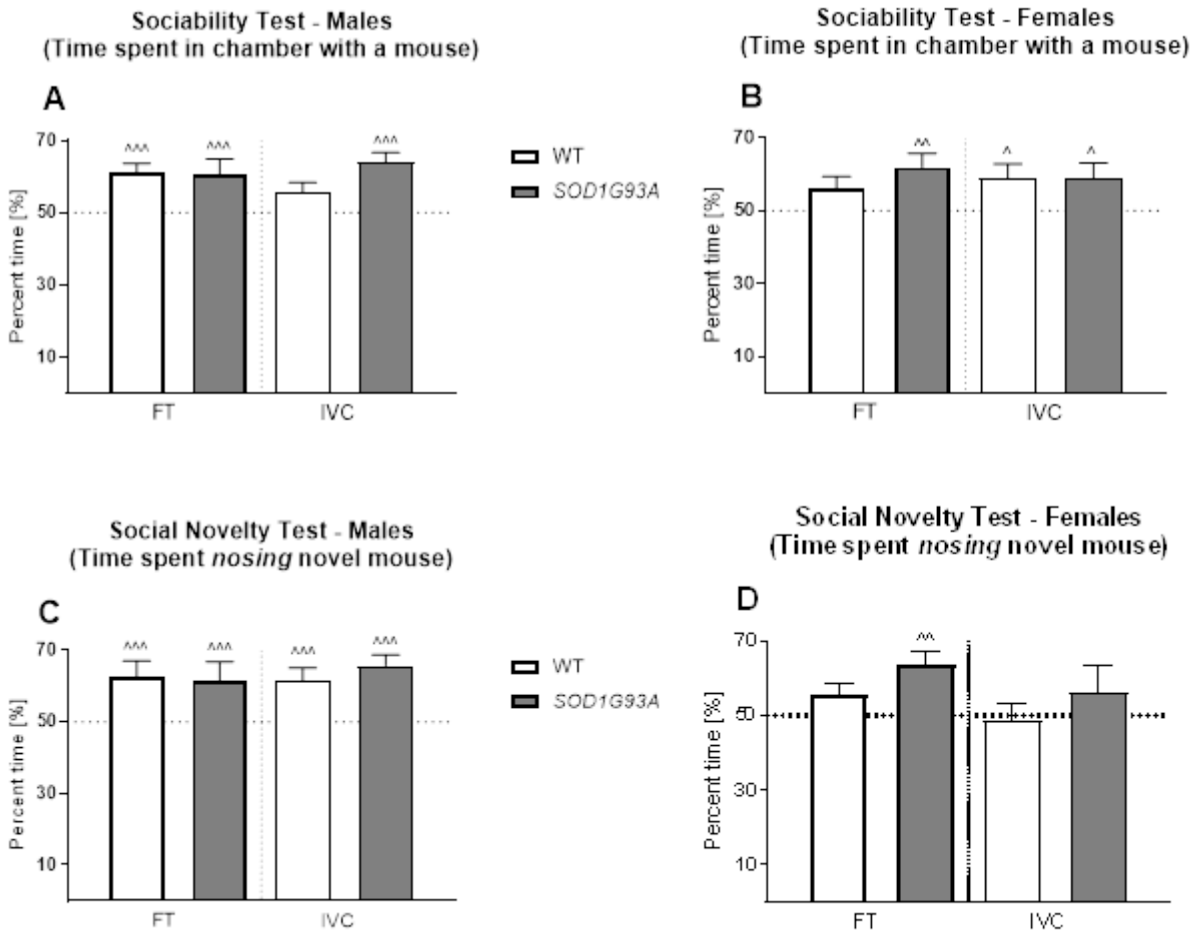

Figure 3. 5A-D Sociability and social recognition memory: (A/B) Percentage time spent interacting with a mouse over an empty cage [%] and **(C/D)** percentage time interacting with a novel mouse over a familiar mouse [%]. Data are shown as mean ± SEM for male **(A/C)** and female **(B/D)** G93A superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates kept in filter top (FT) or individually ventilated cages (IVC). Significant preferences above chance levels (i.e. 50%, indicated by dotted line) are shown as '^' (^^$p < 0.01$, ^^^$p < 0.001$).

3.4 Discussion

We investigated the impact of two different home caging systems on the development of behavioural characteristics of *SOD1*^{G93A} transgenic mice. *SOD1*^{G93A} mice regardless of experimental condition progressively lost body weight, whereas WT mice did not lose any weight. IVC-housed males exhibited a moderately slower body weight loss compared to FT-housed males. Both male and female *SOD1*^{G93A} mice showed degenerating motor performances across tests. The development of an impaired motor performance of *SOD1*^{G93A} females was accelerated when housed in IVCs compared to FT housing. In the pole test, *SOD1*^{G93A} males showed motor deficits earlier than female *SOD1*^{G93A} transgenics. *SOD1*^{G93A} mice presented impaired ASR at 100dB and 120dB (less prominent in female mice at 100dB). Additionally, a PPI deficit was seen in all *SOD1*^{G93A} transgenic mice, which was less severe in *SOD1*^{G93A} females when housed in IVC systems. *SOD1*^{G93A} females exhibited increased contextual as well as cue *freezing*. IVC housing increased cue *freezing* of males over the test period (most evident in the first 2 min of testing). Social preference testing revealed no differences across male test groups, whereas sociability was deficient in FT-housed WT females and social recognition memory was only intact in FT-housed *SOD1*^{G93A} transgenic females.

SOD1^{G93A} transgenic mice exhibited progressively declining body weight over the testing period, which was more pronounced and started earlier in male transgenic mice compared to female *SOD1*^{G93A} mice. This finding is representative of the established phenotype of the *SOD1*^{G93A} mouse model [30,42-44]. We found that IVC-housed males regardless of Genotype showed a delayed increase in body weight across ageing compared to male mice housed in the FT cage system. This delay suggests that food intake or metabolic pathways of mice may be affected in IVCs. Interestingly, studies in wild type-like mice detected IVC-induced changes to body weight development which

did not appear to be related to changes in food intake although increased water intake was observed [12,17,20].

Motor function declined over time in all *SOD1*^{G93A} transgenic mice, which was in line with previous studies. The decline commenced earlier in males than females in a manner similar to what has been reported in most other studies [[42,44,45] but see also [46]]. Indeed, a meta-analysis examining the onset and progression of motor impairments across a variety of *SOD1* transgenic mouse models suggests that whilst sex does seem to play a role in both, the genetic background of the mouse models seems to be critical as to whether sex differences are evident [47]. Importantly, IVC housing moderately accelerated the progression of motor deficits in *SOD1*^{G93A} females when being tested in the accelerod test. This finding could be related to the inherent nature of at least some actively ventilated IVCs to impose chronic cold stress [11]. Importantly, regulation of core temperature when exposed to cold is impaired in the *SOD1* mouse model in the latter stages of the phenotype [48]. *SOD1*^{G93A} transgenic mice also exhibit defective energy homeostasis largely as a consequence of skeletal muscle hypermetabolism [49], a phenomenon which is thought to lead to the destruction of the neuromuscular junction and ultimately neuronal apoptosis [50]. Therefore, chronic housing in a colder environment which innately requires a further increase in energy expenditure could potentially increase motor neuron vulnerability and hasten the progression of the motor impairment phenotype in this model.

The acoustic startle response was impaired in all *SOD1*^{G93A} mice in line with previous findings from our and other laboratories [31,51]. This impaired response may be attributed to hearing deficits, but genotype-related muscle weakness and consequential reduced ability to flinch the body should also be considered as the latter has been implicated in reduced ASR in rats [52]. Importantly, in our study, motor impairments were evident in male *SOD1*^{G93A} mice starting from 16 weeks of age and

less severe in *SOD1*^{G93A} females (significant impairments evident at 18 weeks of age albeit of lesser magnitude than in male mice). In the week of FC and PPI testing (week 17), motor performance was not evaluated making it difficult to clarify if the emerging deficit was already evident in female mice at 17 weeks of age. It is interesting to note that the ASR deficit of transgenic females at 100dB was not of the same magnitude as in male *SOD1*^{G93A} mice. Future research will have to clarify the exact nature of the ASR deficit in more detail, but this phenotype appears to be driven by more than just muscle weakness.

We also detected a pronounced PPI impairment in both male and female *SOD1*^{G93A} animals confirming previous results seen in our lab [31]. The housing condition affected this sensorimotor gating deficit in a sex-dependent manner in an opposite manner as work previously performed in our lab [20], as the PPI deficit of *SOD1*^{G93A} females was less severe but still evident when housed in IVC cages. Interestingly, brain regions such as the superior colliculus (SC), ventral tegmental area (VTA) and caudal pontine reticular nucleus (PnC) have been shown to play key roles in PPI [53]. In line with this, neuronal damage and increased inflammation have been observed in the SC and VTA of *SOD1*^{G93A} transgenic mice [54] and neuronal hypofunction is evident in the PnC of these mice as well [55]. In addition, dopaminergic dysfunction is evident in these brain regions in this ALS model [54] and deregulated dopamine signalling, as well as neuroinflammation has been found to impair sensorimotor gating [56,57]. Furthermore, the stronger effect of IVC housing on PPI in *SOD1*^{G93A} females may be linked to the finding that IVCs have been found to increase c-Fos levels in mice in the limbic region [58] and to ameliorate PPI deficits in a genetic mouse model for schizophrenia [20]. Interestingly, c-Fos is seen as not only a marker for neuronal activity but also a regulator for cellular mechanisms mediating neuronal excitability and survival [59]and is indeed down-regulated in *SOD1*^{G93A} transgenic animals [60]. In line with our findings on sex specificity,

another study also found that 'beneficial' effects of environmental factors (i.e. physical exercise) on disease-relevant parameters are more prominent in female *SOD1*^{G93A} transgenic mice [61]. This effect may not be evident in male mice due to their more rapid degeneration compared to female transgenic mice [62] and the fact that estrogen plays a neuroprotective role in ALS and specifically *SOD1*^{G93A} transgenic animals [63] and that estrogen receptors are abundant in brain regions such as the VTA [64].

SOD1^{G93A} females regardless of housing condition exhibited increased context and cue *freezing*. This is a novel finding as increased *freezing* behaviours have previously only been observed in younger male mice in our lab [31]. Interestingly, abnormal prelimbic and infralimbic neurons were found in the medial prefrontal cortex of *SOD1*^{G93A} mice [65] and correlated with impaired fear extinction memory in younger male mice [65]. Thus, the stronger *freezing* phenotype in the cue test could be related to impaired fear extinction. Interestingly, levels of the insulin-like growth factor-binding protein 2 (IGFBP2), which plays a role in cognitive development and neuronal plasticity [66], are decreased in the hippocampus of symptomatic 18-week-old *SOD1*^{G93A} mice [67] providing a potential additional mechanism behind the increased *freezing* seen in our mice. IVC-housed males exhibited increased cue *freezing* over time, however, this phenotype appeared to be predominantly linked to the first 2 minutes of the test (i.e. no cue presentation). Indeed, average *freezing* during the cue presentation was not different from FT-housed animals. IVC housing is known to interfere with established *freezing* phenotypes. For example, IVCs led to a loss of an impaired fear extinction phenotype previously seen in serotonin transporter knockout rats [21], which was hypothesized to be due to elevated baseline anxiety in these mice caused by increased airflow and noise in this cage system [21]. In line with this, increased anxiety-relevant open field behaviour has been linked to IVC housing [17].

Sociability was intact across experimental conditions except for IVC-housed WT males and FT-housed WT females. All male mice also exhibited intact social recognition memory, but only FT-housed *SOD1*^{G93A} females developed a preference for the novel mouse. Interestingly, younger (PND82) but not older (PND 120) *SOD1*^{G93A} male mice exhibited impaired sociability in our lab [31] suggesting that social impairments of *SOD1*^{G93A} mice may be dependent on age and sex. Sex differences in social paradigms have previously been reported, both in our lab [38] and by others and is thought to be dependent on the methods of social hierarchy formation in males (i.e. competitive) compared to females (i.e. intrinsic) [68]. Further studies should be performed in this area to elucidate the true nature and development of social deficits in *SOD1*^{G93A} transgenic mice of both sexes.

In summary, we found that the IVC cage system can impact on the *SOD1*^{G93A} transgenic mouse model phenotype established in filter top cages and in a sex-dependent manner, though not in a uni-directional manner as this was found to be specific to the test used. These effects were moderate and did not alter the severity of motor degeneration in these mice in a biologically relevant manner. Most important is the effect of IVC housing on sensorimotor gating and fear-associated memory as it may compromise the face validity of the model. Furthermore, potential physiological changes imposed by the cage system used may impact on the testing of interventions, especially those with small effect sizes. Therefore, whilst changing cage system to suit the model may not be a feasible solution, researchers should at least be aware of potential effects when changing cage systems and report the cage system used in their study in detail in line with the recently updated ARRIVE guidelines for reporting on animal research [69] to further enhance the understanding of these effects in the field. This is not only relevant for behavioural studies but also in pharmacological studies (as IVCs can shift drug sensitivity [19]) and endocrinological and

immunological experiments (as IVCs can impact on stress hormones and e.g. pro-inflammatory cytokine levels [22]).

3.5 References

Data availability statement: *Data available on request from the authors.*

1. Burrows EL, McOmish CE, Hannan AJ. Gene-environment interactions and construct validity in preclinical models of psychiatric disorders. *Prog Neuropsychopharmacol Biol Psychiatry.* 2011;35(6):1376-1382.
2. Sorge RE, Martin LJ, Isbester KA, et al. Olfactory exposure to males, including men, causes stress and related analgesia in rodents. *Nature methods.* 2014;11(6):629-632.
3. Gouveia K, Hurst JL. Reducing mouse anxiety during handling: effect of experience with handling tunnels. *PloS one.* 2013;8(6):e66401.
4. Gouveia K, Hurst JL. Optimising reliability of mouse performance in behavioural testing: the major role of non-aversive handling. *Scientific reports.* 2017;7:44999.
5. Hurst JL, West RS. Taming anxiety in laboratory mice. *Nature methods.* 2010;7(10):825-826.
6. Crabbe JC, Wahlsten D, Dudek BC. Genetics of mouse behavior: interactions with laboratory environment. *Science.* 1999;284(5420):1670-1672.
7. Paigen B, Svenson KL, Von Smith R, et al. Physiological effects of housing density on C57BL/6J mice over a 9-month period. *J Anim Sci.* 2012;90(13):5182-5192.
8. Ferrecchia CE, Jensen K, Van Andel R. Intracage ammonia levels in static and individually ventilated cages housing C57BL/6 mice on 4 bedding substrates. *J Am Assoc Lab Anim Sci.* 2014;53(2):146-151.
9. Clough G, Wallace J, Gamble M, Merryweather E, Bailey E. A positive, individually ventilated caging system: a local barrier system to protect both animals and personnel. *Laboratory animals.* 1995;29(2):139-151.

10. Gordon S, Fisher SW, Raymond RH. Elimination of mouse allergens in the working environment: assessment of individually ventilated cage systems and ventilated cabinets in the containment of mouse allergens. *J Allergy Clin Immunol.* 2001;108(2):288-294.

11. David JM, Knowles S, Lamkin DM, Stout DB. Individually ventilated cages impose cold stress on laboratory mice: a source of systemic experimental variability. *Journal of the American Association for Laboratory Animal Science.* 2013;52(6):738-744.

12. Kostomitsopoulos N, Alexakos P, Eleni K, Doulou A, Paschidis K, Baumans V. The effects of different types of individually ventilated caging systems on growing male mice. *Lab Anim (NY).* 2012;41(7):192-197.

13. Mineur YS, Crusio WE. Behavioral effects of ventilated micro-environment housing in three inbred mouse strains. *Physiol Behav.* 2009;97(3-4):334-340.

14. York JM, McDaniel AW, Blevins NA, et al. Individually ventilated cages cause chronic low-grade hypoxia impacting mice hematologically and behaviorally. *Brain Behav Immun.* 2012;26(6):951-958.

15. Kallnik M, Elvert R, Ehrhardt N, et al. Impact of IVC housing on emotionality and fear learning in male C3HeB/FeJ and C57BL/6J mice. *Mamm Genome.* 2007;18(3):173-186.

16. Hawkins P, Anderson D, Applebee K, et al. Individually ventilated cages and rodent welfare: Report of the 2002 RSPCA/UFAW rodent welfare group meeting. *Animal Technology and Welfare.* 2003;2:23-34.

17. Pasquarelli N, Voehringer P, Henke J, Ferger B. Effect of a change in housing conditions on body weight, behavior and brain neurotransmitters in male C57BL/6J mice. *Behav Brain Res.* 2017;333:35-42.

18. Polissidis A, Zelelak S, Nikita M, et al. Assessing the exploratory and anxiety-related behaviors of mice. Do different caging systems affect the outcome of behavioral tests? *Physiology & behavior.* 2017;177:68-73.

19. Logge W, Kingham J, Karl T. Behavioural consequences of IVC cages on male and female C57BL/6J mice. *Neuroscience.* 2013;237:285-293.

20. Logge W, Kingham J, Karl T. Do individually ventilated cage systems generate a problem for genetic mouse model research? *Genes, Brain and Behavior.* 2014;13(7):713-720.

21. Shan L, Schipper P, Nonkes LJ, Homberg JR. Impaired fear extinction as displayed by serotonin transporter knockout rats housed in open cages is disrupted by IVC cage housing. *PloS one.* 2014;9(3):e91472.

22. Mueller FS, Polesel M, Richetto J, Meyer U, Weber-Stadlbauer U. Mouse models of maternal immune activation: Mind your caging system! *Brain, behavior, and immunity.* 2018;73:643-660.

23. Wijesekera LC, Leigh PN. Amyotrophic lateral sclerosis. *Orphanet journal of rare diseases.* 2009;4(1):3.

24. Parton MJ, Broom W, Andersen PM, et al. D90A-SOD1 mediated amyotrophic lateral sclerosis: a single founder for all cases with evidence for a Cis-acting disease modifier in the recessive haplotype. *Human mutation.* 2002;20(6):473-473.

25. Turner BJ, Talbot K. Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. *Prog Neurobiol.* 2008;85(1):94-134.

26. Miller T, Cudkowicz M, Shaw PJ, et al. Phase 1-2 Trial of Antisense Oligonucleotide Tofersen for SOD1 ALS. *N Engl J Med.* 2020;383(2):109-119.

27. Mueller C, Berry JD, McKenna-Yasek DM, et al. SOD1 Suppression with Adeno-Associated Virus and MicroRNA in Familial ALS. *N Engl J Med.* 2020;383(2):151-158.

28. Benatar M. Lost in translation: treatment trials in the SOD1 mouse and in human ALS. *Neurobiol Dis.* 2007;26(1):1-13.

29. Stam NC, Nithianantharajah J, Howard ML, Atkin JD, Cheema SS, Hannan AJ. Sex-specific behavioural effects of environmental enrichment in a transgenic mouse model of amyotrophic lateral sclerosis. *European Journal of Neuroscience.* 2008;28(4):717-723.

30. Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science.* 1994:1772-1772.

31. Kreilaus F, Guerra S, Masanetz R, Menne V, Yerbury J, Karl T. Novel behavioural characteristics of the superoxide dismutase 1 G93A (SOD1G93A) mouse model of amyotrophic lateral sclerosis include sex-dependent phenotypes. *Genes, Brain and Behavior.* 2019:e12604.

32. Kreilaus F, Guerra S, Masanetz R, Menne V, Yerbury J, Karl T. Novel behavioural characteristics of the superoxide dismutase 1 G93A (SOD1(G93A)) mouse model of amyotrophic lateral sclerosis include sex-dependent phenotypes. *Genes Brain Behav.* 2020;19(2):e12604.

33. Karl T, Burne TH, Van den Buuse M, Chesworth R. Do transmembrane domain neuregulin 1 mutant mice exhibit a reliable sensorimotor gating deficit? *Behav Brain Res.* 2011;223(2):336-341.

34. Olaya JC, Heusner CL, Matsumoto M, Shannon Weickert C, Karl T. Schizophrenia-relevant behaviours of female mice overexpressing neuregulin 1 type III. *Behav Brain Res.* 2018;353:227-235.

35. Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology.* 1990:43-46.

36. Perneger TV. What's wrong with Bonferroni adjustments. *Bmj.* 1998;316(7139):1236-1238.

37. Perneger TV. What's wrong with Bonferroni adjustments. *BMJ.* 1998;316(7139):1236-1238.

38. Cheng D, Low JK, Logge W, Garner B, Karl T. Novel behavioural characteristics of female APPSwe/PS1DeltaE9 double transgenic mice. *Behav Brain Res.* 2014;260:111-118.

39. Zieba J, Morris MJ, Weickert CS, Karl T. Behavioural effects of high fat diet in adult Nrg1 type III transgenic mice. *Behavioural brain research.* 2020;377:112217.

40. Olaya JC, Heusner CL, Matsumoto M, Weickert CS, Karl T. Schizophrenia-relevant behaviours of female mice overexpressing neuregulin 1 type III. *Behavioural brain research.* 2018.

41. Zieba J, Morris MJ, Weickert CS, Karl T. Behavioural effects of high fat diet in adult Nrg1 type III transgenic mice. *Behav Brain Res.* 2020;377:112217.

42. Alves CJ, de Santana LP, dos Santos AJ, et al. Early motor and electrophysiological changes in transgenic mouse model of amyotrophic lateral sclerosis and gender differences on clinical outcome. *Brain Res.* 2011;1394:90-104.

43. Peters TM, Kwon YW, Prattis SM, Gurneyț ME. Age-dependent penetrance of disease in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Molecular and Cellular Neuroscience.* 1995;6:349-362.

44. Knippenberg S, Thau N, Dengler R, Petri S. Significance of behavioural tests in a transgenic mouse model of amyotrophic lateral sclerosis (ALS). *Behav Brain Res.* 2010;213(1):82-87.

45. Kirkinezos IG, Hernandez D, Bradley WG, Moraes CT. Regular exercise is beneficial to a mouse model of amyotrophic lateral sclerosis. *Annals of neurology.* 2003;53(6):804-807.

46. Hayworth C, Gonzalez-Lima F. Pre-symptomatic detection of chronic motor deficits and genotype prediction in congenic B6. SOD1G93A ALS mouse model. *Neuroscience.* 2009;164(3):975-985.

47. Pfohl SR, Halicek MT, Mitchell CS. Characterization of the Contribution of Genetic Background and Gender to Disease Progression in the SOD1 G93A Mouse Model of Amyotrophic Lateral Sclerosis: A Meta-Analysis. *J Neuromuscul Dis.* 2015;2(2):137-150.

48. Kandinov B, Korczyn AD, Rabinowitz R, Nefussy B, Drory VE. Autonomic impairment in a transgenic mouse model of amyotrophic lateral sclerosis. *Auton Neurosci.* 2011;159(1-2):84-89.

49. Dupuis L, Oudart H, Rene F, Gonzalez de Aguilar JL, Loeffler JP. Evidence for defective energy homeostasis in amyotrophic lateral sclerosis: benefit of a high-energy diet in a transgenic mouse model. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101(30):11159-11164.

50. Dupuis L, Loeffler JP. Neuromuscular junction destruction during amyotrophic lateral sclerosis: insights from transgenic models. *Curr Opin Pharmacol.* 2009;9(3):341-346.

51. Acevedo-Arozena A, Kalmar B, Essa S, et al. A comprehensive assessment of the SOD1G93A low-copy transgenic mouse, which models human amyotrophic lateral sclerosis. *Disease models & mechanisms.* 2011;4(5):686-700.

52. Blaszczyk J, Tajchert K. Sex and strain differences of acoustic startle reaction development in adolescent albino Wistar and hooded rats. *Acta Neurobiol Exp (Wars).* 1996;56(4):919-925.

53. Rohleder C, Jung F, Mertgens H, et al. Neural correlates of sensorimotor gating: a metabolic positron emission tomography study in awake rats. *Front Behav Neurosci.* 2014;8:178.

54. Jouroukhin Y, Ostritsky R, Assaf Y, Pelled G, Giladi E, Gozes I. NAP (davunetide) modifies disease progression in a mouse model of severe neurodegeneration: protection against impairments in axonal transport. *Neurobiol Dis.* 2013;56:79-94.

55. Browne SE, Yang L, DiMauro JP, Fuller SW, Licata SC, Beal MF. Bioenergetic abnormalities in discrete cerebral motor pathways presage spinal cord pathology in the G93A SOD1 mouse model of ALS. *Neurobiol Dis.* 2006;22(3):599-610.

56. Horvitz JC. Dopamine gating of glutamatergic sensorimotor and incentive motivational input signals to the striatum. *Behav Brain Res.* 2002;137(1-2):65-74.

57. Garay PA, Hsiao EY, Patterson PH, McAllister AK. Maternal immune activation causes age- and region-specific changes in brain cytokines in offspring throughout development. *Brain Behav Immun.* 2013;31:54-68.

58. Bilkei-Gorzo A, Otto M, Zimmer A. Environmental modulation of anxiety-related neuronal activity and behaviors. *Behav Brain Res.* 2008;186(2):289-292.

59. Zhang J, Zhang D, McQuade JS, Behbehani M, Tsien JZ, Xu M. c-fos regulates neuronal excitability and survival. *Nat Genet.* 2002;30(4):416-420.

60. Carrion AM, Link WA, Ledo F, Mellström B, Naranjo JR. DREAM is a Ca 2+-regulated transcriptional repressor. *Nature.* 1999;398(6722):80-84.

61. Veldink JH, Bar PR, Joosten EA, Otten M, Wokke JH, van den Berg LH. Sexual differences in onset of disease and response to exercise in a transgenic model of ALS. *Neuromuscul Disord.* 2003;13(9):737-743.

62. Heiman-Patterson TD, Deitch JS, Blankenhorn EP, et al. Background and gender effects on survival in the TgN(SOD1-G93A)1Gur mouse model of ALS. *J Neurol Sci.* 2005;236(1-2):1-7.

63. Choi CI, Lee YD, Gwag BJ, Cho SI, Kim SS, Suh-Kim H. Effects of estrogen on lifespan and motor functions in female hSOD1 G93A transgenic mice. *J Neurol Sci.* 2008;268(1-2):40-47.

64. Vanderhorst VG, Gustafsson JA, Ulfhake B. Estrogen receptor-alpha and -beta immunoreactive neurons in the brainstem and spinal cord of male and female mice: relationships to monoaminergic, cholinergic, and spinal projection systems. *J Comp Neurol.* 2005;488(2):152-179.

65. Sgobio C, Trabalza A, Spalloni A, et al. Abnormal medial prefrontal cortex connectivity and defective fear extinction in the presymptomatic G93A SOD1 mouse model of ALS. *Genes, Brain and Behavior.* 2008;7(4):427-434.

