Development of High-throughput, Non-invasive Behavioural and Cognitive Tests in Mice to Uncover New Mechanisms of Abnormal Cognition and Behaviour

Thesis

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Development of high-throughput, non-invasive behavioural and cognitive tests in mice to uncover new mechanisms of abnormal cognition and behaviour

Michelle Stewart
PhD - Life and Biomolecular Sciences
MRC Harwell Institute
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Abstract

Cognition is a complex process encompassing a variety of traits, including the ability learn, remember, evaluate situations, make decisions and solve problems. Research over recent decades has begun to elucidate some of the mechanisms through which organisms carry out these complex behaviours, however there is a great deal that is not understood about the basic biology of cognition. Importantly, there is a deficit in our knowledge not only of the underlying biology but also the pathological mechanisms which lead to cognitive disease.

Genetically altered mice have been developed that model some aspects of cognitive disease. However at present these models have helped to elucidate only certain elements of cognitive processes and many have had limited use in drug development.

To further understand cognition and cognitive disease, as well as develop effective treatments, it is critical to have more genetically altered mouse lines that better model the human condition. To find such models we have employed a high-throughput screening approach. Firstly we assessed existing behavioural and cognitive tests for their suitability to high-throughput testing, we then incorporated the selected test, fear conditioning, into a high-throughput pipeline. 289 different genetically altered (GA) lines were screened using the fear conditioning protocol and GA lines with potentially interesting phenotypes selected for further analysis.

Using this screening process Ferric chelate reductase 1-like (Frrs1l) was selected for more in-depth testing. On further investigation we found deletion of Frrs1l to cause increased neonatal mortality and abnormalities in activity, co-ordination, muscle strength, cognition and body weight, as well as seizures. These phenotypes
appear to be caused by a dramatic reduction in AMPA receptor levels at the synapse, as well as mislocalisation of AMPA receptors and alterations in processing. In collaboration with clinicians we corroborated these findings with some newly identified patients with homozygous mutations in \textit{FRRS1L}.
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put up with six years of going to bed early so I can work but has always been supportive and interested in what I’m doing.

Statement of works

Circadian and sleep data collection and collation was carried out by Gareth Banks.

EEG surgery and analysis of traces was carried out by Petrina Lau.

Synaptic Fractionation was carried out by Silvia Corrochano.

Synapse counts were carried out by Enrico Castroflorio.

Once the fear conditioning phenotyping kit was set up and the protocol developed, the majority of GA lines were tested by the Mary Lyon Centre Phenotyping team.

Genotyping was carried out by the GEMS core at MRC Harwell.

All other work was done by myself, including all phenotyping and data analysis of baseline strains, equipment set up, protocol development and all data analysis of IMPC fear conditioning data. All in-vivo phenotyping of Frrs1l line (excepting those tests listed above), qPCR, western blots and glycosylation experiments, cloning, tagging and site directed mutagenesis of Frrs1l, with immunohistochemical staining and imaging.
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1. General Introduction

1.1 Cognition

Cognition is the mental ability to learn, understand and react to one’s surroundings. It encompasses several processes such as perception, the formation of knowledge, memory and working memory, evaluation, reasoning, problem solving, decision making, comprehension and production of language. Cognitive skills are critically important to humans and feature in almost every aspect of day to day life. It is thought to be the high level of cognitive ability, to solve problems, to learn from mistakes or from other humans and to form memories that help in future situations, that sets humans apart from other species. Importantly, these processes are not exclusive to humans, and many aspects or processes of cognition can be found throughout the animal kingdom (Grant, 2016). For example, one aspect of cognition is the ability to teach and learn from others. There are instances of this in animals as well as in humans, such as meerkats teaching offspring to kill scorpions (Thornton and McAuliffe, 2006). However, teaching in animals appears to be limited to food gathering or stalking (Premack, 2007), whereas teaching in humans, although sometimes done for survival reasons, is often done for a more aesthetic or philosophical motivation, such as literacy, language and numeracy. There are many other examples where a broad definition of a cognitive ability is applicable to both humans and animals, but where human function is seemingly more complex or refined. Spatial learning and memory is a good example, humans have an incredible capability to learn where objects are placed and to plan routes to achieve their goals, exemplified by the well known study of taxi drivers in London.
Mice also have an ability to learn spatial tasks, however this is much more limited, with some strains showing difficulty learning a relatively simple radial arm maze (Ammassari-Teule, Hoffman and Rossi-Arnaud, 1993). This suggests that although there are similar basic processes or functionalities, there are species-specific refinements.

On a molecular level, some of the mechanisms known to be involved in learning and memory are highly conserved throughout the animal kingdom. In fact, the cellular machinery involved in synaptic transmission is present in a similar form even in prokaryotes. Despite the fact that prokaryotes are unicellular and do not have synapses, the vesicle release and response mechanisms involve many similar processes (Emes and Grant, 2012). Evolution of this mechanism has lead to a highly complex system that is remarkably similar across all vertebrate species. As such, studying other species can provide useful insights into the basic mechanisms of cognition which are conserved across diverse animal kingdoms, even though the complexity or magnitude of the response maybe different across species.

### 1.2 The Burden of Human Cognitive Disease

There are many diseases that affect cognition, ranging from relatively mild learning disabilities to very severe cognitive decline or impairment. These diseases have a huge, life-limiting, impact for patients as well as a significant economic and social burden. The number of people affected by these diseases, particularly the subset associated with ageing, is increasing dramatically. For example, in 2016 it was reported that 5.4 million people in the US were living with Alzheimer’s disease (AD). This is projected to increase to 7.1 million by 2025 and 13.8 million by 2050.
(Alzheimer Association, 2016). In the UK, a similar rise in cases is expected, from 832,000 people living with dementia in 2014, to more than double that affected by 2050, an estimated 2 million people. The cost of care for patients with dementia is expected to rise from £23.6 billion in the UK in 2014, to £59.4 billion in 2050 (Lewis et al., 2014). This huge increase can be attributed to an ageing population, 94% percent of people with dementia are aged over 65, as the size of this age group increases so does the incidence of these types of disease. Even a small improvement in prognosis could make a significant medical and economic impact. Studies estimate that delaying the onset of dementia by just two years could mean there are almost 400,000 less people with the disease in 2050, saving £12.9 billion in health care in that year alone (Prince et al., 2014). Cognitive disease is not limited to age related decline, there are many other diseases that occur very early in life or at middle-age, such as schizophrenia, Down’s syndrome, autism and dyslexia which also include cognitive deficits symptomology.

Most cognitive diseases result from alterations in neurodevelopment, neurodegeneration or variations in cell signalling, alone or in combination. Diseases such as Rett syndrome and other Autism spectrum disorders are neurodevelopmental diseases, in which symptoms become apparent in the first few years of life. Although many causes of autism remain elusive, Rett syndrome has a defined genetic foundation. Mutations in MeCP2 cause severe mental and physical disability starting from 6 months of age. MeCP2 is a transcriptional regulator which down regulates a variety of genes. When mutated, regulation of gene transcription is dramatically altered and a variety of symptoms develop (Pelka et al., 2006) including developmental differences in brains of affected children such as smaller
brain size over all, closely packed neurons and reduced dendritic spines (Neul and Zoghbi, 2004).

Angelman syndrome is another example of a neurodevelopmental disorder, again symptoms become apparent in the first year of life, with severe learning difficulties, ataxia and seizures developing from 6 months of age. The cause of Angelman syndrome is a variety of deletions in the region of chromosome 15q11-13, which occur in approximately 70% of cases. Other cases of Angelman syndrome are caused by imprinting abnormalities in the same region of chromosome 15, leading to abnormal methylation and gene expression (Clayton-Smith, 2003; Mabb et al., 2011). Developmental defects are apparent in neurons with dendritic spine length and number being significantly decreased (Dindot et al., 2008).

Neurodegenerative diseases are amongst the most common, especially in the ageing population, with Alzheimer’s disease and Parkinson’s disease much more prevalent after the age of 70 (Alzheimer Association, 2016). Again, the causes of these neurodegenerative diseases are not well understood, whilst the pathologies are described in detail, elements that constitute a cause and elements that are consequences are not well defined for diseases such as Alzheimer’s. A common feature of neurodegenerative disease is the presence of inclusions, protein aggregates in brain tissue. In Alzheimer’s disease these inclusions contain amyloid (Ballard et al., 2011), in Parkinson’s diseases they are predominantly alpha-synuclein (Stefanis, 2012) and in ALS they often contain TDP-43 (Wilson et al., 2011). Cell death of neurons is associated with these inclusions, however the underlying mechanism is unclear. As well as inclusions there are other pathologies, for example, in Alzheimer’s Disease neurofibrillary tangles are a major component of the disease and thought by some to be the primary cause of cognitive decline.
Neurofibrillary tangles contain hyperphosphorylated protein TAU which forms tangles and is followed by a cascade of reactions ending in cell death (Maccioni et al., 2010).

Changes in neurodevelopment and neurodegeneration are often entangled with alterations in signalling mechanisms. Several human diseases arise from mutations in glutamate receptor proteins, for example NMDA components Grin1 and Grin2A (Carvill et al., 2013; Chen et al., 2017). Poorly functioning glutamate receptors result in abnormal cell signalling, reduced current response to stimulation and deficits in neuron development. These are categorised as neurodevelopmental diseases, however dysfunctional signalling plays an important role in the disease mechanism.

Similarly, whilst neurodegenerative disorders may have other primary causes, cell signalling alterations have an important impact on symptoms and disease progression. Aβ oligomers in Alzheimer’s disease have been shown to affect synaptic transmission, even before other pathologies are apparent. Aβ appears to be synaptotoxic, causing degradation of synapses and impairment of learning and memory during early stages of disease (Shankar et al., 2008; Guntupalli, Widagdo and Anggono, 2016). Similarly, many of the symptoms of Huntington’s disease are linked to an imbalance of neurotransmitters glutamate and dopamine and the subsequent alterations in synaptic stimulation that follows (André, Cepeda and Levine, 2010; Jiang et al., 2016).

Several new genome wide association studies have been published in recent years which highlight new genes associated with cognitive diseases. Whilst these studies highlight several possible areas that warrant further study, one area that reoccurs
in many studies is the synapse. Schizophrenia related GWAS are enriched for genes in the post synaptic density or post synaptic membrane (Visscher et al., 2017), genes involved in glutamatergic transmission are significantly enriched in GWAS for major depressive disorders (P. H. Lee et al., 2012; Howard et al., 2018) and synaptic regulation features prominently in screens for cognitive function (Lam et al., 2017). The synapse is the key to neuronal cell communication, therefore it follows that components that maintain synaptic function will also be important in cognitive function.

Although some cognitive diseases have pathology that is relatively well described, disease mechanisms and the initial causes of many cognitive diseases still remain elusive. Consequently, many cognitive diseases have few or no drugs available to help lessen the symptoms (Schapira et al., 2014; Briggs, Kennelly and O’Neill, 2016; Castro, Zaman and Holland, 2017). For those diseases for which there are drugs or therapies available, often these only work for a sub-set of the affected patients (Uyanik, Bumin and Kayihan, 2003; Hazell, 2007; Ballon, Girgis and Lieberman, 2011).

There is a clear need for better understanding of the biological mechanisms involved in forming and maintaining cognitive processes. More in-depth knowledge of cognitive systems will improve our ability to produce useful therapeutics which can stop or ultimately reverse cognitive impairment.


1.3 Brief history of Cognitive Neuroscience

How humans think and the processes involved have been studied for many centuries. From the Ancient Greek philosophers Parmenides and Heraclitus who studied truth and deception, to Descartes in the 17th century and his renowned work on the sense of self. However, these were largely philosophical debates with little thought to the biological mechanisms or processes within the brain. More recently in the 1800s, aspects of the study of the mind, psychology, were compartmentalised into the ‘Science of Behaviour’. Research conducted by Pavlov suggested that behaviour was purely a reaction to a stimulus, this was thought to be reflexive and the process through which the stimulus was interpreted in the brain was largely overlooked. This view of ‘stimulus-response’ continued for the next century until the so called ‘Cognitive Revolution’ began in the 1950s (Miller, 2003). In 1956 ‘The Study of Thinking’, published by Jerome Bruner and colleagues, attempted to explain how information is categorised or conceptualised, how humans develop beyond the information given and how they try to employ rationality within the constraints of human ability (for example with the limitations of memory, bias and ignorance) (Bruner, Goodnow and Austin, 1956). This was a seminal publication that helped to develop the idea that behaviour is not simply a response to a stimulus but the result of a more complex system of interpretation and reaction. The ‘Science of Behaviour’ began to be redefined as the ‘Science of Cognition’ (Bruner, Goodnow and Austin, 1956; Arbib et al., 1978). It is not a coincidence that this revolution in cognitive science coincided with the growth in the computer industry. From the very conception of computing, the possibility of artificial intelligence has been debated and the potential to create machines that
‘think’ has led to the need to further define biological cognition (Simon, 1980; Johnson-Laird, 1989).

In 1978 the Sloane Foundation sponsored an event to bring together various disciplines involved in cognitive science and summarise the current thinking in the field. It became apparent that there were many divergent fields, all working on different as well as overlapping areas of cognitive science, the mainstays being Philosophy, Psychology, Linguistics, Computer Science, Anthropology and Neuroscience (which at the time was a relatively new field)(Arbib et al., 1978). As a direct result of this meeting, cross disciplinary funding allowed the initiation of the field now known as Cognitive Neuroscience, the study of biological processes that underlie cognition (Miller, 2003).

1.4 Biological mechanisms of cognition

Cognition is a very complex phenomenon and the biological mechanisms, are yet to be fully revealed. One mechanism, termed Long Term Potentiation (LTP), was discovered as one of the key processes by which memory is acquired and learning takes place. Although it is important to note that whilst LTP is critical for learning and memory, it is also a core brain processing mechanism essential for many brain functions. In 1973 Bliss and Lomo (Bliss and Lomo, 1973) reported that stimulation of the perforant path in the rabbit hippocampus can induce long lasting potentiation of synaptic transmission. Simply put, this means that by repeatedly stimulating a certain network, the synapses involved become stronger, known as Hebbian synapses after Donald Hebb who first proposed this system in 1949. Extensive
work on this pathway over the last four decades has begun to elucidate some of the mechanisms behind this type of potentiation (Nicoll, 2017).

To understand LTP, first the basic mechanism of glutamatergic synaptic transmission must be understood. An action potential is generated in a neuron following depolarization. This action potential travels down the axon and reaches the presynaptic terminal, where it stimulates release of glutamate into the synapse. Glutamate diffuses across the synapse and upon arrival at the post synaptic terminal, binds with N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. AMPA receptors (AMPAR) open, facilitating transport of Na\(^+\) into the post synaptic neurons in exchange for K\(^+\). Initially NMDA receptors do not open, despite being bound by glutamate, as they are blocked by Mg\(^{2+}\). The early depolarization caused by activation of the AMPA receptors, stimulates removal of the Mg\(^{2+}\) block from the NMDA receptors and allows Ca\(^{2+}\) and Na\(^+\) to enter, again in exchange for K\(^+\). This then initiates rapid depolarization and the induction of an action potential in the post synaptic neuron (Lüscher \textit{et al.}, 1999; Kandel, Schwartz and Jessell, 2000; Watkins and Jane, 2006; Shepherd and Huganir, 2007; Nicoll, 2017). This process of synaptic transmission involves release of glutamate, its diffusion across the synaptic cleft and then two complementary mechanisms in the post synaptic membrane, a rapid response from AMPA receptors, followed by a slower but more robust response from the NMDA receptors, which taken together result in a depolarization induced post-synaptic action potential.

Given the above mechanism, the potentiation of the synapse, as described by Bliss and Lomo, could conceivably occur by two scenarios; either through increase in glutamate release at the presynaptic terminal, or increase in response to glutamate
at the post synaptic terminal. Extensive research has shown both of these mechanisms to be true in certain circumstances, however presynaptic changes are relatively minor and infrequent (Emptage et al., 2003; Bliss and Collingridge, 2013) whereas there is increasing evidence that the major role is played in the post synaptic neuron (Lüscher et al., 1999; Soderling and Derkach, 2000; Watt et al., 2004; Granger and Nicoll, 2013).

LTP can be split broadly into two categories, early LTP (E-LTP) which occurs immediately and lasts for several hours, and late LTP (L-LTP), that lasts for longer than 8 hours and requires protein synthesis (Frey et al., 1996; Frey and Morris, 1997). Many factors contribute to the post synaptic response, both immediately in the synapse, as well as secondary messenger systems and other pathways in the spine and dendrite of the neuron. These interactions are complex and many are still to be illuminated, however calcium Calmodulin Kinase II (CAMKII) can be used to illustrate one mechanism which acts through several routes to strengthen the synapse, invoking a greater response to glutamate. Glutamate binding to ionotropic glutamate receptors causes influx of calcium into the post synaptic neuron. Calcium binds to calmodulin initiating a conformational change and promoting binding with CAMKII. CAMKII undergoes autophosphorylation increasing its ability to phosphorylate substrates as well as promoting the subcellular movement of CAMKII to the post synaptic density (PSD). At the synapse, CAMKII phosphorylates the GLUR1 subunit of the AMPA receptors, increasing channel conductance and causing an immediate short term increase in Na\(^+\) transport (Barria et al., 2007). CAMKII also phosphorylates stargazin, a member of the AMPA receptor outer complex. This phosphorylation promotes an interaction with PSD-95, a prevalent protein in the post synaptic density, and causes mobilisation of the AMPA receptor bound to
stargazin, from the cytoplasm to the PSD (Tomita, Stein, et al., 2005; Tsui and Malenka, 2006). Thus, through CAMKII activation, the efficiency of individual AMPA receptors is increased, as well as an increase in the number of AMPA receptors in the synapse, both of which are short term responses to synaptic stimulation.

The above mechanism for the CAMKII response is well evidenced and generally accepted, however other routes are less well defined. It is known that a second response occurs, still within the timeframe of E-LTP, which involves exocytosis of AMPAR vesicles to increase the pool of available AMPAR, and/or lateral movement of AMPA receptors from other areas of the membrane. This process is not yet fully understood, however Makino and Malinow used pH sensitive fluorescent tagging to demonstrate that surface AMPA receptors from non-synaptic sites move laterally into a synapse undergoing LTP (Makino and Malinow, 2010). They also show that exocytosis does occur but that it happens minutes after the initial stimulation near the dendritic shaft and thus is likely to replenish extra synaptic AMPA receptors rather than increase the number of AMPA at the synapse itself. Patterson et al, (Patterson, Szatmari and Yasuda, 2010) similarly demonstrate that increased numbers of AMPA receptors at the synapse is predominantly a result of lateral movement. However they also show that a small proportion, 10-30%, of newly positioned AMPA receptors in the synapse are exocytosed from vesicles within the cytoplasm. Gerges and colleagues (Gerges et al., 2006) demonstrate the role of the exocyst complex in sorting AMPA vesicles and their fusion at the membrane, suggesting that this is a major mechanism for increasing AMPA receptor availability at the synapse during LTP. It is likely that there are several mechanisms involved in increasing AMPA receptors at the synapse and it may be that all the above are true in part. One of the difficulties of studying LTP is that a large proportion of the
work has to be done *in-vitro*, where cell culture conditions and stimulation paradigms often differ between experiments and may be responsible for some of the conflicting observations. What is clear is that the mechanisms for increasing AMPA at the synapse are complex and there is still some work to do to elucidate what is happening during E-LTP.

Finally, for late long term potentiation (L-LTP), the synapse is strengthened through mechanisms that require the synthesis of new proteins (Martin *et al.*, 1997; Scharf *et al.*, 2002; Karpova, 2006). During L-LTP, protein kinases cyclic adenosine monophosphate (cAMP) dependent protein kinase (PKA) and mitogen associated kinase (MAPK) are activated (Ying *et al.*, 2002). This triggers pathways that activate transcription factors including CREB and ELK-1, which in turn modulate the transcription of many other genes. Newly synthesised proteins have different roles in synaptic enhancement. Some, such as BDNF, increase transport of AMPAR to the synapse (Lu, Christian and Lu, 2008), others facilitate remodelling of the synapse or provide cytoskeletal support for new branches, enabling new synapses to form (Fukazawa *et al.*, 2003; Bramham, 2008).

### 1.5 Animal models for the study of cognitive processes

The process of LTP and associated mechanisms explain in part how synaptic alterations can mediate change in response to stimuli. However, further research has relied on the use of animal models to delineate how these cellular processes are related to basic cognitive function.