66. Khan S, Lu X, Huang Q, et al. IGFBP2 Plays an Essential Role in Cognitive Development during Early Life. *Adv Sci (Weinh).* 2019;6(23):1901152.

67. Sim SE, Chung YH, Jeong JH, et al. Immunohistochemical localization of insulin-like growth factor binding protein 2 in the central nervous system of SOD1(G93A) transgenic mice. *J Mol Histol.* 2009;40(2):157-163.

68. van den Berg WE, Lamballais S, Kushner SA. Sex-specific mechanism of social hierarchy in mice. *Neuropsychopharmacology.* 2015;40(6):1364-1372.

69. Percie du Sert N, Hurst V, Ahluwalia A, et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *Journal of Cerebral Blood Flow & Metabolism.* 2020;40(9):1769-1777.

3.6 Acknowledgements

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3.7 Tables

Sex	Male *SOD1*^{G93A}				Female *SOD1*^{G93A}			
Housing	Filter Top (FT)		Individually Ventilated Cage (IVC)		Filter Top (FT)		Individually Ventilated Cage (IVC)	
Age [weeks]	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
15	7.69	15.38	13.33	6.67	0.00	0.00	0.00	0.00
16	23.08	15.38	0.00	13.33	0.00	0.00	0.00	8.33
18	76.92	76.92	53.33	53.33	15.38	30.77	33.33	33.33
19	83.33	91.67	80.00	86.67	38.46	46.15	58.33	66.67
20	100.00	100.00	86.67	93.33	69.23	76.92	100.00	100.00
21	100.00	100.00	93.33	93.33	92.31	92.31	100.00	100.00

Table 3.1: Percentage [%] of male and female superoxide dismutase 1 transgenic mice (SOD1^{G93A}) unable to complete the pole test due to falling from the apparatus (two runs per test week). All WT control mice regardless of sex completed the task successfully.

Sex	Male				Female			
Housing	Filter Top (FT)		Individually Ventilated Cage (IVC)		Filter Top (FT)		Individually Ventilated Cage (IVC)	
Genotype	WT	*SOD1*^{G93A}	WT	*SOD1*^{G93A}	WT	*SOD1*^{G93A}	WT	*SOD1*^{G93A}
Total context *freezing* [s]	85.9 ± 8.48	98.9 ± 14.88	119.4 ± 12.36	106.5 ± 16.16	106.5 ± 12.72	167.2 ± 21.75	96.3 ± 12.25	187.1 ± 18.34
Total cue *freezing* [s]	169.4 ± 21.83	183.5 ± 20.01	229.7 ± 18.62	168.7 ± 20.27	189.7 ± 13.49	277.9 ± 22.48	200.9 ± 12.25	288.5 ± 30.99

Table 3. 2: Total time spent freezing in context (7-min test duration) and cue testing (9-min test duration) of the fear conditioning paradigm (FC). Data are presented as mean ± SEM for male and female superoxide dismutase 1 transgenic mice (SOD1^{G93A}) and non-transgenic wild type-like (WT) control littermates. Main effects of genotype were found in female mice for both context and cue testing (both p's < 0.001). A significant 'Sex' by 'Genotype' interaction was evident for context freezing (p = 0.001). In addition, a main effect of 'Sex' (p = 0.001) and a 'Sex' by 'Genotype' interaction (p = 0.001) were detected for context freezing as well as a main effect of 'Sex' (p = 0.001) for cue freezing.

3.7 Supplementary data

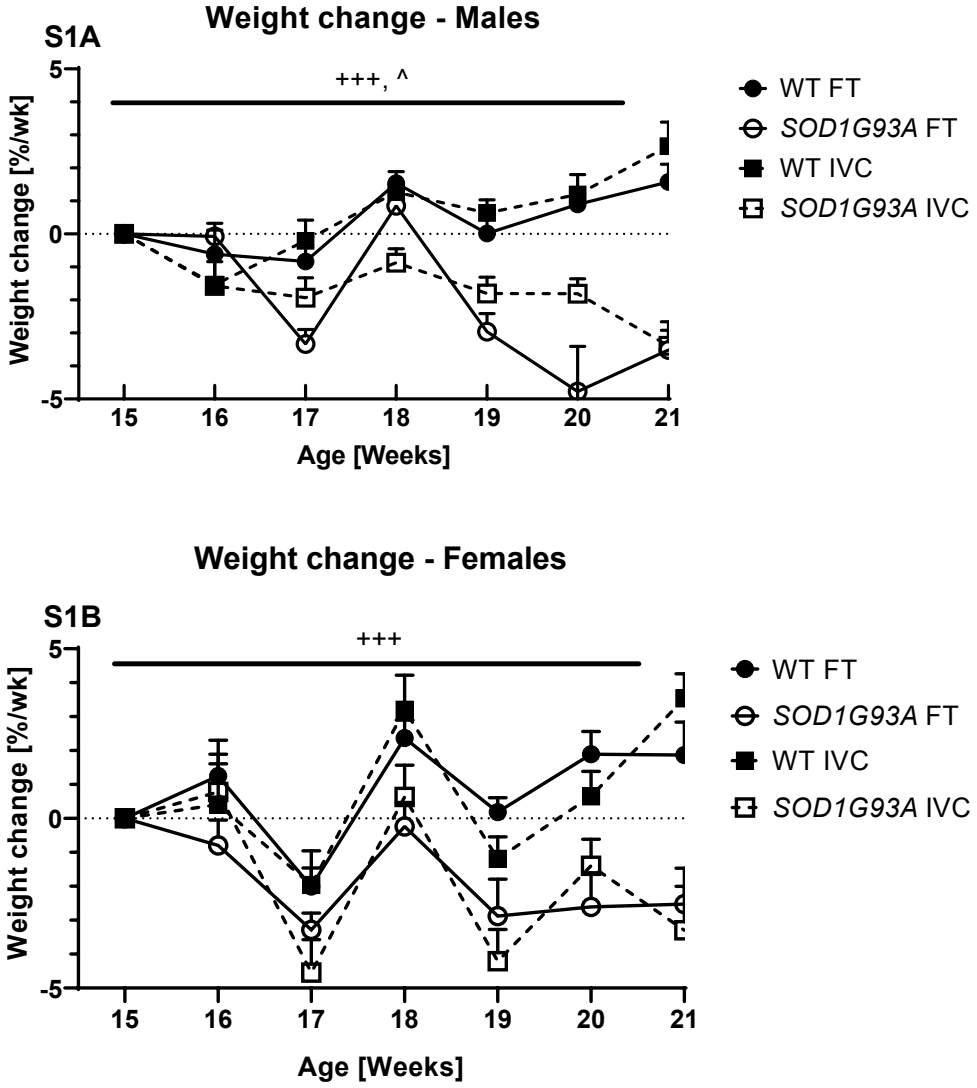


Figure 3S. 1 A-B Percentage rate of body weight change: Body weight change per week [%/wk] is shown for male **(A)** and female **(B)** G93A superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates kept in filter top (FT) or individually ventilated cage systems (IVC). Data are presented as mean ± SEM. 'Week' by 'Genotype' interactions are indicated by $^{+++}p < 0.001$ and a 'week' by 'Housing' interaction is shown by $^{\wedge}p < 0.05$.

Statistical analysis – time spent in chamber during social novelty preference testing: In males, only *SOD1*^{G93A} mice in IVC housing [t(13) = 3.14; p < 0.01] and WT mice in FT housing [trend: t(12) = 2.06; p = 0.06] displayed a preference for the novel mouse) (all other groups: *p's* > 0.08; Fig. S2A). In females, only FT-housed *SOD1*^{G93A} transgenics showed intact social recognition memory [t(12) = 3.3, *p* = 0.006] whereas all remaining experimental groups failed to show a preference for the chamber containing a novel mouse (all *p's* > 0.08) (Fig. S2B).

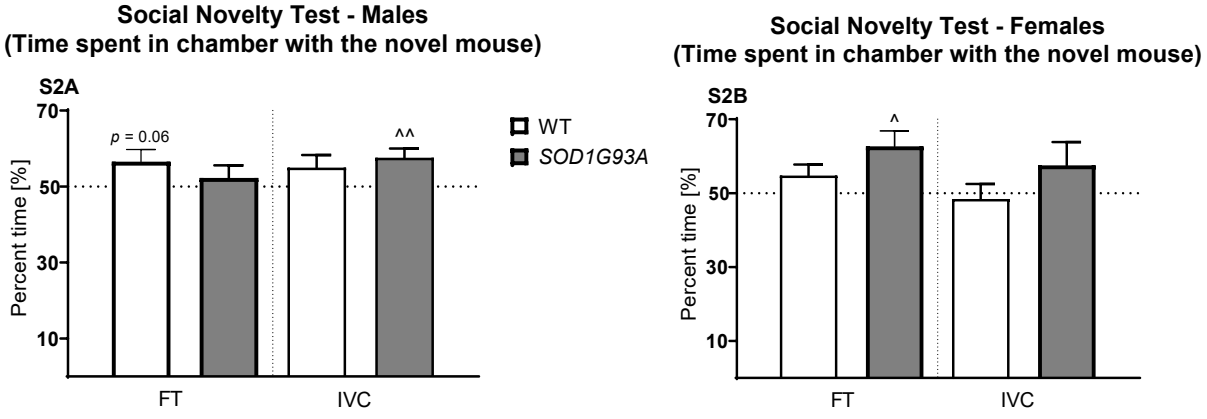


Fig. S2A-B Social recognition memory: Percentage time spent in the chamber containing a novel mouse over a familiar mouse [%]. Data are shown as mean ± SEM for male (A) and female (B) G93A superoxide dismutase 1 transgenic mice (SOD1G93A) and non-transgenic wild type-like (WT) control littermates kept in filter top (FT) or individually ventilated cages (IVC). Significant preferences above chance levels (i.e. 50%, indicated by dotted line) are shown as '^' (^p < 0.05, ^^p <0.01) and a trend is indicated as well. A trend for WT FT males to spend a higher percentage of time in the chamber containing a novel mouse is indicated by 'p = 0.06'.

Chapter 4: Assessment of the potentially disease-modifying effects of a novel *VPS29* polymorphism in the *TDP-43* transgenic mouse model for Amyotrophic Lateral Sclerosis

4.1 Introduction

Traditional genome-wide association studies have typically focused on single loci for causative polymorphisms [1]; however, such a narrow focus likely contributes to the inability to replicate many of the complex features of human disease. Indeed, an analysis of over 600 gene association studies to assess replicability of genetic targets identified found that of those in which the same gene candidate was assessed, only six were able to replicate findings out of the 166 assessed [2]. Thus, it is essential to not only evaluate single gene candidates but to also consider gene-gene interactions (and gene-environment interactions) as those may be equally or more important for disease development than single loci polymorphisms [3]. For example, interactions between fibroblast growth factor 20 and monoamine oxidase B in Parkinson's disease and complex interactions seen with apolipoprotein E in Alzheimer's [4]. In ALS, there is growing evidence of gene-gene interactions [5,6], and due to the large number of potentially causative polymorphisms in both fALS and sALS [7], it is prudent to identify possible gene-gene interactions and evaluate / characterise those potential interactions *in vivo*, for example by using established mouse models for the disease. A large proportion of preclinical research into understanding ALS since 1994 has involved the use of mouse models for *SOD1* polymorphisms, with the most prevalent among them being the *SOD1*^{G93A} mouse model (as outlined in previous chapters). However, in the human ALS population, *SOD1* causative ALS is somewhat rare with mutant *SOD1* being relevant for only 20%

of familial cases and 2% of sporadic ALS cases. Additionally, *SOD1*-related ALS exhibits different pathophysiology, that being the presence of misfolded pathogenic *SOD1* aggregations [8]. however the majority of ALS cases exhibit protein aggregations in motor neurons largely containing pathological or abnormal TAR DNA-binding protein 43 (TDP-43) [9] though mutations in the *TARDBP* gene encoding TDP-43 only account for 4-5% of fALS and approximately 1% of sALS cases [10]. TDP-43 is normally located in the nucleus and is able to shuttle to and from the cytosol, and though the whole gamut of TDP-43's functions is still emerging, it is seen to play key roles in important cellular mechanisms such as splicing, gene expression, neuronal development and embryogenesis [11,12]. Contrary to this, disease inducing mutant TDP-43 in the cytosol is found as a constituent of ubiquitinated protein inclusions in diseased motor neurons and has been linked to a number of cellular dysfunctions such as abnormal neuron function [13], synaptic defects [14] and proteasome dysfunction [15]. The presence of TDP-43-containing protein inclusions are a notable shared pathological feature of ALS and frontotemportal dementia (FTD) contributing significantly to the ALS-Frontotemporal spectrum disorder [16]. Thus, mouse models overexpressing mutant or human wild type TDP-43 are prone to both the cognitive and anxiety phenotypes of FTD and the motor phenotypes of ALS [17].

Recently, a genome-wide association study into rare causes of fALS identified a novel polymorphism in the gene encoding for vacuolar protein sorting-associated protein 29 (VPS29) which was observed to co-localise with mutant TDP-43 in the spinal cord of ALS patients (personal communication with Dr. Shu Yang, Macquarie University, data not published yet).

VPS29, in combination with VPS35 and VPS26, forms what is known as the retromer complex [18] that is structurally conserved across eukaryotic species including mice and humans [19]. Found on the endosomal membrane, the retromer plays a key role in the recycling of transmembrane

receptors [20] and the transport of proteins between the endoplasmic reticulum and the Golgi apparatus [20]. Whilst initially thought to be something of a 'bystander' protein, recent work indicates that VPS29 may potentially serve as a scaffold for the retromer complex and may modulate the sorting response of individual proteins [21]. Interestingly, exome sequencing of an Austrian family found a polymorphism in *VPS35* to be a rare cause of late onset Parkinson's disease [22] indicating that disruption of the retromer complex may have implications for the motor system.

As VPS29 is seen to co-localise with mutant TDP-43 in pathogenic protein inclusions, we hypothesized that there is the potential for disease-modifying interactions (i.e. an earlier onset of, and faster progression of motor impairments) when both are present. Therefore, in this experiment I assessed the disease-modifying potential of overexpressed human wild type and mutant VPS29 via AAV administration on the behavioural phenotype of the iTDP-43^{A315T} transgenic mouse model for ALS.

4.2. Materials and Methods

4.2.1. Animals

Test animals were male (M) and female (F) heterozygous iTDP-43^{A315T} (TDP-43) transgenic mice [*mThy1.2-tTA(41)*] as well as wild type-like (WT) control littermates administered eGFP (GFP), wild type VPS29 (VPS29-WT) or mutant VPS29 (VPS29-MUT) [WT^{GFP}(M = 14, F = 8); TDP-43^{GFP}(M = 6, F = 12); TDP-43$^{VPS29\text{-}WT}$(M = 14, F = 5); TDP-43$^{VPS29\text{-}MUT}$(M =7, F = 9)] inbred on a C57BL/6J background, the creation of which has been described previously [23]. Mice were bred at the animal facility of Macquarie University (Sydney, Australia). Tail tips were taken at PND14 and genotyping was performed by polymerase chain reaction amplification of selective amplicons for tet and iTDP-43^{A315T}. Control and iTDP-43^{A315T} transgenic mice were group-housed (3-5 mice per cage) in Airlaw EVC mouse cages (PPC Moulding services: Villawood, Australia) with corn cob bedding (PuraCob premium: Able Scientific, Perth, Australia) and tissues for nesting material as well as a red igloo (Bioserv, Frenchtown, NJ, USA) and wooden chew stick for enrichment. Mice were kept on a 12 h light/dark cycle (6am - 6pm/6pm – 6am respectively) and food and water were provided *ad libitum*. All research and animal care procedures were approved by the Animal Ethics Committee of Macquarie University (ARA: 2018/019) and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

4.2.2. Adeno-associated virus (AAV) production and administration

Plasmid creation and AAV packaging were performed by the Macquarie University Dementia Research Centre viral core (Sydney, Australia). The wild type (AAV-PHP.B CAG-VPS29-WPRE-bGHpA WT) and mutant (AAV-PHP.B CAG-VPS29-WPRE-bGHpA MUT) forms of *VPS29* were expressed using the CAG promoter of the AAV9.PHP.B capsid as detailed in [24] (hereafter

referred to as VPS29-WT and VPS29-MUT). Similarly, the control vector was expressed using Green Fluorescent Protein (AAV-PHP.B CAG-EGFP-WPRE-bGHpA; referred to as GFP).

For packaging of AAV particles, HEK293T cells were transfected in complete Dulbecco's Modified Eagle Medium (DMEM, Sigma) with 10% foetal bovine serum (FBS) at 70–80% confluence (as described [25]). Culture medium was changed to Iscove's modified Dulbecco's Medium (IMDM, Sigma) with 5% FBS 3 h prior to transfection. Transfection was performed with viral genome-containing plasmid, AAV9-PHP.B plasmid containing rep and cap sequences using polyethyleneimine-Max (PEI-Max, Polysciences) and pFdelta6 as the helper. Cells and supernatant were harvested 72 h post transfection. Supernatant was clarified by adding 40% PEG8000/2.5 M NaCl to a final concentration of 8% PEG8000/0.5 M NaCl and incubated at 4 °C for at least 2 h. Clarified supernatant was centrifuged at 2000g for 30 min. Combined precipitate from clarified supernatant and cell pellet was treated with sodium deoxycholate (final concentration of 0.5%) and benzonase (~ 500 U) at 37 °C for 40 min. After addition of NaCl, incubation at 56 °C for 40 min and freeze–thaw cycle, the solution was centrifuged for 30 minutes at 5000g at a temperature of 4 °C. Purification of supernatants was performed using iodixanol gradient by ultracentrifugation (475,900g for 2 h at 18 °C). AAV particles were concentrated and exchanged into phosphate buffered saline (PBS) in an Amicon 100 kDa 15 ml concentrator at 5000g at a temperature of 4 °C. Titres were determined by quantitative polymerase chain reaction (qPCR) and aliquots were stored at − 80 °C.

AAV vectors were administered by Dr. Annika Van Hummel of the Macquarie University Dementia Research Centre (Sydney, Australia) prior to the commencement of my behavioural testing. 1.2 µl of GFP (5x 10^{13} vg/ml), VPS29-WT (2.39 x 10^{13} vg/ml) or VPS29-MUT (5x 10^{13} vg/ml) vector was injected at 2 sites each bilaterally and the cerebellum into the brains (Fig. 4.i)

of cryo-anesthetised neonatal postnatal day (P) 0 iTDP-43^{A315T} mice as per [26]. Likewise, eGFP was injected into the brains of TDP-43 non-expressing littermates a control.

Figure 4.i Placement of AAV injections into the brain of cryo-anesthetised P0 pups

This methodology has previously been published to show an even spread of AAV throughout the brain [27], however, an increased number of animals were used to account for any variance which may be present.

4.2.3 Behavioural phenotyping

Mice were tested during the first five hours of the light phase (6am-11am) with a minimum inter-test interval of 24 hours. Animals were tested in a number of behavioural tests with relevance to ALS-Frontotemporal spectrum disorder such as motor function in the accelerod, pole test and open field paradigms and anxiety in the elevated plus mas and open field. At completion of testing all test devices were thoroughly cleaned with 70% ethanol. Mice were tested in motor function paradigms fortnightly starting from 6 weeks of age and continued until 14 weeks to track the development and progression of motor symptoms. Mice were also tested in the open field at both 6 and 12 weeks of age and the elevated plus maze was carried out at 13 weeks of age (Fig. ii). Mice were weighed weekly from the ages of 4-13 weeks and bodyweight was analysed across time. Due to the pilot nature of this project, a limited number of tests were selected to allow for a quick turnaround for many animals as well as repeated testing to assess the progression of disease-

relevant phenotypes, and changes thereof, in these animals. At the conclusion of testing, brain and spinal cord tissue were for paraffin sectioning, additionally, cerebellum, hippocampus and cortex were snap frozen proteomics assessment.

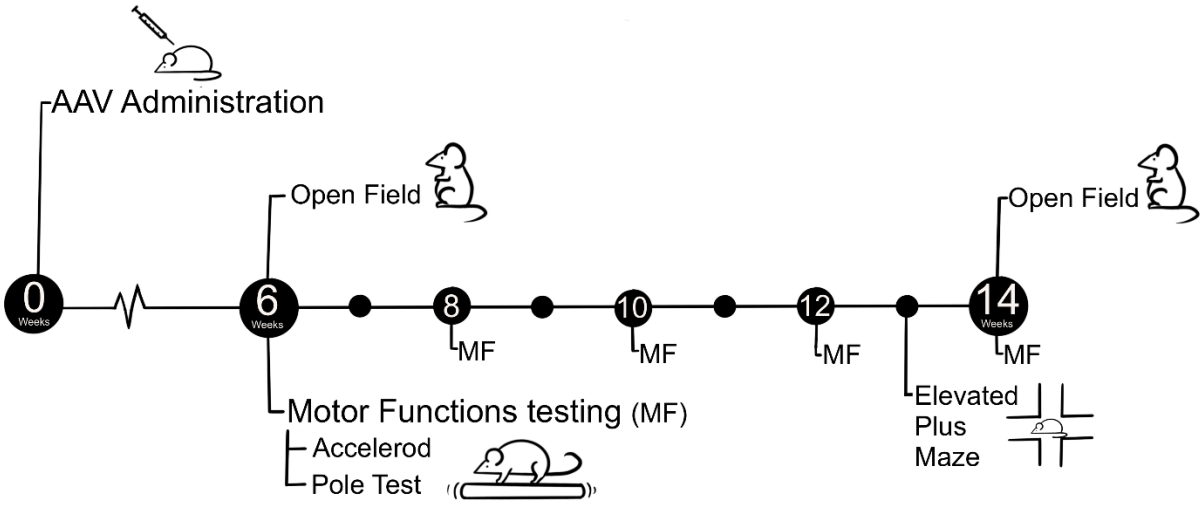


Figure 4.ii Timeline of AAV administration and subsequent behavioural phenotyping

4.2.3.1 Accelerod

To assess motor coordination across time mice were placed on the rotarod apparatus (Ugo Basile, Gemonio, Italy) at a starting speed of 4 RPM, after which an acceleration program of 4-40 RPM ran for 300 seconds (i.e. reaching the top speed at 270 seconds). The latency for mice to fall or loop (defined as when the animal holds on to the rod and spins freely on the rod five consecutive

times) was recorded. The test was performed two times with a 1-hour inter-trial interval (ITI), the average latency from both trials was analysed.

4.2.3.2 Pole test

The pole test was used to assess the degeneration of gross motor co-ordination, balance and motor function in line with our previous paper [28] and what has been observed in this model previously. Mice were placed (facing upwards) on a vertical pole (diameter: 0.8 cm; length: 51 cm) [23]. Mice were allowed 120 s (cut off time) to complete the test. Latency to reach the platform was recorded. Mice who did not complete the test (i.e. were unable to turn, climb down, or fell from the pole) were awarded the maximum test time of 120 s. The test was performed two times with a 1-hour ITI, the total time to complete the test was analysed.

4.2.3.3 Open Field (OF)

To assess locomotion and exploration [29], mice were placed into square red Perspex test chambers for 30 minutes with infrared house lights and infrared cameras for test recording [23]. Test videos were analysed using Any-Maze^{TM} tracking software (Stoelting Co.; Illinois, USA) with the test area divided into a central (25 x 25 cm) and peripheral zone (40 x 40 cm); total distance travelled was automatically measured. In addition, the ratio of distance travelled, time spent in the central zone and velocity over the test duration were used to analyse anxiety-related behaviours.

4.2.3.4 Elevated Plus Maze (EPM)

The EPM measures anxiety-related behaviours as well as exploration and locomotion [30]. Mice were placed in the centre of the apparatus (Ugo Basile, Gemonio, Italy) facing the open arm and

allowed to explore for a total of 5 minutes [23]. The number of entries, total time and total distance spent in the open arms and closed arms was automatically scored using Any-Maze^{TM} tracking software (Stoelting Co.; Illinois, USA). Manual scoring was used for exploratory behaviours (i.e. *head dipping*) and risk assessment behaviour (i.e. stretch-attend postures). Time spent on open arms as well as percentage locomotion on open arms (as a percentage of total time spent on open arms / total distance travelled on arms) were used to assess anxiety-like behaviours. During the course of the testing period, it became apparent that iTDP-43^{A315T} mice were prone to falling off the EPM apparatus. As such, mice who fell were excluded from the test. To be able to analyse some anxiety behaviours, only the first 3 minutes of the test were used for analysis the amended test groups are as follows: WT^{GFP}(M = 14, F = 8); TDP-43^{GFP}(M = 5, F = 11); TDP-43$^{VPS29\text{-}WT}$(M = 7, F = 3); TDP-43$^{VPS29\text{-}MUT}$(M =3, F = 3)

4.2.4 Statistical analysis

Data were initially analysed via two-way ANOVA for 'Sex' and 'Genotype' in addition to three-way repeated measures ANOVA for 'week', 'Sex' and 'Genotype' in the case of motor function testing over time. Where 'Sex' effects and interactions were observed, data were then split for 'Sex' and analysed by one-way ANOVA for 'Genotype' and two-way RM ANOVA for 'week' and 'Genotype' for males and females separately. Overall findings from relevant ANOVA tests are presented below the figure, 'Genotype' effects versus WT^{GFP} are shown in figures as '*' (*p < 0.05, **p < 0.01 and ***p < 0.001). To assess differences between genotypes where a 'Genotype' effect was found, *post-hoc* tests were run using Fisher's LSD test. *Post-hoc* 'Genotype' effects of TDP-43$^{VPS29\text{-}MUT}$ compared to TDP-43$^{VPS29\text{-}WT}$ is indicated in figures as '^' (^p < 0.05, ^^p < 0.01, ^^^p < 0.001), 'Genotype' effects of TDP-43$^{VPS29\text{-}WT}$ and TDP-43$^{VPS29\text{-}MUT}$ compared to TDP-

43^{GFP} is indicated in figures as '#' (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$). All analyses were performed in IBM SPSS Statistics v24.