One of the early experiments in assessing the relationship between synaptic plasticity and behaviour was performed by Kandel and colleagues, using the sea slug *Aplysia* as a model (Castellucci *et al.*, 1970). In this work they showed
conclusively that behavioural responses, in this case the withdrawal of the gill in response to a stimulus, can induce changes in synaptic transmission, either increasing or decreasing the strength of the synapse depending on the paradigm. There has been much further work on animal models, including Morris et al (Morris, 1989) who used NMDA receptor antagonists to assess changes in learning and memory in a Morris Water Maze. Morris et al showed that in the absence of functional NMDA transport, rats have impaired hippocampal spatial memory. Studies carried out on AMPA receptor related proteins show that reduction in functional AMPA receptors causes learning deficits in mice (Menuz and Nicoll, 2008; Sanderson et al., 2011a; Wang et al., 2011). Further work on Calcium Calmodulin Kinase II (CAMKII), has also produced similar results, with modifications in the CAMKII gene having effects on LTP. Cho et al, show that a point mutation in CaMKII in mice reduces LTP dramatically and reduces place cell specificity in the hippocampus (Cho et al., 1998). Further work by Elgersma et al, demonstrates that functional CaMKII is needed for LTP. Two mouse models, one with a non-functional CaMKII and the second with a modified phosphorylation site on CaMKII, which stops inhibition of kinase activity, both have deficits in Morris water maze ability and in LTP. These two models show that fine tuning of the CaMKII response is essential for appropriate learning, with constitutively active or inactive CaMKII having detrimental effects on control of synaptic plasticity (Elgersma et al., 2002). This work, and research from many others has contributed to a large body of evidence which links the mechanism of LTP with learning deficits in animal models. However, there is still much that remains to be elucidated, for example a proteomic approach to assessing AMPA receptor complexes identified twenty one proteins associated with the AMPA receptor complex that had shown no previous association...
Several of the proteomes found in that study have little known or unknown function. Furthermore, a bioinformatics study of proteomes that are known to be highly expressed in the brain, demonstrated that for over 20% of the proteomes there was no neuroscience literature available (Pandey et al., 2014). It is not likely that all of these proteomes are involved in learning and memory, however their precise function in the brain and impact on processes such as cognition is yet to be revealed. These two studies by Schwenk et al and Pandy et al highlight the fact that there are many brain expressed proteomes of which very little is known. Until the roles of these genes are elucidated there will be a significant gap in our knowledge of how the brain functions.

1.6 Why are mice used for genetic neuroscience?

Inbred mice have been particularly beneficial in studying cognition as the effects of background genetic variability on this and other behaviours is profound. In humans, it is thought that the genetic effect, or heritability, of general cognitive ability is around 50% (Plomin, 2000). This heritability is thought to be due to interrelationships between many genes, as such, modifying just one gene may have a very small, perhaps indistinguishable effect, amongst the background noise. Another more recent study by Davies et al, found 148 distinct loci that have impact on general cognitive function in humans (Davies et al., 2018). To study individual gene function it is critical to be able to control the majority of the genetic effects, allowing focus on the manipulated allele(s).

Of course genes are not the only factors that influence cognitive ability, many studies have looked at the effects of nature versus nurture both in childhood and
in adults. Adoption studies have shown that in childhood the environment has a greater effect, perhaps as high as 80%, demonstrated by the increase in cognitive ability when children are adopted away from abusive families (Plomin et al., 1997; Plomin, 2000). It is also clear in some diseases that environmental factors play a large role, one example is the apparent slowing of cognitive decline in Alzheimer’s patients on exercise regimes (Rolland et al., 2007; Intlekofer and Cotman, 2013). It is therefore critical to be able to control the environment, as well as the genetics. Mice are well suited to this type of experimental paradigm as they can be housed according to strict husbandry regimes, with nutrition and home environment standardised across a whole experiment. Some studies have shown significant differences caused by altering the housing, even for short periods, for example Gerdin et al show that splitting mice in to different cages and then rehousing them after three days can have a significant effect on body weight (Gerdin et al., 2012). Even though the consequences of these husbandry changes can be unclear, keeping everything as similar as possible between the controls and the experimental group is likely to reduce any potentially confounding interactions.

Mice as mammals share many biological similarities to humans including the basic body structure, with most major organs and organ systems functioning in a similar way. The central nervous system is no exception although there are some important differences, for example, the cerebral cortex of a human brain is more elaborately developed than that of a mouse brain, not only in overall size but also surface area, human brains have many sulci and gyri creating folds in the surface that are not present in mice. Conversely mice have a more developed olfactory bulb which is not present in the same form in humans. The functional implications of these differences in brain structure are not clear, as the intricacies of brain
function are not yet elucidated. However, it is likely that humans have a greater capacity for cognitive complexity as the surface area of the cortex is dramatically increased. Similarly, due to the reduction in size of the olfactory area humans will have a decreased olfactory repertoire. Nevertheless, despite these differences, there are also many similarities in structure and function of the nervous system, for example the hippocampus is in a similar position in both species, has a similar neuronal structure and is known to function in the same type of processes (e.g. learning and memory). This is demonstrated in studies of comparable hippocampal damage in rodents and humans and the resulting detrimental effect on memory (Amaral et al., 1996; Clark, Zola and Squire, 2000). The same is true of many of the brain areas, the corpus callosum, cerebellum, midbrain, hypothalamus, thalamus, striatum, pons and medulla, all have comparable structure and some similarities in function between mice and humans, although the details of some of the differences are not yet well defined (Piper M. Treuting, Suzanne M. Dintzis, Denny Liggitt, 2012).

### 1.6.1 Genomes and genome editing

Mice and humans are genetically similar, with over 80% of mouse genes having direct orthologues in humans and only 1% of mouse or human genes thought to have no homolog (Lander et al., 2001; Waterston et al., 2002). Regulatory regions are a little more complex, with some being well conserved and others showing significant divergence between species. For example transcription factor networks are very similar, whereas cis-regulatory regions are diverse (Yue et al., 2014).
Although sharing biological similarities is critical, one significant reason the mouse has become the most commonly used model organism for study of genetics, is the relative ease with which the genome can be manipulated compared to other mammals. Since the generation of inbred strains in the early 20th century, genetic differences within inbred strains have been studied. Creating gene-specific alterations however was not possible until Palmiter and Brinster pioneered pronuclear injection techniques, developing protocols to introduce exogenous DNA, (known as transgenes) into single-celled mouse embryos (Palmiter et al., 1982; Palmiter and Brinster, 1986). Further developments in pluripotent stem cells and injection into blastocysts enabled the direct targeting of specific gene sequences. Alleles that can be created through this stem cell approach include null alleles, specific changes within a gene such as point mutations, or tagging a gene with a reporter sequence. (Nagy et al., 1993; Bedell, Jenkins and Copeland, 1997; Carstea, 2009). Since these first types of genetic manipulation, more complex techniques have been developed. Systems such as the cre-lox system can be used to create conditional knockouts in order to study genes in a spatially or temporally controlled way, as is often necessary due to the lethality of the global knockout (Sauer and Henderson, 1988). The conditional deletion approach is particularly useful when pleiotropic effects of a gene may be masking other phenotypes, as well as and allowing the study of the effects of a gene in a specific tissue.

In recent years with the development of CRISPR/Cas9 technology, the range of species which can be genetically manipulated has dramatically increased (Doudna and Charpentier, 2014; Hsu, Lander and Zhang, 2014). Whilst CRISPR/Cas9 allows manipulations in other species, the historical data collected for mice, in combination with easy housing conditions, short generation times and the development of a
range of phenotyping techniques, means the mouse is still the most appropriate model to use for many studies.

1.7 Validity of cognitive phenotyping tests

As part of the generation of new murine models, selection of appropriate phenotyping tests is essential for detecting relevant phenotypes. Several criteria must be evaluated in order to assess how well a test corresponds to human disease: face validity, predictive validity and construct validity (Chadman, Yang and Crawley, 2009). These criteria can be used in terms of the individual mouse model or to assess the usefulness of the phenotyping tests.

1.7.1 Face validity

Face validity refers to the similarity of the phenotype seen in mice with associated behaviour in humans. Thus, assays are investigated to determine if the response of the mouse is similar to the response of a human in a comparable situation. In terms of cognition this is difficult, since the cognitive abilities of the two species are divergent. Recapitulating complex behaviours in mice can be challenging, especially when making comparisons with human behaviours that require elements of communication and sophisticated social interactions. In order to address this problem, aspects of a cognitive disease can be broken down into specific endophenotypes. Endophenotypes comprise individually measured markers or traits that account for a component of a particular disease. Championed in the field of schizophrenia, the concept of endophenotypes is useful across many diseases and has been widely used in studying mouse behaviour (Gottesman and Gould,
An example of an endophenotype for cognitive disease would be impaired spatial memory, this fulfils the definition of an endophenotype as it is well defined, simple and quantifiable.

### 1.7.2 Construct validity

Construct validity refers to how well a test assesses the endophenotype in question, and whether there are confounding factors or alternative interpretations of the data. For example, Brodkin et al. developed a test which automatically measured behaviours such as grooming. To be valid, this grooming test must reliably identify grooming alone without incorrectly designating other behaviours, such as eating. To identify the accuracy of grooming measurements, known groups were used which have been previously published as having increased grooming behaviour (e.g. wet mice vs dry mice). The software showed an increase in grooming in line with previously published data, therefore the equipment was deemed to have passed the test for construct validity (Brodkin et al., 2014).

### 1.7.3 Predictive validity

Finally predictive validity is assessed. This covers the ability to use the specified test to predict future performance. For phenotyping tests one way this can be done is with drugs. Drugs that produce a known reaction in humans should produce the same reaction in mice, and the results of the phenotyping test should change accordingly. Paterson et al. used this method to assess predictive validity of the elevated plus maze. Mice which were dosed with anxiolytic drugs spent more time in the anxiety inducing open arms, indicating the elevated plus maze had good predictive validity for anxiety assessment (Paterson et al., 2010).
1.8 Endophenotypes used to assess cognitive alterations in mice

1.8.1 Anxiety

There is some evidence that anxiety could be a useful endophenotype for cognitive diseases. Many patients, particularly those suffering from degenerative disorders including Alzheimer’s disease, Parkinson’s disease and other dementias have increased levels of anxiety. This anxiety falls into different categories including generalised anxiety disorder, phobic disorders and panic disorders (Chemerinski et al., 1998; Teri et al., 1999; Walsh and Bennett, 2001; Porter et al., 2003). It is thought that excess anxiety could be one of the early symptoms of dementia and several studies have focussed on the ability to predict the development of Alzheimer’s disease based on the anxiety levels of patients with mild cognitive impairment. These studies are conflicting, with some suggesting a link and others finding no correlation (Sinoff and Werner, 2003; Devier et al., 2009). Typically studies of human anxiety can be difficult to interpret, with various anxiety scales, which do not always correlate, being used to quantify symptoms. Although it is not clear whether this link is causal or simply correlated there is much evidence, that incidence of anxiety associated with Alzheimer’s disease, is much more prevalent than anxiety in non-demented patients (Forsell, Palmer and Fratiglioni, 2003; Porter et al., 2003; Devier et al., 2009).

Using the mouse to study anxiety-related disorders in humans has some relevance. Firstly, on a macroscopic scale, the brain regions thought to be relevant to anxiety are similar in both species, namely the amygdala, hippocampus, thalamus and hypothalamus, although other areas, such as the cortex, are more developed in
humans (Sartori, Landgraf and Singewald, 2011). For some forms of anxiety there are clearer links between the physiology of mice and humans. One study by Cohen et al shows that early life stress has a similar effect on amygdala development in both mice and humans, leading to anxiety in later life for both species due to changes in neurodevelopment (Cohen et al., 2013).

More evidence of conserved anxiety mechanisms come from research into specific neurotransmitters. Several papers have shown that serotonin reuptake inhibitors (SSRIs) reduce anxiety in humans. In mice, similar reductions in anxiety are seen from the deletion of the serotonin transporter gene, which leads to the same physiological consequence of SSRI’s in humans, an increase in serotonin levels at the synapse (Gross et al., 2002; Holmes, Murphy and Crawley, 2003).

In mice, anxiety can be assessed using a variety of behavioural tests, two of the most commonly used being open field and light dark box.

The open field test is a simple test that investigates how much a mouse moves around an open arena for a defined period of time (Cummins and Walsh, 1976; Brown, Chambon and Hrabé de Angelis, 2005; Solberg et al., 2006; White et al., 2013). The arenas are not covered and are brightly lit, therefore creating an anxiogenic environment which, in wild-type mice, provokes responses such as thigmotaxis, with only occasional excursions into the more anxiety-inducing centre of the arena. A proxy for the anxiety levels of individual mice is calculated based on how long and how frequently they are in the centre of the arena, with anxious mice spending less time in the centre. However, there are several factors that could confound this test, including the motivation for the mouse to explore, general activity levels such as hypo activity, which could be related to deteriorating health,
or hyperactivity as a result of a neurological disorder. Hyperactivity can be difficult to untangle from anxiety since anxious mice can often be agitated and hyperactive, in some cases it is not clear whether the hyperactivity is a direct result of the anxiety or an independent phenotype (Tohda, Nakanishi and Kadowaki, 2009; Carpenter, Saborido and Stanwood, 2012; Beraldo et al., 2015). In addition to these challenges of interpretation, open field has disadvantages in that it is easily affected by previous testing regimes and other environmental influences. McIlwain et al. showed that changing the order in which behavioural tests are carried out has a more significant effect on open field results than on other phenotyping tests (McIlwain et al., 2001). This is further exemplified by Simon et al. (M. Simon et al., 2013) in a large study across multiple institutes where different results were seen in open field tests despite testing the same strain, working to the same protocol and harmonising as much as possible in terms of husbandry regimes.

An alternative test for murine anxiety is the light dark box. This involves placing the mouse in an arena, divided into dark and light compartments and recording the time spent in each area, as well as the number of transitions between them. The ratio of the time spent in each compartment as well as the number of transitions can indicate different anxiety states, with anxious mice likely to spend more time in the dark compartment with fewer transitions (Crawley and Goodwin, 1980; Hascoët and Bourin, 2009).

Although the mice have to move from one area to the other, the analysis does not rely so heavily on how much distance the mice have moved, and therefore general activity level may have less influence on the results than in the open field test. Data shows that prior handling and test history have less effect on light-dark box than it does on open field (McIlwain et al., 2001).
Finally, to assess whether these tests have suitable predictive validity, mice treated with drugs known to have anxiolytic or anxiogenic effects in humans, are tested and changes in behaviour in these tests noted. This has been demonstrated for both open field and light dark box. Administration of anxiolytic drugs diazepam and clonazepam increase exploratory behaviour in both tests, characterised by increased movement across the centre in open field and increased number of transitions in the light dark box (Crawley and Goodwin, 1980; Crawley, 1981; Jones et al., 1988; Siemiatkowski et al., 2000).

Taken together with the assessment of face and construct validity, open field and light-dark box can both provide insightful information into the cognitive endophenotype of anxiety. However confounding factors in open field may make interpretation more difficult and care needs to be taken to mitigate these as much as possible.

1.8.2 Spatial and episodic memory

Deficits in spatial and episodic memory are key symptoms in many diseases with a cognitive component, such as pre-motor Huntington’s Disease (Possin et al., 2017), Alzheimer’s Disease (Hof and Bouras, 1991; Laatu et al., 2003), schizophrenia (Park et al, 1992) and Parkinson’s Disease (Morris et al., 1988). Patients are often unable to name or recall objects they have recently been shown (de Toledo-Morrell et al., 2000), they cannot replicate sequences of events they have just witnessed (Guariglia, 2007) or they are unable to search in a systematic manner, revisiting places they have already looked, more often than healthy subjects (Owen et al., 1997).
Whilst there is little known about memory storage and retrieval for many types of memory, for spatial memory in particular there has been significant research which has begun to illuminate some of the mechanisms. Seminal work from O’Keefe and Nadel show that certain cells in the hippocampus, termed ‘place cells’, fire action potentials more often when an animal is in a specific place. Firing of different cells as the animal moves around creates a cognitive map of their surroundings (O’Keefe and Nadel, 1978; O’Keefe et al., 1998). Further work by Moser et al. described the role of grid cells, these cells are found in the dorsocaudal medial entorhinal cortex and, like place cells, fire in accordance with location but at regular intervals, creating a map which resembles a grid. Moser et al. also discovered head direction cells, which fire in accordance with the direction the animal is facing (Fyhn et al., 2004; Moser, Rowland and Moser, 2015). Much of this work, initially carried out in rats, has been recapitulated in mice (Wilson and Tonegawa, 1997). In humans, similar recordings are more difficult but it is clear that the hippocampus plays a major role, as patients with specific hippocampal damage have deficits in spatial memory (Amaral et al., 1996; Astur et al., 2002). Damage to the hippocampus, along with memory deficits is also reported in Alzheimer’s and Parkinson’s disease (Lehéricy et al., 1994; Braak et al., 1995; Fleischman et al., 2005) and Huntington’s Disease (Walker, 2007; Roos et al., 2010).

This behaviour is well represented in mouse models, as mice with mutations in genes linked to Parkinson’s disease, Alzheimer’s disease, and Huntington’s disease all show deficits in spatial working memory. King et al and Deacon et al independently studied the Tg2576 mouse model carrying the Swedish family Alzheimer’s mutation, which results in an over production of amyloid (King and Arendash, 2002; Deacon et al., 2008). Both found that the transgenic mice
performed poorly in spatial recognition tasks. Studies of other models, such as the R6/1 mouse model of Huntingdon’s disease and mice with deletion of the Parkin gene also showed similar deficits in spatial memory (Ransome and Hannan, 2012; Rial et al., 2014). However the prevalence of this phenotype is model dependent, as not all mice thought to model the above diseases show deficits in spatial working memory. This is demonstrated by Filali et al and Karl et al who both find no deficits in two other lines with modified Alzheimer related genes, APP/PS1 double mutants and the J20 model (Filali, Lalonde and Rivest, 2009; Karl et al., 2012a).

The face validity of spatial and episodic memory as an endophenotype for cognition is well evidenced. It is to be expected that not all models of these diseases will have the same phenotypes. The fact that spatial memory is not affected in some transgenic mouse lines, implies that the genes modified in those cases are involved in other disease mechanisms, or perhaps the phenotyping tests were not sensitive enough to determine differences.

There are several tests that can be used to evaluate spatial and episodic memory in mice such as open field habituation, novel object recognition and spontaneous alternation.

Open field habituation uses the same equipment as the test described above (1.8.1), but with a modification to the protocol to allow assessment of memory. A habituation protocol involves performing open field tests on consecutive days, or for longer periods on one day, and evaluating any changes in behaviour. For one day trials, exploratory behaviour in mice decreases as the environment becomes familiar, therefore activity in the first five minutes should be greater than the last five minutes of an hour session. For multiple day protocols, mice will behave
differently in the familiar environment on the second day, as opposed to the novel environment on day one, with decreased activity evident in most mice.

Mouse models for various cognitive disorders show alterations in open field habituation, these include Parkin deficient mice (Rial et al., 2014), the Tg2576 Alzheimer’s model (Deacon et al., 2009) and the YAC128 model of Huntington’s Disease (Van Raamsdonk, 2005).

Whilst this test can reveal convincing changes in behaviour with habituation and memory, there are still issues of interpretation, as the results are sensitive to confounds such as the previous experience of the mouse and to any motor deficits.

Novel object recognition is used to assess the ability of mice to remember whether they have encountered an object before. In general, after an initial phase of increased anxiety, mice will explore a novel object, when they are presented with the object for a second time they show less interest. There are many variants of novel object recognition paradigms in mice, a commonly used paradigm comprises one session familiarising a mouse to two objects, then replacing one for a novel object in a subsequent session and evaluating changes in exploration (Sanderson et al., 2011a). The hypothesis being that if the object is remembered, exploration of that object will be less than on the first occasion.

Novel object recognition has been widely used to assess models of Alzheimer’s disease and whilst some research shows phenotypic deficits such as the Tg2576 Swedish mutation line (Taglialatela et al., 2010; Miedel et al., 2017), other studies show no difference in the same model (Hale and Good, 2005). Other Alzheimer’s models, J20 and APPswe/PS1 double mutants show no difference from wild-type controls (Karl et al., 2012b; Cheng et al., 2014). Worryingly there is also evidence
of some inconsistencies between novel object phenotypes in baseline strains. Sik and Lad independently show evidence of object memory in C57BL/6J mice, as the novel object is explored at levels greater than chance, whereas Van Gaalen shows no preference for the novel object in the same strain and Frick shows sex differences that are not apparent in other studies (van Gaalen and Steckler, 2000; Frick and Gresack, 2003; Şık et al., 2003; Lad et al., 2010). This is concerning as a reproducible and stable baseline is necessary to be able to assess differences in genetically altered (GA) lines. However, these inconsistent results may reflect the use of different protocols, equipment and facilities with different husbandry and testing environments, all of which are known to affect novel object recognition (Tang et al., 2001; Kazlauckas et al., 2011). The validity of this test in terms of specificity for detecting memory function is high, however there is evidence for concerns that the paradigms are not robust enough to produce consistent results.

A further extensively employed test for assessing working memory function is spontaneous alternation (Contet, Rawlins and Deacon, 2001). In one example of this, using a three arm ‘Y maze’ or ‘T-maze’, mice that explore the left arm during the first trial of a test, will chose to explore the right arm on the second trial at a frequency greater than chance. This requires the mouse to process spatial information in the short term, in order to choose the alternate arm on the second trial. Spatial working memory (SWM), as assessed in spontaneous alternation, can be further split into two areas, egocentric, which relies on self-based co-ordinates, or allocentric, which relies on the environment. These two types of working memory are thought to rely on the different brain areas of the caudate and hippocampus respectively (Possin et al., 2017). Spontaneous alteration tasks in mice can be
adjusted to test one or both of these types of working memory, allowing more specificity in the interpretation of the response.

Mice with mutations in cognition related genes have shown deficits in this test. Models of Huntington’s disease (Giralt et al., 2012) Alzheimer’s disease (King and Arendash, 2002) and glutamate receptor dysfunction (Sanderson et al., 2010) all show reduced alternation in a three arm maze. Data for spontaneous alternation appears to be fairly robust with results recapitulated for the same model. For example the Alzheimer’s model Tg2576 showed similar deficits in performance when assessed by several groups (King and Arendash, 2002; Deacon et al., 2008; Mitani et al., 2013).

Although the behaviour to alternate is not an innate human response, the ability to remember and react to the environment, which is a key feature of this test, correlates well with human spatial working memory. As with most tests there are potential confounding factors such as lack of motivation to explore or motor deficits that limit movement. However these can be assessed by running other tests in parallel which measure these potentially confounding behaviours. Evidence of any confounding effect can then be taken into consideration when interpreting alternation behaviour. This evidence suggests that this test is suitable for assessing the endophenotype of spatial or episodic memory.

Assessing the predictive validity of the three tests listed above, open field habituation, spontaneous alternation and novel object recognition, is more difficult. For spatial and episodic memory there are no drugs available that can reliably improve spatial memory, and therefore it is not possible to assess whether mutant mice with deficits can show improvement after drug administration. We can
however assess the converse, as drugs are available that can reduce the ability to perform in spatial memory tasks. Amphetamine, scopolamine and morphine all reduce alternation and exploratory behaviour (Anisman and Kokkinidis, 1975; Kitanaka et al., 2015). Scopolamine also reduces object recognition, as does diazepam and alprazolam (Dodart, Mathis and Ungerer, 1997; Bertaina-Anglade et al., 2006). Scopolamine blocks the cholinergic system, which is known to be affected in Alzheimer’s disease (Kása, Rakonczay and Gulya, 1997; Mufson et al., 2009) whereas diazepam increases GABAergic signalling causing general reduction in excitability in many brain areas. All of these drugs are likely to be non-specific, possibly having effects on many brain functions, rather than just memory alone. Therefore the usefulness of using these drugs to assess validity of cognitive tests is unclear since they are not acting on a single mechanism but causing widespread disruption. Nevertheless, it has been demonstrated that these tests can provide assessment of spatial memory and that GA lines with modifications that are predicted to alter spatial memory can show phenotypes in this test.

1.8.3 Hippocampal function

Hippocampal function incorporates a collection of endophenotypes including memory and anxiety that relate to cognition. Elements of hippocampal function, such as spatial memory, have already been covered in the previous section, however there are other less specific tests which can be used to examine function in general, such are marble burying and digging.

A study by Deacon et al (Deacon and Rawlins, 2005) shows that damage to the hippocampus in mice alters species specific behaviours such as digging, measured by the removal of burrowing substrate from a tube or burying measured by how
mice cover marbles with sawdust. It is suggested that deficits in these behaviours could be connected to hippocampal damage seen in the early stages of Alzheimer’s. The hippocampus is a complex region that carries out many different roles and as such controls many different behaviours including learning, memory, place recognition, anxiety, depression, stress and sociability (Leuner and Gould, 2010). Clearly this wide range of functions in humans do not all correlate to digging behaviour in mice. Conversely, repetitive behaviours such as digging can also be indicative of other changes, such altered brain function in areas other than the hippocampus.

However, it is possible that a measure such as digging behaviour is useful as a simple primary assessment of broad hippocampal function, since it is clear that damage to the hippocampus affects this behaviour. Jirkof and Deacon both suggest that, as well as being modified by hippocampal damage, digging behaviour can be affected by other conditions impacting general wellbeing, and whilst this test could identify problems with the hippocampus it could also highlight other non-specific problems such as general ill health (Deacon, 2009; Jirkof, 2014).

Hippocampal function as determined by digging has promising predictive validity, with anxiolytic drugs alprazolam and diazepam, reducing the number of marbles buried (Nicolas, Kolb and Prinssen, 2006), as do anti-depressant drugs citalopram and fluoxetine (Takeuchi, Yatsugi and Yamaguchi, 2002; Huang, Bannerman and Flint, 2008). Reduction in digging in response to anti-depressant drugs shows that modifying neurotransmitter signalling has effects on marble burying and indicates this could be a useful simple test to assess brain function of an undefined cause.
1.8.4 Non-declarative memory

Non-declarative or implicit memory is often thought of as unconscious memory, the ability to react to a stimulus without thinking, a learned reaction rather than a conscious recollection. Non-declarative memory is often compromised in cognitive disease. In Alzheimer’s disease, deficits in implicit memory are demonstrated by the inability to remember primed word lists (Fleischman et al., 2005) and in Parkinson’s by the inability to improve the time spent finding objects in a contextual cueing task (van Asselen et al., 2009). Implicit learning and memory is also impaired in several neurodevelopmental disorders, for example Prader-Willi syndrome (PWS). PWS is a disorder which involves abnormal imprinting or deletion of a region of chromosome 15. PWS patients have impairments in implicit memory tasks such as the Tower of London task. Similarly, patients with Williams syndrome, another neurodevelopmental disorder caused by deletion of a chromosomal region, this time on chromosome seven, have deficits in implicit learning and memory. Williams syndrome patients are slower to learn procedural tasks and reaction time to serial learning tasks does not improve with practice (Vicari, Bellucci and Carlesimo, 2001; Vicari, Verucci and Carlesimo, 2007).

Another method of assessing implicit memory is to use fear conditioning. Fear conditioning uses the association of a conditioned stimulus with an unconditioned aversive stimulus. In humans this is done by quantifying skin conductance, a measure of autonomic arousal which changes in response to an event. Subjects learn to associate a certain shape, the conditioned stimulus, to a loud noise, the unconditioned stimulus. Deficits in this fear conditioning have been associated with dementia in humans, as Hamann et al demonstrated (Hamann, Monarch and Goldstein, 2002). Patients with AD do not learn to associate the image of a shape
with a loud noise, whilst unaffected controls show a clear change in behaviour. However fear conditioning is a test mostly developed for use in animal models with only one other study published using a similar paradigm in humans with dementia. This second study shows a similar lack of non-declarative memory in patients suffering from frontal temporal lobar dementia (FTD) (Hoefer et al., 2008). Both studies by Hoefer et al and Hamann et al are limited in terms of samples size with only small patient groups being involved, however the conclusions are clear, non-declarative memory measured through fear conditioning is affected in both Alzheimer’s and FTD. Non-declarative memory seems to be a clear choice for an endophenotype to assess cognitive disease due to its appropriate face validity.

In mice, non-declarative memory can be assessed using a fear conditioning paradigm with a similar premise to the test used on humans. In this case the paradigm requires the mice to form an association between a conditioned stimulus, such as a sound pulse, and an un-conditioned aversive stimulus, such as a foot shock.

Despite the lack of fear conditioning testing in humans the test has been used widely in animal model research, and specifically transgenic models of AD such as APPV717F, J20 and APPswe (Gerlai et al., 2002; Bonardi et al., 2011; Karl et al., 2012a). These studies find fear conditioning deficits in several Alzheimer’s models. It has also been shown that fear conditioning phenotypes in mice correlate with other learning deficits, such as impaired alternation (Corcoran et al., 2002), and with mice that have genetic alterations in Alzheimer’s related genes such as Tg2576 which shows not only fear conditioning deficits but also deficits in spatial learning and memory (Barnes and Good, 2005; Deacon et al., 2008). In terms of neurodevelopmental disorders, several mouse models lacking genes involved in
these disorders show deficits in fear conditioning. For example, mice lacking *Ube3a*, a gene associated with Angelman’s syndrome, have deficits in contextual but not cued fear conditioning (Jiang et al., 1998) and mice with deletion of *Cyln2*, which causes Williams syndrome, show deficits in contextual fear conditioning for both homozygous and heterozygous deletions (Hoogenraad et al., 2002).

Fear conditioning (FC), like most cognitive tests in mice, can be confounded by the differing inherent activity levels. The test relies on recording freezing behaviour which can be altered significantly if the mice are hypo or hyper active. However, baseline readings of activity taken at the start of the test can somewhat control for this and reduce the risk of incorrectly identifying a cognitive phenotype, giving this test good construct validity.

Finally, to evaluate predictive validity, the use of pharmacological agents or specific brain damage, on fear conditioning behaviour has been assessed. Saxe et al. showed that when hippocampal neurogenesis is ablated using focal radiation, contextual fear conditioning is impaired. Interestingly in this study they do not see any effect in y-maze or Morris water maze, implying that neurogenesis is needed for non-declarative but not spatial memory (Saxe et al., 2006). They also find that only the contextual conditioning is altered, the cued fear conditioning is normal. There is evidence indicating that cued fear conditioning is linked to the amygdala rather than the hippocampus, therefore ablation of parts of the hippocampus would be expected to have no effect on cued response (Phillips and LeDoux, 1992). Other work by Bardgett et al., shows that NMDA receptor antagonists, which are known to reduce LTP, reduce the response of both contextual and cued fear conditioning in mice. It is likely that this affects both cued and contextual FC as the reduction
in NMDA response is not limited to the hippocampus but is throughout the brain (Bardgett et al., 2003).

1.9 Unknowns in the genetics of cognitive disease

The brief summary of behavioural phenotyping tests above, gives an outline of some of the tests currently used in cognitive research in mice, how they can detect phenotypes relevant to cognitive disease and some of the inherent strengths and weaknesses associated with each.

Nevertheless, although there is a growing number of mouse lines available, there are still many unknowns in our knowledge of cognition and the mechanisms through which these complex processes function. This gap in knowledge has been highlighted in the plethora of genome wide association studies (GWAS) conducted over the last decade. For schizophrenia alone 108 GWAS loci have been identified as needing further investigation (O’Donovan et al., 2008; Sullivan et al., 2008; Kanazawa et al., 2017) with a further 28 being associated with bi-polar disorder (K. W. Lee et al., 2012). GWA studies for Alzheimer’s diseases have produced similar results, with many uncharacterised genes being indicated as possible contributors to disease susceptibility (Waring and Rosenberg, 2008; Bertram and Tanzi, 2009; Lambert et al., 2013; Gusareva et al., 2014). For example Lambert et al, found 11 genes with links to various systems including the immune system, synapse function and axonal transport. Many of the genes highlighted in these screens have little known of their normal function or potential role in disease. Similarly GWAS for autism spectrum disorders also highlight several genes about which little is known such as SEMA5A and MACROD2, or indicates genes where part
of the function is known but the disease causing mechanism is unclear, *TRIM33* or *CDH9* are just two of many examples where this is the case. There is also growing evidence that, rather than one gene causing one phenotype, for some cognitive disorders, it maybe that one genetic variation can cause a variety of cognitive or neurological phenotypes, dependent on environmental factors and/or genetic background. As shown by Qin et al and Steinhausen et al who both show that families of patients with schizophrenia have a higher incidence of epilepsy and anxiety disorders (Qin et al., 2005; Steinhausen et al., 2009). It is not clear what effect the environment has on these cases but it is clear that there are complex interactions that are not yet understood.

As well as the relatively common disorders, such as schizophrenia, anxiety and dementia, there are also a large number of rare cognitive diseases. In recent years, rare disease consortia have been set up to work together in an attempt to increase success in diagnosing and treating some of these disorders. As of 2016, there were over 7000 rare diseases named by the International Rare Disease and Research Consortium (IRDiRC). Of these, only around half have a known genetic cause and more than half have no known mechanism (Lochmüller et al., 2017). Whilst many of these rare diseases are not cognition related, a large proportion of them are, with 7 of the 22 consortia in IRDiRC focussing solely of neurological disorders, including synaptopathies, neuropathies, frontal temporal lobar degeneration and Rett related disorders. There is also overlap of cognitive symptoms in other IRDiRC consortia, such as mitochondrial disease and dystonia.

This evidence from novel genes found in GWAS of common disorders, to rare diseases with no known underlying genetic mutation, demonstrates a clear need
for more in-depth understanding of the genetics of cognition and associated cognitive disorders.

Although many mouse models have been made over the last decade, there is still a huge portion of the genome of which little is known and no models are available. A recent commentary article revealed that although there are approximately 20,000 genes in the human genome, a subset of only 100 genes make up a quarter of the published papers concerning gene function (Dolgin, 2017). In addition, in 2014 Pandey et al. assessed the literature available for genes that were highly expressed in the brain and discovered that 5% of the genes were responsible for 70% of the papers, and that 20% of the genes had no literature about them at all (Pandey et al., 2014). These remarkable reports highlight an extensive gap in our knowledge of which genes are involved in neurological processes and how they function individually and in networks.

Crucially, the mouse models that are widely studied have not given rise to useful therapeutics, with very few drugs currently available to treat many types of cognitive disorder (Schapira et al., 2014; Briggs, Kennelly and O’Neill, 2016). It is possible that the mouse models used in the past have not resulted in useful therapeutics because they are the wrong models; it maybe that the current models have been focussed on a subset of genes which may not be critical in the disease process, or they are compromised by uncontrolled genetic background or poor study design (Begley and Ioannidis, 2015; Justice and Dhillon, 2016).

There is a desperate need for new models of cognitive disease, not only in genes that are currently known in order to elucidate mechanisms thought to be disease
causing in humans, but also across genes that have no known function. Studying these uncharacterised genes may illuminate new mechanisms or highlight previously unlinked pathways, which could in turn lead to a greater understanding of diseases pathogenesis and potential new drug targets.

New mouse models of cognitive disorders are essential to further our understanding of cognitive processes, to find new targets for drugs and to assess treatments for the many currently untreatable cognitive diseases.

1.10 Assessing mammalian gene function

To address the lack of comprehensive data, a greater assessment of mammalian gene function is needed to understand in more detail the proteins and pathways that have already been studied, and also to discover the roles of novel proteins and genes in cognitive function.

The two leading methods for assessing mammalian gene function using animal models are the approaches of forward and reverse genetics (Takahashi, Pinto and Vitaterna, 1994). Reverse genetics involves selecting a gene to study, based on various factors including any known function, relationship to known pathways, expression patterns, similarity to other genes, data from other species and GWAS studies. A genetically altered (GA) strain carrying the mutation of choice is created, and the mouse line undergoes an in-depth phenotyping study.

Forward genetics is the opposite of this, starting with a phenotype and then looking for the gene responsible. This involves screening of many animals through a phenotyping pipeline. The animals used in this approach have often been produced
through chemical mutagenesis, for example N-nitroso-N-ethyl-urea, which creates multiple point mutations in each founder animal. These point mutations are inherited in offspring of founder animals, who are then assessed through phenotyping pipelines. This technique has the advantage of being completely unbiased and entirely novel gene functions have been uncovered using this approach (De Angelis et al., 2000; Nolan et al., 2000; Potter et al., 2016). A disadvantage of this has always been the mapping of the mutation. With the advent of Next Generation Sequencing, this has become much easier, however identifying the mutation can still take time and each potential genetic alteration has to be fully validated.

With the huge increases in efficiency of genome engineering in the last decade there is now another option that provides a method for unbiased screening. Rather than using chemical mutagens to randomly mutate the genome, large ES cell resources (Skarnes et al., 2011), and now CRISPR/Cas9 technology (Doudna and Charpentier, 2014; Hsu, Lander and Zhang, 2014) can be used to generate genetic alterations on a scale unimaginable a decade ago. This is a significant undertaking requiring large scale resources, however many facilities across the globe are now generating knockout lines, and specific mutations, for many hundreds of genes every year (Brown and Moore, 2012b). Some of these facilities have formed consortia and have been systematically knocking out genes and assessing these GA lines through phenotyping pipelines (Ayadi et al., 2012) as a way of carrying out unbiased screens and finding novel gene function. These large screens do not create libraries of point mutations or complex modifications, most of these screens are for knockouts only. Whilst this knockout data is useful, it does omit the subtleties of point mutation alleles that alter gene function in a multiplicity of ways.
Hypomorphs, hypermorphs, antimorphs and neomorphs can be incredibly useful, especially when full deletions causes lethality (De Angelis and Balling, 1998). Often mutations generated by chemical mutagenesis resemble human disease mutations more closely than complete deletions.

The other limitation of this type of high-throughput approach is that the phenotyping assessment is a screen only. The tests do not probe deeply into any one phenotyping area, rather they are designed to provide broad systematic information into a variety of body systems. However, a great advantage is that this method produces large phenotype datasets on many different genetically altered lines, and provides a large resource of GA lines available to the scientific community. These data can help inform future in-depth research which would then be carried out by more specialist laboratories using the models made available through these consortia (Ayadi et al., 2012; Bradley et al., 2012; Dickinson et al., 2016; Meehan et al., 2017).

1.10.1 The International Mouse Phenotyping Consortium

One such high-throughput screen currently underway at MRC Harwell is that of the International Mouse Phenotyping Consortium (IMPC). The IMPC is a worldwide consortium with 19 research institutions across 11 countries and 5 national funders. The aim of the IMPC project is to create a knockout line for all protein coding genes in the mouse genome and phenotype them through a comprehensive phenotyping platform (Brown and Moore, 2012a). MRC Harwell is funded to generate and phenotype at least 800 GA lines as part of the IMPC programme. Generation of lines is already underway and new lines are available to phenotype continuously, therefore presenting a unique opportunity to screen these newly
generated GA lines for potential cognitive deficits. The IMPC is an excellent resource producing data on many different biological systems. However, at the outset of this work, the IMPC phenotyping pipeline did not include a test that was suitable to assess cognition. Numerous tests exist which are capable of assessing complex cognitive function, however many of these take a long time to perform or include protracted training periods. If a test could be distinguished which would provide useful data on cognitive function, whilst being robust enough to withstand high-throughput constraints, then a great wealth of information could be gathered about these new GA lines.

### 1.10.1.1 New mouse models

The opportunity to screen a plethora of new GA mouse lines for cognitive and behavioural disorders may lead to discovery of new models for cognitive disease. However, to be of use to the research community these models must be an improvement on the models already available. The IMPC hopes to provide better models through several mechanisms, firstly comprehensive QC of either ES cells or alleles generated through CRISPR/Cas9 technology, to ensure that the genetic makeup of the models is thoroughly characterised (Mianné et al., 2017). Where possible, mice will be generated using the knockout first cassette (Skarnes et al., 2011), which allows assessment of the constitutive deletion as well as the potential to create a conditional deletion to further delineate gene function. Mouse models generated through either the knockout first approach or through CRISPR/Cas9 will have disadvantages, as mentioned above (1.10), these models will be deletions rather than point mutations so may lack subtle changes that are seen in humans. However, for novel genes, a deletion is likely to produce a useful initial indication
of the gene function and highlight whether the gene is suitable for further study. Moreover, in high-throughput studies, small changes are difficult to delineate therefore the extremes of complete deletions may provide more robust data.

Throughout IMPC genetic background will be consistent, with all lines across the consortia being generated on a coisogenic C57BL/6N background. Finally, data generated will be made freely available on web portals and crucially new GA lines will be made available from repositories throughout the world.

Several papers have been published in the last 12 months on the initial data from the IMPC screen. So far these data have revealed that around 35% of genes are essential for survival, with the homozygous knockouts being lethal or sub-viable (Dickinson et al., 2016). Analysis of data on auditory function for 3006 GA lines has found 67 lines with hearing deficits, 52 of which are in novel genes with no previous link to deafness (Bowl et al., 2017). Furthermore analysis of data on metabolism for 2016 GA lines found that 974 lines had a strong metabolic phenotype, 429 of which has some known function but had no link to metabolism and 51 genes had no functional annotation at all. Human orthologues of 23 of these 51 genes were subsequently found to be associated with metabolic disease in human GWAS studies (Rozman et al., 2018).

These papers show the range of data that has been produced by the IMPC screen so far and the range of phenotyping models that have been generated. A large hit rate of 48% was found in the metabolism study, whereas only 3% of genes were found to have deafness phenotypes. However, it is clear that in both of these areas, new and interesting models have been generated and, with further study, these
models could provide important insight into the mechanisms underlying deafness or metabolic disease.