4.3 Results

4.3.1 Bodyweight per week

Three-way RM ANOVA revealed an interaction of 'Age' by 'Sex' by 'Genotype' [$F(27,477) = 5.37$, $p < 0.001$], therefore data were analysed split by 'Sex'. An 'Age' by 'Genotype' interaction [$F(27,252) = 13.16$, $p < 0.001$] was seen for bodyweight per week in male mice and the data suggest that iTDP-43^{A315T} mice gained less weight as they aged over the test period compared to WT^{GFP} (all *p's* < 0.001). When data were split by 'Genotype' all experimental groups gained weight over the testing period [RM ANOVA for 'age': WT^{GFP} $F(9,90) = 167.7$, $p < 0.001$; TDP-43^{GFP} $F(9,27) = 74.24$, $p < 0.001$; TDP-43$^{VPS29\text{-}WT}$ $F(9,81) = 138.7$, $p < 0.001$; TDP-43$^{VPS29\text{-}MUT}$ $F(9,54) = 65.57$, $p < 0.001$]. Importantly, when data were split by 'Age', one-way ANOVA revealed a main effect of 'Genotype' in each week (all *p's* < 0.001). Further *post-hoc* analysis using Fisher's LSD indicated that iTDP-43^{A315T} groups regardless of vector condition were lighter than WT^{GFP} males from 5 weeks of age onward (all *p's* < 0.001; Fig. 4.1A). Furthermore, TDP-43$^{VPS29\text{-}WT}$ mice were lighter than all other iTDP-43^{A315T} groups from 4 weeks until the end of testing (Fig. 4.1A), no difference was seen between TDP-43^{GFP} and TDP-43$^{VPS29\text{-}MUT}$ mice at any of the weeks analysed (all *p's* > 0.05).

Similarly, an 'Age' by 'Genotype' interaction [$F(27,225) = 2.14$, p 0.013] was detected for bodyweight per week in female mice suggesting that all iTDP-43^{A315T} groups gained weight slower than WT^{GFP} littermates. When data were split by 'Genotype' all females were seen to gain weight over the testing period [RM ANOVA for 'Age': WT^{GFP} $F(9,45) = 98.49$, $p < 0.001$; TDP-43^{GFP} $F(9,72) = 90.5$, $p < 0.001$; TDP-43$^{VPS29\text{-}WT}$ $F(9,36) = 65.49$, $p < 0.001$; TDP-43$^{VPS29\text{-}MUT}$ $F(9,72) = 108.5$, $p < 0.001$]. Split by 'Age', an effect of 'Genotype' was seen at each age (all *p's* < 0.001; Fig. 4.1B). Further *post-hoc* analysis revealed that TDP-43^{GFP} and TDP-43$^{VPS29\text{-}WT}$ females

weighed less than WT^{GFP} littermates at all ages (all $p < 0.047$; Fig. 4.1B) whereas this was only the case from 8-13 weeks of age for TDP-$43^{VPS29\text{-}MUT}$ mice. Furthermore TDP-$43^{VPS29\text{-}WT}$ females weighed less than all other iTDP-43^{A315T} groups over the testing period; Fig. 4.1B).

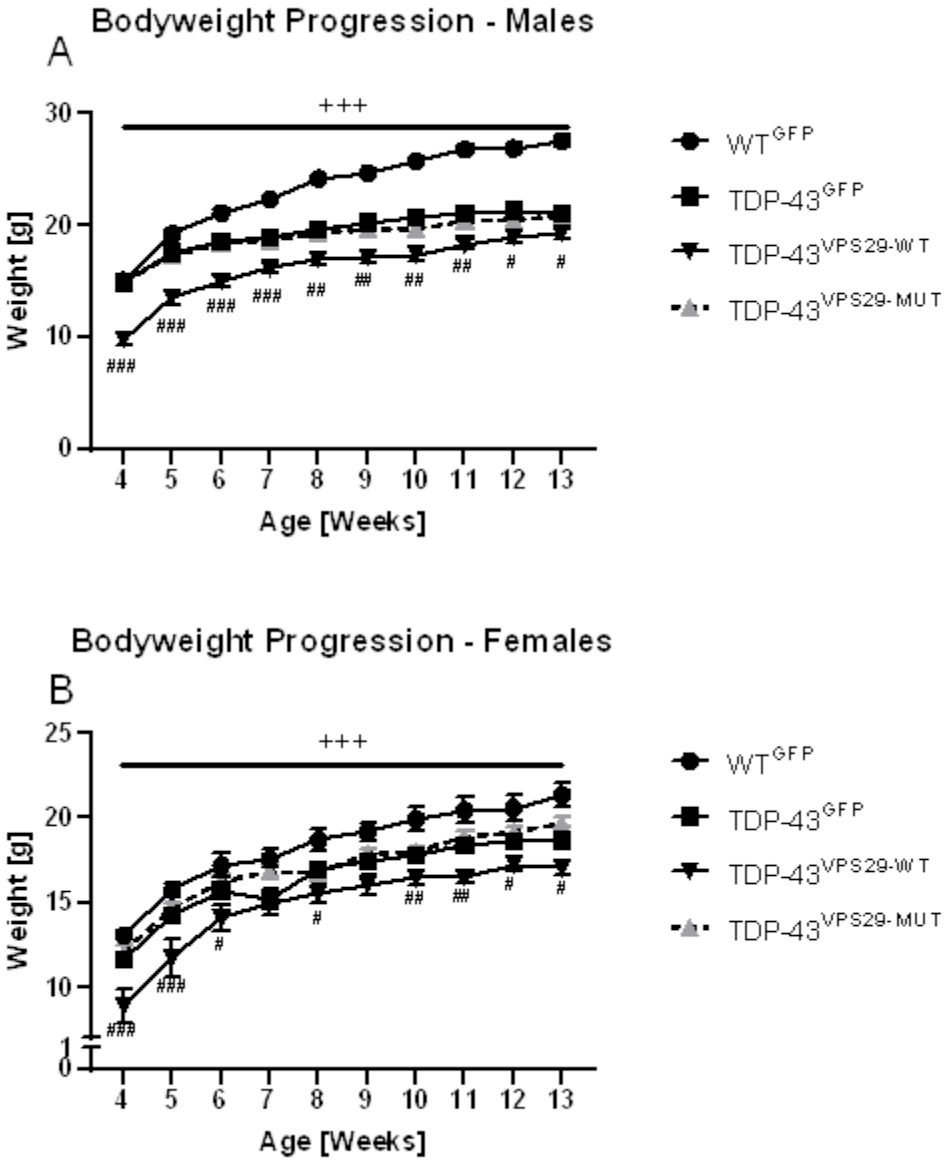


Figure 4.1 A-B Bodyweight progression over testing period: Average weight [g] for male **(A)** and female **(B)** mice over the testing period. Data are shown for green fluorescent protein (GFP), wild type VPS-29 (VPS29-WT) and mutant VPS-29 (VPS29-MUT) administered iTDP-43^{A315T} transgenic mice (TDP-43) and non-transgenic wild type-like (WT) control littermates. For bodyweight analysis cohort numbers were as such WT^{GFP}(Males = 11, Females = 6); TDP-43^{GFP}(Males = 4, Females = 9); TDP-43$^{VPS29\text{-}WT}$(Males = 10, Females = 5); TDP-43$^{VPS29\text{-}MUT}$(Males = 7, Females = 9). Data are presented as mean ± SEM. Interactions of 'Age' by 'Genotype' (as iTDP-43^{A315T} mice gained less weight than WT^{GFP}) are indicated by $^{+++}p < 0.001$. Post-hoc 'Genotype' effects of TDP-43^{GFP} and TDP-43$^{VPS29\text{-}MUT}$ compared to TDP-43$^{VPS29\text{-}WT}$ are indicated as '#' (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$).

4.3.2 Motor function

Accelerod:

Three-way RM ANOVA did not detect an interactions of 'Sex' by 'Age' [$F(4,268) = .259$, $p = 0.90$] or 'Sex' by age' by 'Genotype' [$F(12,268) = 1.19$, $p = 0.29$] for average latency to fall from the accelerod so data were analysed across sex.

When analysing the average latency to fall from the accelerod across test ages, two-way RM ANOVA revealed an interaction of 'Age' and 'Genotype' [$F(12,268) = 1.97$, $p = 0.027$]. Split by 'Genotype', the motor performance of groups did not significantly change across test weeks for WT^{GFP}, TDP-43^{GFP} or TDP-$43^{VPS29\text{-}WT}$ (all p's > 0.09). However, TDP-$43^{VPS29\text{-}MUT}$ mice tended to improve their motor performance in the accelerod over the testing period [RM ANOVA for 'Age': $F(4,56) = 2.37$, $p = 0.064$]. Split by 'Age', one-way ANOVA revealed an overall 'Genotype' effect for each age (all $p < 0.001$). When further assessed by Fisher's LSD tests, all iTDP-43^{A315T} mice regardless of vector condition exhibited lower latencies to fall from the accelerod compared to WT^{GFP} in all test weeks (all p's < 0.006) except from 6 weeks of age when TDP-$43^{VPS29\text{-}WT}$ mice did not show a difference to WT^{GFP} ($p = 0.089$). Furthermore, TDP-$43^{VPS29\text{-}MUT}$ mice exhibited lower latency to fall compared to TDP-$43^{VPS29\text{-}WT}$ at 6 weeks of age (all p's > 0.09 for all other test weeks).

Pole test:

Three-way RM ANOVA detected an 'Age' by 'Sex' by 'Genotype' interaction [$F(12,268) = 1.91$, $p = 0.034$] for the latency to reach the platform. Thus, data were split by sex. A trend for an interaction was seen for 'Age' by 'Genotype' in male mice [$F(12,148) = 1.74$, $p = 0.064$] as iTDP-43^{A315T} mice (in particular TDP-$43^{VPS29\text{-}WT}$ and TDP-$43^{VPS29\text{-}MUT}$) tended to exhibit a more rapidly increasing latency to reach the platform compared to WT^{GFP} mice. However, all male mice needed

increasingly longer to reach the platform over the testing period regardless of vector condition [RM ANOVA for 'Age': WT^{GFP} $F(4,52) = 2.7$, $p = 0.041$; $TDP\text{-}43^{GFP}$ $F(4,20) = 8.25$, $p < 0.001$; $TDP\text{-}43^{VPS29\text{-}WT}$ $F(4,52) = 11.3$, $p < 0.001$; $TDP\text{-}43^{VPS29\text{-}MUT}$ $F(4,24) = 15.65$, $p < 0.001$] (Fig. 4.2A). When data were split by 'Age', an effect of 'Genotype' was seen for each week when analysed by one-way ANOVA indicating male $iTDP\text{-}43^{A315T}$ mice exhibited increased latency to reach the platform compared to WT^{GFP} mice. Further *post-hoc* testing revealed that this was from the age of 8 weeks onwards, with a notable exception at 10 weeks of age at which $TDP\text{-}43^{VPS29\text{-}WT}$ ($p = 0.055$) and $TDP\text{-}43^{VPS29\text{-}MUT}$ ($p = 0.051$) only tended to exhibit higher latency to reach the platform compared to WT^{GFP} and $TDP\text{-}43^{GFP}$ (Fig. 4.2B).

In females, a significant 'Age' by 'Genotype' interaction was found [$F(12,120) = 2.93$, $p = 0.001$] (Fig. 4.2C). Importantly, split by 'Genotype', all $iTDP\text{-}43^{A315T}$ mice (strong trend in $TDP\text{-}43^{VPS29\text{-}MUT}$) regardless of vector condition had worsening latency to reach the platform over the test period [RM ANOVA for 'age': $TDP\text{-}43^{GFP}$ $F(4,44) = 16.1$, $p < 0.001$; $TDP\text{-}43^{VPS29\text{-}WT}$ $F(4,16) = 11.2$, $p < 0.001$; $TDP\text{-}43^{VPS29\text{-}MUT}$ $F(4,32) = 2.61$, $p = 0.054$] whereas this motor performance decline was not evident in WT^{GFP} mice [$F(4,28) = 2.14$, $p = 0.1$]. Split by 'Age', an overall 'Genotype' ANOVA effect was seen at each age starting at age 8 weeks (all $p < 0.037$). Fisher's LSD testing revealed that female $iTDP\text{-}43^{A315T}$ mice exhibited a significantly longer latency to reach the platform compared to WT^{GFP} mice,from the age of 8 weeks old onwards. Only exception was week 10 in which only $TDP\text{-}43^{VPS29\text{-}MUT}$ females exhibited higher latency to reach the platform compared to WT^{GFP} (Fig. 4.2C)

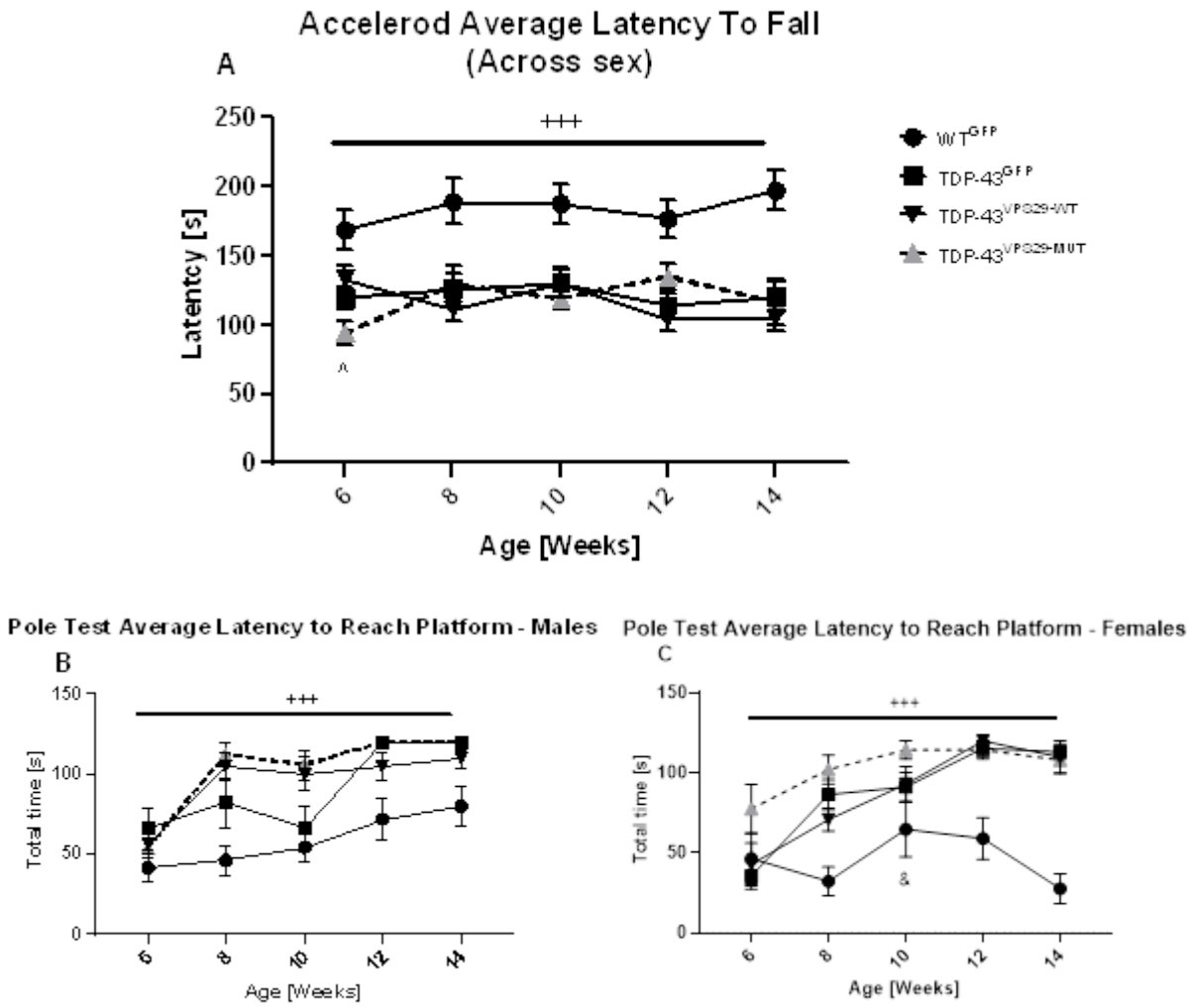


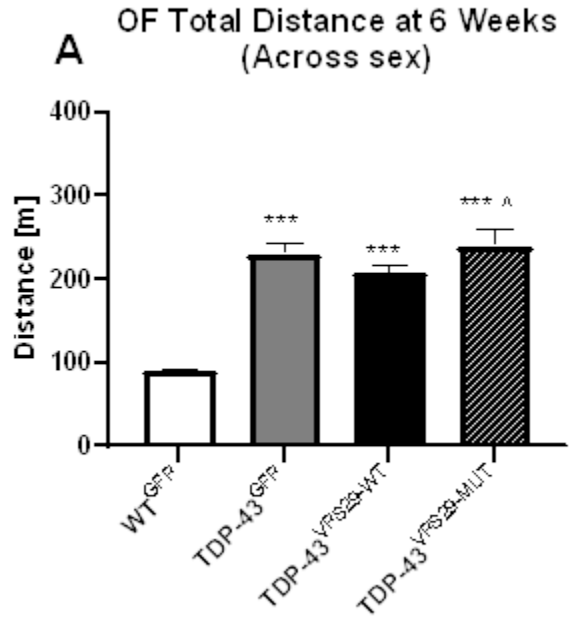
Figure 4.2 A-C Motor function assessment over testing period: (A) Average latency to fall from the accelerod [s] **(B,C)** Average total test time in the pole test [s]. Data are shown for green fluorescent protein (GFP), wild type VPS-29 (VPS29-WT) and mutant VPS-29 (VPS29-MUT) administered iTDP-43^{A315T} transgenic mice (TDP-43) and non-transgenic wild type-like (WT) control littermates. Data are presented as mean ± SEM. Interactions of 'Age' by 'Genotype' as iTDP-43^{A315T} mice exhibited worsening performance compared to WT^{GFP} are indicated as '+' ($^{+++}p < 0.001$). A post-hoc effect of 'Genotype' is indicated by '^' ($^{\wedge}p < 0.05$) as TDP-43 $^{VPS29-MUT}$ mice exhibited lower latency to fall compared to WT^{GFP} and TDP-43$^{VPS29-WT}$ mice at 6 weeks **(A)**. A 'Age' by 'Sex' by 'Genotype' interaction was found for total time in the pole test. **(C)** A trend for female TDP-43$^{VPS29-WT}$ ($p = 0.055$) and TDP-43 $^{VPS29-MUT}$ ($p = 0.051$) to exhibit marginally higher latency to reach the platform compared to WT^{GFP} is indicated by '&'

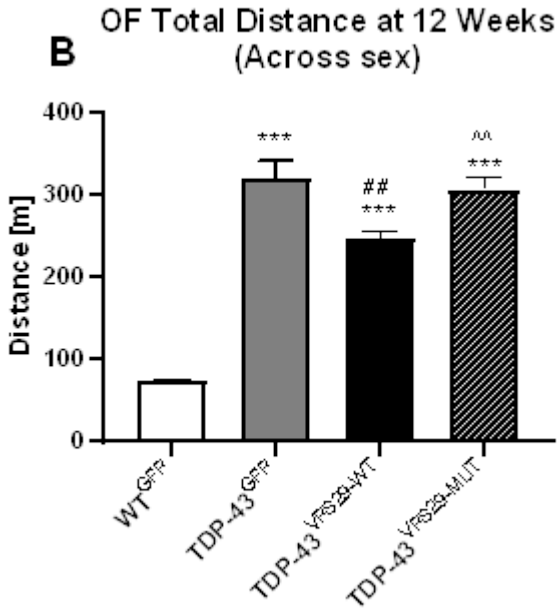
4.3.3 Open field

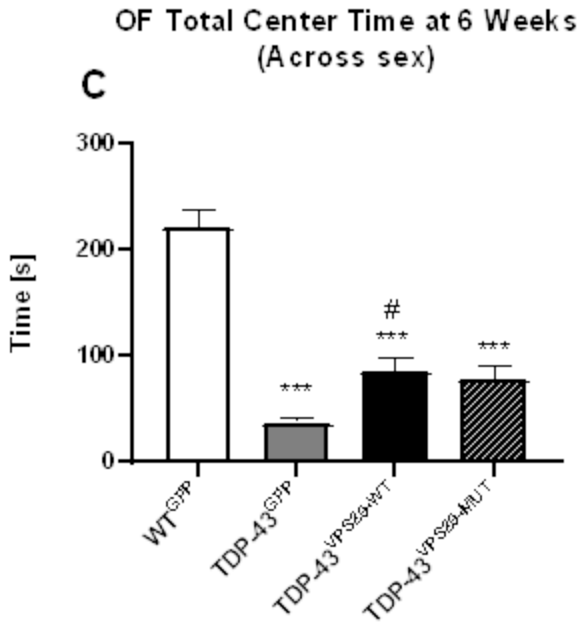
Locomotion: Two-way ANOVA did not detect a main effect of 'Sex' for the total distance travelled in the OF carried out at six [$F(1,67) = 0.45$, $p = 0.50$] and twelve [$F(1,67) = 0.012$, $p = 0.91$] weeks of age so data were analysed across sex. A main effect of 'Genotype' was seen at both ages [six weeks: $F(3,67) = 35.31$, $p < 0.001$ (Fig. 4.3A) - twelve weeks: $F(3,67) = 64.58$, $p < 0.001$ (Fig. 4.3B]. Fisher's LSD tests showed that all iTDP-43^{A315T} mice regardless of vector condition exhibited hyperlocomotion compared to WT^{GFP} mice at six weeks of age (Fig. 3A). Furthermore, TDP-43$^{VPS29\text{-}MUT}$ mice exhibited significantly higher levels of locomotion compared to TDP-43$^{VPS29\text{-}WT}$ (Fig. 4.3A). Similarly, at twelve weeks of age, all iTDP-43^{A315T} mice regardless of vector condition exhibited hyperlocomotion compared to WT^{GFP} mice (Fig. 4.3B) and TDP-43$^{VPS29\text{-}MUT}$ mice had significantly higher locomotion scores than TDP-43$^{VPS29\text{-}WT}$ animals. In addition, the TDP-43$^{VPS29\text{-}WT}$ mice exhibited lower locomotion compared to TDP-43^{GFP} mice at that age (Fig. 4.3B).

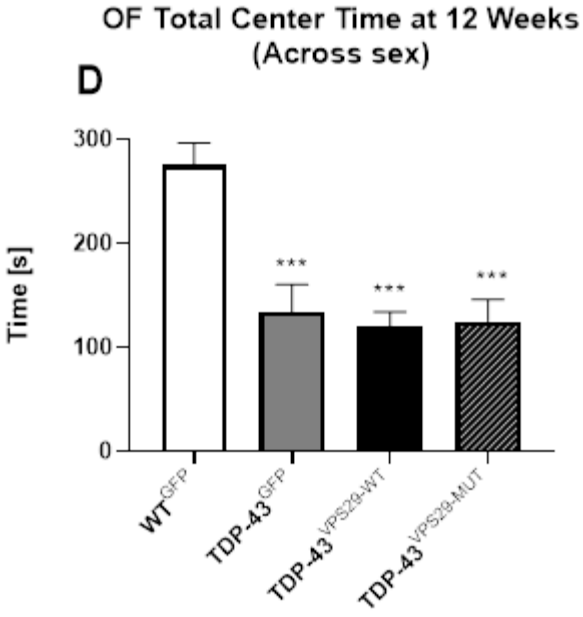
Anxiety behaviours: Two-way ANOVA did not detect main effects of 'Sex' for anxiety-related OF behaviours at either age tested (all *p's* > 0.327) so data were analysed across sex. A main effect of 'Genotype' for total time spent in the centre zone was evident at both six weeks [$F(3,67) = 34.14$, $p < 0.001$ (Fig. 4.3C)] and twelve weeks [$F(3,67) = 11.55$, $p < 0.001$ (Fig. 3D)]. Post hoc testing confirmed that all iTDP-43^{A315T} mice regardless of vector condition spent less time in the OF centre zone compared to their WT^{GFP} counterparts at both ages tested. In addition, TDP-43$^{VPS29\text{-}MUT}$ mice spent more time in the centre zone than TDP-43^{GFP} at six weeks of age (Fig. 4.3C) whereas no further group differences were detected at twelve weeks (Fig. 4.3D). Similarly, a main effect of 'Genotype' was also evident for OF centre distance ratio at both six weeks [$F(3,67) = 136.27$, $p < 0.001$ (Fig. 4.3E)] and twelve weeks [$F(3,67) = 50.33$, $p < 0.001$ (Fig. 4.3F)] of age and *post-hoc*

testing confirmed that all iTDP-43^{A315T} mice regardless of vector condition exhibiting reduced centre locomotion ratios compared to WT^{GFP} mice. No other *post-hoc* differences were detected (all p's > 0.39).


A
OF Total Distance at 6 Weeks
(Across sex)
Distance [m]
400
300
200
100
0
***
***
*** ^
WT GFP
TDP-43 GFP
TDP-43 VPS29-WT
TDP-43 VPS29-MUT


B
OF Total Distance at 12 Weeks
(Across sex)
Distance [m]
400
300
200
100
0
***
##
***
^^
***
WT GFP
TDP-43 GFP
TDP-43 VPS29-WT
TDP-43 VPS29-MUT


C
OF Total Center Time at 6 Weeks
(Across sex)
Time [s]
300
200
100
0
***
#
***
***
WT GFP
TDP-43 GFP
TDP-43 VPS29-WT
TDP-43 VPS29-MUT


D
OF Total Center Time at 12 Weeks
(Across sex)
Time [s]
300
200
100
0
***
***
***
WT GFP
TDP-43 GFP
TDP-43 VPS29-WT
TDP-43 VPS29-MUT

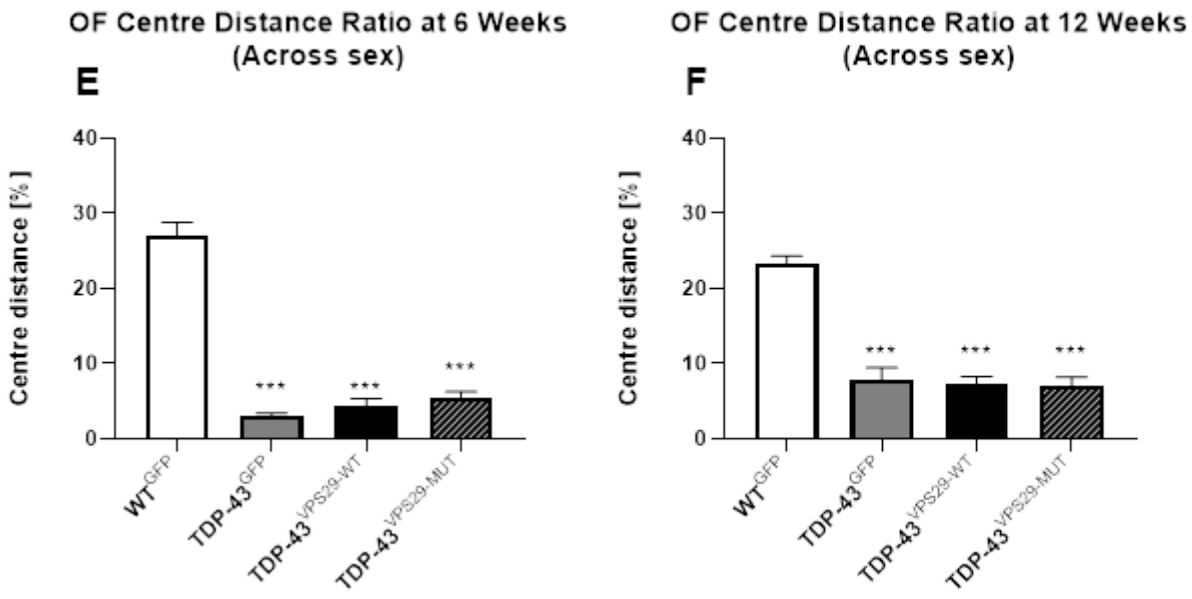


Figure 4.3 A-F Open field assessment: Average total distance [m] travelled at **(A)** 6 and **(B)** 12 weeks of age. Average time spent in the centre zone [s] at **(C)** 6 and **(D)** 12 weeks of age. Average percentage of time spent in the centre zone [%] at **(E)** 6 and **(F)** 12 weeks of age. Data are shown for green fluorescent protein (GFP), wild type VPS-29 (VPS29-WT) and mutant VPS-29 (VPS29-MUT) administered iTDP-43^{A315T} transgenic mice (TDP-43) and non-transgenic wild type-like (WT) control littermates. Data are presented as mean ± SEM. Effects of genotype versus WT^{GFP} are indicated by '*' (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Post-hoc 'Genotype' effects of TDP-$43^{VPS29\text{-}MUT}$ compared to TDP-$43^{VPS29\text{-}WT}$ are indicated in figures as '^' (^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$), 'Genotype' effects of TDP-$43^{VPS29\text{-}WT}$ and TDP-$43^{VPS29\text{-}MUT}$ compared to TDP-43^{GFP} are indicated in figures as '#' (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$).