1.10.1.2 Comprehensive data adds value to cognitive phenotyping assessment

One of the advantages of broad based phenotyping platforms is that a single mouse can be compared across multiple tests. Even though the other tests may not be directly related to the field of interest they can often help with interpretation of the phenotype. SHIRPA (SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine at St Mary’s; Royal London Hospital, St Bartholomew’s and the Royal London School of Medicine; Phenotype Assessment) is an example that provides useful data for interpreting results from cognitive tests, either by revealing confounding factors or by highlighting other phenotypes which may be relevant. SHIRPA consists of several levels of phenotype assessment, the most commonly used is the primary screen for behavioural observations (Rogers et al., 1997; Nolan et al., 2000) which is now frequently used on its own as a modified-SHIRPA protocol (Masuya et al., 2005; Filali, Lalonde and Rivest, 2009; Ayadi et al., 2012; Brown and Moore, 2012b). The modified-SHIRPA protocol employed as part of the IMPC pipeline assesses the following:

- Contact righting
- Aggression
- Vocalisation
- Head bobbing
• Unexpected behaviours
• Startle response
• Trunk curl
• Limb grasp
• Gait
• Locomotor activity
• Tail elevation
• Activity in viewing jar
• Transfer arousal
• Tremors


Many of the phenotypes highlighted in the SHIRPA test are often present in mice with cognitive dysfunction. For example, mice with motor neuron disorder or Huntington’s disease show cognitive deficits alongside gait abnormalities and tremors (Corrochano et al., 2012; Mandillo et al., 2014).

Similarly, data from the grip strength and pre-pulse inhibition (PPI) tests can add useful insight. Mice with neurodegenerative disorders often show muscle weakness, and mice with schizophrenic like tendencies can show discrepancies in PPI, both with or without co-presentation of cognitive deficits (Geyer, Mcilwain and Paylor, 2002; Powell, Zhou and Geyer, 2009). That is not to say mice will always show phenotypes in every test, but rather if there are multiple phenotypes across several tests, a more accurate interpretation of the data can be made.

As well as in-vivo phenotyping, the IMPC pipeline also includes some ex-vivo analysis that could prove useful to the interpretation of data from cognitive tests.
The majority of IMPC alleles are knockout first lines derived from modified ES cells (Skarnes et al., 2011) (Figure 1).

Figure 1 The tm1a ‘knockout-first’ allele contains an IRES:lacZ trapping cassette, alongside a promoter driven neo cassette, and a floxed critical exon from the selected gene. When crossed to a Flp recombinase, the tm1a allele can be converted to the tm1c allele, which restores gene activity and leaves the floxed critical exon, ready for excision with tissue or time specific cre. Cre deletion of the tm1a allele removes the promoter-driven neo selection cassette and the floxed critical exon. This generates a global knockout of the selected gene leaving a lacZ- tagged allele (tm1b). Grey squares denote exons, in this case exon three is floxed in tm1a and tm1c alleles and deleted in tm1b and tm1d.

The mice generated for phenotyping have been through a cre excision step, creating the tm1b allele. This tm1b allele has a splice site acceptor at a critical exon that fuses the murine gene to a LACZ element before halting transcription. Therefore LACZ is transcribed, and consequently the protein β-galactosidase is present in all tissues where the original gene would have been expressed. This
allows assessment of gene expression using X-gal staining to indicate β-galactosidase activity.

Again, this does not allow the assessment of cognitive function, but provides useful insight into gene function by highlighting areas of the brain where the gene in question is expressed. This approach is limited, as with many tests in a high-throughput setting, a balance has to be struck between gathering useful data and capacity. As such gene expression is only assessed at two time points, embryonic day 12.5 and adult (over 8 weeks of age). Although this does not provide a complete picture of the gene expression it does provide a useful snapshot during development and again in adulthood which nonetheless can be very informative.

Data from the above tests, taken together with the results of the new test for cognition, will allow assessment of phenotypes of many, as yet uncharacterised, GA lines.

1.11 Aims of this project

- Current cognitive phenotyping tests will be developed and validated followed by assessment of their suitability in high-throughput screening. This assessment will be carried out by testing several baseline strains and performing statistical analysis on all data.
• Once a suitable test is established, this will be incorporated into the International Mouse Phenotyping Consortium (IMPC) pipeline, which is already underway at MRC Harwell but currently lacks a suitable test for cognition.

• Knockout lines generated as part of the IMPC programme will be screened using the assigned cognitive assessment.

• Any mice showing a phenotype will be considered for further in-depth testing after triage based on cognitive phenotyping, results from other phenotyping tests and known information about the gene.

• One line will be taken forward for further molecular analysis with the aim to develop a new model to provide insight into cognitive mechanisms and disease.
2.0 Materials and Methods

2.1 Animals

2.1.1 Housing conditions and licensing
All mice were kept and studied in accordance with UK Home Office legislation and local ethical guidelines issued by the Medical Research Council (Responsibility in the Use of Animals for Medical Research, July 1993; Home Office licenses 30/2890 and 30/3384). Mice were fed ad libitum on a commercial diet (SDS Rat and Mouse No. 3 Breeding diet, RM3) and had free access to water (9–13 ppm chlorine). Mice were kept under controlled light (light 7am–7pm, dark 7pm–7am), temperature (21±2°C) and humidity (55±10%) conditions. All mice were bred in house and maintained in Techniplast Blueline caging with 542cm² floor area. All cages had aspen wood chip bedding, one cardboard tunnel and white paper shavings (Datesand, Manchester). Mice were housed in same sex groups of 3-5.

2.1.2 Mice for cognitive phenotyping assessment
Five inbred strains were used for assessment of high-throughput cognitive tests (Chapter 3): 129S9/SvEvH, C57BL/6J, C57BL/6NTac, C3H/HeH and FVB/NCrl.

2.1.3 Mice for IMPC phenotyping screen
All mice were coisogenic on the C57BL/6NTac background. Mice were derived from ES cells carrying the Knockout first cassette (Figure 1). These mice carrying the tm1a allele were converted to the tm1b allele (and therefore a global deletion) by
cre recombination to remove the selection cassette and the floxed critical exon (Skarnes et al., 2011). In the initial stages of the project the cre recombination involved breeding mice carrying the tm1a allele to mice carrying a ubiquitously expressed cre (C57BL/6NTac-Tg(ACTB-cre)3Mr/H). Offspring from this cross carried the converted allele, now tm1b, and the cre recombinase. A further breeding step to C57BL/6NTac was carried out to remove the cre allele before bulk up and intercrossing for experimental cohorts. In later stages of the project a soluble cell permeable cre was used to perform the recombination on embryos in-vitro (see 2.1.4). Finally, in the latter part of the screen an increasing number of lines were generated using CRISPR/Cas9 technology to create null alleles.

2.1.4 Generation of Frrs1l

Frrs1l<sup>tm1a/+</sup> mice were derived from C57BL6/NTac ES cells. Frrs1l<sup>tm1a/+</sup> was converted to the null tm1b alleles by carrying out an IVF using Frrs1l<sup>tm1a/+</sup> sperm and C57BL6/NTac oocytes. Soluble cell permeable cre (TAT-Cre (Tat-NLS-Cre, HTNC, HTNCre), Excellegen, Rockville, USA) was added to two cell Frrs1l<sup>tm1a/+</sup> embryos to convert them to embryos that express the Frrs1l<sup>tm1b/+</sup> allele. The cre excised the selection cassette and floxed critical exon, creating a null allele. Following washing to remove the soluble cre, the IVF procedure was completed as normal. The tm1b mice were crossed to C57BL6/NTac and heterozygous progeny then intercrossed to create Frrs1l<sup>tm1b/tm1b</sup>, Frrs1l<sup>tm1b/+</sup> and Frrs1l<sup>+/+</sup>mice.
2.1.5 Conditional deletion of Frrs1l

The Frrs1l knockout first (tm1a) line was utilized to create a conditional allele by crossing Frrs1l\textsuperscript{tm1a/+} to a GA line which expresses flp recombinase maternally, (Gt(ROSA)26Sor \textsuperscript{tm2(CAG-flpo, -EYFP)Ics}). This expression pattern enables the conversion of the tm1a allele to tm1c in utero. Frrs1l\textsuperscript{tm1c/+} flp negative mice were next crossed to B6.Cg-Tg(UBC-cre.EsR1)1Ejb/J which enables the translocation of ubiquitously expressed cre to the nucleus on administration of tamoxifen. Frrs1l\textsuperscript{tm1c/+ cre positive males were crossed to Frrs1l\textsuperscript{tm1c/+ cre negative females to produce phenotyping cohorts. Frrs1l\textsuperscript{tm1c/tm1c cre positive mice when dosed with tamoxifen should convert to the tm1d allele to make Frrs1l\textsuperscript{tm1d/tm1d}, the knockout allele. Since the cohort is still in testing and deletion to generate the tm1d allele has not been confirmed by qPCR, this group will be referred to as ‘Frrs1l\textsuperscript{tm1c/tm1c, cre positive, tamoxifen dosed’.

2.2 Phenotyping

2.2.1 Behavioural phenotyping

For all behavioural phenotyping tests, mice were taken into the phenotyping room at least 20 minutes before the test to acclimatise.

2.2.2 Assessment of high-throughput cognitive tests

2.2.2.1 Mice used in high-throughput cognitive test assessment

129S9/SvEvH, C57BL/6J, C57BL/6NTac, C3H/HeH and FVB/NCrl mice were group housed in cages of five from weaning. Testing started at 10 weeks of age +/- 4
days and mice were subjected to a pipeline comprising open field test, novel object test, light dark box test, marble burying test, spontaneous alternation test and digging test. Tests were carried out at intervals of between 3 and 7 days but these intervals were consistent between groups and tests. Fear conditioning was carried out on a subset of each strain (n=10 for each strain/sex) at approximately 12 months of age. FVB mice were not tested in fear conditioning due to the inability of the current tracking system to distinguish white mice.

### 2.2.2.2 Open field habituation

This test is used to assess behaviour in a novel environment, including exploration, anxiety and motor function.

Four square arenas (44x44cm) were set up in a square in a small testing room with a video camera overhead. Lighting in arenas was set at 150-200 lux. A minimum of two and a maximum of four mice were tested at one time, one mouse per arena. Arenas were cleaned with 70% ethanol before and after each trial.

Each mouse was placed in the corner of an arena and recorded using video tracking software (Ethovision, Noldus, Netherlands). After 30 minutes, each mouse was removed from the arena and placed back in its home cage. The test was repeated after 24 hours. All open field testing was carried out in the morning, between 8am and 12am.

Videos were analysed using Ethovision software. Each arena was divided into three zones, periphery (8 cm from the edge of the arena), centre (an 11x11cm box in the centre of the arena), and the remaining intermediate zone. Parameters measured for each zone were; total distance moved, latency to first entry, duration, frequency and velocity (Cummins and Walsh, 1976).
2.2.2.3 Open field test

For standard open field one trial test, protocol was as above however only one trial of 20 minutes was carried out on a single day.

2.2.2.4 Novel object test

Arenas were cleaned with 70% ethanol, dried and lux level set to 150-200 luc. Mice were habituated to the arenas for 10 minutes a day for three days immediately prior to the novel object test day. On the day of the test (the fourth day), two identical novel objects were placed in each open field arena, in two corners, 10cm from the two corner sides. The mice were placed in the arena near the wall opposite the objects and facing away from them. Behaviour was recorded using video tracking software (Ethovision). After 10 minutes the mice were removed from the arenas and placed back in their home cage, the objects were also removed and the arenas cleaned down with 70% ethanol. Two more objects, one familiar from trial one and one novel, were placed in the arenas in the same locations as previously. After four minutes in their home cage the mice were placed back in the arena and
video recorded for five minutes. Objects and arenas were cleaned with 70% ethanol between trials. Video tracking was analysed using Ethovision software. Each arena was split into four quarters, one for each of the objects and two empty. Data was analysed per zone to give parameters; duration, frequency, velocity and distance moved (Sanderson et al., 2011b). All mice were tested between 8am and 12pm.

### 2.2.2.5 Marble burying test

9 marbles were placed, in an evenly spaced pattern, in Techniplast blueline cage bases containing approximately 4cm depth of sawdust. One mouse was placed in each cage and plastic IVC lids were positioned on top. After 30 minutes the number of marbles more than two thirds buried were counted (Deacon, 2009). Tests was carried out in the afternoon between 12pm and 4pm.

### 2.2.2.6 Light dark box test

The test arena consisted of a high sided Perspex box split into two areas, one third with black walls and a black lid, enclosed except for a small opening that led to the light side, and two thirds consisting of clear walls, a white floor and no lid. Total size of the arena was 40x30. Light levels in the open side of the light dark box were set at 100 lux. One mouse at a time was placed in the open side of the box facing away from the door. Video tracking was started. After 5 minutes the mouse was removed and placed back into its home cage. The light dark box was cleaned before each run with 70% alcohol. Video tracking was analysed using Ethovision software and parameters analysed included duration in light side, duration in dark side and
number of crosses between light and dark zones (Hascoët and Bourin, 2009). All testing was carried out between 8am and 12pm.

2.2.2.7 Spontaneous alternation test

Mice were taken into the phenotyping room at least 20 minutes before the test to acclimatise. Light level was approximately 80 lux within the maze. A T-maze made of black acrylic, total arm length 75cm each, width 12cm and height 20cm, had a thin covering of clean sawdust placed in the bottom with some dirty bedding from the home cage of the mice to be tested evenly spread along the arms. Mice were put in the long end of the T, facing away from the rest of the maze. The operator stood out of sight and the mouse was left until it entered one of the arms at the other end of the T. A divider was placed at the end of the arm and the mouse kept there for 30 seconds. The divider was then removed, the mouse picked up and placed back in the starting position and the process repeated. After two choices the mouse was placed back in its home cage (Lalonde, 2002). This was performed.
morning (8am-11am) and afternoon (1pm-4pm) for five consecutive days and presence or absence of alterations were recorded.

### 2.2.2.8 Digging test

Mice were placed singly in techniplast blueline cages containing sawdust, a small amount of shredded paper (for nesting) and a plastic tube 68mm wide and 200mm long, with a small stand on one end to hold the tube on an angle and stop it from rolling. Approximately 185g of food pellets were placed inside the tube. Mice were housed in these cages between 2pm and 3pm and left for two hours. The weight of the food left in the tube was then weighed and recorded. The food was placed back in the tube and replaced in the cage overnight (Deacon, 2006). The next morning the food left in the tube was weighed again. The mice were then housed back in cages with their previous cage mates.

### 2.2.2.9 Fear conditioning test

Mice were placed individually into square arenas (17x17 cm, height 25cm) with metal grid floors (Ugo Basile, Italy) (Figure 4); lux in each arena was set at 26 +/- 1. A conditioning protocol was initiated using AnyMaze software (Version 4.5, Stoelting, USA) and consisted of 150s baseline recording, a 5s 90db tone (conditioned stimulus, CS), immediately followed by a 0.5s 0.5mA shock (unconditioned stimulus, UCS). The paired CS and UCS were repeated twice more at 150s intervals. 150s after the last UCS (total of 616.5s in the arena), the mice were removed and placed back in their home cage(Nobili et al., 2017). Arenas were cleaned with 70% alcohol and dried between each trial. Conditioning trial was carried out between 8am and 1pm.
Figure 4 Fear conditioning set up. Mice are placed in the arena on day one where CS and UCS pairings are administered. Mice are placed in same arena on day two to measure the degree of freezing to context.

On day two, mice were placed back in the same arena to assess response to context, no tones or shocks were administered. This was carried out between 8am and 12pm. Four hours after the context trial, mice were place in round arenas, with a plastic floor, black and white walls, reduced lux to 10 and scent of vanillin added. Mice were recorded for three minutes with no tones or shocks and then for the next three minutes with a 5s tone (identical to the tone on day one) sounding every 60s.

At all times mice were video tracked using AnyMaze software and time freezing calculated using AnyMaze freezing detection parameters.
2.2.2.10 Weights

Mice were weighed before midday to limit within day variability. An average weight was taken over 5 seconds using an Ohaus Adventurer Pro balance (Ohaus Europe GmbH, Nänikon, Switzerland).

2.2.3 Additional phenotyping tests

2.2.3.1 Y-maze forced alternation test

A forced alternation variation of the y-maze test was used to assess working memory. Mice were placed in a y-maze (38x8cm per arm, height 15cm) with one arm blocked off, they were then allowed to explore the start arm and the 'familiar' arm for ten minutes. Mice were returned to the home cage for a two minute inter trial interval, during which the maze was cleaned to remove odour cues. The mice were then returned to the maze and allowed access to all three arms for five minutes. Mice were video tracked at all times using Ethovision software (Noldus, Netherlands) Protocol modified from Sanderson et al. 2007. Parameters measured were time spent in each arm (s) and frequency in each arm. Mice were tested between 8am and 12pm.

2.2.3.2 Motor function (MO) wheel-running test

Female mice were tested in motor function cages as in (Mandillo et al., 2014). Briefly, mice were singly housed and placed in Techniplast blueline IVC bases at 50-100 lux. Each cage containing aspen bedding and a standard running wheel. Time running, number of runs, distance, rotations and velocity were recorded for two weeks using Phenomaster software (TSE systems, Bad Homburg, Germany). On the third week the standard wheel was replaced with a complex wheel with
rungs missing at uneven intervals. The addition of the complex wheel allows for assessment of co-ordination and/or motor learning, as well as general wheel running activity. Data were recorded for a further week with the complex wheel.

![Motor function caging set up (TSE,Bad Homburg, Germany). Mice were placed in a cage with a standard wheel (A) for two weeks, subsequently the standard wheel was replaced with a complex wheel (B) for a further week.](image)

### 2.2.3.3 Grip strength

Grip strength was assessed using a Grip Strength Test (BioSeb, Chaville, France). Readings were taken from all four paws, three times per each mouse at each time point, as per manufacturer’s instructions. Parameter measured is grip strength (g) (Joyce et al., 2016). Mice were tested between 8am and 4pm.

### 2.2.3.4 Paw placement gait analysis

Paw placement was analysed using Locotronic® (Intelli-Bio, France) (124x28cm, height 20cm). Briefly, animals moved down a corridor consisting of a horizontal ladder, from a light starting area, to a darker finish area (bars: 3 mm diameter;
spaced by 7 mm), 250 lux in the centre of the corridor. Infrared sensors above and below each bar space, recorded any errors of placement. A trial was discounted if the mouse took more than 30s to move to the finish after exiting the start area. First complete trial for each mouse was used in analysis. Parameters measured are fore paw and hind paw errors (which are summed for analysis) and time taken to complete trial (Watson-scales et al., 2018). Mice were tested between 8am and 4pm.

### 2.2.3.5 Rotarod

To assess co-ordination and motor learning, mice were placed on an accelerating rotarod (Ugo Basile), speed increased from 4rpm up to 40rpm over a five minute period. The time taken for the mouse to fall from the rod was recorded (Acevedo-arozena et al., 2011). This was repeated three times with a 15 minute inter-trial interval. Testing was carried out between 9am and 4pm.

### 2.2.3.6 Home cage assessment (HCA)

Group housed mice were tagged with RFID microchips and left to recover for three days in the home cage. Chipped mice were then placed in the Home Cage Analysis system (Actual Analytics, Edinburgh). Video data was captured as well as location tracking using RFID co-ordinates (Bains et al., 2016). Mice were tested for 72 hours at each age and movement data for either the light or dark period was summed over the 72 hours. Parameters are distanced moved (cm). Data was extracted into csv format by Sonia Bains.


2.2.3.7 **Sleep assessment**

Mice were singly housed and analysed for immobility defined sleep using video tracking analysis as described in (Fisher et al., 2012). During light periods lux was set at 100. Data was recorded using AnyMaze software (Version 4.5, Stoelting, USA), each >40 second period of immobility was defined as sleep, as described in Banks et al. 2015. Parameters measured are time in immobility defined sleep (s) and distance moved (cm). Sleep analysis was carried out by Gareth Banks.

2.2.3.8 **Passive infra-red sleep and circadian screening**

Mice were analysed for circadian activity and immobility defined sleep via the COMPASS system as described in Brown et al (Brown et al., 2016). Mice were individually housed and data captured for 5 days in a 12:12 LD cycle, followed by 9 days in constant darkness. Data analysis was performed using custom python scripts and excel spreadsheet macros, developed in house (Banks et al, in preparation). Circadian analysis was performed by converting activity data from PIR to AWD files for analysis on Clocklab (Actimetrics, Illinois)) or Actiwatch Sleep analysis software (CamNtech, Cambridge). Sleep analysis carried out by Gareth Banks.

2.2.3.9 **Acoustic startle and pre-pulse inhibition**

Mice were analysed for sensorimotor gating deficits using the startle and pre-pulse inhibition test. Mice were assessed for an acoustic startle response, recorded as an exaggerated flinch, which is measured by an accelerometer. Mice were placed in a Perspex tube inside a sound proof chamber in a commercially available startle and
PPI system (Med Associate Inc. Vermont, USA). The mice were exposed to a pre-pulse, a weaker stimulus 80ms before the startle tone, reaction to the subsequent startle tone was assessed. The startle response should be attenuated by the pre-pulse, this is termed ‘pre-pulse inhibition’ (Powell, Zhou and Geyer, 2009). Mice were exposed to randomised trials of pre-pulse tones of 55, 65, 70 and 75 dB with a background level set at 50dB and a startle tone at 110dB (Geyer, Mcilwain and Paylor, 2002). The maximum (positive peak) value for the movement of the mouse is recorded by the accelerometer after the startle tone. Values for background movement are deducted from the startle value prior to analysis. Testing was carried out between 8am and 12pm.