4.3.4 Elevated plus maze

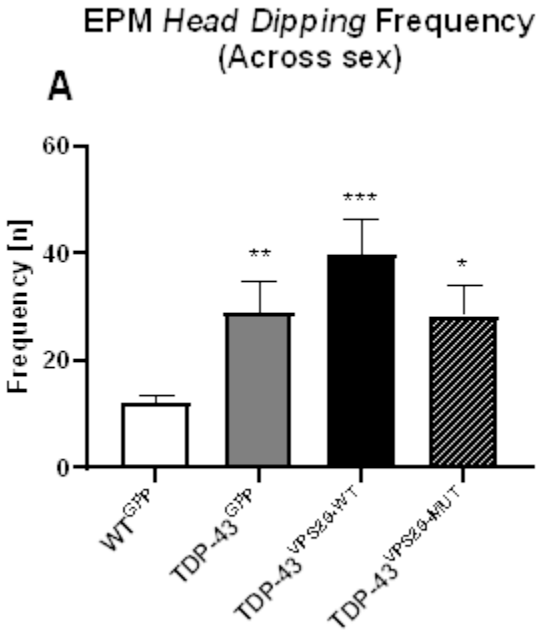
Exploration: Two-way ANOVA did not detect main effects of 'Sex' for exploration-related behaviours in the EPM (all *p's* > 0.55) so data were analysed across sex. A main effect of 'Genotype' [$F(3,46) = 6.83$, $p = 0.001$] for frequency of *head dipping* was evident. *Post-hoc* testing indicated that all iTDP-43^{A315T} mice regardless of vector condition showed more exploration than WT^{GFP} mice (Fig. 4.4A). No difference in *head dipping* frequency was evident between iTDP-43^{A315T} groups (all *p's* > 0.05). Furthermore, a main effect of 'Genotype' [$F(3,46) = 4.46$, $p = 0.008$] was seen for time spent *head dipping* and *post-hoc* testing revealed that TDP-$43^{VPS29\text{-}MUT}$ mice spent significantly more time *head dipping* compared to all other experimental groups (Fig. 4.4B).

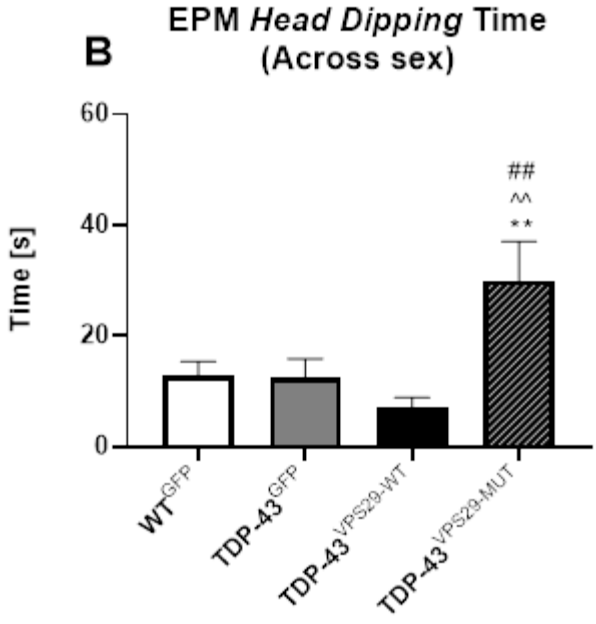
Anxiety behaviours: Two-way ANOVA did not detect main effects of 'Sex' for *stretch attend postures* in the EPM (all *p's* > 0.052) so data were analysed across sex. A main effect of 'Genotype' [$F(3,46) = 4.07$, $p = 0.012$] was detected for the frequency of *stretch attend postures*. Fisher's LSD testing revealed that TDP-$43^{VPS29\text{-}WT}$ mice showed less *stretch attend postures* compared to WT^{GFP} and TDP-43^{GFP} controls (Fig. 4.4C). When analysing time spent in *stretch attend postures* a 'Genotype' was evident [$F(3,46) = 7.37$, $p < 0.001$] with *post-hoc* testing revealing that all iTDP-43^{A315T} mice spent less time in *stretch attend postures* compared to WT^{GFP} counterparts (Fig. 4.4D).

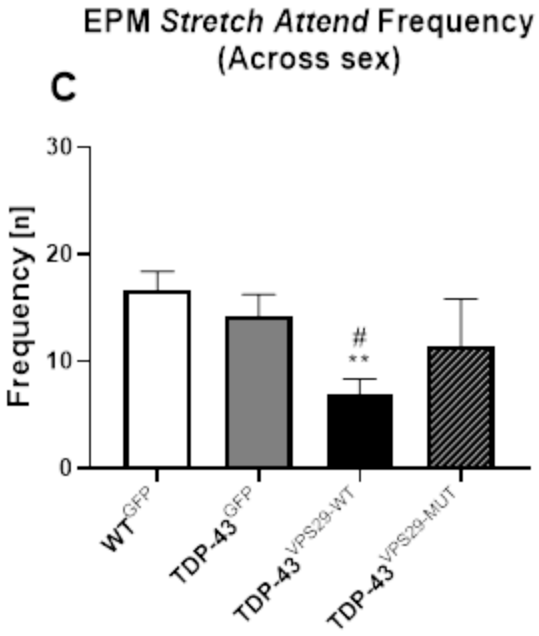
Behavioural performance on the open arms was also analysed. Two-way ANOVA detected a 'Sex' by 'Genotype' interaction for percentage time spent on the open arms of the EPM [$F(3,46) = 3.91$, $p = 0.014$] (Fig. 4.4E-F). Thus, data were split by sex. In male mice, an overall effect of 'Genotype' on percentage open arm time was evident [$F(3,25) = 6.93$, $p = 0.001$]. *Post-hoc* testing revealed that all iTDP-43^{A315T} spent more time on the open arms compared to WT^{GFP} (Fig. 4.4E). In female

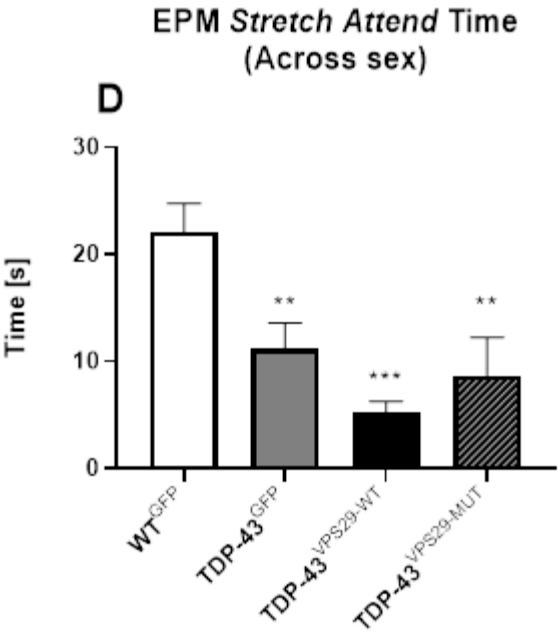
mice, 'Genotype' had a similar effect [F(3,21) = 7.58, p = 0.001] with TDP-43^{GFP} and TDP-43$^{VPS29\text{-}WT}$ mice spending more time on the open arms compared to WT^{GFP} mice and TDP-43$^{VPS29\text{-}WT}$ females spending more time on the open arms compared to all other iTDP-43^{A315T} groups (Fig. 4.4F).

When analysing percentage distance on the open arms, two-way ANOVA detected a 'Sex' by 'Genotype' interaction [F(3,46) = 5.38, p = 0.003] (Fig. 4.4G-H). Thus, data were split by sex. A 'Genotype' effect was evident for male mice [F(3,29) = 7.37, p = 0.001]. Further *post-hoc* testing indicated that only TDP-43$^{VPS29\text{-}MUT}$ mice showed significantly increased open arm locomotion compared to all other groups. No main effect of 'Genotype' was observed in female mice ['Genotype': F(3,21) = 0.7, p = 0.56] (Fig. 4.4H).


EPM *Head Dipping* Frequency
(Across sex)
A
Frequency [n]
60
40
20
0
**
***
*
WT GFP
TDP-43 GFP
TDP-43 VPS29-WT
TDP-43 VPS29-MUT


EPM *Head Dipping* Time
(Across sex)
B
Time [s]
60
40
20
0
##
^^
**
WT GFP
TDP-43 GFP
TDP-43 VPS29-WT
TDP-43 VPS29-MUT


EPM *Stretch Attend* Frequency
(Across sex)
C
Frequency [n]
30
20
10
0
#
**
WT GFP
TDP-43 GFP
TDP-43 VPS29-WT
TDP-43 VPS29-MUT


EPM *Stretch Attend* Time
(Across sex)
D
Time [s]
30
20
10
0
**
***
**
WT GFP
TDP-43 GFP
TDP-43 VPS29-WT
TDP-43 VPS29-MUT

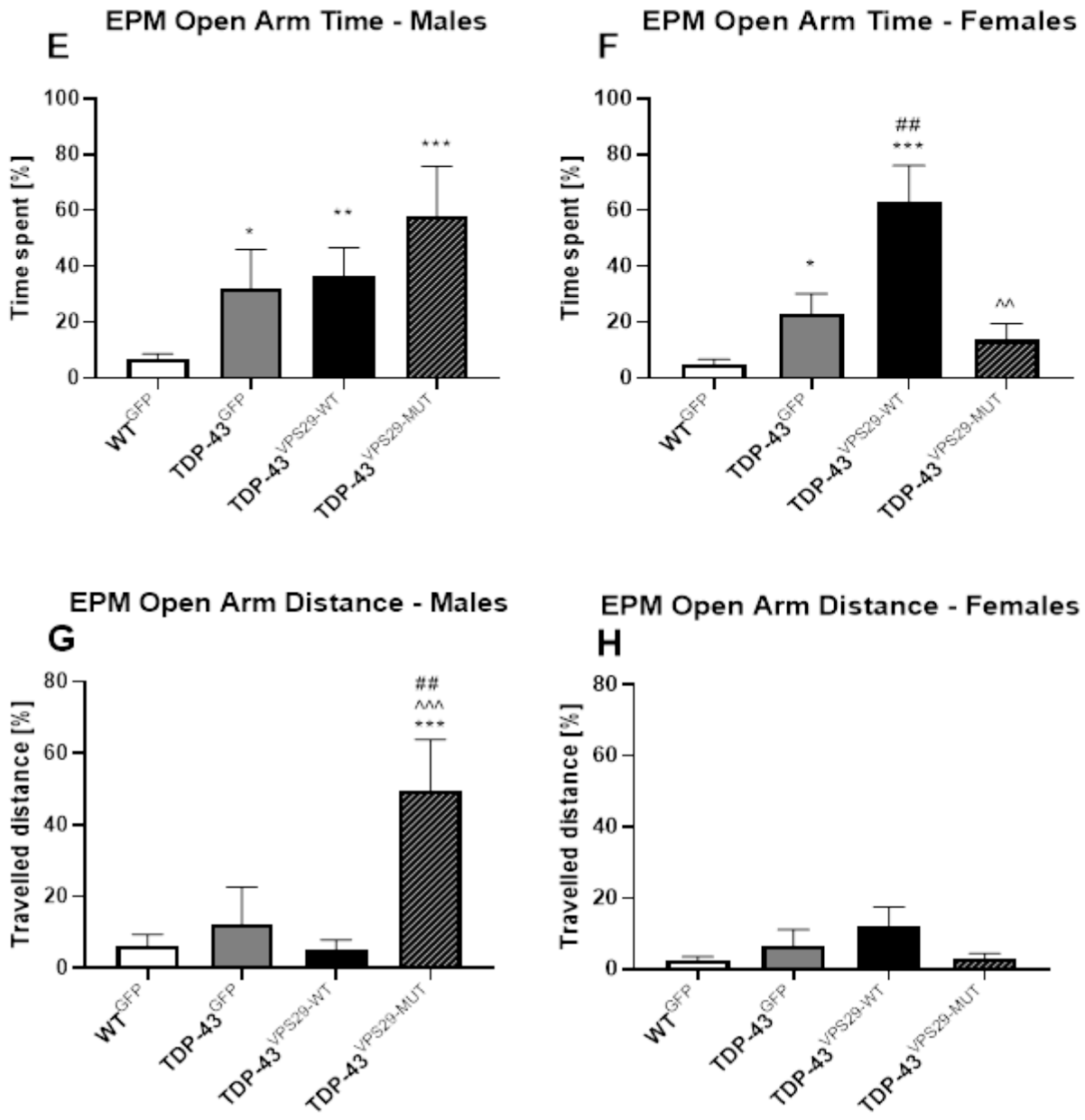


Figure 4.4 A-H Elevated plus maze assessment: Average **(A)** head dipping instances [n] and **(B)** time spent head dipping [s]. Average **(C)** stretch attend instances [n] and **(D)** time spent in stretch attend posture [s]. **(E,F)** Average percentage of total test time spent on the open arm [%]. **(G,H)** Average percentage of arm distance travelled on the open arm [%]. Data are shown for green fluorescent protein (GFP), wild type VPS-29 (VPS29-WT) and mutant VPS-29 (VPS29-MUT) administered iTDP-43^{A315T} transgenic mice (TDP-43) and non-transgenic wild type-like (WT) control littermates. Data are presented as mean ± SEM. Effects of Genotype versus WT^{GFP} are indicated as '*' (*p < 0.05, **p < 0.01, ***p < 0.001). Post-hoc 'Genotype' effects of TDP-43$^{VPS29-MUT}$ compared to TDP-43$^{VPS29-WT}$ are indicated in figures as '^' (^p < 0.05, ^^p < 0.01, ^^^p < 0.001), 'Genotype' effects of TDP-43$^{VPS29-WT}$ and TDP-43$^{VPS29-MUT}$ compared to TDP-43^{GFP} are indicated in figures as '#' (#p < 0.05, ##p < 0.01, ###p < 0.001). A 'Sex' by 'Genotype' interaction was found for percentage time spent on the open arm **(E/F)** and percentage distance travelled on the open arm **(G/H)**.

4.4. Discussion

In this study I evaluated potentially disease-modifying gene-gene interaction of human wild type and mutant VPS29 on the behavioural phenotype of iTDP-43^{A315T} transgenic mice while also considering sex effects. All iTDP-43^{A315T} experimental groups gained less weight compared to WT^{GFP} across age. TDP-43$^{VPS29\text{-}WT}$ mice of both sexes were lighter and gained less weight than other iTDP-43^{A315T} groups. All iTDP-43^{A315T} mice exhibited motor function impairments on the accelerod with TDP-43$^{VPS29\text{-}MUT}$ mice exhibiting more severe motor deficits early compared to TDP-43$^{VPS29\text{-}WT}$ littermates. In the pole test, all iTDP-43^{A315T} mice exhibited worsening motor performance across test weeks, although this was observed in all male mice. All iTDP-43^{A315T} groups regardless of sex exhibited OF hyperlocomotion. Importantly, this phenotype was more prominent in TDP-43$^{VPS29\text{-}MUT}$ mice at six weeks of age compared to other iTDP-43^{A315T} groups. Interestingly, opposing anxiety phenotypes were detected in the OF and EPM as iTDP-43^{A315T} mice displayed an anxiogenic OF phenotype which was less prominent in younger TDP-43$^{VPS29\text{-}WT}$ mice compared to TDP-43^{GFP}. All iTDP-43^{A315T} mice showed lower anxiety behaviour as measured by *protected stretch attend postures* compared to WT^{GFP}. However, open arm activity was sex dependent as all iTDP-43^{A315T} males spent longer on the open arm compared to WT^{GFP}, whereas this was only seen in TDP-43$^{VPS29\text{-}WT}$ females. Moreover, TDP-43$^{VPS29\text{-}MUT}$ males travelled more on the open arm compared to all other male groups, this phenotype was not seen in female mice.

When analysing bodyweight progression, all iTDP-43^{A315T} experimental groups did not gain as much weight and were therefore lighter than their WT^{GFP} littermates regardless of sex. This well-established difference in bodyweight gain in iTDP-43^{A315T} mice compared to controls has been

linked to progressive muscular atrophy in the transgenic mice [23] and is also found in other models including iTDP-43^{M337V} transgenic mice [31] as well as alternate mouse models for the TDP-43^{A315T} mutation [32]. Interestingly, TDP-43$^{VPS29\text{-}WT}$ overexpressing animals did not gain as much bodyweight as the other transgenic groups. This is a novel finding as studies focusing on disease mediated alterations to the retromer complex have not reported alterations to murine bodyweight, though it must be noted that this measure was not explicitly investigated. Importantly, the retromer complex plays a role in the transport of glucose transporter isoform 4 (Glut4) in adipocytes [33] and lowered expression of VPS35 is observed in mouse models for type 2 diabetes [34] indicating that alterations to retromer subunit expressions may impact on bodyweight as mice were seen to be obese. The finding that the wild type from of human VPS29 appears to play a role in bodyweight development in mice requires further investigation, such as the investigation of diabetes-relevant metabolic endpoints and post-mortem fat-pad analysis should be used to further understand VPS29's function in metabolism and bodyweight gain.

iTDP-43^{A315T} mice of both sexes exhibited motor impairments in the accelerod across weeks. Interestingly, at six weeks of age (start of testing), TDP-43$^{VPS29\text{-}MUT}$ mice exhibited a lower latency to fall than TDP-43$^{VPS29\text{-}WT}$ counterparts, which is first *proof of concept* that mutant VPS29 may affect behavioural domains in interaction with the TDP-43^{A315T} mutation. Motor function deficits in the accelerod are a characteristic of the iTDP-43^{A315T} mouse model [23], however, our findings provide novel insights into this phenotype as the original publication on these mice found impairments only at twelve weeks but not four weeks of age [23] (the original study did not test mice at six weeks). Though it must be noted that the test facility may have impacted the advanced development of this motor phenotype for two reasons: firstly, the protocol that was used in the current experiment involved more of a prolonged acceleration of the rotation speed compared to

the initial study (i.e. 4-40 RPM over 300 seconds versus 5-60 RPM over 120 seconds [23]) which is less challenging for the mice but may be more exhausting as the test duration is significantly longer. Indeed, acceleration rate and ultimate speed influence the sensitivity of the accelerod test, for example, a more rapidly accelerating paradigm unsurprisingly led to lower latencies to fall compared to slower acceleration protocols when tested in Withdrawal Seizure-Control mice [35]. Importantly, rapid acceleration may compress fall latency data and hide small effects sizes of motor deficits. Moreover, the effect of test experience or motor learning over trials is seen to increase latency to fall [35]. In line with this, the lower average latency of TDP-43$^{VPS29\text{-}MUT}$ mice to fall at six weeks of age as a result of gene-gene interactions in these mice may be indicative of either an earlier onset of motor deficits or delayed motor learning, as their latency to fall improved once these mice were experienced with the test paradigm. In this context it should be noted that the early deficit observed in the current study may not have been observed in the initial experiments due to the 'best run of five' being recorded on the testing day, so motor learning may have already taken place. Interestingly, whilst overt motor dysfunction in the accelerod at young age has not been observed in this model, gait irregularities are present in these mice as early as at four weeks of age [36]. Another reason for the detection of motor impairments at six weeks of age could be based on differences between test laboratory environments of the two relevant studies. The current experiment was conducted in the Macquarie university (MQ) animal facility whereas the initial behavioural studies were conducted at the University of New South Wales (UNSW). Importantly, there are differences in the home cage systems used between these two test sites. Mice at UNSW were housed in Allentown XJ cages (Allentown Inc.) with wire bars running the length of the cage which allowed for increased climbing opportunities. Housing at MQ is in Airlaw EVC mouse cages (PPC Moulding services: Villawood, Australia) which only include vertical wire bars from

the food hopper thereby reducing climbing opportunities. Thus, the comparatively sedentary conditions, which provide less opportunity for training motor functions in the home cage, may have played a role in the early motor function impairments seen in TDP-43$^{VPS29\text{-}MUT}$ mice in this experiment. Indeed, when 10-week-old C57BL/6 mice were housed in cages that prevented lid climbing, impairments in motor coordination, grip strength and muscle stamina were observed when tested five and ten weeks later [37]. This suggests that a more 'depriving', i.e. less motor behaviour-stimulating, environment may trigger an earlier onset of motor deficits in TDP-43 transgenic mice, in particular if these mice are challenged further by exposing them to another risk variant for the disease. In line with this rationale, previous studies of forced and voluntary running wheel exercise in the *SOD1*^{G93A} transgenic mouse model have shown that motor skill 'training' can delay the onset and progression of disease-relevant phenotypes [38-40] as well as preserve motor neurons when initiated in 70-day-old pre-symptomatic *SOD1*^{G93A} mice [41]. Thus, opportunities to 'practice' motor skills constitute an important confounding factor for the development and progression of ALS-like phenotypes and their severity and onset in mouse models.

iTDP-43^{A315T} mice also exhibited a progressively worsening motor phenotype in the pole test similar to what has been reported in the initial studies on the pole test [23]. Importantly, all male mice including WT^{GFP} mice exhibited increased latencies to reach the platform whereas in females, only iTDP-43^{A315T} female mice progressively worsened across weeks. The progressive impairment in iTDP-43^{A315T} mice of both sexes is in line with the literature [23,36]. The worsening motor performance in male but not female WT^{GFP} mice may be due to the effects of the home cage on motor co-ordination and stamina of male mice in particular (as discussed above) as they are consistently heavier than female mice and bodyweight can negatively impact on the motor performance in mice [42]. It should also be noted here that past studies in the iTDP-43^{A315T} mice have

thus far have only assessed motor function at two discrete ages, those being four and twelve weeks of age [23,36], as such, the current study is the first in which motor function has been assessed longitudinally in these mice allowing for a more in-depth look at how motor function progresses in the iTDP-43^{A315T} mouse model.

iTDP-43^{A315T} mice regardless of sex exhibited a hyperlocomotive OF phenotype. This behaviour was decreased in TDP-43$^{VPS29\text{-}WT}$ mice at six weeks of age compared to TDP-43$^{VPS29\text{-}MUT}$ mice and at twelve weeks of age compared to TDP-43^{GFP} and TDP-43$^{VPS29\text{-}MUT}$ animals. Notably, the lower levels of locomotion in TDP-43$^{VPS29\text{-}WT}$ mice indicate a gene-gene interaction of VPS29-WT with iTDP-43^{A315T} that serves to moderately reverse the hyperlocomotion evident across iTDP-43^{A315T} experimental groups. Similar to the motor phenotype findings, the initial studies into the iTDP-43^{A315T} mouse model found a hyperlocomotive phenotype at twelve weeks but not four weeks of age [23]. Thus, the detection of hyperlocomotion already in 6-week-old iTDP-43^{A315T} mice is novel and highly relevant for the potential planning of early intervention studies as early treatment initiated prior to phenotype onset show the most favourable therapeutic outcomes [43].

iTDP-43^{A315T} animals also exhibited altered exploratory behaviours across tests with transgenic mice showing circling behaviours in the periphery of the OF test arena and increased exploratory behaviours on the EPM evidenced by increased *head dipping* frequency, compared to WT^{GFP}. Though abnormal circling exploration in the OF was noticed in the original study [23], this gives credence to the presence of an abnormal exploration phenotype in these mice as has been observed in the C9orf72 [44] mouse model for FTD, which present with TDP-43 pathology. This is the first time exploration on the EPM has been assessed in iTDP-43^{A315T} mice and notably, only TDP-43$^{VPS29\text{-}MUT}$ mice spent considerably more time *head dipping* compared to all other mice. This indicates that overexpression of mutant VPS29 alters the exploratory phenotype in the iTDP-

43^{A315T} model giving evidence of a gene-gene interaction on this behavioural domain. Increased exploratory behaviour could be thought of as disinhibited or impulsive behaviour and may potentially be a sign of impaired risk assessment behaviour [45] (in line with the findings on reduced *protected stretch attend postures* in the EPM discussed in the following paragraph). Interestingly, some studies do report impulsivity in the EPM being linked to less EPM anxiety [46] as is also evident in our study, however, other studies found reduced anxiety-like behaviours in addition to lower impulsivity (as measured by entries in to the EPM centre zone) [47]. These are important considerations as disinhibitory behaviour is a key symptom of frontotemporal dementia [48] and notably mutations in TDP-43 are observed to sit on of the spectrum between ALS and FTD [49]. Moreover, acute stress is seen to increase impulsivity and reduce inhibition in human FTD patients [50], thus the FTD-like disinhibition seen in this model on the EPM looks to be highly exacerbated and interact with mutant VPS29.