2.2.3.10 Telemetry EEG

Electroencephalography (EEG) measurements were conducted in male mice, around 30g in weight, and aged from 4-6 months (n=3 Frrs1+/+ and Frrs1−/−). Mice were anesthetized with 4 – 5 % isoflurane in 1L oxygen pre minute, and implanted with a transmitter that measures electroencephalography (EEG) at two positions. After surgical recovery, the animals were able to freely move while assessed in their home cage environment for at least 2 weeks. EEG analysis was conducted after at least 3 or more post-surgery recovery days to ensure no residual anaesthesia effect. EEG surgery and analysis conducted by Petrina Lau.

2.2.3.11 Pathology

P0 mice were culled at approximately 6-12 hours after birth by overdose of anaesthetic (euthatal). After death was confirmed pups were placed in containers
with 10% formalin for fixation. Tissues were then embedded in paraffin followed by staining with haematoxylin and eosin.

Adult mice were perfused with 4% PFA and brains dissected before being embedded in paraffin. Sections were stained with haematoxylin and eosin (H&E) or luxol blue.

2.3 Tamoxifen dosing

Tamoxifen (Cambridge Bioscience, Cambridge, UK) was made up to a concentration of 20mg/ml in corn oil and 1% ethanol. Mice were dosed daily by oral gavage for five days, with a total dose of 500 mg/kg over 5 days.

2.4 Quantitative PCR analysis

RNA extraction from P0 brain tissue or cerebellum of 14 month old mice was performed using an RNeasy kit (Qiagen) (n=5 Frrs1l+/+; n=5 Frrs1l−/− at P0, n=4 Frrs1l+/+; n=4 Frrs1l−/− at 14 months). cDNA synthesis was performed using the High Capacity cDNA RT kit (ThermoFisher Scientific) starting with 2 µg of total RNA. cDNA for qPCR amplification was used at a final concentration of 10 ng per well. All the reactions were run in triplicate. Fast Sybr Green mastermix from ThermoFisher Scientific was used and the reactions had a final volume of 20 µl. Primers were at a final concentration of 360 nM. Primers were designed to span exon-exon boundaries and are listed in Table 1. Fold changes were calculated using the 2-ddCt method using the 7500 Software v2.0.6 and normalized using S16 endogenous reference genes relative to WT genotype (Livak and Schmittgen, 2001).
2.5 Western blot

Adult cerebellum or P0 whole brain samples were bisected and one fraction homogenized in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5) with phosphatase and protease inhibitor cocktails (Roche), using lysing matrix tubes D (MP Biomedicals, Germany) and a Fast-Prep-24 homogenizer at 4°C. Homogenates were centrifuged at 12,000 g 4°C for 20 min. 30 μg of soluble fractions were resolved by SDS–PAGE (NUPAGE system, Invitrogen) and transferred to Nitrocellulose membranes (Millipore) for western-blot analysis.

After incubation with primary antibodies, protein was visualized using anti-mouse or anti-rabbit secondary antibodies IRDye® (Li-Cor Biosciences) at 1:10000 dilutions and quantified using the scanning infrared Odyssey imaging system CX (Li-Cor Biosciences).

2.6 Glycosylation assay

Bisected adult cerebellum was homogenised in RIPA buffer as above. 60 ug of the soluble fraction was denatured with glycoprotein denaturing buffer for 10 minutes at 100 °C, followed by immediate immersion in ice. Samples were divided into three aliquots and glycobuffer added, followed by Endo-H (QABio, E-EH02), PNGase (New England Biolabes, P0704S) or water. All samples were incubated at 37 °C for 1 hour. Half of each sample, containing 10 μg of protein, was resolved by SDS–PAGE (NUPAGE system, Invitrogen) and transferred to Nitrocellulose membranes for Western blot analysis.
2.7 Post-Synaptic Fractionation enrichment

Synaptic fractionation was conducted following a modified protocol using a single cerebral hemisphere (after removing the cerebellum). Samples were homogenized in Syn-PER™ Synaptic Protein Extraction Reagent (Thermo Scientific, IL, USA) with phosphatase and protease inhibitor cocktails (Roche) using a Dounce homogenizer. Homogenates were cleared by centrifugation at 1200 x g for 10 min and then at 15000 x g for 20 min at 4 °C. The supernatant is the cytosolic fraction and the pellet the crude synaptosomal fraction. The pellet was resuspended in syn-PER lysis buffer with 0.1 mM CaCl2 and 2% Triton-X, 40 mM Tris, pH 6 and incubated on ice, with gentle agitation for 30 minutes (solubilises (mostly) non-synaptic proteins). Following a centrifugation at 40,000 x g for 30 minutes, at 4 °C, to remove the solubilised extra junction proteins, the pellet was washed with 1% Triton-X, 20 mM Tris, pH 6. The sample was centrifuged again and resuspended in 1% Triton x-100, 20 mM Tris, pH 8 and incubated on ice, with gentle agitation for 30 minutes (solubilises (mostly) pre-synaptic proteins). After another centrifugation at 40,000 x g for 30 minutes, at 4°C, the pellet containing the post-synaptic density was resuspended in 1% Triton x-100, 20 mM Tris, pH8, and precipated by adding 10 times volume of ice cold acetone at -20°C overnight and a final centrifugation at 15,000 x g for 30 minutes, at 4 °C. The pellet containing the post-synaptic fraction was resuspended in 5 % SDS. After protein quantification with a DC assay (BioRad), 15 μg of protein from the post-synaptic fraction and 20 μg from the cytoplasmic fractions were resolved in precast SDS-PAGE gels and transferred to Nitrocellulose membrane (Invitrogen) for Western blot analysis. Synaptic fractionation carried out by Silvia Corrochano.
2.8 Mammalian expression vector cloning

2.8.1 RNA extraction

RNA was isolated from adult C57BL/6NTac brain using QIAzol reagents and the recommended protocol for extraction from fatty tissue. Adult brain was dissected into four, weighed and placed in four Eppendorfs. 1 ml of lysis buffer (QIAzol lysis reagent) was added to each Eppendorf and samples were shaken for 30 s at 40 °C before being incubated for 5 minutes at room temperature. 0.2 ml of chloroform was added to each Eppendorf, tubes were shaken for 15 seconds and incubated at room temperature for 3 minutes. Tubes were centrifuged at 12000 g for 15 minutes at 4 °C. Supernatant was transferred to clean eppendorfs and 500 µl of isopropanol added per tube followed by 40 minutes incubation at room temperature. Tubes were centrifuged at 12000 g for 10 minutes at 4 °C and supernatant removed. 1ml of 70% ethanol was added to each tube, before centrifuging again at 7500 g for 5 minutes at 4 °C. Supernatant was removed and the pellet dried in air for 5 minutes. RNAse free water was added and the concentration of re-suspended RNA measured by NanoDrop.

2.8.2 cDNA synthesis for sub cloning

cDNA was synthesised using the TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Cat no. 4310299) and following manufacturer’s instructions. PCR reaction for cDNA synthesis contained 7.8 µl RNA free water, 0.8 µl of RNA sample, 1 µl Oligo DT and Taqman RT1 enzyme.
2.8.3  PCR amplification of Frrs1l

A blunt end Frrs1l product was amplified from cDNA with a proof reading enzyme, to ensure accuracy of amplification and subsequent cloning. This Frrs1l blunt ended PCR product was generated using Platinum™ SuperFi™ PCR Master Mix kit (Thermofisher, Cat no. 1235820) and following manufacturer's instructions. PCR reaction was set up using 12.5 µl master mix, 1.25 µl Frrs1l forward primer, 1.25 µl Frrs1l reverse primer (table 1), 8 µl water, 2 µl cDNA.

2.8.4  Gel extraction

Agarose gels were prepared using 100 ml TAE buffer and 0.8 g agarose (Sigma, Cat no. A9539 number), plus 4 µl of SYBR safe DNA gel stain (ThermoFisher, Cat no. S33102). 4 µl of DNA loading dye (Thermo Scientific, Cat no. R1161) was added to each PCR reaction and 10 µl of reaction was loaded into the gel, followed by electrophoresis at 80 V for 50 minutes.

Bands of the correct size for Frrs1l cDNA were cut from the gel, weighed and placed in an Eppendorf.

DNA was extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, Cat No. 28704) following manufacturer’s instructions.

2.8.5  Addition of adenosine overhang

Adenosine overhang was required to clone the PCR product into a TA vector. Purified DNA was incubated with dATP to add an A-overhang to the blunt ended Frrs1l PCR product. The reaction was set up as follows: 4µl of 10x buffer, 0.24µl of Taq polymerase, 1.4 µl of dATP, 3.16 µl of water, 1.2 µl of MgCl₂, 30 µl of purified DNA. Samples were incubated for 20 minutes at 72 °C.
DNA was purified using QIAquick PCR Purification kit (Qiagen, cat no. 28104) following manufacturer’s instructions. Purified DNA was stored for subsequent cloning.

2.8.6 TA cloning system

2.8.6.1 Cloning into pcDNA™ 3.1/V5-His TOPO™

Frrs1l PCR product with adenosine overhang, as generated above, was cloned into V5-His TOPO™ vector using the pcDNA™ 3.1/V5-His TOPO™ TA Expression Kit (ThermoFisher, cat no. K480040). Manufacturer’s instructions were followed to clone Frrs1l into the vector and then transform One Shot TOP10 Chemically Competent E.coli. Briefly, the reaction mix contained 1 µl salt solution, 1 µl pcDNA3.1/v5-His-TOPO vector, 2 µl of DNA and 2 µl of water. This reaction was mixed gently and incubated at room temperature for 5 minutes, then placed on ice. 2 µl of cloning reaction were added to One Shot TOP10 Chemically Competent E.coli and mixed gently before incubating on ice for 30 minutes. Reactions were heat shocked at 42 °C for 30 seconds, then immediately transferred back to ice. 250 µl of SOC medium was added and the tubes were shaken at 37 °C for one hour. Each transformation reaction was spread onto antibiotic selection plates and incubated overnight at 37 °C.
2.8.6.2 Colony PCR and selection

On the following day, successful transformation was confirmed by colony PCR.

PCR reactions were set up containing 22.5 µl PCR mix, 10 µl Frrs1l forward primer and 10 µl Frrs1l reverse primer. Colonies were selected and inoculated onto a replica plate, then into the PCR reaction mix. PCR was carried out as follows:

2 minutes at 94 °C, then 25 cycles of [15 s 94 °C, 30 seconds at 62.5 °C, 1 minute at 72 °C] then hold at 4 °C. PCR products were run on agarose gels as above.

Each replica plate was incubated overnight at 37 °C and the following day liquid cultures set up from confirmed clones as follows. Kanamycin antibiotic was added.
to LB (1:100) to a final dilution of 50µg/ml. Each colony was selected with a pipette tip and the tip ejected into 5mls of LB/kanamycin solution, which was then incubated overnight at 37 °C.

2.8.6.3 Miniprep of pcDNA3.1 Frrs1l V5-His plasmid from liquid culture

DNA was purified using the PureYield™ Plasmid Miniprep System (Promega, cat no. A1223) as per manufacturer’s instructions. Eluted DNA was stored at -20 °C.

2.8.6.4 Restriction digest of pcDNA3.1 Frrs1l V5-His plasmid

To confirm presence of backbone and Frrs1l, a restriction digest was set up. DNA concentration from minipreps were analysed using NanoDrop. Restriction digests were set up as follows: 1 µl 10x buffer, 1 µl HindIII, Xhol1 1 µl, then 1-2 µl of DNA, made up to 10 µl with water. Reactions were incubated at 37 °C for 1 hour, then on ice. Reactions were run on an agarose gel for 1 hour at 90 V.

Samples which produced bands of the correct insert size were sent for sequencing to determine the full Frrs1l product had been incorporated in the correct orientation.

2.8.7 Cloning - gateway cloning system

To create an N-terminally GFP tagged protein the Gateway™ pcDNA™-DEST53 Vector was used (ThermoFisher, cat no. 12288015).

2.8.7.1 Entry vector

Firstly, Frrs1l DNA was amplified from cDNA as described above (as in 2.10.1). After gel purification (as in 2.10.3) the PCR product was cloned into the D-TOPO
entry vector using the pENTR™/D-TOPO™ Cloning Kit, with One Shot TM TOP10 Chemically competent *E.coli* (ThermoFisher, cat no. K240020).

Figure 7 Plasmid map of pENTR™/D-TOPO entry vector into which Frrs1l was cloned ready for sub cloning into gateway destination plasmid.

Transformation was carried out as per manufacturer’s instructions and confirmation by colony PCR was carried out as above (in 2.10.5). Samples were sent for sequencing to confirm correct insertion of *Frrs1l* into the entry vector. Following sequence confirmation, mini-preps were carried out to prepare plasmids for sub cloning (as in 2.10.4.1).
2.8.7.2 Destination vector

Using the D-TOPO entry-Frrs1l clone, Frrs1l was sub cloned into the destination vector, Gateway™ pcDNA™ –DEST53 and transformation into DH5a cells carried out as per manufacturer’s instructions.

![Plasmid map of pcDNA-DEST53 destination vector](image)

Figure 8 Plasmid map of pcDNA-DEST53 destination vector which, on expression, will result in an N-terminally GFP tagged FRRS1L protein.

Colony PCR was carried out on the following day to confirm presence of Frrs1l in the backbone. Samples showing correctly sized bands were sent for sequencing, after miniprep (as in 2.8.6.2 and 2.8.6.3).
For both pcDNA™ 3.1/V5-His TOPO™ TA and Gateway™ pcDNA™ –DEST53, aliquots of correct clones confirmed by sequencing were stored in glycerol at -80 °C. 500 µl of bacteria solution from liquid preps were added to 250 µl glycerol and 250 µl water.

### 2.9 Site directed mutagenesis

Site directed mutagenesis was used to introduce a point mutation equivalent to human G321X in the N-terminal GFP plasmid. Primers were designed using Agilent QuikChange Primer Design Tool ([https://www.genomics.agilent.com/primerDesignProgram.jsp](https://www.genomics.agilent.com/primerDesignProgram.jsp)) (See Table 1). Mutagenesis was carried out using QuikChange II XL Site-Directed Mutagenesis Kit using XL10-Gold Ultra competent cells as per manufacturer’s instructions. Reaction mix contained 5 µl 10X reaction buffer, 20 ng of dsDNA template, 2.5 µl of forward primer, 2 µl of reverse primer (Table 1), 1 µl of dNTP mix and ddH₂O up to 50 µl, finally adding 1 µl of PfuUltra HF DNA polymerase (2.5U/µl). Sub-cloning and sequencing carried out as above (2.8.6.2 and 2.8.6.3).

### 2.10 Transfection of Neuro2A cells

Cells were seeded at 2.5 X10^5 cells per well in 6-well plates with coverslips. On the following day, when cells had reached around 70-80% confluence, the cells were transfected with expression plasmids (1 ug per well) using Lipofectamine 3000 (Invitrogen). After 30 hours post-transfection, the cells were fixed with PFA 4 % for 15 min and then washed and prepared for immunostaining. Positive and negative staining controls were processed alongside test samples.
2.11 Primary neurons

24 hours prior to embryo harvest, in preparation for primary culture, coverslips were placed in 24 well plates (Greiner Bio One, Frickenhausen, Germany) and coated with poly-D-lysine (Sigma, St. Louis, Missouri, US) and laminin (Thermo Scientific, Waltham, USA). Embryos were harvested at 15.5 dpc and cortex dissected in cold PBS. 0.25% trypsin (Thermo Scientific, Waltham, USA) was added and tissue was digested for 20 minutes at 37°C. After spinning and washing twice with PBS, cells were resuspended by pipetting up and down in neuronal growth medium. Neuronal growth medium consisted of neurobasal medium (Thermo Scientific, Waltham, USA), 1% L-glutamine (Thermo Scientific, Waltham, USA), 1% penicillin and streptomycin, (Thermo Scientific, Waltham, USA), and 2% B-27 supplement (Thermo Scientific, Waltham, USA).

Excess poly-D-lysine and laminin was removed from the wells and coverslips were washed once with neurobasal media. Cells were counted and diluted in 500µl of neuronal growth medium before being plated at a concentration of 50,000 cells per well.

Cells were grown at 37 °C and 5 % CO₂ for six days, with 350 µl of neuronal growth medium changed every 2-3 days.

Cells were washed with PBS, then fixed with 4 % PFA for 8 minutes. After removal of PFA, cells were washed with PBS three times and stored in PBS at 4 °C prior to immunohistochemistry.
2.12 Immunohistochemistry on primary neurons and transfected Neuro2A cells

Fixed cells were washed with TBS, then blocked for 1 hr with normal goat serum. Primary antibodies were added, followed by incubation overnight at 4 °C. The following day, primary antibodies were removed and the cells washed 3 times with PBS. Secondary antibodies were added and incubated at room temperature for 1-2 hours. Cells were washed with PBS 3 times, then mounted onto glass slides using ProLong™ Gold Antifade Mountant with DAPI (ThermoFisher, P36935). Cells were visualised using Zeiss LSM 700 confocal microscope (Carl Zeiss AG) and images taken using a 63x objective for Neuro2A and a 20x objective for primary neurons.

2.13 Synapse Counts

Formalin fixed wax embedded sections of whole brain were dewaxed and the antigen was unmasked using sodium citrate buffer solution (pH 6.0) at 80 °C for 30 minutes. Sections were next washed in phosphate-buffered saline (PBS, pH 7.4) and processed for immunofluorescence (as in 2.15) Confocal z-stacks covering the whole depth of the slices were acquired at 63x magnification using Zeiss LSM 700 confocal microscope (Carl Zeiss AG). VGLUT1 positive puncta were analysed on confocal images using Fiji software. Caudal sections were used to analyse both stratum oriens and radiatum of the CA1 region of the hippocampus. Images from the cerebellum were acquired from the fourth/fifth lobe to measure the VGLUT1 puncta in the molecular layer of the cerebellar cortex. Imaging of synapses carried out by Enrico Castroflorio.
2.14 Sholl analysis

Sholl analysis was carried out on primary neurons generated as in 2.11. Following blocking with normal goat serum. Antibodies for Tubulin were added to illuminate neurons. Primary antibodies were removed and cells washed with TBS, followed by addition of secondary antibody to Tubulin. Isolated primary neurons with one long axon and dendrites were selected and imaged. Confocal z-stacks covering the depth of the neuron were acquired at x20 magnification using Zeiss LSM 700 confocal microscope (Carl Zeiss AG).

Neurons were analysed using the Fiji ImageJ simple neurite tracer plugin (Longair, Baker and Armstrong, 2011) followed by analysis using the Sholl analysis plugin (Ferreira et al., 2014). Neurites were binned into 1um segments and all measurements calculated from the axon hillock.

2.15 Antibodies

Rabbit monoclonal anti-b-actin (1/3000 Sigma); mouse anti-α tubulin (1/3000 Abcam), rabbit anti-GluR1 (1/1000 Millipore 1504) mouse anti-GluR2 (1/800 Millipore MAB397), rabbit anti-GluR4 (1/1000 Millipore AB1508), mouse anti-CAMKII (1/200 Proteintech 20665-1) mouse anti-SNAP25 (1/500 Biolegend 836304), rabbit anti-PSD-95 (1/1000 Cell Signalling 3450T), and mouse anti-GRIN1 (for NMDA receptor, 1/1000 Novus, NB300-118). Guinea pig anti-VGLUT1 (1:200, Synaptic System).

For immunoblots protein was visualized using anti-mouse or anti-rabbit secondary antibodies IRDye® (Li-Cor Biosciences) at 1:10000 dilutions and quantified using the scanning infrared Odyssey imaging system CX (Li-Cor Biosciences).
For fluorescence immunochemistry, protein was visualised using AlexaFluro-488 mouse conjugated antibody and AlexaFluro-594 rabbit conjugated antibody.

### 2.16 Genotyping

All genotyping was carried out using qPCR. Genotyping for tm1a, tm1b and tm1c alleles was carried out using a selection of at least two of the assays listed below dependant on the allele/construct; Neomycin – designed around the neomycin resistance cassette present in tm1a alleles, LacZ – designed around the LacZ cassette, present in tm1a and tm1b alleles, Promoter driven tm1b – designed to detect the recombined sequence after conversion of a tm1a to a tm1b allele. FRT-loxP – designed to detect the recombined sequence after conversion from a tm1a to a tm1c allele. BP-LOA – break point loss of allele, designed around the loxP site of the cassette and should be present in tm1a/tm1b/tm1c and tm1d alleles, CR-LOA – critical region loss of allele based on the critical region that is flanked by loxP sites, this will be absent in tm1b and tm1d alleles and present in tm1a, tm1c and wild-type.

![Figure 9 Schematic of genotyping primer location used to determine presence and zygosity of tm1a, tm1b or tm1c alleles for knockout first lines.](image-url)
CRISPR alleles were genotyping using two assays, one to detect the wildtype sequence and one to detect the sequence with the deletion.