Interestingly iTDP-43^{A315T} mice presented with dichotomous anxiety-like behaviours, spending less time in the centre zone of the open field indicating an anxiogenic-like phenotype but exhibiting reduced anxiety on the EPM as they spent less time in *protected stretch attend* posture and showed more activity on the open arms compared to WT^{GFP} littermates. This anxiety-like phenotype in the OF has been observed in the initial iTDP-43^{A315T} mouse study [23], but again only at twelve but not four weeks of age. Importantly, the increased time spent and distance travelled on the open arms of the EPM were sex-specific and are novel in that no such sex effects were seen in initial studies. In line with the current finding, in the TDP-43^{Q311K} knock-in mouse model, behavioural deficits in female transgenic mice presented later in life and with less severity than their male counterparts [51]. This sex specificity could be linked to estrogen-mediated neuroprotection. Estrogen receptors are abundantly expressed in the central nervous system and play a role in cognitive maintenance

and injury response [52] potentially delaying the degeneration in female TDP-43 transgenic mice. Importantly, only male TDP-43$^{VPS29\text{-}MUT}$ mice travelled further on the open arms compared to all other male experimental groups, an effect that was not seen in any female mice. This indicates that the gene-gene interactions from overexpression of mutant VPS29 might prove to have some sex-dependent characteristics as the overexpression of human wild type VPS29 in these mice exacerbated the development of behavioural deficits in male animals only. However, that sex effects should be considered in more detail in future studies.

Finally, some considerations for potential mechanisms involved in the observed gene-gene interactions on behavioural domains. The retromer complex, of which VPS29 forms a key component, plays a role in the intracellular transport of sortilin [53]. Sortilin is a type I membrane bound glycoprotein abundantly expressed in the central nervous system [54] where it functions as a protein transporter between the plasma membrane, endo/lysosome and Golgi apparatus [55]. It also acts as a receptor and mediator for the endocytosis of progranulin, a protein which is seen to aid the clearance of TDP-43 from the cytosol [56]. Haploinsufficiency of progranulin has been linked to the causation of TDP-43-linked FTD [57]. This is important as *in vitro* knockdown of VPS29 led to retromer fragility and easier degradation by proteasomes [58]. Furthermore, loss of the retromer complex has been shown to increase sortilin in the endosome [59]. Thus, the co-localization of pathogenic TDP-43 and the mutant VPS29 modelled in the current study may impact on retromer function and lead to lower membrane levels of sortilin causing a reduction in intracellular PGRN and a more robust FTD-like phenotype in the TDP-43$^{VPS29\text{-}MUT}$ mice.

In conclusion, the overexpression of a novel VPS29 mutation in the established iTDP-43^{A315T} mouse model for ALS-frontotemporal spectrum disorder did not exacerbate the ALS-relevant phenotype but may impact on motor learning. Moreover, the exaggerated disinhibitory behaviour

observed in mutant VPS29 overexpressing mice in the elevated plus maze may indicate a gene-gene interaction that shifts the iTDP-43^{A315T} phenotype further to the FTD end of the disease spectrum and therefore this is a proof of concept for this model and further behavioural testing for ALS/FTD relevant domains such as memory and cognition should be assessed in the future in this model.

4.5 References

1. Weiss KM, Terwilliger JD. How many diseases does it take to map a gene with SNPs? *Nature genetics.* 2000;26(2):151-157.
2. Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K. A comprehensive review of genetic association studies. *Genetics in medicine.* 2002;4(2):45-61.
3. Cordell HJ. Detecting gene–gene interactions that underlie human diseases. *Nature reviews Genetics.* 2009;10(6):392-404.
4. Fan J, Tao W, Li X, et al. The Contribution of Genetic Factors to Cognitive Impairment and Dementia: Apolipoprotein E Gene, Gene Interactions, and Polygenic Risk. *Int J Mol Sci.* 2019;20(5):1177.
5. Kim NC, Andrews PC, Asselbergs FW, et al. Gene ontology analysis of pairwise genetic associations in two genome-wide studies of sporadic ALS. *BioData mining.* 2012;5(1):1-6.
6. Majoor-Krakauer D, Willems P, Hofman A. Genetic epidemiology of amyotrophic lateral sclerosis. *Clinical genetics.* 2003;63(2):83-101.
7. Mancuso R, Navarro X. Amyotrophic lateral sclerosis: Current perspectives from basic research to the clinic. *Progress in neurobiology.* 2015;133:1-26.
8. Pare B, Lehmann M, Beaudin M, et al. Misfolded SOD1 pathology in sporadic Amyotrophic Lateral Sclerosis. *Sci Rep.* 2018;8(1):14223.
9. Suk TR, Rousseaux MWC. The role of TDP-43 mislocalization in amyotrophic lateral sclerosis. *Mol Neurodegener.* 2020;15(1):45.
10. Alsultan AA, Waller R, Heath PR, Kirby J. The genetics of amyotrophic lateral sclerosis: current insights. *Degenerative neurological and neuromuscular disease.* 2016;6:49.

11. Buratti E, Baralle FE. Multiple roles of TDP-43 in gene expression, splicing regulation, and human disease. *Front Biosci.* 2008 Jan 1;13:867-78.

12. Sephton CF, Good SK, Atkin S, et al. TDP-43 is a developmentally regulated protein essential for early embryonic development. *Journal of Biological Chemistry.* 2010;285(9):6826-6834.

13. Godena VK, Romano G, Romano M, et al. TDP-43 regulates Drosophila neuromuscular junctions growth by modulating Futsch/MAP1B levels and synaptic microtubules organization. *PloS one.* 2011;6(3):e17808.

14. Lin M-J, Cheng C-W, Shen C-KJ. Neuronal function and dysfunction of Drosophila dTDP. *PloS one.* 2011;6(6):e20371.

15. Estes PS, Boehringer A, Zwick R, Tang JE, Grigsby B, Zarnescu DC. Wild-type and A315T mutant TDP-43 exert differential neurotoxicity in a Drosophila model of ALS. *Human molecular genetics.* 2011;20(12):2308-2321.

16. Janssens J, Van Broeckhoven C. Pathological mechanisms underlying TDP-43 driven neurodegeneration in FTLD-ALS spectrum disorders. *Hum Mol Genet.* 2013;22(R1):R77-87.

17. Gendron TF, Petrucelli L. Rodent models of TDP-43 proteinopathy: investigating the mechanisms of TDP-43-mediated neurodegeneration. *J Mol Neurosci.* 2011;45(3):486-499.

18. Seaman MN. The retromer complex–endosomal protein recycling and beyond. *Journal of cell science.* 2012;125(20):4693-4702.

19. Harbour ME, Seaman MN. Evolutionary variations of VPS29, and their implications for the heteropentameric model of retromer. *Communicative & integrative biology.* 2011;4(5):619-622.

20. Bonifacino JS, Rojas R. Retrograde transport from endosomes to the trans-Golgi network. *Nature reviews Molecular cell biology.* 2006;7(8):568-579.

21. Banos-Mateos S, Rojas AL, Hierro A. VPS29, a tweak tool of endosomal recycling. *Curr Opin Cell Biol.* 2019;59:81-87.

22. Zimprich A, Benet-Pagès A, Struhal W, et al. A mutation in VPS35, encoding a subunit of the retromer complex, causes late-onset Parkinson disease. *The American Journal of Human Genetics.* 2011;89(1):168-175.

23. Ke YD, van Hummel A, Stevens CH, et al. Short-term suppression of A315T mutant human TDP-43 expression improves functional deficits in a novel inducible transgenic mouse model of FTLD-TDP and ALS. *Acta Neuropathol.* 2015;130(5):661-678.

24. Deverman BE, Pravdo PL, Simpson BP, et al. Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nat Biotechnol.* 2016;34(2):204-209.

25. Harasta AE, Power JM, von Jonquieres G, et al. Septal Glucagon-Like Peptide 1 Receptor Expression Determines Suppression of Cocaine-Induced Behavior. *Neuropsychopharmacology.* 2015;40(8):1969-1978.

26. von Jonquieres G, Mersmann N, Klugmann CB, et al. Glial promoter selectivity following AAV-delivery to the immature brain. *PloS one.* 2013;8(6):e65646.

27. Ittner A, Chua SW, Bertz J, et al. Site-specific phosphorylation of tau inhibits amyloid-beta toxicity in Alzheimer's mice. *Science.* 2016;354(6314):904-908.

28. Kreilaus F, Guerra S, Masanetz R, Menne V, Yerbury J, Karl T. Novel behavioural characteristics of the superoxide dismutase 1 G93A (SOD1(G93A)) mouse model of amyotrophic lateral sclerosis include sex-dependent phenotypes. *Genes Brain Behav.* 2020;19(2):e12604.

29. Olaya JC, Heusner CL, Matsumoto M, et al. Overexpression of Neuregulin 1 Type III Confers Hippocampal mRNA Alterations and Schizophrenia-Like Behaviors in Mice. *Schizophrenia Bulletin.* 2017.

30. Olaya JC, Heusner CL, Matsumoto M, Shannon Weickert C, Karl T. Schizophrenia-relevant behaviours of female mice overexpressing neuregulin 1 type III. *Behav Brain Res.* 2018;353:227-235.

31. Xu Y-F, Zhang Y-J, Lin W-L, et al. Expression of mutant TDP-43 induces neuronal dysfunction in transgenic mice. *Molecular neurodegeneration.* 2011;6(1):1-14.

32. Stribl C, Samara A, Trumbach D, et al. Mitochondrial dysfunction and decrease in body weight of a transgenic knock-in mouse model for TDP-43. *The Journal of biological chemistry.* 2014;289(15):10769-10784.

33. Yang Z, Hong LK, Follett J, et al. Functional characterization of retromer in GLUT4 storage vesicle formation and adipocyte differentiation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2016;30(3):1037-1050.

34. Morabito MV, Berman DE, Schneider RT, Zhang Y, Leibel RL, Small SA. Hyperleucinemia causes hippocampal retromer deficiency linking diabetes to Alzheimer's disease. *Neurobiol Dis.* 2014;65:188-192.

35. Rustay NR, Wahlsten D, Crabbe JC. Influence of task parameters on rotarod performance and sensitivity to ethanol in mice. *Behav Brain Res.* 2003;141(2):237-249.

36. van Hummel A, Chan G, van der Hoven J, et al. Selective Spatiotemporal Vulnerability of Central Nervous System Neurons to Pathologic TAR DNA-Binding Protein 43 in Aged Transgenic Mice. *Am J Pathol.* 2018;188(6):1447-1456.

37. Roemers P, Hulst Y, van Heijningen S, et al. Inducing Physical Inactivity in Mice: Preventing Climbing and Reducing Cage Size Negatively Affect Physical Fitness and Body Composition. *Front Behav Neurosci.* 2019;13:221.

38. Kirkinezos IG, Hernandez D, Bradley WG, Moraes CT. Regular exercise is beneficial to a mouse model of amyotrophic lateral sclerosis. *Annals of neurology.* 2003;53(6):804-807.

39. Mahoney DJ, Rodriguez C, Devries M, Yasuda N, Tarnopolsky MA. Effects of high-intensity endurance exercise training in the G93A mouse model of amyotrophic lateral sclerosis. *Muscle Nerve.* 2004;29(5):656-662.

40. Carreras I, Yuruker S, Aytan N, et al. Moderate exercise delays the motor performance decline in a transgenic model of ALS. *Brain Res.* 2010;1313:192-201.

41. Deforges S, Branchu J, Biondi O, et al. Motoneuron survival is promoted by specific exercise in a mouse model of amyotrophic lateral sclerosis. *The Journal of physiology.* 2009;587(14):3561-3572.

42. McFadyen M, Kusek G, Bolivar V, Flaherty L. Differences among eight inbred strains of mice in motor ability and motor learning on a rotorod. *Genes, Brain and Behavior.* 2003;2(4):214-219.

43. Benatar M. Lost in translation: treatment trials in the SOD1 mouse and in human ALS. *Neurobiol Dis.* 2007;26(1):1-13.

44. Chew J, Gendron TF, Prudencio M, et al. C9ORF72 repeat expansions in mice cause TDP-43 pathology, neuronal loss, and behavioral deficits. *Science.* 2015;348(6239):1151-1154.

45. Bortolato M, Godar SC, Davarian S, Chen K, Shih JC. Behavioral disinhibition and reduced anxiety-like behaviors in monoamine oxidase B-deficient mice. *Neuropsychopharmacology.* 2009;34(13):2746-2757.

46. Ueno K-i, Togashi H, Mori K, et al. Behavioural and pharmacological relevance of stroke-prone spontaneously hypertensive rats as an animal model of a developmental disorder. *Behavioural pharmacology.* 2002;13(1):1-13.

47. Binder E, Droste SK, Ohl F, Reul JM. Regular voluntary exercise reduces anxiety-related behaviour and impulsiveness in mice. *Behav Brain Res.* 2004;155(2):197-206.

48. Zamboni G, Huey E, Krueger F, Nichelli PF, Grafman J. Apathy and disinhibition in frontotemporal dementia: insights into their neural correlates. *Neurology.* 2008;71(10):736-742.

49. Ling S-C, Polymenidou M, Cleveland DW. Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis. *Neuron.* 2013;79(3):416-438.

50. Dorfman HM, Meyer-Lindenberg A, Buckholtz JW. Neurobiological mechanisms for impulsive-aggression: the role of MAOA. *Neuroscience of aggression.* 2013:297-313.

51. Watkins J, Ghosh A, Keerie AF, Alix JJ, Mead RJ, Sreedharan J. Female sex mitigates motor and behavioural phenotypes in TDP-43 Q331K knock-in mice. *Scientific Reports.* 2020;10(1):1-10.

52. Zarate S, Stevnsner T, Gredilla R. Role of Estrogen and Other Sex Hormones in Brain Aging. Neuroprotection and DNA Repair. *Front Aging Neurosci.* 2017;9:430.

53. Kim E, Lee Y, Lee HJ, et al. Implication of mouse Vps26b-Vps29-Vps35 retromer complex in sortilin trafficking. *Biochem Biophys Res Commun.* 2010;403(2):167-171.

54. Nykjaer A, Willnow TE. Sortilin: a receptor to regulate neuronal viability and function. *Trends Neurosci.* 2012;35(4):261-270.

55. Nielsen MS, Madsen P, Christensen EI, et al. The sortilin cytoplasmic tail conveys Golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein. *EMBO J.* 2001;20(9):2180-2190.

56. Beel S, Herdewyn S, Fazal R, et al. Progranulin reduces insoluble TDP-43 levels, slows down axonal degeneration and prolongs survival in mutant TDP-43 mice. *Mol Neurodegener.* 2018;13(1):55.

57. Cruts M, Gijselinck I, van der Zee J, et al. Null mutations in progranulin cause ubiquitin-positive frontotemporal dementia linked to chromosome 17q21. *Nature.* 2006;442(7105):920-924.

58. Fuse A, Furuya N, Kakuta S, et al. VPS29–VPS35 intermediate of retromer is stable and may be involved in the retromer complex assembly process. *FEBS letters.* 2015;589(13):1430-1436.

59. Canuel M, Lefrancois S, Zeng J, Morales CR. AP-1 and retromer play opposite roles in the trafficking of sortilin between the Golgi apparatus and the lysosomes. *Biochemical and biophysical research communications.* 2008;366(3):724-730.

Chapter 5: The therapeutic potential of cannabidiol in the *superoxide dismutase 1* transgenic mouse model for Amyotrophic Lateral Sclerosis – a pilot study

5.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a rapidly progressing neurodegenerative disease which damages both upper and lower motor neurons leading to muscular atrophy and ultimately death. However, as a result of unclear causative mechanisms the condition is poorly treated with only one widely available disease-modifying drug, Riluzole, though it is only minimally effective in extending life by a clinically insignificant 2 months[1] in the terminal phase of the disease; and one recently approved treatment, Edaravone, that seemingly slows the progression of the disease but only when initiated early [2].

Whilst a significant number of other neuroprotective and immune-modifying drugs have shown initially promising results in mice expressing pathological *SOD1*, there have been no successful candidates thus far coming out of phase II and III human clinical trials [5], with some such as minocycline even accelerating disease progression [6]. Cannabinoids have recently been identified as a novel treatment option for diseases similar in nature to ALS such as multiple sclerosis (MS), due to their anti-excitotoxic and neuroprotective effects and the symptomatic management of the disease [7]. For example, *Sativex* is a cannabis-derived therapy, which contains a close to equal mix of the phytocannabinoids Δ^9-tetrahydrocannabinol (THC: dose of ~27 mg/ml) and cannabidiol (CBD: dose of ~25 mg/ml) and has shown efficacy in the management of neuropathic pain and muscle spasticity in MS [8,9]. Thus, cannabinoids may potentially provide valuable candidates for

the treatment of ALS. Supporting this idea, the two main endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) have been found to be elevated in the spinal cord of *SOD1* and *TDP-43* transgenic mouse models for ALS [10,11]. Furthermore, increase in cannabinoid receptor 2 (CB_2) expression is seen in human forms of ALS [12] as well as in the preclinical mouse models [13] and treatment with a "*Sativex*-like" (containing a mixture of THC and CBD) formulation increased CB_2 receptor proliferation in male and female *SOD1*^{G93A} mice [14]. Indeed, naturally occurring cannabinoids extracted from the *cannabis sativa* plant (i.e. phytocannabinoids) as well as synthetic cannabinoids have been found to have potential as disease-modifying therapeutic agents in ALS mouse models. In the *SOD1*^{G93A} transgenic mouse model, THC not only delayed disease progression, but to also marginally increase the survival of these mice [15]. Additionally, the synthetic CB_2 receptor-specific agonist AM1214 prolonged survival of mice when treatment was started at symptom onset [13].

Importantly, CBD, the primarily non-psychoactive extract from the *cannabis sativa* plant which elicits anti-convulsive [17], anxiolytic [18], anti-nausea [19] and anti-inflammatory actions [20] has not been tested alone in animal models for ALS. Thus, I ran a pilot study assessing the potential therapeutic benefit of remedial CBD in the *SOD1*^{G93A} transgenic mouse model. CBD treatment with a dose of 50mg/kg started at symptom onset and *SOD1*^{G93A} transgenic mice were tested weekly for their motor performance and bodyweight to assess the potential for chronic CBD treatment to reverse or slow down the progression of ALS-relevant phenotypes of this transgenic mouse model. For this pilot study, I focused on female *SOD1*^{G93A} mice due to the well documented later symptom onset and slower rate of motor function degeneration in females compared to male *SOD1*^{G93A} mice [21], which enables a more sensitive assessment of potentially small treatment effects on this ALS transgenic model.

5.2 Materials and Methods

5.2.1 Animals

Test animals were female heterozygous *superoxide dismutase 1 G93A* transgenic mice [*SOD1*^{G93A}: B6-Tg(SOD1-G93A)1GUr/j; The Jackson Laboratory Stock Number: 004435] as well as wild type-like control littermates (n = 6-7). Mice were bred at Australian BioResources (ABR Moss Vale, Australia). Genotyping was performed post weaning (postnatal day 21) by polymerase chain reaction amplification of selective amplicons for the knockout allele. Breeding colonies at ABR were housed in individually ventilated cages (Type Mouse Version 1; Airlaw, Smithfield, Australia; air change: 90-120 times per hour averaged; passive exhaust ventilation system). The test mice were transported to the animal facility at Western Sydney University (School of Medicine, Campbelltown, Australia) at six weeks of age. Control and *SOD1*^{G93A} transgenic mice were group-housed (2-3 mice per cage) in a quasi-randomised manner in IVC cages (GM500 Green, Techniplast Australia Pty Ltd, Rydalmere, Australia; system uses active airflow of 75 air exchanges per hour) with corn cob bedding (PuraCob premium: Able Scientific, Perth Australia) and tissues and 'crinkle nest' for nesting material. Mice were kept on a 12h light dark cycle (9:00-21:00) and food and water were provided *ad libitum*. All research and animal care procedures were approved by the Western Sydney University Animal Care and Ethics Committee (#A12905) and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

5.2.2 Drug preparation and administration

Powdered cannabidiol was initially dissolved in 100% ethanol followed by Tween80 in equal parts and finally diluted with 0.9% biological saline. Ethanol and Tween80 comprised 10% of the final volume. The vehicle control was made via the same method without the addition of powdered

CBD. Vehicle (VEH) or CBD at a dose of 50 mg/kg were administered via intraperitoneal (i.p.) injection using a volume of 10 ml/kg. The dose of 50 mg/kg was chosen as it had shown efficacy in previous studies performed in our lab in a transgenic model for Alzheimer's disease [22].

WT and *SOD1*^{G93A} female mice were assigned in a quasi-random manner to either VEH or CBD (WT-VEH n = 7; WT-CBD n = 6; *SOD1*^{G93A} -VEH n = 6; *SOD1*^{G93A} -CBD n = 6) and treated daily commencing at 15 weeks ± 1 week of age and continuing until the completion of the experiments in week 22 ± 1 (total of 49 days of treatment). Injections were administered in the afternoon between 1400-1600 to avoid acute effects of CBD during behavioural testing, which was carried out in the morning (09:00-14:00; i.e. first half of the light phase). Mice were weighed every six days to ensure correct CBD dosage.

5.2.3 Behavioural phenotyping

At 15 weeks (±1 week) of age mice were initially tested in all behavioural paradigms to establish a baseline phenotype (drug-free, VEH / CBD administration only started after completion of the initial motor phenotype assessment in week 15). Following this, all mice received treatment for 3 weeks (i.e. week 15 – week 18) before behavioural testing was resumed at 18 weeks of age. Behavioural paradigms were run weekly (day 1: accelerod; day 2: pole test, PaGE test) and ran concurrently with daily treatments (testing in the morning, treatment in the afternoon) until 21 weeks of age (Fig 5.i.). Mice were tested during the first five hours of the light phase (09:00-14:00) with a minimum inter-test interval of 24 hours. Animals were tested in a number of behavioural test with relevance to motor impairments in ALS which are established in our lab [23]. Mice were assigned to treatment groups prior to the start of testing as bodyweights were required to determine injection volumes, weight was then recorded every six days to inform these volumes. Age and

order of testing are described in Table 1. At completion of testing all test devices were thoroughly cleaned with 80% ethanol.

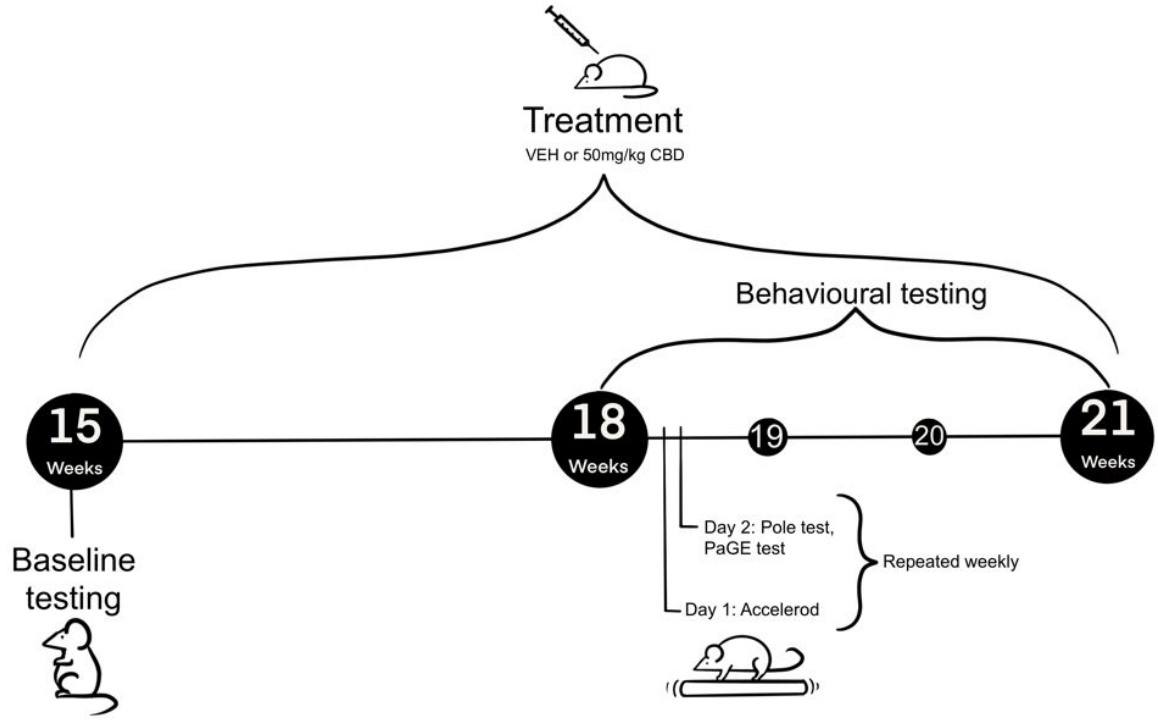


Figure 5.i Timeline of CBD treatment and behavioural testing.

5.2.3.1 Accelerod

To assess the potential for CBD to improve motor coordination across time in the *S* ALS model, mice were first trained on the rotarod apparatus (ENV-574M, MED Associates Inc., St Albans, VT, USA) at a fixed speed of 12 rotations per minute (RPM) for two minutes similar to work previously performed in our lab [23]. This training was repeated a total of three times one day prior to the first test to ensure that the mice understood the task. On the test day, mice were placed on the rotarod apparatus at a starting speed of 4 RPM, after which an acceleration program of 4-40

RPM ran for 300 seconds (i.e. reaching the top speed at 270 seconds). The latency for mice to fall from the rod was recorded. The test was performed three times with a 1-hour inter-trial interval (ITI), the average run time from both trials was analysed.

5.2.3.2 Pole test

The pole test was used to assess the degeneration of gross motor co-ordination, balance and motor function in line with our previous paper [24]. Mice were placed (facing upwards) on a vertical pole (diameter: 1 cm; length: 51 cm) similar to work previously performed in our lab [23]. Mice were allowed 60 s (cut off time) to complete the test. Latency to reach the platform was recorded. Mice who did not complete the test (i.e. were unable to turn or climb down, or fell from the pole) were awarded the maximum test time of 60 s. The test was performed three times with a 1-hour ITI, the average time to turn on the pole and average total test time were analysed.

5.2.3.2 Paw grip endurance (PaGE) test

To mor specifically assess the effects of CBD treatment on grip strength and grip endurance, the PaGE test was adapted from Weydt Et al. [25]. Mice were initially placed on a 1 cm x 1 cm wire grid and gently pulled by the base of the tail to ensure gripping of the bars. Once attached, mice were inverted on a frame ~70cm above an empty cage containing fresh bedding. Mice were assessed for the duration they were able to hold on to the wire grid with a maximum allowed time of 90 seconds. Mice who did not complete the test (i.e. were unable to grip the wire grid or hold until the timer was started) were awarded the minimum test time of 0 seconds. The test was performed three times with a 1-hour ITI, the average of the three runs was analysed.

5.2.4 Statistical analysis

Data were initially analysed using two-way analysis of variance (ANOVA) for the main between test parameters ‘Genotype’ and ‘treatment’ as well as three-way repeated measures (RM) ANOVA

for the within measures: ‘age’ (weekly body weight development and motor function tests across age). Where significant interactions were found data were split for corresponding factors and further analyses carried out. Mice were analysed in their assigned treatment groups from the beginning of the experiment prior to CBD administration. Data are shown as means ± standard error of the mean (SEM). F-values and degrees of freedom are presented for ANOVAs and significant ‘Genotype’ effects *versus* corresponding WT females are shown in figures and tables as ‘*’ (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). Treatment effects *versus* corresponding VEH group are indicated using ‘#’ ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$) and interactions between ‘Genotype’ and ‘treatment’ are shown as ‘+’ ($^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$). All analyses were performed in IBM SPSS Statistics v24.

5.3. Results

5.3.1 Bodyweight development

Prior to the start of treatment (at 15 weeks of age), two-way ANOVA revealed a main effect of 'Genotype'. *SOD1*^{G93A} transgenic mice had a lower initial bodyweight [$F(1,21) = 5.36$, $p = 0.031$] compared to their WT littermates (Table 5.1), no effect of assigned future treatment was observed ($p > 0.87$).

Across the experimental treatment period, three-way RM ANOVA revealed an interaction of 'age' by 'Genotype' [$F(8,180) = 13.1$, $p = 0.002$] as *SOD1*^{G93A} animals lost weight during the seven weeks of testing whereas WT mice did not (Figure 5.1). This difference was confirmed when data were split by 'Genotype' - an overall effect of 'Age' on bodyweight was seen in *SOD1*^{G93A} animals [two-way RM ANOVA: $F(8,72) = 7.67$, $p = 0.009$] but not WT control mice [$F(8,88) = 7.67$, $p = 0.57$]. Two-way ANOVA split by 'Age' revealed that *SOD1*^{G93A} mice displayed significantly lower bodyweights each week from an age of 15 weeks onwards compared to control littermates (Figure 1). CBD treatment did not affect the bodyweight development of either experimental group over the treatment period (no main 'Treatment' effect and no interactions with 'Age' or 'Genotype' or both: all p's > 0.1).

Figure 1

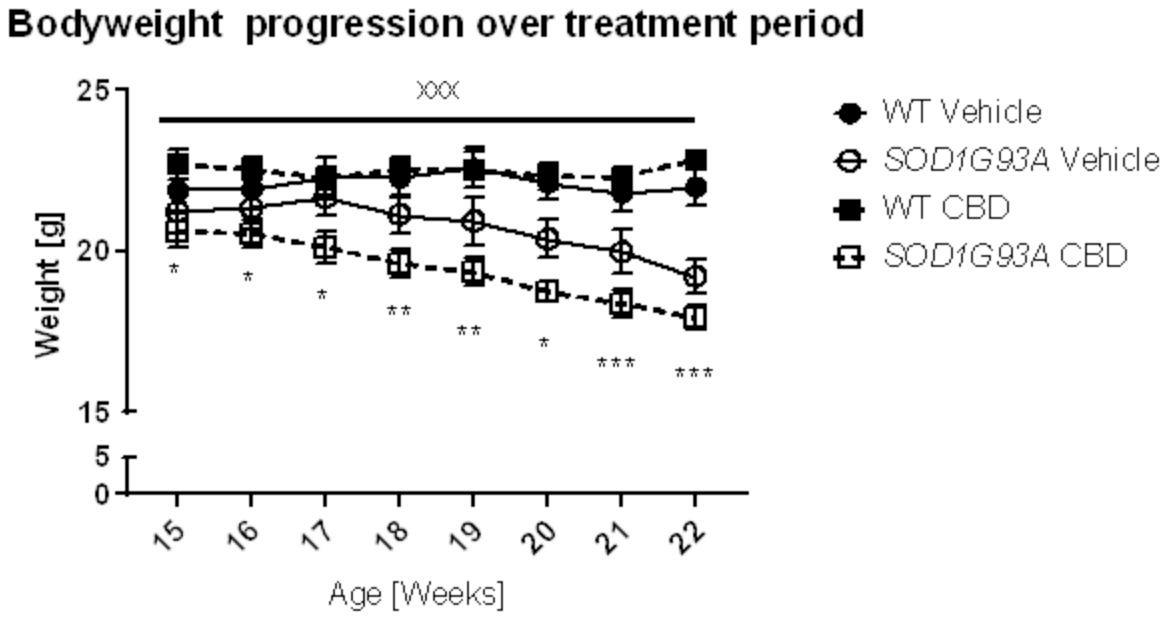


Figure 5. 1- Bodyweight development across time: Bodyweight per week [g] for vehicle-treated (VEH) and cannabidiol-treated (CBD) female superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates. Data are presented as mean ± SEM. Two-way ANOVA main effects of 'Genotype' versus corresponding WT females are shown as '*' (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). A significant three-way RM ANOVA interaction between 'Age' and 'Genotype' is shown as $^{xxx}p < 0.001$.

5.3.2 Motor function

3.2.1 Baseline – prior to start of CBD treatment:

Two-way ANOVA for latency to fall from the accelerod prior to the commencement of CBD/vehicle treatment revealed a main effect of 'Genotype' as *SOD1*^{G93A} transgenic mice exhibited worse motor performance on the accelerod compared to WT littermates [$F(1,21) = 47.1$, $p < 0.001$] (Table 5.1), no main effects or interactions for pre-assigned 'Treatment' groups were observed ($p > 0.65$). In the pole test, when assessing average time to turn on the pole, two-way ANOVA revealed a main effect of pre-assigned treatment [$F(1,21) = 6.14$, $p = 0.022$] as mice assigned to the future CBD treatment group showed an initially faster time to turn compared to those assigned to receive vehicle (Table 5.1). No effects of or interactions with genotype were seen (all $p > 0.05$). Analysis of the average latency to reach the platform revealed no main effects or interactions of 'Genotype' or pre-assigned treatment (Table 1). Finally, in the PaGE test, *SOD1*^{G93A} mice exhibited worse grip endurance compared to WT littermates [$F(1,21) = 11.75$, $p = 0.003$] (Table 5.1). No main effect of CBD or interactions with pre-assigned 'Treatment' were observed ($p > 0.92$).

Treatment	Vehicle		Cannabidiol (CBD)	
Genotype	**WT**	***SOD1^{G93A}***	**WT**	***SOD1^{G93A}***
Bodyweight				
Pre-treatment bodyweight [g] *	21.871 ± .629	21.217 ± .681	22.673 ± .466	20.603 ± .523
Accelerod				
Latency to fall [s] ***	301.62 ± 5.034	200.944 ± 17.38	291.83 ± 9.738	203.83 ± 19.78
Pole test				
Time to turn [s] #	15.92 ± 5.734	16.68 ± 3.935	7.25 ± 2.643	5.60 ± 0.868
Time to reach platform [s]	23.16 ± 5.774	23.71 ± 3.716	14.18 ± 2.994	19.42 ± 5.337
PaGE test				
Latency to fall from grid [s] **	89.76 ± 0.238	57.29 ± 12.73	87.94 ± 2.056	57.46 ± 14.18

Table 5. 1- Baseline phenotype: Bodyweight and motor function for all experimental groups prior to commencement of CBD treatment at 15 ± 1 weeks of age. Data are presented as mean ± SEM for female superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates. Two-way ANOVA main effects of 'Genotype' are shown as '*' (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) and 'Treatment' effects are shown as '#' (#$p = 0.022$).

3.2.2 After 3 weeks of CBD administration

Accelerod: Three-way RM ANOVA revealed that *SOD1*^{G93A} mice developed progressively worsening motor performance over weekly testing compared to WT littermates ['Age' by 'Genotype': $F(3,63) = 11.87$, $p < 0.001$]. This was confirmed when data were split by 'Genotype'. *SOD1*^{G93A} transgenic females exhibited a significant decline in latency to fall off the accelerod across weeks [$F(3,30) = 26.94$, $p < 0.001$] whereas WT did not [$F(3,33) = 0.53$, $p = 0.67$]. When data were split by 'Age' a significant effect of 'Genotype' was seen for all weeks after the initial treatment phase (all p's < 0.01) (Figure 5.2). No main effect of CBD or interactions with 'Genotype' or 'age' were observed (all p's > 0.68).

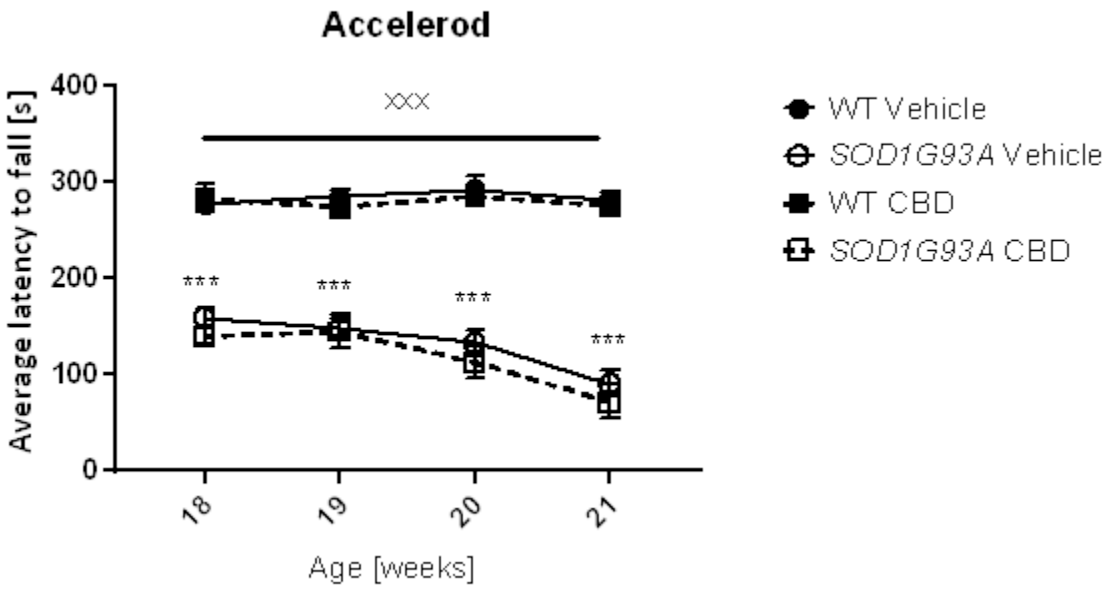


Figure 5. 2- Accelerod assessment over testing period: Average latency to fall from the accelerod [s] after the initial 3-week CBD treatment period. Data are shown for vehicle-treated (VEH) and cannabidiol-treated (CBD) female superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates. Two-way ANOVA main effects of genotype versus corresponding WT of the same week are shown as ***$p < 0.01$. A significant three-way RM ANOVA interaction between 'Age' and 'Genotype' is shown as $^{xxx}p < 0.001$.

Pole test: In the pole test, a significant 'Age' by 'Genotype' interaction was found [$F(3,63) = 6.14$, $p = 0.005$] as $SOD1^{G93A}$ mice took progressively longer to turn compared to WT littermates with increasing age (Figure 5.3A). Indeed, split by 'Genotype', a main effect of 'age' [$F(3,30) = 5.8$, $p = 0.003$] was evident in $SOD1^{G93A}$ females whereas WT females did not change performance across time [$F(3,33) = 1.61$, $p = 0.21$]. When data were split by 'Age' a significant two-way ANOVA main effect of 'Genotype' was seen for all weeks (all p's < 0.01). No CBD main effects or interactions with 'Genotype' or 'Age' were seen (all p's > 0.05).

Analysing the average latency to reach the platform over the testing period, three-way RM ANOVA revealed a strong trend for an 'Age' by 'Genotype' interaction [$F(3,63) = 2.68$, $p = 0.055$] as $SOD1^{G93A}$ transgenic females tended to take increasingly longer to finish the test than their WT littermates (Figure 5.3B). When data were further split by 'Age', a significant effect of 'Genotype' was seen for each week after the initial 3-week CBD treatment phase (all p's < 0.01). No CBD main effect or interactions with 'age' or 'Genotype' were observed (all p's > 0.05).

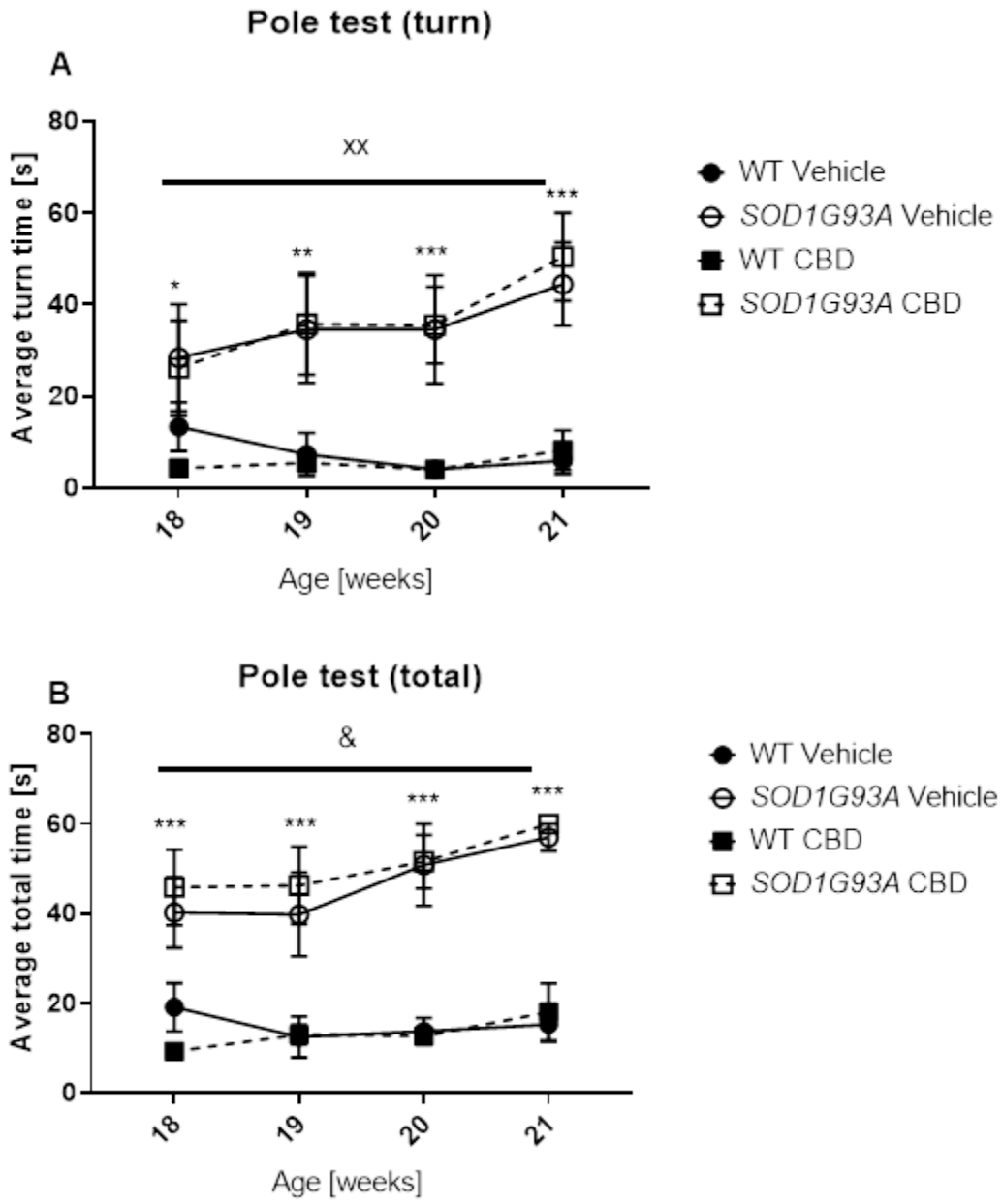


Figure 5.3A-B - Pole test assessment over testing period: Average latency [s] to turn on **(A)** and reach the platform **(B)** in the pole test after the initial 3-week CBD treatment period. Data are shown for vehicle-treated (VEH) and cannabidiol-treated (CBD) female superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates. Two-way ANOVA main effects of Genotype versus corresponding WT of the same week are shown as '*' (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). A significant three-way RM ANOVA interaction between 'Age' and 'Genotype' is indicated by $^{xx}p < 0.01$. A trend interaction between 'Age' and 'Genotype' is shown as $^{\&}p = 0.055$.

PaGE test: In the PaGe test, *SOD1*^{G93A} transgenic females showed progressively worsening grip endurance as measured by the latency to fall from the wire grid over the testing period [three-way RM ANOVA 'Age' by 'Genotype' interaction: $F(2,42) = 19.37$, $p < 0.001$]. Indeed, split by genotype, *SOD1*^{G93A} mice worsened over weeks [$F(2,20) = 16.18$, $p < 0.001$] whereas WT counterparts did not show changes to their grip endurance performance across test weeks [$F(2,22) = 2.15$, $p = 0.14$] (Figure 5.4). When data were split by 'age', a significant effect of 'Genotype' was seen for all weeks after the initial 3-week treatment phase (all p's < 0.01). Once again, no CBD main effect or interactions with 'age' or 'Genotype' were observed (all p's > 0.05). PaGE test was only assessed until 20 weeks of age as at 21 weeks of age *SOD1*^{G93A} mice were unable to grasp the wire grid for the test to start.

Figure 4

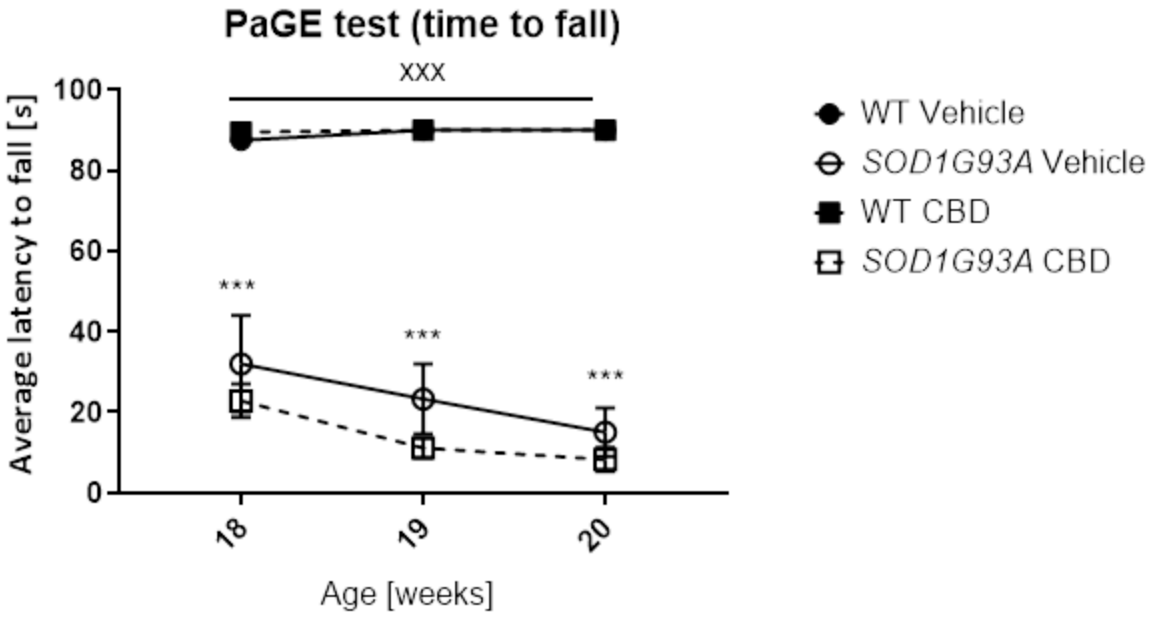


Figure 5.4- Grip test assessment over testing period: Average latency to fall from the the grip strength test [s] after the initial treatment period. Data are shown for vehicle-treated (VEH) and cannabidiol-treated (CBD) female superoxide dismutase 1 transgenic mice ($SOD1^{G93A}$) and non-transgenic wild type-like (WT) control littermates. Two-way ANOVA main effects of genotype versus corresponding WT of each week are shown as ***$p < 0.001$. A significant interaction between 'age' and 'Genotype' is indicated by $^{xxx}p < 0.001$.

5.4 Discussion

In this pilot study I evaluated the ability of remedial chronic CBD to delay or ameliorate bodyweight decline and / or motor degeneration in female *SOD1*^{G93A} transgenic mice. Behavioural testing prior to the commencement of CBD treatment at around 15 weeks of age revealed initially lower bodyweight as well as impaired motor abilities in *SOD1*^{G93A} mice compared to WT littermates. Across the 6-week CBD treatment period, *SOD1*^{G93A} transgenic mice lost bodyweight and showed progressively worsening motor performance in the accelerod, pole test and PaGE test compared to WT littermates. CBD did not change any of the test parameters recorded in this pilot study and also did not change any *SOD1* genotype-dependent differences in bodyweight development or motor test performance. Ultimately, chronic treatment with 50mg/kg CBD at symptom onset was not effective in ameliorating or delaying the ALS-like phenotype in the *SOD1*^{G93A} mouse model.

At 15 weeks of age *SOD1*^{G93A} females exhibited lower bodyweight compared to WT littermates, which progressively declined further with increasing age. This decline in bodyweight is one of the key characteristics of *SOD1*^{G93A} transgenic mice[26] but also other mouse models of ALS including Ubiquilin-2 (*UBQLN2)* [27] and *Profilin1* (*PFN1*) [28]. The rapid weight loss of ALS transgenic mice is thought to be a consequence of a combination of muscle hypermetabolism and disrupted energy homeostasis [29] in these mice. Furthermore, oral motor dysfunction and dysphagia contribute to weight loss in human ALS patients [30] and similar phenotypes have indeed also been observed in *SOD1*^{G93A} mice [31]. Chronic CBD treatment did not influence the bodyweight development of mice regardless of genotype. There is contention in the field as to the effect of cannabinoids on appetitive behaviours in mice. Previous studies have shown that acute CBD administration (i.p., 10 mg/kg CBD) in male C57BL/6 mice [32] as well as a sub-chronic CBD treatment (for 14 days,

i.p., 5 mg/kg CBD) of male Wistar rats [33] led to a significant although small reductions in food intake and bodyweight gain. Interestingly, these effects were absent when evaluating chronic CBD treatment (for 42 days, i.p., 20 mg/kg CBD) in C57BL/6J mice, which was argued to be a result of developing tolerance to chronic cannabinoid exposure [34]. Interestingly, CBD appears to be a non-competitive negative modulator of the CB_1 receptor [35]. CB_1 receptors can be found in adipocytes and are seen to play a role in peripheral energy homeostasis and in hepatic lipogenesis in response to high fat diets. Furthermore, agonism of CB_1 receptors by leptin increases food intake in C57BL/6 mice but not their CB_1 receptor knockout transgenic littermates [36]. Tolerance to chronic cannabinoid exposure is mediated by downregulation of CB_1 receptor expression and reduced G-protein coupled receptor sensitivity [37] which may account for the lack of effect of chronic CBD treatment on bodyweight.

Female *SOD1*^{G93A} mice showed progressively worsening motor performances across test paradigms. The latency to fall from the accelerod decreased in ALS transgenic females across age compared to WT mice [23]. A similar decline in motor abilities was also observed for both the pole test, in which *SOD1*^{G93A} mice were unable to turn without falling, and the PaGE test which was required to be stopped one week early as ALS transgenic mice were not able to even hold on to the wire anymore which is a requirement for the test to start. The findings are in line with previous reports about the progression of motor deficits in *SOD1*^{G93A} mice [23] regardless of sex when assessed previously in our laboratory and a feature of the model in general [25]. Rapid motor function degeneration is a major characteristic of the *SOD1*^{G93A} mouse model due to aggregation of misfolded SOD1 protein ultimately leading to progressive motor neuron cell death [38].

An effect of 'treatment' group was observed for the latency to turn on the pole test and this was evident prior to the start of any actual CBD treatment. This is an interesting finding as every

attempt was made to ensure this experiment was as standardised as possible, e.g. same cage system for all mice, standardised test protocols shown to be effective previously in our laboratory [23], and treatment administration post testing, unfortunately, the results in this experiment were only analysed after test and treatment completion. As such, the unexpected bias seen between treatment groups could not be adjusted for. However, these initial 'treatment' effects on motor function were not evident once CBD treatment had actually started and also did not affect any other motor paradigm, one of the reasons why a comprehensive motor testing strategy had been applied. Finally, as animals were quasi-randomized across treatment groups, this 'pseudo-treatment' effect is likely related to the phenomenon that even laboratory mice of inbred strains can exhibit individual behavioural responses. Indeed, whilst the expectation is for genetically identical inbred mice to behave identically, this is often not the case as uncontrollable factors such a pre- [39] and post-natal [40] nutrition as well as social status [41] can be different across individuals of one litter and thereby impact on individual behavioural responses later in life.

Ultimately, in this study, treatment with 50 mg/kg CBD did not impact on any aspect of motor coordination or function tested. However, this dose has previously shown beneficial effects on the phenotype of 12-month-old AβPPswe/PS1ΔE9 Alzheimer's disease model mice [22]in our lab. Conversely, whilst not as comprehensively investigated, CBD's inability to alter motor impairment phenotypes has previously been observed in the TAU58/2 transgenic mouse model for dementia where CBD was administered chronically at the same 50 mg/kg dose as used in the current experiment [42]. Furthermore, acute CBD effects on motor function were not to be expected in this study as previous investigation has revealed no change in open field locomotor activity when administered at the same dose [43]. CBD's potential to alter motor impairments is of interest as the phytocannabinoid displays agonistic activity at the peroxisome proliferator-activated receptor-γ

(PPAR-γ) to enact anti-inflammatory and neuroprotective effects [44]. Importantly, activation of PPAR-γ (e.g. by synthetic agonists such as pioglitazone) significantly delays the onset and progression of the *SOD1*^{G93A} mouse model motor phenotype and extends life expectancy when the treatment is started prior to noticeable hind-limb tremors [45]. Another PPAR-γ agonist, the cannibigerol quinone derivative VCE-003.2, also delays disease onset and progression, ameliorates bodyweight decline, and extends the lifespan of *SOD1*^{G93A} mice when administered chronically from PND60 onwards [46]. Furthermore, a meta-analysis of studies in which pharmaceutical candidates were therapeutically successful in preclinical testing (often in the *SOD1*^{G93A} mouse) but failed to translate in human clinical trials suggests that the age at which treatment starts is a major factor for treatment success; animals treated prior to the onset of clinical symptoms (e.g., hind limb tremor, abnormal gait, abnormal hind leg splaying) had improved odds of later onset, slower phenotype progression and longer survival compared to animals for which treatment started at or after symptom onset (the latter being similar to our treatment design, which was chosen for logistical reasons as our breeding and test facilities are in different locations) [47].