Genotyping was carried out using Taqman probes and ABI GTX Taqman master mix. Reactions are run on an ABI 7500 and analysed using ABI software CopyCaller. Each samples is run in duplicate with 7 controls of known copy number and a no template control.

All genotyping was carried out by the Genotyping core at MRC Harwell.

2.17 Statistics

Gaussian data were assessed with ANOVA, with Tukey post hoc analysis when computing multiple pairwise comparisons (used for baseline pilot screen - open field, novel object, light dark box, spontaneous alternation, digging). Sidak’s post hoc analysis was used when comparing two variables (such as open field trial 1 vs trial 2) and Dunnett’s post hoc analysis when comparing multiple control groups to one experimental group used for the conditionally deleted mice and controls (open field, HCA, grip strength, rotarod, locotronic, startle and pre-pulse inhibition, sleep (time, number of bouts, bout length)).

Repeated measures ANOVA was used for tests with repeated measures on the same animal (grip strength, rotarod, motor function, PIR sleep over time for Frrs1l⁻/− data) followed by appropriate post-hoc test as specified above.

T-test was used to assess two groups with normally distributed data (immunoblot quantification, qPCR, synaptic counts))

Categorical data was analysed with Fisher’s exact test (used for SHIRPA).
Non Gaussian data was analysed with non-parametric Kruksal-Wallis ANOVA with Dunn’s multiple comparisons (baseline data for marble burying and fear conditioning between strains differences, Frrs1<sup>-/-</sup> data for open field) or with Mann-Whitney test (Frrs1<sup>-/-</sup> y-maze)

Paired data was assessed with paired t-test (pilot open field habituation and fear conditioning within strain comparison).

Mortality data was assessed with chi-squared test.

All power calculations were performed using online power and sample size calculator (Chow et al., 2017; Chow, Shao and Wang, 2018).

Data from multiple lines was compared to a rolling baseline of C57BL/6NTac mice for the IMPC screen. Initial lines were selected for further analysis by t-test against baseline that had been tested up to that point. Later in-depth analysis of IMPC fear conditioning data was carried out using mixed models and a likelihood ratio test. Firstly a null model for each line was calculated with date of experiment as a random effect. A second model was calculated using both date of experiment as a random effect and genotype as a fixed effect. Likelihood ratio test was carried out by running an ANOVA on the two models to ascertain whether including genotype as an effect made the models significantly different. Benjamini-Hochberg correction was carried out on the p-values from the likelihood ratio test to reduce the false discovery rate.

Statistical analysis was carried out using Graphpad-Prism or R.

All data is plotted with bars showing standard deviation unless otherwise specified.

*<0.05, **<0.01, ***<0.001, ****<0.0001.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gria 1 Fwd</td>
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</tr>
<tr>
<td>Gria 1 Rev</td>
<td>AAGCCGCATGTTCCTGTGATT</td>
<td></td>
</tr>
<tr>
<td>Gria 2 Fwd</td>
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<tr>
<td>Gria 2 Rev</td>
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</tr>
<tr>
<td>Gria 3 Fwd</td>
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<tr>
<td>Gria 3 Rev</td>
<td>ACGTGGTAGTTCAAATGGAAGG</td>
<td>For qPCR to check expression</td>
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<td>Gria 4 Fwd</td>
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<tr>
<td>Frrs1l Ex1-2 Fwd</td>
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<tr>
<td>Frrs1l Ex1-2 Rev</td>
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</tr>
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<tr>
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<tr>
<td>Frrs1l Ex4-5 Rev</td>
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<td>Frrs1l with point mutation Fwd</td>
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<td>For site directed mutagenesis</td>
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<tr>
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<tr>
<td>T7 Fwd</td>
<td>TAATACGACTCACTATAGGG</td>
<td>pcDNA3.1 and pcDNA-DEST53</td>
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<td>Primers for C-terminal tag Fwd</td>
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<tr>
<td>Primers for N-terminal tag Rev</td>
<td>TTAAGGGGTTCCCCCATCAGGCTAGAA</td>
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</tbody>
</table>

Table 1 Primer sequences for expression analysis and cloning.
3.0 High-throughput behavioural test analysis

3.1 Introduction

Performing high-throughput analysis using a whole organism, such as mice, is an invaluable tool to identify novel genes causative of cognitive and behavioural phenotypes. The identification of new mouse models of cognitive disease will allow us to better elucidate mechanisms and provide alternative targets for therapies. Here we employ a high-throughput approach as a method to discover new mouse models of cognitive disease. The primary advantage of this method is that many GA lines can be screened in a short period of time. However, as this is a primary screen, each gene is not assessed in-depth for a particular phenotype. Some results are likely to be only an indication of a particular disease mechanism which will require further comprehensive assessment. Assessment of many genes means that tests have to conform to particular constraints, for example they must take a short amount of time to allow a reasonable throughput and they must be able to detect a phenotype with a relatively small sample size. The success of this type of screen critically depends on the capacity of the test of choice to identify or recognize specific phenotypes related to abnormal cognition and behaviour.

3.1.1 Selection of tests for High-throughput screen.

Several criteria have been identified as being important for this screen:

1) Tests should be relevant to cognitive phenotypes in humans.

2) Tests should be of sufficient power to identify phenotypes from cohorts of 8 males and/or 8 females.

3) The test should be robust enough to produce reproducible data.
4) Test should not rely on qualitative or subjective assessment by individuals.

5) The data should not be easily affected by multiple experimenters.

Several tests were selected (Table 2) based on how well they might comply with the above criteria and other practical factors including availability of equipment.

<table>
<thead>
<tr>
<th>Test</th>
<th>Phenotype assessed</th>
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</thead>
<tbody>
<tr>
<td>Open field</td>
<td>Anxiety and spatial memory</td>
</tr>
<tr>
<td>Novel object</td>
<td>Object recognition and memory</td>
</tr>
<tr>
<td>Marble burying</td>
<td>Obsessive compulsive tendencies/digging/hippocampal function</td>
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<tr>
<td>Light dark box</td>
<td>Anxiety</td>
</tr>
<tr>
<td>Spontaneous alternation</td>
<td>Spatial working memory</td>
</tr>
<tr>
<td>Digging</td>
<td>Digging/hippocampal function</td>
</tr>
<tr>
<td>Fear conditioning</td>
<td>Non-declarative memory</td>
</tr>
</tbody>
</table>

Table 2 Tests selected for assessment of suitability to high-throughput screening.

### 3.1.2 Open field habituation

The open field test is used to assess both anxiety and spatial memory by means of placing a mouse in an open arena for a defined period of time and assessing
locomotion and exploratory behaviour (Cummins and Walsh, 1976; Carola et al., 2002). Ratio of distance moved in the centre of the arena vs distance moved in total, can give an indication of anxiety, as anxious mice will not often visit the centre of the arena. However, this can be confounded by other phenotypes such as locomotor deficits or hypo activity. Open field can also be used to assess spatial memory if a two day habituation trial is employed. Mice will behave differently in the novel environment on day one, compared to the familiar environment on the second day. This change in behaviour acts as an indicator of memory from the previous day (Contet, Rawlins and Deacon, 2001; Crawley, 2008; Deacon et al., 2009) and is a simple method to assess non-associative learning.

Data is collected and analysed through video tracking software (Noldus, Ethovision, Netherlands) removing user variability in terms of scoring the tests and reducing analysis time.

3.1.3 Novel object recognition

This test is used to assess the ability of the mouse to discriminate between a novel and a familiar object, an ability that is often lacking in humans with cognitive impairment (Hof and Bouras, 1991; Laatu et al., 2003). In this paradigm, mice are placed in a familiar arena and are given access to two identical objects. After a period of exploration the mouse is placed back in the home cage and the objects are changed for a one familiar and one novel object. Exploration of the objects is evaluated (Sanderson et al., 2011a). It is known that this test is susceptible to environmental influences. Tang and Lazlauckas independently showed that differences in cage enrichment can affect exploratory activity in this test (Tang et al., 2001; Kazlauckas et al., 2011) and that reduced exploratory behaviour
correlates with inability to distinguish a novel object. Therefore, care must be taken with this test to ensure the environment is kept as consistent as possible so there is no environment induced variability between mice, which could mask the exposition of phenotypes. Finally, if the mice are in an anxious environment or have not had sufficient enrichment they may not explore at all, rendering the test ineffective. These confounding factors must be controlled to achieve reproducible results if this test is to be useful in a high-throughput setting. As with open field, data is collected and analysed through video tracking software (Noldus, Ethovision, Netherlands) with similar effects on user variability and analysis time.

### 3.1.4 Marble burying and Digging

Marble burying and digging were both selected as a means to measure non-specific hippocampal function. Marble burying involves placing a mouse in a cage with 9 marbles and assessing how many are buried in a 30 minute period. Digging assessment requires the mouse to be placed in a cage with a tube containing food pellets, the amount of food removed from the tube after two hours is recorded.

The link with human cognitive disease is less clear but it has been shown that mice with hippocampal lesions do not perform well in marble burying and digging tasks (Deacon, 2009). Digging and marble burying are both included in this screen as it is not clear that the same process is measured by both assays (Deacon and Rawlins, 2005). In addition, a disadvantage of marble burying is the subjective assessment of how many marbles are buried, as this requires the experimenter to decide if a marble is more than two thirds covered or not. Digging is not subjective as it only requires the amount of substrate left in the container to be weighed.
There is a high capacity for many mice to be tested using these assays in a single week and, although the data is collected manually, it requires little analysis.

### 3.1.5 Light dark box

Light dark box is included as a second test for anxiety due to the link between cognitive diseases and anxiety in humans (Chemerinski *et al.*, 1998; Porter *et al.*, 2003).

A one trial open field is already included in the standard IMPC pipeline, however it is important to use a separate test for anxiety to corroborate any phenotypes differences in open field and perhaps find phenotypes not present using the open field paradigm. When using a test such as open field it can be difficult to distinguish between emotional and non-emotional contributions to a phenotype, e.g. anxiety and motor function. Anxiety is biologically complex and is likely to involve many different pathways depending on specific situations. There is some evidence that anxiety as measured in open field and light dark box could be reflecting different aspects of emotional response (Ramos, 2008). Therefore it is possible that an anxiety related phenotype could be present in one test but not the other, although this is not always the case (Holmes, Murphy and Crawley, 2003).

Light dark box paradigms are generally short and data is collected and analysed computationally, therefore it is likely to fit well into a high-throughput pipeline if the results are favourable.
3.1.6 Spontaneous alternation

The spontaneous alternation test takes advantages of the natural behaviour of the mouse to explore unfamiliar environments. As such, it can be used to assess spatial working memory (Contet, Rawlins and Deacon, 2001), which is impaired in many human cognitive diseases such as Huntington’s Disease (Possin et al., 2017), Alzheimer’s Disease (Guariglia, 2007), Schizophrenia (Park et al., 1992) and Parkinson’s Disease (Owen et al., 1997). In this paradigm mice are placed in a T-maze, and observed until they make a choice of arm, they are then placed back at the start of the maze and observed for their second arm choice. Mice will most often alternate between arms to explore the novel environment which requires the short term processing of spatial information (Lalonde, 2002). However, for mice to behave in this way, the environment must not be anxiogenic, since anxious mice will be less willing to explore novel environments. Therefore, the test environment has to be well controlled to avoid any confounding environmental influences.

Spatial working memory (SWM) can be further split into two areas, egocentric, or allocentric. In the context of a high-throughput screen where high level phenotypes are distinguished, the test is intended to be able to assess both types of memory, but not distinguish between them. This gives us the ability to potentially detect more phenotypes that can be delineated in more in-depth testing during a future study.

This spontaneous alternation test, although potentially time consuming, could provide valuable information on the ability of mouse models to process spatial information.
3.1.7 Fear conditioning

Fear conditioning was selected as a means to assess non-declarative or implicit memory, which is found to be deficient in many cognitive disorders including dementia, Parkinson’s Disease and amnesia (Tranel et al., 1994; Fleischman et al., 2005; van Asselen et al., 2009).

In the FC paradigm employed in this screen, the mouse is exposed to three pairings of tone and shock. The following day the mouse is exposed to the same environment without the conditioned stimuli (tones), then later to a new environment with the tones. Freezing in each condition is measured as an indication of anxiety and therefore a memory of the aversive stimuli from the previous day.

Freezing before any tones or shock can also be used as an indication of anxiety, as anxious mice will generally freeze more (van Gaalen and Steckler, 2000). This lends another level of utility to this test as both non-declarative memory and, to a certain extent, anxiety, can be assessed.

Data is recorded using video tracking equipment and analysed using freezing detection software (Anymaze, Wood Dale, USA), limiting the effect of the user on the results.

Fear conditioning appears to lend itself well to high-throughput, meeting many of the criteria stipulated above, including potential throughput, ease of analysis and links to human disease.
3.1.8 Assessment of established behavioural tests for high-throughput screening

To establish the suitability of the above tests for high-throughput screening, 20 male and 20 female mice from five different inbred strains were tested; C57BL/6J (B6J), C57BL/6N (B6N), C3H/HeH (C3H), 129S9/SvEvH (129SVEV), and FVB/NCrl (FVB).

Data suggests that sample sizes of 12-15 are usually sufficient depending on the behavioural test (Sukoff Rizzo and Crawley, 2017). However, these pilot data are intended to provide an indication of the population variance in order to calculate the statistical power of each test. To this end, a larger sample size was selected as this may capture any greater variability within the population of mice tested for this project. A sample size of twenty was chosen that represents an increase from the minimum sample size and the maximum number that could logistically be tested.

Starting at 10 weeks of age, mice were tested consecutively through a pipeline of open field, novel object, light dark box, marble burying, spontaneous alternation, digging and fear conditioning. Cohort sizes were reduced to 8-10 per sex and strain for fear conditioning due to operational issues. FVB mice were excluded from fear conditioning entirely due to the inability of the software to accurately track albino mice. These strains were selected for several reasons; firstly, they are all common laboratory strains that have been used extensively in the past. 129SVEV and FVB were commonly used for making transgenic mice due to the efficient germline competency of 129SVEV embryonic stem (ES) cells (Carstea, 2009) and the prevalence of a prominent pronucleus in FVB fertilised one-cell embryos, making them ideal for pronuclear injection (Taketo et al., 1991). Although technologies
have developed towards using other strains for genetic manipulation, some of the available models are still on these backgrounds. B6J have been the strain of choice for many researchers across the globe, and whilst many genetically altered lines may have been made on other strains, a significant proportion of them have been backcrossed onto B6J. GA lines on B6N are becoming more common since the development of JM8 ES cells and subsequently the large ES cell resources containing thousands of knockout cells on this background (Skarnes et al., 2011).

Finally C3H have been widely used in the past for N-Ethyl-N-nitroso urea (ENU) mutagenesis screens (De Angelis et al., 2000; Nolan et al., 2000).

It is also important to establish and compare baseline data for the selected tests in the different strains. This is due to the strains having diverse characteristics therefore allowing the sensitivity of the test to be investigated (Blake et al., 2017). For example, FVB, 129SVEV and C3H are known to behave markedly differently from one another in many tests (Bolivar, Pooler and Flaherty, 2001; Mineur and Crusio, 2002; Bothe et al., 2004; Moy et al., 2007, 2008; Lad et al., 2010). In contrast, B6N and B6J are quite similar in some of their behaviours with more subtle differences than the other selected strains (M. Simon et al., 2013). This may help to assess the extent to which these tests can differentiate changes of a smaller magnitude and the ability of the selected tests to determine distinctive differences in the particular strains.

Once determined the selected test for cognition will be included in a high-throughput pipeline which is underway at MRC Harwell, with plans to screen 1000 knockout lines on a C57BL/6NTac co-isogenic background over a ten year period.
3.2 Results

3.2.1 Open field habituation

Mice were tested in an open field arena for 30 minutes on two consecutive days, comparison between days and strains was assessed.

The object of this test was to assess two questions:

1. Is the test able to distinguish differences between day one and day two within each strain?

2. Is this test able to distinguish differences among strains?

<table>
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<th>B6J males</th>
<th>B6N males</th>
<th>C3H males</th>
<th>FVB males</th>
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<th>B6J females</th>
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Table 3 Heat map of open field habituation p values for all parameters for day one compared to day two, by paired t-test for each sex and strain. For most parameters B6J, B6N and C3H show a significant difference between day one and day two, in comparison to 126SVEV and FVB which show few differences between the two trials. Light green p<0.05, dark green p<0.01. NB: N/A indicates insufficient data on day two due to 129SVEV not entering the centre of the arena.

1. Table 3 shows that there are significant within-strain differences between day one and day two in almost all parameters for three of the strains assessed. B6N and B6J exhibit a decrease in activity on day two and C3H show an increase. The change in activity from day one to day two was
not consistent across different strains. 129SVEV activity is very low regardless of test day and FVB activity levels are high on both days, with little alteration between day one and day two (Figures 10 and 11).

2. There are clear and dramatic differences between the baseline strains (Tables 4 and 5). 129SVEV and FVB are highly significantly different to B6N, B6J and C3H in terms of overall activity, showing significantly reduced or significantly increased activity respectively. C3H differ from B6N, B6J, FVB and 129SVEV on many parameters. However B6J and B6N show a very similar pattern and do not differ significantly from each other on any of the parameters measured.

<table>
<thead>
<tr>
<th>Multiple comparisons between strains</th>
<th>Centre Total distance moved (cm)</th>
<th>Centre Duration in zone (s)</th>
<th>Centre Latency to first (s)</th>
<th>Centre Velocity (cm/s)</th>
<th>Whole arena Total distance moved (cm)</th>
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Table 4 P value heat map for multiple comparisons between females of five inbred strains tested in open field. Data analysed by ANOVA and Tukeys post hoc analysis. Significant differences are seen between 129SVEV and FVB compared to B6N, B6J and C3H on almost all parameters, with 129SVEV being hypoactive and FVB being hyperactive in comparison to B6N, B6J and C3H. B6N and B6J have similar results with no significant differences between them. P<0.05 light green, p<0.01 medium green, p<0.001 dark green.
<table>
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<tr>
<th>Multiple comparisons between strains</th>
<th>Centre Total distance moved (cm)</th>
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<th>Centre Latency to first (s)</th>
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<th>Whole arena Total distance moved (cm)</th>
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Table 5 P value heat map for multiple comparisons between males of five inbred strains tested in open field. Data analysed by ANOVA and Tukey post hoc analysis. Male data follows a similar pattern to female data with significant differences seen when comparing FVB to B6N, B6J and C3H. However although 129SVEV males are significantly different to B6J and B6N, in contrast to the female data, 129SVEV males and C3H males have significant differences in only two parameters. 129SVEV males and FVB males show similar tendencies to hypo and hyper activity respectively. P<0.05 light green, p<0.01 medium green, p<0.001 dark green.
Figure 10 Open field habituation centre parameters for female baseline strains. B6J and B6N show decreased movement in the centre of the arena on day two, whereas C3H show increased movement in the centre (A). B6J and B6N show decreased time in the centre of the arena, again in contrast to C3H which spend increased time in the centre on day two (B). Latency to enter the centre is decreased on day two for C3H only, all other strains show no difference (C). 129SVEV and FVB show no
difference in any of the centre parameters between day one and day two. (*<0.05, **<0.01, ***<0.001), n=20 per strain and sex.

Figure 11 Open field habituation centre parameters for male baseline strains. B6J and B6N show decreased movement in the centre of the arena on day two, whereas C3H show increased movement.
in the centre (A). B6J and B6N show decreased time in the centre of the arena, in contrast to C3H which spend increased time in the centre on day two (B). Latency to enter the centre is increased on day two for B6J, B6N and FVB with no difference in C3H and 129. (C) (*<0.05, **<0.01, ***<0.001), n=20 per strain and sex.

Figure 12 Open field habituation total distance moved in the whole arena for baseline strains males (A) and females (B). B6J and B6N show decrease in movement on day two compared to day one for both females (A) and males (B). C3H show in increase in movement on day two in males only with no difference in the females (B) 129SVEV and FVB show no differences between day one and day two for both males and females (*<0.05, **<0.01, ***<0.001).
Power calculations were carried out on the difference between run one and run two for male and female B6N mice on parameter ‘total distance moved in the whole arena’. This showed that with a power of 0.8 the sample size needed to detect a phenotype with mean difference between runs of 20%, is 9 for females and 10 for males.

3.2.2 Novel object

To assess short term memory, mice were tested using a novel object paradigm. During habituation to two identical objects, C3H and FVB mice spend more time in the zones with the objects (Z1 and Z2) than in the empty zones (Z3 and Z4). B6N and B6J mice spend less time in the object zones and more time in the empty zones. 129SVEV data is highly variable and mice show no preference to any particular zone. No strain shows a preference for the placement of one object over the other with no difference between Z1 and Z2 (Figure 13A and Figure 14A).

During the second phase of the test, one of the identical objects was replaced with a new object of different size, shape, colour and texture. In this trial B6N and B6J again show little interest in the objects with more time spent in the empty zones, Z3 and Z4, and no preference for the novel object. C3H and FVB mice, explore the objects but do not show a preference for the novel object. 129SVEV mice show no zone preference (Figure 13B and Figure 14B).
Figure 13 Novel object data from female baseline. During habituation, trial (A) Z1 and Z2 contain two identical objects. During novel trial (B) Z1 contains a copy of the object from the habituation trial and Z2 contains a novel object. Z3 and Z4 are empty throughout. Habituation data (A) shows no zone preference in 129SVEV, with B6J and B6N spending more time in the empty zones and C3H and FVB spending more time within object containing zones. The pattern is comparable during the novel trial (B), with no strain showing a preference for the novel object in Z2. n=20 per strain and sex.
Figure 14 Novel object data from male baseline. During habituation trial (A) Z1 and Z2 contain two identical objects. During novel trial (B) Z1 contains a copy of the object from the habituation trial and Z2 contains a novel object. Z3 and Z4 are empty throughout. Habituation data (A) shows no zone preference in 129SVEV, with B6J and B6N spending more time in the empty zones and C3H and FVB spending more time within object containing zones. The pattern is similar during the novel trial (B), with no strain showing a preference for the novel object. n=20 per strain and sex.

Data is consistent for males and females, with the exception of C3H males, which do not show a preference for the objects during habituation, whereas female C3H do.
Taken together this data shows that this paradigm did not produce the expected results with no strain showing a preference to explore the novel object over the familiar object.

<table>
<thead>
<tr>
<th>Females</th>
<th>129SVEV</th>
<th>B6J</th>
<th>B6N</th>
<th>C3H</th>
<th>FVB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z₁ vs Z₂</td>
<td>0.9098</td>
<td>0.1191</td>
<td>0.6371</td>
<td>0.5913</td>
<td>0.782</td>
</tr>
<tr>
<td>Z₃ vs Z₄</td>
<td>0.9901</td>
<td>0.603</td>
<td>0.8939</td>
<td>0.6904</td>
<td>0.9431</td>
</tr>
<tr>
<td>Z₁ vs Z₃</td>
<td>0.8371</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Z₁ vs Z₄</td>
<td>0.6849</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0087</td>
<td>0.0003</td>
</tr>
<tr>
<td>Z₂ vs Z₃</td>
<td>0.9939</td>
<td>0.0065</td>
<td>0.0011</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td>Z₃ vs Z₄</td>
<td>0.9397</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>0.0007</td>
<td>0.0019</td>
</tr>
</tbody>
</table>

**Habituation**

| Z₁ vs Z₂ | 0.317  | 0.1124 | 0.9992 | 0.9903 | 0.9844 |
| Z₃ vs Z₄ | 0.815  | 0.3241 | 0.9995 | 0.9975 | 0.9335 |
| Z₁ vs Z₃ | 0.9965  | 0.0021 | 0.0002 | 0.0028 | 0.0002 |
| Z₁ vs Z₄ | 0.6899  | 0.0003 | 0.0001 | 0.0026 | 0.0042 |
| Z₂ vs Z₃ | 0.7096  | 0.0212 | 0.0004 | 0.0029 | <0.0001 |
| Z₃ vs Z₄ | 0.9968  | 0.0012 | <0.0001 | 0.0006 | 0.0001 |

**Novel**

**Table 6** Novel object data from female baseline. Heat map of p values for zone comparisons using one way ANOVA followed by Tukey post hoc analysis. During both habituation and the novel trial, females of all strains show no preference between the two empty zones or the two zones containing the objects. Absence of preference for Z₂ over Z₁ during the novel trial indicates no memory of the object from trial one, or more likely in this case, inability of this paradigm to elicit a difference. All
Table 7 Novel object data from male baseline. Heat map of p values for zone comparisons using one way ANOVA followed by Tukey post hoc analysis. During both habituation and the novel trial, males of all strains show no preference between the two empty zones or the two zones containing the objects. Absence of preference for Z2 over Z1 during the novel trial indicates no memory of the object from trial one, or more likely in this case, inability of this paradigm to elicit a difference. B6J, B6N and FVB show a difference between time spent in the empty zones (Z3 and Z4) versus time spent in the object zones (Z1 and Z2) in both trials. C3H males display a difference between time spent in Z1 and Z4 but not Z3 during habituation but this difference is not present in the novel trial. 129SVEV show no preference for any zones other than a limited preference for Z3 over the object zones during

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>129SVEV</th>
<th>B6J</th>
<th>B6N</th>
<th>C3H</th>
<th>FVB</th>
</tr>
</thead>
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<td><strong>Habituation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z1 vs Z2</td>
<td>0.9959</td>
<td>0.9983</td>
<td>0.991</td>
<td>&gt;0.9999</td>
<td>0.9901</td>
<td></td>
</tr>
<tr>
<td>Z3 vs Z4</td>
<td>0.9999</td>
<td>0.9851</td>
<td>0.9748</td>
<td>0.3087</td>
<td>0.737</td>
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</tr>
<tr>
<td>Z1 vs Z3</td>
<td>0.6834</td>
<td>0.0012</td>
<td>0.0001</td>
<td>0.4127</td>
<td>0.0064</td>
<td></td>
</tr>
<tr>
<td>Z1 vs Z4</td>
<td>0.7889</td>
<td>0.0007</td>
<td>0.0004</td>
<td>0.0267</td>
<td>0.0363</td>
<td></td>
</tr>
<tr>
<td>Z2 vs Z3</td>
<td>0.7218</td>
<td>0.0015</td>
<td>&lt;0.0001</td>
<td>0.4741</td>
<td>0.0051</td>
<td></td>
</tr>
<tr>
<td>Z3 vs Z4</td>
<td>0.7488</td>
<td>0.0005</td>
<td>0.0001</td>
<td>0.012</td>
<td>0.0277</td>
<td></td>
</tr>
<tr>
<td><strong>Novel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z1 vs Z2</td>
<td>0.9349</td>
<td>0.9998</td>
<td>0.232</td>
<td>0.3501</td>
<td>0.3252</td>
<td></td>
</tr>
<tr>
<td>Z3 vs Z4</td>
<td>0.1565</td>
<td>0.5206</td>
<td>0.6554</td>
<td>0.9963</td>
<td>0.996</td>
<td></td>
</tr>
<tr>
<td>Z1 vs Z3</td>
<td>0.0404</td>
<td>0.0149</td>
<td>0.0136</td>
<td>0.0044</td>
<td>0.0288</td>
<td></td>
</tr>
<tr>
<td>Z1 vs Z4</td>
<td>&gt;0.9999</td>
<td>0.0033</td>
<td>0.0007</td>
<td>0.0022</td>
<td>0.0399</td>
<td></td>
</tr>
<tr>
<td>Z2 vs Z3</td>
<td>0.0177</td>
<td>0.0371</td>
<td>0.0056</td>
<td>&lt;0.0001</td>
<td>0.0017</td>
<td></td>
</tr>
<tr>
<td>Z3 vs Z4</td>
<td>0.9564</td>
<td>0.0028</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>
the novel trial. \( P<0.05 \) light green, \( P<0.01 \) medium green, \( P<0.001 \) dark green. \( n=20 \) per strain and sex.

### 3.2.3 Marble burying

As a general assessment of hippocampal function, mice were assessed using the marble burying test. Mice were placed singly in cages containing 9 marbles for a 30 minute period. Number of marbles more than 2/3 buried was assessed, limited differences between strains were found.

The only significant differences in the males data was between FVB and 129SVEV \( (P<0.001) \) and FVB and B6J \( (P<0.05) \). Data from females shows B6J burying significantly less than C3H \( (P<0.001) \) and FVB \( (P<0.01) \) (Figure 15).

Power equations for B6N males and females show that with a baseline average of 7.25 and 7.1, and standard deviations of 2.13 and 1.4 respectively, to differentiate a mutant phenotype with a mean of 5, with a power of 0.8 and a type 1 error rate of 5\%, would need 15 males and 7 females.
Figure 15 Marble burying data for females (A) and males (B). Significant differences in females are seen between B6J and C3H, and also between B6J and FVB. In males, FVB are significantly different to B6J and 129SVEV, data was analysed using Kruskal-Wallis non-parametric ANOVA with Dunns multiple comparisons post hoc analysis (*<0.05, **<0.01, ***<0.001). n=20 per strain and sex.
3.2.4 Light dark box

To assess anxiety, mice were placed in a box that was 1/3 dark and 2/3 light and assessed for time spent in each area over a five minute period, with more anxious mice expected to spend less time in the light compartment. Data from light dark box shows significant differences between the five strains. Both FVB and C3H females spent significantly more time in the light than B6N and B6J (p<0.05). Differences in males were less pronounced, with the only significant difference being that FVB mice spent more time in the light than both B6N and 129SVEV (p<0.05) (see Figure 16).

To further assess the ability to distinguish phenotypes, power calculations were carried out. For B6N the mean time spent in the dark for females is 77.63, and for males is 72.49. Power calculations show that with a power of 0.8, a mean of 55 can be differentiated with an n of 8 for females, however the male data is more variable and the same difference would need an n of 18 males.
Figure 16 Light dark box data for females (A) and males (B). In females significant differences were detected between C3H and B6N, as well as C3H and B6J. FVB were also significantly different from B6N and B6J. In males the only significant differences between strains were FVB vs B6N and 129SVEV. B6J and B6N strains spent an average of less than 40% in the light, whereas C3H and FVB spent an almost equal amount of time in each zone. 129SVEV data is highly variable. (*<0.05, **<0.01, ***<0.001). n=20 per strain and sex.
3.2.5  Spontaneous alternation

Spontaneous alternation was used to assess short term spatial memory. Mice were tested in a T-maze for two trials per day on five consecutive days. Each trial consisted of the mouse being placed in the long arm and moving into one of the two short arms, it was then placed back at the beginning and allowed to select an arm for the second time. Alternations between the arms were recorded.

Data shows that C3H females performed better than the other strains with an average percentage alternation of >80%. 129SVEV females had an average percentage alternation of around 60%, with B6N and B6J having averages of 65% and 73% respectively. The males showed a greater propensity to alternate, with C3H males having an average of >80%, and the B6N, B6J and 129S6 all having >70% alternations. FVB mice from both sexes displayed approximately 50% alternation, which is not above chance levels and indicates no memory of the previous trials.

C3H females were significantly different to 129SVEV, B6J and FVB (p<0.01) and FVB males were significantly different to all other strains (p<0.01) (see Figure 17)

Spontaneous alternation testing demonstrated a percentage alternation of >70% in most strains. This indicates that if a mutant line had random alternation (i.e. 50%) this would be detectable and infer a memory deficit. Power calculations confirm this, with sample sizes of 4 and 6 needed to detect a mean of 50% using B6N female and male data respectively, using a power of 0.8.
Figure 17 Spontaneous alternation data from five baseline strains, females (A) and males (B). C3H females alternate significantly more than B6J, 129SVEV and B6N (p<0.01). FVB males alternate significantly less than all other strains (p<0.01). All baseline strains except FVB show alternation behaviour at a rate greater than chance (50%). (*<0.05, **<0.01, ***<0.001). n=20 per strain and sex.

3.2.6 Digging

As a second test to assess broad hippocampal function, mice were assessed for general digging behaviour. Mice were placed in a cage containing a large tube with
approximately 180g of food pellets. The amount of pellets removed from the tube in two hours was assessed.

![Graph A](image1)

![Graph B](image2)

Figure 18 Data from females (A) and males (B) for digging test. C3H and FVB females dig significantly more food pellets than 129SVEV and B6J (p<0.01). In males 129SVEV and B6J showing lower levels of digging than C3H and FVB (P<0.01). n=20 per strain and sex.

Over the two hour period differences in digging behaviour were seen between most strains (Figure 18). C3H and FVB females showed increased digging behaviour and
removed significantly more food pellets than 129SVEV and B6J (p<0.01). Results in the males were similar, with 129SVEV and B6J showing lower levels of digging than C3H and FVB (p<0.01).

To detect a reduction in digging behaviour by a third in the B6N strain, with a power of 0.8, an n of 11 would be needed for females but an n of 21 for males.

3.2.7  **Fear conditioning**

A fear conditioning protocol was used to assess non-declarative learning and memory. Mice were placed in an arena with a grid floor and exposed to three pairings of tone and foot shock. Mice were then placed back in the same arena the next day to measure response to the same context, and in a different arena in the afternoon of the next day to measure response to the tone. Time freezing as an indicator of anxiety was measured, with increased anxiety in the context and cue parts of the test being associated with memory of a noxious stimulus on the previous day.

Cohort sizes were reduced for this test to 8-10 per strain and sex for operational reasons. This test was carried out several months after the previous behavioural phenotyping when the mice were 12 months old. FVB mice were not tested due to the inability of the video tracking software to distinguish albino mice.

During fear conditioning mice reacted as expected, with an increase in freezing seen between baseline freezing and freezing in the same context 24 hours later, and between pre and post cue freezing for all strains and sexes (p<0.01) with the exception of C3H males. In C3H males freezing in context did not increase
significantly compared to baseline, although there was a trend in that direction. C3H male response to cue however was similar to other strains, with an increased freezing associated with tone delivery (P<0.01). Smaller sample sizes in this test meant that some groups did not fall within a normal distribution, as assessed by the Shapiro-Wilk test, therefore within strain comparison between pre-shock freezing and context were carried out using the Wilcoxon Rank Sum test.

Figure 19 Fear conditioning data for four baseline strains, females (A) and males (B). Data are baseline percentage time freezing, taken before any exposure to tones or shocks, and context
percentage time freezing, recorded on day two, 24 hours after three pairings of tone and shock. All strains and sexes show increased freezing during context (p<0.01) with the exception of C3H males. n=8-10 per strain and sex.

In context, differences between strains were seen between 129SVEV females and all other strains, with 129SVEV females showing increased freezing (p>0.05). In males differences were seen between C3H and 129SVEV as well as C3H and B6J, in both cases C3H males showing reduced freezing.
Figure 20 Fear conditioning data for four baseline strains, females (A) and males (B). Data are percentage time freezing in the cue trial on day two before and after presentation of tones. All strains and sexes show increased freezing after exposure to the tone (p<0.01). n=8-10 per strain and sex.

In the cue trial, there were no strain differences between females, however in males significant differences were seen between 129SVEV and both B6N and C3H (p<0.01) (figure 20). All inter-strain comparisons were carried out using non-parametric Kruskal-Wallis ANOVA followed by Dunns’s multiple comparisons.

Fear conditioning data revealed that all strains, with the exception of C3H males, learned to associate a context and cue with an aversive stimulus. This indicates the fear conditioning test could be suitable to assess non-declarative memory in mice. To assess this further, power calculation were used to determine sample size needed for both context and cue trials using B6N data. Average difference in percent time freezing between baseline and context is 26 and 21 for females and males respectively. To distinguish an increase in percentage time freezing of 9, with a power of 0.8, sample sizes need to be 7 and 8 respectively. Similarly, for cue, average difference in percent time freezing between cue trial pre tone and cue trial with tone presented is 21 or 17 for females and males respectively. To detect an increase in percentage time freezing of 9, this would need a sample size of only 4 for females, and 8 for males.

3.3 Discussion

3.3.1 Tests suitable for immediate further assessment

Open field
Habituation data shows that B6N, B6J and C3H mice display a change in activity on day two. This is consistent with other baseline open field habituation screens on similar strains (Bothe et al., 2004; Bolivar, 2009). Both B6N and B6J strains show a reduction in activity on day two, this is hypothesised to be due to habituation to the arena and the lack of necessity to explore what is now a familiar environment. As defined by O’Keefe, animals make a cognitive map of their environment and need to explore to create the internal representation of their surroundings (O’Keefe and Nadel, 1978). Once this map is complete, the animal no longer has such a drive to explore and activity reduces. The activity pattern of C3H is quite different, with much lower activity over all compared to B6N and B6J and, conversely, an increase in activity on day two. This reversed behavioural pattern on day two may be due to the low activity on day one. It could be speculated that the C3H did not explore on day one, which is evident from the low level of activity, so on day two, when the environment is no longer so anxiogenic, they are explore further in order to form their cognitive map of the surroundings. The reasons behind the behavioural changes are open to interpretation; however it is clear that for the B6J, B6N and C3H that these strains form some memory of the environment, since behaviour on day two is significantly altered from day one.

129SVEV and FVB mice display no differences in behaviour between day one and day two. The tendency for 129SVEV and FVB to be hypoactive and hyperactive, respectively, is a confounding factor for this assay. The lack of alteration in movement between day one and day two for these two strains could be due to the extremes of their baseline activity levels and is not necessarily an indication of poor memory.
These results show that open field habituation is a good candidate test to be included in the high-throughput pipeline. Power equations show that for some parameters a sample size of 9 will be enough to detect a phenotype with a reasonable amount of confidence, with a power of 0.8 and type one error rate of 5%. Although the data variation on some parameters is quite high, such as latency to enter the centre, it was decided this test was suitable for further assessment and possible inclusion in the high-throughput pipeline.

Fear conditioning

Fear conditioning data shows differences between strains, nevertheless all strains exhibited evidence of learning and memory on day two. The variability of these data were low and power calculations suggested that samples sizes of 8 could be suitable. This test will also undergo further testing for possible inclusion in the high-throughput phenotyping screen.

Although there were other tests that produced useful baseline data and may be suitable for high-throughput screening, the above two tests (open field habituation and fear conditioning) were chosen not only because they best meet the criteria listed above, but most importantly, as they have a clear link to learning and memory. In both tests, mice clearly show a change in response after having learned the environment from day one, either a change in exploratory behaviour, as seen in open field, or a change in freezing behaviour, as demonstrated in fear conditioning.

It is, however, not a straightforward correlation with the exact clinical condition in human patients. As previously discussed, fear conditioning paradigms are rarely
carried out with patients, although some assessment of Alzheimer’s and frontal temporal dementia patients has been carried out and shown an impaired fear response (Hamann, Monarch and Goldstein, 2002; Hoefer et al., 2008). However, fear conditioning has been found to be a useful assay to test learning and memory in mice and has been employed for many different studies. Much of this research has found phenotypes in GA mice that correlate to memory loss in humans with similar genetic differences (Van Dam et al., 2000; Siegmund and Wotjak, 2007; Costa, Scott-McKean and Stasko, 2008).

Similarly, it is not a direct extrapolation of the open field test results in mice into a clinical condition in patients. Although open field is thought to be a measure of spatial learning and memory in the mice, which is often impaired in human cognitive disease (Moss et al., 1986; Owen et al., 1997; Guariglia, 2007). Defects in open field habituation have been observed in correlation with other defects in spatial learning and memory, as well as in situations known to affect spatial memory in mice (e.g., after genetic alterations or induced hippocampal damage) (Van Raamsdonk, 2005; Deacon et al., 2009; Rial et al., 2014).

Another useful feature of both of these tests is their ability to assess not only learning and memory capacity in mice, but also their activity and anxiety levels. Open field has long been used to evaluate anxiety, (Cummins and Walsh, 1976; Lipkind et al., 2004; Lalonde and Strazielle, 2008) with results such as, decreased time spent in the centre, or decreased ratio of distance moved in the centre versus the whole arena thought to correlate with anxious behaviour.

Fear conditioning (FC) may also be indicative of anxiety, as increased freezing on day one before any stimuli, may imply an increase in anxiety. However, this needs
to be investigated further as FC is primarily used to assess non-declarative memory and is not a validated test for anxiety.

### 3.3.2 Tests suitable for future further assessment

**Light dark box**

The data obtained in the light-dark box test showed that B6J and B6N strains have a clear preference for the dark side.

Unfortunately, there is significant amount of variability in light dark box data, making this test inappropriate for high-throughput screening. Male data is universally variable, with little difference in the level of variability across strains, with the exception of FVB which are more consistent. The female data in general is less variable but there is a substantial difference in the 129SVEV females, with the data separating into two distinct groups. 12 of the 129SVEV females spend less than 30% of their time in the light, whereas the remaining 7 spend above 70% in the light. Analysis of the metadata shows that 129SVEV females were tested in two batches, 7 of the 9 mice tested in the first batch were the mice that spent >70% of time in the light. This leads to a significant effect of the day of test on the variability. This could be due to various environmental differences such as handling or noise in the testing room. It is difficult to be more specific but it is clear that this test is sensitive to certain environmental factors, which it make it less robust.

Interestingly, the data for light dark box does not correlate well with the anxiety assessment in open field. For females, the strains that spent the most time in the light during the test, which therefore indicates that they are the least anxious, are C3H and FVB. However, the mice that spend proportionally more time in the centre of the arena in open field, again suggesting they are the least anxious, are B6J.
Light dark box testing in the males showed that FVB mice tend to spend more time in the light than B6N and 129SVEV mice. However, during the open field test, B6J and B6N spend proportionally more time in the centre. These tests suggest that female B6J are less anxious in open field but more anxious in light dark box. Whereas B6J and B6N males, show less anxiety in open field but little difference to the other strains in light dark box. These differences could be explained by the potentially divergent emotional responses elicited by the two different paradigms (Ramos, 2008).