One previous preclinical study evaluated the properties of cannabinoid therapy for ALS. Sativex, a 1:1 combination treatment of Δ^9-THC and CBD at a dose of 40 mg/kg (20 mg/kg each of Δ^9-THC and CBD), administered i.p. in *SOD1*^{G93A} mice from 10 weeks of age onwards resulted in a delay of ALS-relevant phenotype onset and also a slower disease progression (as measured by clinical score) to the disease end stage although the treatment did not affect the overall lifespan [48]. It is interesting to speculate whether the observed therapeutic effect is based on synergistic effects of the two cannabinoids combined or linked to the findings in some but not all studies that CBD potentiates the actions of Δ^9-THC by delaying its degradation thereby allowing for longer lasting action [49]. However, it is important to mention here that this CBD-mediated potentiation appears

highly dependent on the ratio and overall dosage of CBD and Δ^9-THC. As an example, CBD has been seen to potentiate locomotor suppression and attenuate hypothermia induced by threshold levels of Δ^9-THC at a ratio of 1:1 CBD:Δ^9-THC [50] but potentiate both of these effects when administered at a ratio of 10:1 and 50:1 [51].

Furthermore, CBD has been seen to enhance the signalling of the endocannabinoid system by moderately delaying the breakdown of anandamide and alleviating psychotic symptoms when tested in human schizophrenia patients [52], this is important as increased levels of anandamide have also been seen to help control muscle spasticity in the 'chronic relapsing experimental allergic encephalomyelitis' (CREAE) model for multiple sclerosis [53], a key feature in both human ALS and models for the disease.

Interestingly, treatment with phytocannabinoids such as Δ^9-THC [15] and CBN [16], as well as synthetic cannabinoids such as the CB_2 receptor agonist AM1241 [13] and the cannabinoid receptor agonist WIN55,212-2 [54] have proven to effectively delay progression in the *SOD1*^{G93A} transgenic mouse model when administered alone around the onset of hind-limb tremors. This ability to impede disease progression at such a late stage indicates that activation of the main cannabinoid receptors ($CB_{1/2}$) is required for this effect. Indeed, inquest into the pharmacodynamics and pharmacokinetics of cannabinoids has revealed that Δ^9-THC, CBN and AM1241 are potent agonists of the CB_2 cannabinoid receptor [55], with WIN55,212-2 acting centrally as a potent agonist at CB_1 receptors [56] indicating that the activation of these receptors (at which CBD shows only weak inverse agonism[57]) may be key to ameliorate the ALS-like phenotype seen in these mice.

In conclusion, this pilot study provides first insights into the effect of chronic 50 mg/kg CBD treatment in female *SOD1*^{G93A} mice, when started at symptom onset. CBD did not affect bodyweight decline or motor function impairments in this ALS mouse model. However, this may

be due to multiple factors inherent to this study such as the dosage used and the time at which treatment was initiated. In future studies, it would be interesting to evaluate the therapeutic effects of additional doses of CBD thereby also considering preventative treatment strategies.

5.5 Limitations

As the largest benefit has been seen in treatment regimens that are initiated prior to the onset of ALS-like symptoms [47], it is possible that CBD treatment in the current study was started too late in this experiment to adequately prevent degeneration and protect the remaining vulnerable motor neurons. Further limitations of this study include the use of only female mice in a model with demonstrated sex-specific phenotypes. Indeed, the CB_2 receptor agonist AM1241 was effective in delaying motor function degeneration in male but not female mice [58] which was thought to be due to the already slower disease progression seen in female mice [59] and the potential estrogenic effects of cannabinoids [60]. Additionally, the effect of CBD treatment on cognitive impairments seen in the *SOD1*^{G93A} mouse model [23] were not assessed due to the pilot nature of this study, limiting the potential for this experiment to discuss therapeutic aspects of CBD in the context of ALS-frontotemporal spectrum disorder. Finally, this study was not pursued any further (i.e. increasing animal numbers or indeed testing male mice) due to animal welfare concerns at the later stages of the experiments as safe injections in *SOD1*^{G93A} mice became increasingly difficult as mice lose significant body fat and display hind limb clasping and rigidity, increasing the potential for inaccurate placement of the needle.

5.6 References

1. Miller RG, Mitchell JD, Moore DH. Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *Cochrane database of systematic reviews.* 2012(3).
2. Hardiman O, van den Berg LH. Edaravone: a new treatment for ALS on the horizon? *Lancet Neurol.* 2017;16(7):490-491.
3. Song J-H, Huang C-S, Nagata K, Yeh JZ, Narahashi T. Differential action of riluzole on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. *Journal of Pharmacology and Experimental Therapeutics.* 1997;282(2):707-714.
4. Distad BJ, Meekins GD, Liou LL, Weiss MD, Carter GT, Miller RG. Drug therapy in amyotrophic lateral sclerosis. *Physical medicine and rehabilitation clinics of North America.* 2008;19(3):633-651.
5. Zhu Y, Fotinos A, Mao LL, et al. Neuroprotective agents target molecular mechanisms of disease in ALS. *Drug discovery today.* 2015;20(1):65-75.
6. Gordon PH, Moore DH, Miller RG, et al. Efficacy of minocycline in patients with amyotrophic lateral sclerosis: a phase III randomised trial. *The Lancet Neurology.* 2007;6(12):1045-1053.
7. Perras C. Sativex for the management of multiple sclerosis symptoms. *Issues Emerg Health Technol.* 2005(72):1-4.
8. Novotna A, Mares J, Ratcliffe S, et al. A randomized, double-blind, placebo-controlled, parallel-group, enriched-design study of nabiximols (Sativex®), as add-on therapy, in subjects with refractory spasticity caused by multiple sclerosis. *European Journal of Neurology.* 2011;18(9):1122-1131.

9. Barnes MP. Sativex®: clinical efficacy and tolerability in the treatment of symptoms of multiple sclerosis and neuropathic pain. *Expert opinion on pharmacotherapy.* 2006;7(5):607-615.

10. Bilsland LG, Dick JR, Pryce G, et al. Increasing cannabinoid levels by pharmacological and genetic manipulation delay disease progression in SOD1 mice. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2006;20(7):1003-1005.

11. Witting A, Weydt P, Hong S, Kliot M, Mőller T, Stella N. Endocannabinoids accumulate in spinal cord of SOD1G93A transgenic mice. *Journal of neurochemistry.* 2004;89(6):1555-1557.

12. Yiangou Y, Facer P, Durrenberger P, et al. COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord. *BMC neurology.* 2006;6(1):12.

13. Shoemaker JL, Seely KA, Reed RL, Crow JP, Prather PL. The CB2 cannabinoid agonist AM-1241 prolongs survival in a transgenic mouse model of amyotrophic lateral sclerosis when initiated at symptom onset. *Journal of neurochemistry.* 2007;101(1):87-98.

14. Moreno-Martet M, Espejo-Porras F, Fernández-Ruiz J, Lago E. Changes in Endocannabinoid Receptors and Enzymes in the Spinal Cord of SOD1G93A Transgenic Mice and Evaluation of a Sativex®-like Combination of Phytocannabinoids: Interest for Future Therapies in Amyotrophic Lateral Sclerosis. *Cns neuroscience & therapeutics.* 2014;20(9):809-815.

15. Raman C, McAllister SD, Rizvi G, Patel SG, Moore DH, Abood ME. Amyotrophic lateral sclerosis: delayed disease progression in mice by treatment with a cannabinoid.

Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases. 2004;5(1):33-39.

16. Weydt P, Hong S, Witting A, Möller T, Stella N, Kliot M. Cannabinol delays symptom onset in SOD1 (G93A) transgenic mice without affecting survival. *Amyotrophic Lateral Sclerosis.* 2005;6(3):182-184.
17. Carlini EA, Cunha JM. Hypnotic and antiepileptic effects of cannabidiol. *Journal of clinical pharmacology.* 1981;21(S1):417S-427S.
18. Zuardi A, Guimares F. Cannabidiol as an Anxiolytic and Antipsychotic in Cannabis: The Medicine Plant McFarland & Co. London; 1991.
19. Parker LA, Mechoulam R, Schlievert C. Cannabidiol, a non-psychoactive component of cannabis and its synthetic dimethylheptyl homolog suppress nausea in an experimental model with rats. *Neuroreport.* 2002;13(5):567-570.
20. Hampson AJ, Grimaldi M, Axelrod J, Wink D. Cannabidiol and (-)Delta9-tetrahydrocannabinol are neuroprotective antioxidants. *Proceedings of the National Academy of Sciences of the United States of America.* 1998;95(14):8268-8273.
21. Pfohl SR, Halicek MT, Mitchell CS. Characterization of the Contribution of Genetic Background and Gender to Disease Progression in the SOD1 G93A Mouse Model of Amyotrophic Lateral Sclerosis: A Meta-Analysis. *J Neuromuscul Dis.* 2015;2(2):137-150.
22. Watt G, Shang K, Zieba J, et al. Chronic Treatment with 50 mg/kg Cannabidiol Improves Cognition and Moderately Reduces Aβ 42 Levels in 12-Month-Old Male AβPP swe/PS1ΔE9 Transgenic Mice. *Journal of Alzheimer's Disease.* 2020(Preprint):1-14.

23. Kreilaus F, Guerra S, Masanetz R, Menne V, Yerbury J, Karl T. Novel behavioural characteristics of the superoxide dismutase 1 G93A (SOD1G93A) mouse model of amyotrophic lateral sclerosis include sex-dependent phenotypes. *Genes, Brain and Behavior.* 2019:e12604.

24. Kreilaus F, Guerra S, Masanetz R, Menne V, Yerbury J, Karl T. Novel behavioural characteristics of the superoxide dismutase 1 G93A (SOD1(G93A)) mouse model of amyotrophic lateral sclerosis include sex-dependent phenotypes. *Genes Brain Behav.* 2020;19(2):e12604.

25. Weydt P, Hong SY, Kliot M, Moller T. Assessing disease onset and progression in the SOD1 mouse model of ALS. *Neuroreport.* 2003;14(7):1051-1054.

26. Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science.* 1994:1772-1772.

27. Le NT, Chang L, Kovlyagina I, et al. Motor neuron disease, TDP-43 pathology, and memory deficits in mice expressing ALS-FTD-linked UBQLN2 mutations. *Proceedings of the National Academy of Sciences of the United States of America.* 2016;113(47):E7580-E7589.

28. Fil D, DeLoach A, Yadav S, et al. Mutant Profilin1 transgenic mice recapitulate cardinal features of motor neuron disease. *Hum Mol Genet.* 2017;26(4):686-701.

29. Dupuis L, Oudart H, Rene F, Gonzalez de Aguilar JL, Loeffler JP. Evidence for defective energy homeostasis in amyotrophic lateral sclerosis: benefit of a high-energy diet in a transgenic mouse model. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101(30):11159-11164.

30. Mazzini L, Corra T, Zaccala M, Mora G, Del Piano M, Galante M. Percutaneous endoscopic gastrostomy and enteral nutrition in amyotrophic lateral sclerosis. *J Neurol.* 1995;242(10):695-698.

31. Lever TE, Gorsek A, Cox KT, et al. An animal model of oral dysphagia in amyotrophic lateral sclerosis. *Dysphagia.* 2009;24(2):180-195.

32. Riedel G, Fadda P, McKillop-Smith S, Pertwee RG, Platt B, Robinson L. Synthetic and plant-derived cannabinoid receptor antagonists show hypophagic properties in fasted and non-fasted mice. *British journal of pharmacology.* 2009;156(7):1154-1166.

33. Ignatowska-Jankowska B, Jankowski MM, Swiergiel AH. Cannabidiol decreases body weight gain in rats: involvement of CB2 receptors. *Neurosci Lett.* 2011;490(1):82-84.

34. Schleicher EM, Ott FW, Muller M, et al. Prolonged Cannabidiol Treatment Lacks on Detrimental Effects on Memory, Motor Performance and Anxiety in C57BL/6J Mice. *Front Behav Neurosci.* 2019;13:94.

35. Laprairie R, Bagher A, Kelly M, Denovan-Wright E. Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *British journal of pharmacology.* 2015;172(20):4790-4805.

36. Di Marzo V, Goparaju SK, Wang L, et al. Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature.* 2001;410(6830):822-825.

37. Martin BR, Sim-Selley LJ, Selley DE. Signaling pathways involved in the development of cannabinoid tolerance. *Trends Pharmacol Sci.* 2004;25(6):325-330.

38. Higgins CM, Jung C, Xu Z. ALS-associated mutant SOD1 G93A causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes. *BMC neuroscience.* 2003;4(1):16.

39. Raygada M, Cho E, Hilakivi-Clarke L. High maternal intake of polyunsaturated fatty acids during pregnancy in mice alters offsprings' aggressive behavior, immobility in the swim test, locomotor activity and brain protein kinase C activity. *J Nutr.* 1998;128(12):2505-2511.

40. Barnard CJ, Behnke JM, Gage AR, Brown H, Smithurst PR. Maternal effects on the development of social rank and immunity trade-offs in male laboratory mice (Mus musculus). *Proc Biol Sci.* 1998;265(1410):2087-2093.

41. D'Amato FR. Effects of male social status on reproductive success and on behavior in mice (Mus musculus). *J Comp Psychol.* 1988;102(2):146-151.

42. Watt G, Chesworth R, Przybyla M, et al. Chronic cannabidiol (CBD) treatment did not exhibit beneficial effects in 4-month-old male TAU58/2 transgenic mice. *Pharmacology Biochemistry and Behavior.* 2020;196:172970.

43. Long LE, Chesworth R, Huang XF, McGregor IS, Arnold JC, Karl T. A behavioural comparison of acute and chronic Delta9-tetrahydrocannabinol and cannabidiol in C57BL/6JArc mice. *Int J Neuropsychopharmacol.* 2010;13(7):861-876.

44. Esposito G, Scuderi C, Valenza M, et al. Cannabidiol reduces Aβ-induced neuroinflammation and promotes hippocampal neurogenesis through PPARγ involvement. *PloS one.* 2011;6(12):e28668.

45. Kiaei M, Kipiani K, Chen J, Calingasan NY, Beal MF. Peroxisome proliferator-activated receptor-gamma agonist extends survival in transgenic mouse model of amyotrophic lateral sclerosis. *Exp Neurol.* 2005;191(2):331-336.

46. Rodriguez-Cueto C, Santos-Garcia I, Garcia-Toscano L, et al. Neuroprotective effects of the cannabigerol quinone derivative VCE-003.2 in SOD1(G93A) transgenic mice, an

experimental model of amyotrophic lateral sclerosis. *Biochem Pharmacol.* 2018;157:217-226.

47. Benatar M. Lost in translation: treatment trials in the SOD1 mouse and in human ALS. *Neurobiol Dis.* 2007;26(1):1-13.

48. Moreno-Martet M, Espejo-Porras F, Fernández-Ruiz J, de Lago E. Changes in Endocannabinoid Receptors and Enzymes in the Spinal Cord of SOD 1G93A Transgenic Mice and Evaluation of a Sativex®-like Combination of Phytocannabinoids: Interest for Future Therapies in Amyotrophic Lateral Sclerosis. *CNS neuroscience & therapeutics.* 2014;20(9):809-815.

49. Klein C, Karanges E, Spiro A, et al. Cannabidiol potentiates Δ 9-tetrahydrocannabinol (THC) behavioural effects and alters THC pharmacokinetics during acute and chronic treatment in adolescent rats. *Psychopharmacology.* 2011;218(2):443-457.

50. Todd S, Arnold J. Neural correlates of interactions between cannabidiol and Δ9-tetrahydrocannabinol in mice: implications for medical cannabis. *British journal of pharmacology.* 2016;173(1):53-65.

51. Hayakawa K, Mishima K, Hazekawa M, et al. Cannabidiol potentiates pharmacological effects of Δ9-tetrahydrocannabinol via CB1 receptor-dependent mechanism. *Brain research.* 2008;1188:157-164.

52. Leweke FM, Piomelli D, Pahlisch F, et al. Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. *Transl Psychiatry.* 2012;2(3):e94.

53. Baker D, Pryce G, Croxford JL, et al. Endocannabinoids control spasticity in a multiple sclerosis model. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2001;15(2):300-302.

54. Bilsland LG, Dick JR, Pryce G, et al. Increasing cannabinoid levels by pharmacological and genetic manipulation delays disease progression in SOD1 mice. *The FASEB journal.* 2006;20(7):1003-1005.

55. Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther.* 1997;74(2):129-180.

56. Ferraro L, Tomasini MC, Gessa GL, Bebe BW, Tanganelli S, Antonelli T. The cannabinoid receptor agonist WIN 55,212-2 regulates glutamate transmission in rat cerebral cortex: an in vivo and in vitro study. *Cereb Cortex.* 2001;11(8):728-733.

57. McPartland JM, Duncan M, Di Marzo V, Pertwee RG. Are cannabidiol and Delta(9) - tetrahydrocannabivarin negative modulators of the endocannabinoid system? A systematic review. *Br J Pharmacol.* 2015;172(3):737-753.

58. Kim K, Moore DH, Makriyannis A, Abood ME. AM1241, a cannabinoid CB2 receptor selective compound, delays disease progression in a mouse model of amyotrophic lateral sclerosis. *Eur J Pharmacol.* 2006;542(1-3):100-105.

59. Veldink JH, Bar PR, Joosten EA, Otten M, Wokke JH, van den Berg LH. Sexual differences in onset of disease and response to exercise in a transgenic model of ALS. *Neuromuscul Disord.* 2003;13(9):737-743.

60. Lee SY, Oh SM, Chung KH. Estrogenic effects of marijuana smoke condensate and cannabinoid compounds. *Toxicol Appl Pharmacol.* 2006;214(3):270-278.

Chapter 6: General Discussion

6.1 Validity of preclinical research into ALS

For preclinical research to provide insight into the mechanisms, presentation, and potential treatments of human diseases, both *in vitro* and *in vivo* modelling must present a high degree of validity for the disease in question and consider all factors potentially impacting this validity. Thus, appropriate model selection and experimental design including test protocol selection are of utmost importance at the experimental planning stages.

6.1.1 Mouse model validity in the context of research into ALS

Assessing mouse model validity for diseases with multitude impacted mechanisms and heterogeneity in presentation such as ALS is a complex prospect, further compounded by recent advances in the understanding of the existence of a ALS-Frontotemporal spectrum disorder [1]. Indeed, recent research in my lab which I was involved in identified that the *SOD1*^{G93A} transgenic mouse model, thought of as a model presenting a 'pure' ALS-relevant mouse phenotype, exhibited impairments in spatial memory and associative learning [2] which are both relevant to FTD [3]. Indeed in a studied Istro-Romanian family, the leu144phe mutation in the *SOD1* gene was observed to lead to a rare case of ALS coupled with dementia [4] and a prospective case study in which cognitive decline was observed in a patient expressing the *SOD1*^{I113T} mutation 2 years prior to the onset of motor function degeneration [5]. It is therefore prudent to assess aspects of both diseases in preclinical research targeting risk factors or new therapeutics, as the human patient population is likely to experience aspects of both sides of the spectrum, for example: ALS patients are likely to exhibit executive dysfunction manifested as impairments in cognitive flexibility [6], working memory [6] and reading of social cues [7], and a non-insignificant number of FTD patients are likely

to suffer from concomitant motor neuron pathology leading to fasciculations and mild muscle wasting [8] during the course of their disease. Presently, the *Superoxide Dismutase 1* mouse model for ALS [9], has led the way for investigations into ALS development and pathophysiology and is still the most widely used model in preclinical ALS research [10]. However, onset and progression of disease-relevant phenotypes can differ from what is seen in the human disease. For example, $SOD1^{A4V}$, the most commonly observed mutation in *SOD1*-causative human fALS with a rapidly progressing phenotype (survival average of 1.2 years compared to 2.5) [11,12], exhibits a slow progressing phenotype in mouse models for the mutation [13]. These differences are likely due to the need for overexpression of the mutant genes 10-30 times in excess of what is observed in human ALS patients[13] in addition to large differences seen in the upper motor neurons between humans and murine models [14].

A number of models of the ALS/Frontotemporal spectrum disorder (or any other disorder) display an endophenotype for the disease rather than the full spectrum of symptoms evident in humans. For example, transgenic mouse models for TDP-43 exhibit ubiquitinated inclusions in neurons [17], rapid motor function deficits, and low survival but no motor neuron loss [18]. Notably, disease-relevant endophenotypes in mouse models for other neurodegenerative disorders such as schizophrenia are seen to be influenced by environment [19] and sex [20]. Thus, these often under-reported factors are potentially key to understanding the differences seen between studies using the same mouse model. For example environmental enrichment was observed to accelerate ALS-relevant phenotypes in $SOD1^{G93A}$ female mice but not males prompting my investigation into potentially confounding factors in Chapter 1 and 2 of this thesis.

6.2 Factors affecting the validity of preclinical mouse models.

To echo the quotation of the statistician George Box in [21], "Remember that all models are wrong; the practical question is how wrong do they have to be to not be useful?" [22], certainly an important question when it comes to the validity of transgenic models and how both controllable (i.e. home cage conditions [23,24], animal husbandry [25,26] and test selection [27,28], inter-investigator variation [29,30]) and uncontrollable (i.e. social hierarchy [31]) factors affect the desired phenotypes in these models. These effects are further compounded when assessing complex models of human disease as often these models are susceptible to gene-environment interactions [32] so the conditions in which the animals are kept and tested may in some cases be as important as the genetic polymorphism(s) which are investigated in those models as has been observed in both my lab [33] and others [34]. In the following, I will outline in more detail, how some of these environmental factors impact on behavioural domains and thereby potentially confound the face validity of ALS-relevant transgenic mouse models.

6.2.1 Handling

As has been discussed in the first experimental Chapter of this thesis, I found that handling using the tunnel method increased locomotion speed and exploration as well as reduced anxiety-like behaviours in a sex-dependent manner in the open field and elevated plus maze. This is an interesting finding as whilst it did replicate many of the findings of non-aversive handling methods found in initial studies on C57Bl6 and BALB/c inbred and ICR(CD-1) outbred mouse strains [35-38], this is the first evidence of sex specificity of handling effects as this was not seen in previous studies in this domain [35,36,39]. Also interesting is that results gained from the open field and elevated plus maze test can be somewhat likened to what is seen in studies focusing on environmental enrichment (such as attenuation of anxiety and stress [40]). This implies that less aversive handling

can have positive effects in these animals and thus are recommended to be a permanent feature of the cage environment as this is when the strongest anxiolytic effects of non-aversive handling have been observed [36].

My research also provided first evidence that handling methods can affect more complex behavioural processes such as Pavlovian fear conditioning and sensorimotor gating. It is likely that at least some effects of handling seen on these domains are linked to the effects handling has on stress circuits, as standard laboratory handling of C57BL/6J mice was seen to vastly increase plasma corticosterone levels [41]. Thus, the method of handling used should be considered during project planning for example, if stress is relevant for domains to be tested, consider the use of less aversive handling methods or habituate test animals to handling prior to the start of testing.

6.2.2 Housing and Environmental Enrichment

The cage system in which laboratory animals are housed can have an impact on not only the behavioural characteristics of a validated mouse model (for example, significant changes to the phenotype of an established genetic mouse model for schizophrenia, i.e. *neuregulin 1* mutant mice, when filter top housing was compared to individually ventilated cages [33]), but can also alter important neurotransmitter turnover rates [42] as well as physiological factors such as blood thickness [43] and white cell count [43]. As explored in Chapter 3 of this thesis, whilst the main motor phenotype of *SOD1*^{G93A} transgenic mice was not seen to be affected the cage systems evaluated, the moderate impacts of these systems on the prepulse inhibition and fear conditioning tests indicate that the assessment of housing conditions in models with a stable phenotype is still valid and the characterization and reporting of these effects will allow for better inter-study comparisons in particular when disease-relevant phenotypes are investigated more comprehensively (as they should for *SOD1*^{G93A} transgenic mice as outlined in earlier study from our laboratory [2]).

To add to this discussion more broadly, the effects that items/objects added to these cage systems, known as 'environmental enrichment (EE)', may have on both the physiology and behaviour of laboratory rodents has been widely studied. As such, the addition of EE leads for example to increased overall activity in the home cage [44] and reduced exploration in the open field and T-Maze [45,46]. Physiologically, environmental complexity has been seen to increase astrocytes and oligodendrocytes [47] and increase dendritic spine density and connections in rats [48,49]. With relevance to transgenic mouse models, EE leads to slower disease progression [50] and ameliorates protein deficits [51] in R6/2 and R6/1 Huntington's disease mice respectively. This effect of home cage setup was important for my work as even a loss of climbing opportunities in the home cages of mice in my investigation into gene-gene interactions in TDP-43 mice may have impacted on motor performance over the testing period.

6.2.3 Standardization in preclinical research

In light of the vast number of variables that have been observed to impact on the validity and reproducibility, standardization of both animal facilities and testing environment was initially proposed to improve the comparison of data within and between laboratories as well as reduce variation seen in the field. However, even when exhaustively controlled for as many variables as possible, including shipping times, housing, test protocols and apparatus, across three facilities, Crabbe et. al [52] saw differences in behaviours across test sites when animals of the same strain were tested [52]. Thus, the rigid control of variables surrounding the environment mice are kept and tested in is unlikely to lead to more comparable results. Additionally, one of the contentious issues surrounding the variability of preclinical data is the reliability of the tests themselves. A review by Wahlsten, D. [53] on the subject of behavioural testing standardization, noted that for tests such as the open field, elevated plus maze and the Morris water maze there is a wide array of differing

apparatus configurations for what are regarded as 'common' or 'standard' tests in the field [53] Such differences are no doubt a confounding factor when attempting to compare studies. Therefore, more comprehensive reporting of all factors involved in mouse testing will allow preclinical researchers to sort through the noise and find those that really make a difference to better inform experimental design and potentially improve translatability from animal studies. With regard to models of ALS, specifically the *SOD1* model, the priority measure thus far has been decline in weight and gross motor function, however there is evidence to show that alterations to gait appear weeks prior to these more noticeable symptom presentations [54], thus ALS models may need to be studied sooner than what would be expected. In light of this, the collection and collation of national and international data on mouse models of disease comparing the severity and progression of phenotypes in the context of housing and testing conditions would prove an interesting and valuable future endeavor.

6.3 Investigation into Risk Factors and Therapeutics using mouse models for ALS

6.3.1 Translational value of preclinical research into ALS

In recent years there has been something of a 'crisis of translation' in which up to 96% of novel treatment candidates identified in preclinical research from multiple fields were not successful when evaluated in human clinical trials [55]. Failures of translation have also been extensively seen from preclinical research into novel ALS treatments as in the 25 years since the regulatory approval of Riluzole, only one other drug (Edaravone [56]) has recently been approved for the treatment of ALS. Thus, almost all of the potentially novel treatments have failed to make it into clinical use usually failing in phase 2 or 3 clinical trials [57,58] after showing initial preclinical success. One of the major arguments surrounding this relatively poor translation from bench-to-bedside, is the irreproducibility of findings within and across laboratories. The variability seen in how

environmental factors inherent to animal housing facilities [59,60], animal husbandry and investigator [61], and biologically relevant test and protocol selection [53] influence the endophenotype in preclinical research models is important to therapeutic evaluation which often use such measures for therapeutic efficacy as differences across test sites/studies can impact on findings. In line with this it must also be stated that recently the issue of publication bias and thereby the overstating of positive preclinical treatment results is a further challenge for translational research. For example, of 525 unique publications selected in meta-analysis for the preclinical assessment of animal stroke studies, only 6 (1.2%) did not report a significant finding [62].

6.3.2 Preclinical evaluation of gene-gene interactions

One avenue of addressing the translatability of modern animal models of ALS is the creation of models with a high level of validity for the human disease. As previously mentioned, this is a complex prospect due to the multi-faceted nature of mutations and resultant phenotypes in the ALS-frontotemporal dementia spectrum. Such challenges are usually present due to differential penetrance of familial-associated mutations as well as heterogeneity in presentation within families, for example the P506S mutation in *ubiquilin-2 (UBQLN2)* presented with rapid decline in motor function and cognitive impairments in one female family member whereas two male members (brother and cousin) presented only with less severe motor phenotypes [63]. Thus, the problem is not inherent to mouse model studies only.

The existence of potential gene-environment interactions in ALS is still controversially discussed in the field due to inconsistent reporting surrounding impact on the presentation and progression of ALS/FTD-relevant phenotypes in both human patients and hard to model factors for mouse models for the disease[64]. Regardless, the investigation of gene-gene interactions may be another good strategy to improve the validity of current mouse models for ALS and could pave the way

for more translatable models for the disease in the future. Presently, many studies of gene-gene interactions in the field are centered around the potential benefit of gene-modifications in antisense oligonucleotides [65] and small interfering RNA [66], however, even with the large number of causative mutations known, studies of disease-modifying interactions in multi-transgenic models are somewhat rare and predominantly in models of various *SOD1* mutations [67-69]. Thus, further assessments of these interactions in other than *SOD1* transgenic mouse models, e.g. in models of more commonly observed ALS-relevant mutations, such as mutant TDP-43 are important. This rationale is in line with the strategy chosen for Chapter 4 where I investigated the effects of overexpressing mutant (as well as wild type) VPS29 on the behavioural phenotype of iTDP-43^{A315T} transgenic mice. I found several gene-gene interactions for a variety of behavioural domains with partial relevance for ALS and FTD (i.e. altered motor learning and anxiolytic-like behaviours) with some of those being sex-specific. This proof of concept provides a rationale to better model the heterogeneity seen in ALS/Frontotemporal spectrum disorder in future studies and to evaluate gene-gene interactions more often thereby also considering sex effects which will allow for better characterization of the human disease and potentially more translatable therapeutics.

Recent widespread application of RNA-seq has allowed for the investigation into differences in gene expression in transgenic mouse models of ALS. Most commonly seen is the upregulation of proinflammatory apoptotic genes in *SOD1* and FUS/TDP-43 mice[70,71]. Importantly, the differential expression of genes in *SOD1* mice does appear to be mutation and age related. RNA-seq analysis of pre-symptomatic *SOD1*^{G85R} mice revealed few genes with altered regulation that did not impact on the disease state [72], whereas early microarray analysis of *SOD1*^{G93A} mice indicated that increased expression of lipid and carbohydrate genes as well as those serving translation and transcription function at the pre-symptomatic stage (60 days) whereas at late stage (120 days) these

functions were downregulated [73]. These findings indicate that gene-gene interactions are highly variable depending on the model used and mutation studies, and therefore should be considered during the phenotyping of novel models and testing of novel treatments.

6.3.3 Preclinical evaluation of potential new therapeutics

Ultimately, the goal for mouse models of human disease is to provide a model system to understand mechanisms behind the disease, identify risk factors for the disease, and test potential new treatment candidates. Recently, ALS treatment trial candidates have met with mixed success as phase 2 and 3 trials in erythropoietin [74] and creatine [75] did not alter the disease progression in ALS patients whereas trials in the Anti-glutamatergic drug Talampanel [76] and the skeletal muscle troponin activator Tirasemtiv [77] while not halting disease progression, have shown promise to delay motor decline in early trials. In 2019 a phase III clinical trial was initiated in Queensland investigating the therapeutic potential for cannabis-based medical extracts in ALS patients using a high ratio CBD:THC (25:<2) extract given three times daily for a total daily dose of 375mg after the initial 2-week titration period. The rational for this trial was the preclinical efficacy of cannabinoids such as Δ^9-THC [78], Sativex [79] and AM1241 [80] to delay the onset and progression of ALS-relevant phenotypes in mice. Importantly, no studies to date have investigated the therapeutic potential of CBD alone in ALS transgenic mouse models. As such, I took the opportunity to run a pilot study assessing the chronic administration of 50 mg/kg CBD in symptomatic female *SOD1*^{G93A} mice. I found that at this dose, when started at symptom onset, CBD did not affect the ALS-relevant phenotype in these mice focusing only motor phenotypes as well as body weight progression.

This is especially important in models with rapidly progressing phenotypes such as ALS as the factors of background strain, sex and animal age at treatment start are all implicated in the

effectiveness of preclinical treatment testing [81]. Interestingly a computer-modelled re-analysis of treatment candidates which were effective in ALS mice but subsequently failed testing in the clinic found that often the relevant mouse studies were underpowered (n = 4 - 10)and conclusions that were drawn were due to 'noise' in the data as a result of slower disease progression mice expressing a lower number of transgene copies compared to the rest of the cohort and the recording of deaths that were not as a result of the ALS-like phenotype [82]. Ideally, treatment of patients with ALS should start prior to the onset of symptoms, and while this is possible in familial ALS with a known family history, this is not an option for the vast majority of cases which present sporadically and often with unknown or multiple mutations being involved in the development of ALS. Thus, until the factors predisposing human patients to sporadic ALS are elucidated, treatments which are able to halt or at least drastically slow disease progression in mouse models for familial ALS are still the only system available to evaluate new candidates. However, this will only be possible with more comprehensive reporting of factors surrounding preclinical testing in addition to timely testing and characterization of gene-gene/gene-environment interactions affecting these models.

6.4 Limitations

The experimental strategies for the various chapters in this thesis were affected by some limitations. Firstly, I was unable to make comparisons of the effects of handling on the behaviour of ALS mouse models as the breeding of the *SOD1*^{G93A} transgenic animals that were used in subsequent chapters was still in the early stages based on an adverse event at our breeding facility (mouse colony needed to be re-established) and thus *SOD1*^{G93A} transgenic mice couldn't be used for this first experiment. Secondly, in Chapter 4, the disease-modifying potential of mutant or wild type VPS29 in the iTDP-43^{A325T} mice was impacted by low sample size and in a limited number

of tests. However, this was done to allow repeated testing of mice across age and to minimize the number of tests for each mouse. Furthermore, this project was carried out late in my candidature but provided an excellent opportunity to generate first insights into the role of the newly discovered VPS29 variant in ALS. Furthermore, it gave me the opportunity to gain insights into working with the iTDP-43^{A325T} mouse model and to carry out a research project in another laboratory environment. Thirdly, my assessment of CBD was limited in that only female animals at the age of 15 weeks were used for treatment and testing (due to limited availability of test animals at the time – see above). Thus, sex-specific responses to the treatment could not be analysed, which is important to note as sex differences in the behavioural response to cannabinoids have previously been documented [83,84]. Furthermore, the commencement of CBD treatment after the onset of the ALS phenotype in these mice could have impacted on the potential efficacy of the CBD treatment to reduce ALS-relevant behavioural deficits. Finally, the i.p. administration of CBD in this experiment was challenging during the end stages of the experiment, as *SOD1*^{G93A} transgenic mice at that age not only show significant bodyweight loss but also hind limb spasticity which made it difficult to deliver CBD safely (as the hind limbs clasped when mice were handled for injections limiting the accessibility of the abdomen). For animal welfare reasons we decided against increasing the sample sizes of these experimental cohorts further. This is relevant as computer modelling of power and effect sizes in therapeutic studies recommended a minimum of 24 mice per condition when using ALS transgenic mice [82].

6.5 Future directions

In the context of the findings and limitations of the work presented in this thesis further relevant work in the domains investigated should follow up on the effect of handling methods in the

SOD1^{G93A} transgenic mouse model. Furthermore, the sample size of experiments in Chapters 3 and 4 could be increased and more behavioural domains tested (using separate test cohorts) to better understand gene-gene interactions of VPS29 and TDP-43, and further investigate the therapeutic properties of CBD in the *SOD1*^{G93A} mouse model in both male and female mice. For the latter project, treatment should commence as early as possible as a preventative intervention and an oral administration route should be considered.

6.6 Overall Conclusion

In conclusion, I found that environmental factors such as handling methods and the home cage system used have the potential to influence mouse behaviours in domains relevant to ALS-Frontotemporal spectrum disorder and often in a way that is sex-dependent and should therefore be a consideration for experimental planning. Furthermore, overexpression of mutant VPS29 interacts with mutations of TDP-43 to influence FTD-related behavioural domains, however, if these are relevant to 'pure' ALS as well requires further investigation. And finally, CBD as evaluated to date in this thesis has no therapeutic effect on the progression of ALS-relevant phenotypes in *SOD1*^{G93A} females, however, limitations of the study design may have impacted on this and thus require further testing.

6.8 References

1. Ling S-C, Polymenidou M, Cleveland DW. Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis. *Neuron.* 2013;79(3):416-438.
2. Kreilaus F, Guerra S, Masanetz R, Menne V, Yerbury J, Karl T. Novel behavioural characteristics of the superoxide dismutase 1 G93A (SOD1G93A) mouse model of amyotrophic lateral sclerosis include sex-dependent phenotypes. *Genes, Brain and Behavior.* 2019:e12604.
3. Eslinger PJ, Dennis K, Moore P, Antani S, Hauck R, Grossman M. Metacognitive deficits in frontotemporal dementia. *J Neurol Neurosurg Psychiatry.* 2005;76(12):1630-1635.
4. Mase G, Ros S, Gemma A, et al. ALS with variable phenotypes in a six-generation family caused by leu144phe mutation in the SOD1 gene. *J Neurol Sci.* 2001;191(1-2):11-18.
5. Katz JS, Katzberg HD, Woolley SC, Marklund SL, Andersen PM. Combined fulminant frontotemporal dementia and amyotrophic lateral sclerosis associated with an I113T SOD1 mutation. *Amyotroph Lateral Scler.* 2012;13(6):567-569.
6. Strong M, Abrahams S, Goldstein L, et al. Amyotrophic lateral sclerosis–frontotemporal spectrum disorder (ALS-FTSD): revised diagnostic criteria. Amyotroph Lateral Scler Frontotemporal Degener 18: 153–174. 2017.
7. Beeldman E, Raaphorst J, Klein Twennaar M, de Visser M, Schmand BA, de Haan RJ. The cognitive profile of ALS: a systematic review and meta-analysis update. *J Neurol Neurosurg Psychiatry.* 2016;87(6):611-619.
8. Burrell JR, Kiernan MC, Vucic S, Hodges JR. Motor neuron dysfunction in frontotemporal dementia. *Brain : a journal of neurology.* 2011;134(Pt 9):2582-2594.

9. Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science.* 1994:1772-1772.
10. Lutz C. Mouse models of ALS: Past, present and future. *Brain Res.* 2018;1693(Pt A):1-10.
11. Siddique T, Deng HX. Genetics of amyotrophic lateral sclerosis. *Hum Mol Genet.* 1996;5 Spec No(Supplement_1):1465-1470.
12. Rosen DR, Bowling AC, Patterson D, et al. A frequent ala 4 to val superoxide dismutase-1 mutation is associated with a rapidly progressive familial amyotrophic lateral sclerosis. *Human molecular genetics.* 1994;3(6):981-987.
13. Andersen PM. Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. *Curr Neurol Neurosci Rep.* 2006;6(1):37-46.
14. Marshall KL. *Axonal Regeneration of ALS Patient iPSC-derived Motor Neurons*, Johns Hopkins University; 2021.
15. Weydt P, Hong SY, Kliot M, Moller T. Assessing disease onset and progression in the SOD1 mouse model of ALS. *Neuroreport.* 2003;14(7):1051-1054.
16. Cudkowicz M, McKenna-Yasek D, Sapp P, et al. Epidemiology of mutations in superoxide dismutase in amyotrophic lateal sclerosis. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society.* 1997;41(2):210-221.
17. Neumann M, Sampathu DM, Kwong LK, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science.* 2006;314(5796):130-133.
18. Xu YF, Gendron TF, Zhang YJ, et al. Wild-type human TDP-43 expression causes TDP-43 phosphorylation, mitochondrial aggregation, motor deficits, and early mortality in transgenic mice. *J Neurosci.* 2010;30(32):10851-10859.

19. Burrows EL, Hannan AJ. Cognitive endophenotypes, gene-environment interactions and experience-dependent plasticity in animal models of schizophrenia. *Biol Psychol.* 2016;116:82-89.

20. Eltokhi A, Kurpiers B, Pitzer C. Behavioral tests assessing neuropsychiatric phenotypes in adolescent mice reveal strain- and sex-specific effects. *Sci Rep.* 2020;10(1):11263.

21. Tadenev AL, Burgess RW. Model validity for preclinical studies in precision medicine: precisely how precise do we need to be? *Mammalian Genome.* 2019;30(5-6):111-122.

22. Box GE, Draper NR. *Empirical model-building and response surfaces.* John Wiley & Sons; 1987.

23. Wolfer DP, Litvin O, Morf S, Nitsch RM, Lipp HP, Wurbel H. Laboratory animal welfare: cage enrichment and mouse behaviour. *Nature.* 2004;432(7019):821-822.

24. Wurbel H. Ideal homes? Housing effects on rodent brain and behaviour. *Trends Neurosci.* 2001;24(4):207-211.

25. Meijer M, Kramer K, Remie R, Spruijt B, Van Zutphen L, Baumans V. Effect of routine procedures on physiological parameters in mice kept under different husbandry procedures. *Animal Welfare.* 2006;15(1):31-38.

26. Balcombe JP, Barnard ND, Sandusky C. Laboratory routines cause animal stress. *Contemp Top Lab Anim Sci.* 2004;43(6):42-51.

27. McIlwain KL, Merriweather MY, Yuva-Paylor LA, Paylor R. The use of behavioral test batteries: effects of training history. *Physiology & behavior.* 2001;73(5):705-717.

28. Paylor R, Spencer CM, Yuva-Paylor LA, Pieke-Dahl S. The use of behavioral test batteries, II: effect of test interval. *Physiology & behavior.* 2006;87(1):95-102.

29. Sorge RE, Martin LJ, Isbester KA, et al. Olfactory exposure to males, including men, causes stress and related analgesia in rodents. *Nature methods.* 2014;11(6):629-632.

30. van Driel KS, Talling JC. Familiarity increases consistency in animal tests. *Behav Brain Res.* 2005;159(2):243-245.

31. Horii Y, Nagasawa T, Sakakibara H, et al. Hierarchy in the home cage affects behaviour and gene expression in group-housed C57BL/6 male mice. *Sci Rep.* 2017;7(1):6991.

32. Post AM, Weyers P, Holzer P, et al. Gene-environment interaction influences anxiety-like behavior in ethologically based mouse models. *Behav Brain Res.* 2011;218(1):99-105.

33. Logge W, Kingham J, Karl T. Do individually ventilated cage systems generate a problem for genetic mouse model research? *Genes, Brain and Behavior.* 2014;13(7):713-720.

34. Shan L, Schipper P, Nonkes LJ, Homberg JR. Impaired fear extinction as displayed by serotonin transporter knockout rats housed in open cages is disrupted by IVC cage housing. *PloS one.* 2014;9(3):e91472.

35. Hurst JL, West RS. Taming anxiety in laboratory mice. *Nature methods.* 2010;7(10):825-826.

36. Gouveia K, Hurst JL. Reducing mouse anxiety during handling: effect of experience with handling tunnels. *PloS one.* 2013;8(6):e66401.

37. Gouveia K, Hurst JL. Optimising reliability of mouse performance in behavioural testing: the major role of non-aversive handling. *Scientific reports.* 2017;7:44999.

38. Gouveia K, Hurst JL. Improving the practicality of using non-aversive handling methods to reduce background stress and anxiety in laboratory mice. *Scientific Reports.* 2019;9(1):1-19.

39. Ghosal S, Nunley A, Mahbod P, et al. Mouse handling limits the impact of stress on metabolic endpoints. *Physiol Behav.* 2015;150:31-37.

40. Fox C, Merali Z, Harrison C. Therapeutic and protective effect of environmental enrichment against psychogenic and neurogenic stress. *Behav Brain Res.* 2006;175(1):1-8.

41. Benedetti M, Merino R, Kusuda R, et al. Plasma corticosterone levels in mouse models of pain. *Eur J Pain.* 2012;16(6):803-815.

42. Pasquarelli N, Voehringer P, Henke J, Ferger B. Effect of a change in housing conditions on body weight, behavior and brain neurotransmitters in male C57BL/6J mice. *Behav Brain Res.* 2017;333:35-42.

43. York JM, McDaniel AW, Blevins NA, et al. Individually ventilated cages cause chronic low-grade hypoxia impacting mice hematologically and behaviorally. *Brain Behav Immun.* 2012;26(6):951-958.

44. Simonetti T, Lee H, Bourke M, Leamey CA, Sawatari A. Enrichment from birth accelerates the functional and cellular development of a motor control area in the mouse. *PloS one.* 2009;4(8):e6780.

45. Zhu SW, Yee BK, Nyffeler M, Winblad B, Feldon J, Mohammed AH. Influence of differential housing on emotional behaviour and neurotrophin levels in mice. *Behav Brain Res.* 2006;169(1):10-20.

46. van de Weerd HA, Baumans V, Koolhaas JM, van Zutphen LF. Strain specific behavioural response to environmental enrichment in the mouse. *J Exp Anim Sci.* 1994;36(4-5):117-127.

47. Szeligo F, Leblond C. Response of the three main types of glial cells of cortex nad corpus callosum in rats handled during suckling or exposed to enriched, control and impoverished

environments following weaning. *Journal of Comparative Neurology.* 1977;172(2):247-263.

48. Globus A, Rosenzweig MR, Bennett EL, Diamond MC. Effects of differential experience on dendritic spine counts in rat cerebral cortex. *J Comp Physiol Psychol.* 1973;82(2):175-181.

49. Volkmar FR, Greenough WT. Rearing complexity affects branching of dendrites in the visual cortex of the rat. *Science.* 1972;176(4042):1445-1447.

50. Hockly E, Cordery PM, Woodman B, et al. Environmental enrichment slows disease progression in R6/2 Huntington's disease mice. *Ann Neurol.* 2002;51(2):235-242.

51. Spires TL, Grote HE, Varshney NK, et al. Environmental enrichment rescues protein deficits in a mouse model of Huntington's disease, indicating a possible disease mechanism. *J Neurosci.* 2004;24(9):2270-2276.

52. Crabbe JC, Wahlsten D, Dudek BC. Genetics of mouse behavior: interactions with laboratory environment. *Science.* 1999;284(5420):1670-1672.

53. Wahlsten D. Standardizing tests of mouse behavior: reasons, recommendations, and reality. *Physiol Behav.* 2001;73(5):695-704.

54. Wooley CM, Sher RB, Kale A, Frankel WN, Cox GA, Seburn KL. Gait analysis detects early changes in transgenic SOD1(G93A) mice. *Muscle Nerve.* 2005;32(1):43-50.

55. Hingorani AD, Kuan V, Finan C, et al. Improving the odds of drug development success through human genomics: modelling study. *Scientific reports.* 2019;9(1):1-25.

56. Abe K, Aoki M, Tsuji S, et al. Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. *The Lancet Neurology.* 2017;16(7):505-512.

57. Petrov D, Mansfield C, Moussy A, Hermine O. ALS clinical trials review: 20 years of failure. Are we any closer to registering a new treatment? *Frontiers in aging neuroscience.* 2017;9:68.

58. Wobst HJ, Mack KL, Brown DG, Brandon NJ, Shorter J. The clinical trial landscape in amyotrophic lateral sclerosis-Past, present, and future. *Med Res Rev.* 2020;40(4):1352-1384.

59. Mo C, Renoir T, Hannan AJ. What's wrong with my mouse cage? Methodological considerations for modeling lifestyle factors and gene-environment interactions in mice. *J Neurosci Methods.* 2016;265:99-108.

60. Jirkof P, Abdelrahman A, Bleich A, et al. A safe bet? Inter-laboratory variability in behaviour-based severity assessment. *Lab Anim.* 2020;54(1):73-82.

61. Lewejohann L, Reinhard C, Schrewe A, et al. Environmental bias? Effects of housing conditions, laboratory environment and experimenter on behavioral tests. *Genes Brain Behav.* 2006;5(1):64-72.

62. Sena ES, van der Worp HB, Bath PM, Howells DW, Macleod MR. Publication bias in reports of animal stroke studies leads to major overstatement of efficacy. *PLoS Biol.* 2010;8(3):e1000344.

63. Vengoechea J, David MP, Yaghi SR, Carpenter L, Rudnicki SA. Clinical variability and female penetrance in X-linked familial FTD/ALS caused by a P506S mutation in UBQLN2. *Amyotroph Lateral Scler Frontotemporal Degener.* 2013;14(7-8):615-619.

64. Al-Chalabi A, Hardiman O. The epidemiology of ALS: a conspiracy of genes, environment and time. *Nat Rev Neurol.* 2013;9(11):617-628.

65. Lagier-Tourenne C, Baughn M, Rigo F, et al. Targeted degradation of sense and antisense C9orf72 RNA foci as therapy for ALS and frontotemporal degeneration. *Proceedings of the National Academy of Sciences.* 2013;110(47):E4530-E4539.

66. Rizvanov AA, Mukhamedyarov MA, Palotás A, Islamov RR. Retrogradely transported siRNA silences human mutant SOD 1 in spinal cord motor neurons. *Experimental brain research.* 2009;195(1):1-4.

67. Mitsui S, Otomo A, Nozaki M, et al. Systemic overexpression of SQSTM1/p62 accelerates disease onset in a SOD1 H46R-expressing ALS mouse model. *Molecular brain.* 2018;11(1):1-16.

68. Van Damme P, Braeken D, Callewaert G, Robberecht W, Van Den Bosch L. GluR2 deficiency accelerates motor neuron degeneration in a mouse model of amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol.* 2005;64(7):605-612.

69. Son M, Puttaparthi K, Kawamata H, et al. Overexpression of CCS in G93A-SOD1 mice leads to accelerated neurological deficits with severe mitochondrial pathology. *Proceedings of the National Academy of Sciences of the United States of America.* 2007;104(14):6072-6077.

70. Polymenidou M, Lagier-Tourenne C, Hutt KR, et al. Long pre-mRNA depletion and RNA missplicing contribute to neuronal vulnerability from loss of TDP-43. *Nat Neurosci.* 2011;14(4):459-468.

71. Liu W, Venugopal S, Majid S, et al. Single-cell RNA-seq analysis of the brainstem of mutant SOD1 mice reveals perturbed cell types and pathways of amyotrophic lateral sclerosis. *Neurobiol Dis.* 2020;141:104877.

72. Bandyopadhyay U, Cotney J, Nagy M, et al. RNA-Seq profiling of spinal cord motor neurons from a presymptomatic SOD1 ALS mouse. *PloS one.* 2013;8(1):e53575.

73. Ferraiuolo L, Heath PR, Holden H, Kasher P, Kirby J, Shaw PJ. Microarray analysis of the cellular pathways involved in the adaptation to and progression of motor neuron injury in the SOD1 G93A mouse model of familial ALS. *J Neurosci.* 2007;27(34):9201-9219.

74. Lauria G, Dalla Bella E, Antonini G, et al. Erythropoietin in amyotrophic lateral sclerosis: a multicentre, randomised, double blind, placebo controlled, phase III study. *J Neurol Neurosurg Psychiatry.* 2015;86(8):879-886.

75. Pastula DM, Moore DH, Bedlack RS. Creatine for amyotrophic lateral sclerosis/motor neuron disease. *Cochrane Database Syst Rev.* 2012;12(12):CD005225.

76. Pascuzzi RM, Shefner J, Chappell AS, et al. A phase II trial of talampanel in subjects with amyotrophic lateral sclerosis. *Amyotroph Lateral Scler.* 2010;11(3):266-271.

77. Shefner JM, Wolff AA, Meng L, et al. A randomized, placebo-controlled, double-blind phase IIb trial evaluating the safety and efficacy of tirasemtiv in patients with amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Frontotemporal Degener.* 2016;17(5-6):426-435.

78. Raman C, McAllister SD, Rizvi G, Patel SG, Moore DH, Abood ME. Amyotrophic lateral sclerosis: delayed disease progression in mice by treatment with a cannabinoid. *Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases.* 2004;5(1):33-39.

79. Moreno-Martet M, Espejo-Porras F, Fernández-Ruiz J, de Lago E. Changes in Endocannabinoid Receptors and Enzymes in the Spinal Cord of SOD 1G93A Transgenic

Mice and Evaluation of a Sativex®-like Combination of Phytocannabinoids: Interest for Future Therapies in Amyotrophic Lateral Sclerosis. *CNS neuroscience & therapeutics.* 2014;20(9):809-815.

80. Kim K, Moore DH, Makriyannis A, Abood ME. AM1241, a cannabinoid CB2 receptor selective compound, delays disease progression in a mouse model of amyotrophic lateral sclerosis. *Eur J Pharmacol.* 2006;542(1-3):100-105.

81. Benatar M. Lost in translation: treatment trials in the SOD1 mouse and in human ALS. *Neurobiol Dis.* 2007;26(1):1-13.

82. Scott S, Kranz JE, Cole J, et al. Design, power, and interpretation of studies in the standard murine model of ALS. *Amyotroph Lateral Scler.* 2008;9(1):4-15.

83. Tseng AH, Craft RM. Sex differences in antinociceptive and motoric effects of cannabinoids. *Eur J Pharmacol.* 2001;430(1):41-47.

84. Craft RM. Sex differences in behavioral effects of cannabinoids. *Life Sci.* 2005;77(20):2471-2478.