There may also be other factors affecting these results, for example, C3H mice are known to have retinal degeneration due to a mutation in the Pde6b gene, resulting in impaired vision from around 3 weeks of age (Farber and Lolley, 1974). FVB mice also carry the same retinal degeneration gene allele as C3H (Puk, De Angelis and Graw, 2013). It is conceivable that differences in visual perception could have some effect on these behaviour tests. The edge of the arena in open field can be detected by whiskers in the absence of sight, whereas it may be more difficult to distinguish between the light and dark sides of the light dark box. Finally, we can see that light dark box is sensitive to environmental influences due to the unusual spread of data from 129SVEV females. It is possible that despite efforts to keep the environment consistent, there are still changes occurring that can affect how mice behave in this test.

Light dark box data was significantly different across strains and power equations suggest that, to distinguish relevant statistical differences on a B6N background, samples sizes of 8 are suitable for females, however numbers needed for males are higher than logistically possible for high-throughput screening. This test may work well in a high-throughput environment with some possible modifications to
the protocol. However, at this stage we have decided not to follow through with further assessment of this test due to the promising data from open field and fear conditioning, both of which may allow assessment of learning and memory as well as anxiety.

**Spontaneous alternation**

The alternations in the baseline for all the strains assessed were high, with most strains alternating more than 70% of the time. FVB mice did not show any propensity to alternate, with random arm choice of 50% in both males and females. This indiscriminate choice of destination by FVB could be explained by the open field results for this strain, where FVB mice demonstrate marked hyperactivity. During spontaneous alternation testing, FVB mice run throughout the maze, immediately running into an arm as soon as they are placed at the starting point, with no apparent exploration. It is likely that the hyperactivity of FVB mice may be a confounding factor in this test.

Using the B6N data, to identify a strain with no alternation only a very small sample size, as low as 4, may be needed. Alternation in rodents relies on many brain areas (Lalonde, 2002) and is also a measure of spatial reference memory, as the mouse has to remember the previous arm in order to alternate on the second trial. Although this data from spontaneous alternation would provide useful insights as part of the high-throughput screen, it has been decided not to continue with this test, as the data collection time is too great to enable the screening of high numbers of mutants. This protocol consisted of two trials per day for five consecutive days. It is likely that shorter paradigms can be employed and may be
able to provide the same informative data but with a reduced investment in time. However, as this test needs further optimising we will not continue with it at present.

### 3.3.3 Tests not suitable for high-throughput phenotyping

#### Novel object

The novel object protocol used here did not demonstrate the preference to novelty that was expected in these strains. This was surprising as the protocol had been validated elsewhere and shown a difference in at least one of the strains tested (Sanderson et al., 2011a). However, it is clear that this type of variability exists in other laboratories, with other groups being unable to replicate the work of others (Şık et al., 2003) (Frick et al, 2003). Tests of subtle behaviour, such as novel object, are highly susceptible to many environmental factors. Much of the inconsistency between phenotypes across different groups could be the result of other elements that are unrelated to the genetic alteration. Specifics of the protocol, video tracking parameters, location of testing room, personnel carrying out the test, breeding strategy and housing environment are just a few of the potential confounders. For example there are many variations of the novel object protocol itself, with one of the inconsistencies between laboratories being the inter trial interval; this can be 2 minutes (Sanderson et al., 2011a) to 7 days (Tang et al., 1999); the objects used also vary dramatically in shape, colour and texture.

The inability for this protocol to elicit a preference for the novel object makes it unsuitable for further use; however with some changes it may be possible to create
the correct environment for the mice to show a preference. At this time we decided not to follow up on this test as we have several other promising avenues to explore.

**Marble burying and digging tests**

These tests produced some promising results, with differences seen among strains and relatively low variability for females. The sample sizes needed for females were realistic for high-throughput in both digging and marble burying, being 11 and 7 respectively. However males needed greater numbers, with at least 15 males for marble burying and 21 for digging. These high sample size numbers make these tests unsuitable for high-throughput screening unless they can be modified to reduce variability. Moreover, the link between these tests and specific endophenotypes shared with humans is more tenuous than for open field and fear conditioning. Both open field and fear conditioning have some correlation with human symptoms, both mice and humans have evidence of spatial memory and both can condition to fearful stimuli. However, digging is a species-specific behaviour and does not have an obvious parallel in humans. We know there are some behavioural links as hippocampal lesions affect digging behaviour in both marble burying and digging tests (Deacon and Rawlins, 2005). Nevertheless, this correlation is quite non-specific and the pathways involved have not been defined. It is possible that the phenotype associated with the hippocampal lesion is actually more related to general well-being of the mouse; a so-called ‘luxury behaviour’ that is only engaged in when the mouse is well and not under stress (Jirkof, 2014). Perhaps the best human correlate for this is ‘Activities of Daily Living’, as personal hygiene and house work are often the first things to be disrupted when feeling
unwell. However, alterations in this type of behaviour could be due to many reasons and many different pathways, not necessarily linked to cognitive disease or dementia. This can be seen in the literature, alterations in digging behaviour in the mouse have been linked to numerous problems, including Alzheimer’s disease, bacterial or viral infection, post-surgical pain and inflammatory bowel disease (Arras et al., 2007; Filali, Lalonde and Rivest, 2009; McLinden et al., 2012; Tarr et al., 2012; Jirkof et al., 2013). It is clear that digging and burrowing are useful indicators of well-being in mice. However, there are already potential tests of cognition (OF and FC) that we can consider for high-throughput. Consequently, as the cause of any modified digging behaviour could be many things, not necessarily related to cognition, we have decided not to take these tests any further.

3.4 Summary

In conclusion, many of the tests carried out as part of the baseline screen have yielded informative data.

- Open field and fear conditioning are selected for further assessment and potential inclusion in the high-throughput pipeline. Expected sample size needed is 9 for females and 10 for males in open field, 8 for females and 10 for males in contextual fear conditioning.

- Light-dark box and spontaneous alternation are not to be continued at present but may be included in the future if opportunities arise to optimise protocols. Expected sample size needed is 8 and 18 for light
dark box, and 4 and 6 for spontaneous alternation, females and males respectively.

- Digging and marble burying are non-specific with high variability in males and in light of more informative tests will not be included. Expected sample size needed is 11 and 21 for digging, 8 and 18 for marble burying, females and males respectively.

- Novel object did not work well and will be excluded from this study in its present form.
4.0 Test establishment and high-throughput screening

4.1 Introduction

In previous work (Chapter 3), a study was carried out to select tests that may be suitable for assessing elements of cognition as part of a high throughput screen. After analysing data from five inbred mouse strains it was decided to further characterise open field habituation and fear conditioning to confirm applicability for high-throughput screening. Initial data suggested that both of these tests may be suitable as a first-pass to identify mice with learning/memory deficits and anxiety-relevant traits. Power equation calculations of sample sizes from the pilot study allowed us to establish that small cohorts of 8 would be sufficient to detect abnormal phenotypes expressing learning deficits. Furthermore, data collected in these tests is neither subjective nor is there a need for time consuming curation or manual analysis of the data. Initial assessments, therefore, would suggest that these tests are suitable for high-throughput screening. Nevertheless, further testing is required to establish that test outcomes do not vary considerably when testing multiple batches and that when incorporating the test into a screening battery, that the test does not affect, nor is it affected by, upstream and downstream tests.

After further assessment of robustness and reproducibility, it is expected that one of these tests will be adopted by the International Mouse Phenotyping Consortium (IMPC) testing pipeline (figure 19). The IMPC is a worldwide consortium aiming to create null alleles for every gene in the mouse genome and characterise mice
carrying those alleles using a systematic high-throughput pipeline of phenotyping tests (see 1.10.1). As the IMPC screen is already established at MRC Harwell, this project represents a unique opportunity to assess a large number of coisogenic knockout mouse strains for cognitive phenotypes. Additionally there is the potential to compare the qualitative and quantitative effects of cognitive screening and establish whether comorbidities are inherited with other phenotypic domains. As a high-throughput screen, the IMPC pipeline is designed to investigate several body systems in the same test cohorts. It is not intended to provide an in-depth assessment in any one domain, rather a primary assessment of each body system in order to define potential gene-phenotype associations. As a first step in a hierarchical assessment of phenotype, this initial assessment needs to be augmented by detailed phenotyping in specific research areas. The premise of the cognition test would be the same, to highlight a potential interesting phenotype for further in-depth assessment.

Statistical analysis for high-throughput datasets is complex as volume of data is continually growing both in terms of baseline data and new genetically altered lines. In the initial part of the project data is analysed using standard t-tests for statistical significance, however as the dataset increases, more complex models can be applied to better interrogate the dataset as a whole.

Cognition is an essential component of behaviour that has not yet been incorporated into this high-throughput screen, despite there being so many human disorders where cognitive deficits are a symptom. One of the unique benefits of this type of screen is that the same cohorts are assessed through a wide range of tests, allowing correlation of any cognitive phenotypes with those affecting other systems, such as changes in metabolism or sensory deficits.
4.1.1 Further assessment of selected cognitive tests for suitability for high throughput screening

4.1.1.1 Placement of cognitive tests within the IMPC pipeline

The cognitive tests selected for further investigation, open field habituation (OFH) and fear conditioning (FC), should be compatible with the already established IMPC pipeline. In addition, testing should have minimal effects on the outcome of tests downstream, as well as minimal reaction to previous tests.

![IMPC pipeline for the first phase of the project.](image)

4.1.1.2 Open field

Open field (OF) is known to be affected by test order. McIlwain and Paylor et al (McIlwain et al., 2001; Paylor et al., 2006) both demonstrate that even a non-invasive test such as a neurological examination, which includes handling and placement in an arena, can result in decreased locomotor activity and decreased
vertical activity in an open field when compared to naïve controls. Susceptibility of OF performances to prior assessment is test specific. For example, Lad (Lad et al., 2010) showed that there is no effect on open field performance when following in a barrier test, where mice are tested in a home cage with an additional barrier to climb over. It seems likely that the more stressful the test, the greater effect it could have on open field performance. To reduce any of these confounding factors, open field was placed earliest in the IMPC pipeline. However, this is not strictly the first test as several weeks of weight assessment are carried out prior to this. The open field test paradigm included in the IMPC pipeline consists of a single trial of twenty minutes duration. To cause minimum disruption to already established IMPC tests, and to enable suitable throughput, open field habituation was placed at the start of the pipeline and was modified to two twenty minute trials, rather than two thirty minute trials as in the primary screen. Day one consisted of the standard twenty minute open field protocol as already established; this was followed by a second twenty minute trial on the next day. Reducing the duration of the test may result in different phenotypic outcomes, however shorter open field paradigms can still be used to measure habituation. Holmes and McIlwain detected habituation over a single 30 minute trial (McIlwain et al., 2001; Holmes et al., 2002) whilst Bolivar used three trials lasting 15 minutes on three consecutive days (Bolivar, 2009) and was able to detect habituation in C57BL/6J on day two. There is significant gains in terms of throughput if a shorter protocol can be validated, and based on the literature it should still be possible to detect habituation.
4.1.1.3 Fear conditioning

Fear conditioning was more difficult to position in the pipeline, potentially being affected by previous tests and also influencing the results of later assays. The existing IMPC pipeline was designed taking into account effects of test order and age and has been established already at many centres around the world. Therefore it was not logistically possible to move the order of any existing tests, and therefore necessary to fit FC into the established pipeline. It was desirable that FC should not be placed before any of the behavioural tests in the existing pipeline, as having a stressful test that included a foot shock could potentially induce behavioural changes in these other tests. It is also generally agreed that behavioural tests should be carried out in series starting with the least stressful and ending with the most stressful (Crawley and Paylor, 1997; Crawley, 2008). Taking this into account, FC could not be placed before any of the other behavioural phenotyping tests in weeks 9-10 (figure 21). Equipment availability issues meant that FC could not be carried out in week 11 with calorimetry or in week 12 with the heart phenotyping tests. X-ray, ABR and body composition analysis (week 14) involve an injectable anaesthetic which is quite stressful in itself and it was not clear whether this would influence behaviour in FC. Therefore it was decided that FC would initially be placed in week 13 alongside IPGTT (intraperitoneal glucose tolerance test) before the imaging tests that require injectable anaesthesia.

Stress can have effects on glucose homeostasis. Tabata et al. show that cage movement and handling immediately before IPGTT causes an increase in plasma glucose (Tabata, Kitamura and Nagamatsu, 1998). It is also known that the autonomic nervous system can control secretion of insulin and glucagon. The autonomic nervous system is associated with the fight or flight response, with
processes such as digestion being suppressed in times of stress (Thorens, 2011). FC is a potentially stressful test and, whilst it is unlikely that the stress response would last several days, it was decided to carry out IPGTT first in week 13.

### 4.1.1.4 Assessment of suitability

To further assess the suitability of each test within the context of the IMPC pipeline, separate cohorts of C57BL/6NTac mice were subjected to the entire pipeline with either OF habituation or FC included at the selected time point. Data were analysed for each of the cognitive tests as well as tests carried out downstream.

### 4.2 Results

#### 4.2.1 Open field

Four batches, each containing 3–5 B6N females or 5 B6N males were subjected to the IMPC pipeline. Open field (week nine) was extended to include a second twenty minute trial on the following day, a modification of the standard IMPC single twenty minute trial on day one.

The results of this study were inconsistent with the original results of the baseline assessment (3.2.1). During initial primary assessment, B6N males and females showed highly significant differences between day one and day two in parameters measuring total distance moved in the centre, duration in the centre and total distance moved in the whole arena. Using the new protocol, we no longer see any change in activity between day one and day two, with all parameters having no significant differences between the two trials (figure 22). Data on day one are
comparable to the pilot screen, however values on day two show no reduction in activity (Figure 22) when analysed by paired t-test. Total distance moved in the centre (p=0.149 and p=0.459), duration in centre (p=0.195 and p=0.566) and total distance moved in whole arena (p=0.467 and 0.493) for females and males respectively.
Figure 22 Open field habituation parameters for female and male B6N assessed in two twenty minute open field tests on consecutive days. Males and females show no significant difference between run one and run two on any of the parameters (total distance moved in the centre (p=0.149 and p=0.459), duration in centre (p=0.195 and p=0.566) and total distance moved in whole arena (p=0.467 and 0.493)) for females and males respectively. Primary screen data shown for comparison, for consistency, primary screen data is shown for the first 20 minutes of each 30 minutes trial only. Data analysed by paired t-test. n=20 per strain and sex for initial screen, n=15 females and 20 males for pipeline screen.

ANOVAs with Sidak post hoc multiple comparisons were performed, comparing each batch within each trial and showed no significant differences between batches with one exception; total distance moved in females was significantly different between batches two and four (Table 8 and Figure 23).

<table>
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Table 8 ANOVA to assess effect of batch on open field habituation results. Four batches of females and three batches of males were assessed. No significant differences were found between batches with the exception of whole arena distance moved, where post hoc comparisons showed that female batches two and four were significantly different (p=0.0451).
Figure 23 Open field parameters split by batch (one-seven). Batches one to four are female, batches five to seven are male. Batches were analysed by ANOVA to assess whether variance could be attributed to batch, however no significant differences between batches were seen except between female batches two and four, for whole arena distance moved in trial 1 (p=0.047) (ANOVA results for remainder of batch comparisons shown in Table 8).

4.2.2 Fear conditioning

6 batches each containing five B6N males were tested using a FC paradigm in week 13 of the IMPC high-throughput pipeline. Males only were selected as females were already being tested in a plethysmography test in the same week and were therefore unavailable.

Data for B6N mice showed a similar trend and distribution to the original baseline assessment (see 3.2.7). Mice display an approximate 20% increase in time freezing when assessing contextual conditioning on day two and a similar increase during assessment of cued conditioning later in the afternoon of the same day. Power calculations have determined that a sample size of n=7 can identify mutant cohorts where freezing does not increase by more than 5% in contextual or cued trials (power 0.8, type I error rate 5%).
Figure 24 Fear conditioning data from six batches of five males tested through the full IMPC pipeline (secondary screen) plotted with baseline screen data for reference (primary screen). Percentage time freezing prior to conditioning (baseline) and percentage time freezing to context (Context) are shown in (A). Percentage times freezing before and after the cue are shown in (B). Wilcoxon matched-pairs signed rank test determine p values of p=0.002 for primary screen males and p<0.0001 for secondary screen (A), and p=0.008 for primary screen cue with p<0.001 for secondary screen cue (B). n=8 for initial screen, n=30 for pipeline screen.
The tests carried out after FC were evaluated to determine whether FC in week 13 of the pipeline had any impact on test outcome. The final test in the IMPC pipeline is a terminal bleed to measure a number of clinical chemistry and haematology parameters. This includes the measurement of a number of parameters that are sensitive to prolonged stress and might indicate whether the inclusion of FC in the pipeline has such an adverse effect. The key parameter to assess was fructosamine as this can be elevated in animals that are chronically stressed (Surwit, Schneider and Feinglos, 1992; Reusch et al., 1993). Fructosamine levels were unaffected after introduction of fear conditioning (p=0.075 by t-test) therefore there was no reason to expect inclusion of FC would negatively effects tests that follow it.

Figure 25 Clinical chemistry measurement of fructosamine. No difference in fructosamine data is seen after introduction of fear conditioning into the IMPC pipeline (indicated by black line) (p=0.075 analysed by t-test). n=31 pre fear conditioning, n=49 post fear conditioning.
Fear conditioning screening of IMPC lines

Fear conditioning was selected as the cognitive test to use in the IMPC pipeline. From January 2014, knockout lines from the IMPC were screened using this test. FC was carried out at 13 weeks of age and males only were tested. Mice were bred so that a cohort of a minimum of eight males would be tested in 1-2 batches over a short period of time (usually two weeks). In some cases where lines did not breed as expected there was an increase in smaller batches in order to reach a sample size of eight. All viable lines were screened as homozygotes. Heterozygote animals were only screened if the homozygotes were not viable (i.e. no homozygotes survived to weaning), or if homozygotes were sub-viable, defined as less than 50% of expected number of homozygotes surviving at weaning. In some cases, heterozygous and homozygous cohorts were screened for sub-viable lines. All mutant lines are co-isogenic, being generated and maintained on a B6N background. Throughout the study, controls were screened in weekly batches of 5 B6N mice, selected from the inbred colony in the facility.
Figure 26 Fear conditioning data for B6N control mice tested over 3 years, binned by month. Individual values for % time freezing before any tones or shocks shown as ‘Baseline’ (blue) and data for % time freezing in the same context the following day shown as ‘Context’ (yellow) (A). Baseline freezing is generally lower, with an increase in freezing in the same context the following day. Freezing during the cue trial is shown in (B), with % time freezing in the new context before any tones shown as ‘Cue pre tone’ (blue), and % time freezing over 180s period during which three tones sound, shown as ‘Cue post tone’ (red). Freezing generally increases after presentation of the tone.
Data for baseline mice shows an overall increase in freezing in the same context on the day following conditioning, when compared to baseline freezing on day one (baseline freezing mean = 5.29, context freezing mean = 22.85, standard deviation=7.3 and 14.3 respectively, p<0.001 by paired t-test) (Figure 26A). However, there are a proportion of mice that have high freezing on day one, and a separate proportion that show little or no increase in freezing on day two. This was less apparent in the original baseline screen, which is likely to be due to the smaller number tested and the increased in variability with a larger sample size. Cue data is similar, with an overall increase in freezing seen after presentation of the cue tone, however variability is increased for both pre and post cue freezing (pre-tone freezing mean = 29.22, post tone freezing mean =51.26, standard deviation 14.0 and 12.16 respectively, p<0.0001 by paired t-test) (Figure 26B).

The origin of this increased variability is unclear, though it is likely to be due to a number of factors including handling and prior experience of the mice. It is possible that previous tests could be making the mice more anxious on day one, therefore they show increased freezing during baseline. Conversely in other mice the increased handling by multiple handlers and increased testing may make them less susceptible to the conditioned stimuli, since they have been in several potentially anxiety inducing tests in the preceding weeks and may be becoming acclimatized to handling. Despite this variability there is still an overall increase in freezing in both the context and cue conditions.

In summary, 289 GA lines were screened using the fear conditioning protocol, of these, 179 lines were screened as homozygotes and 124 as heterozygotes (including 14 lines that were screened as both heterozygotes and homozygotes due to sub-viability of homozygotes).
Figure 27 Scatter plot of baseline percentage time freezing (blue) and freezing in the same context on the day following conditioning (yellow) for all GA lines tested as heterozygotes. Lines are ordered alphabetically. The majority of mice show low levels of freezing on day one, before any tones or shocks, and an increase in freezing on day two after fear conditioning.
Figure 28 Scatter plot of baseline percentage time freezing (blue) and freezing in the same context on the day following conditioning (yellow) for all GA lines tested as homozygotes.
Figure 29 Freezing during the cued conditioning trial for all GA lines tested as heterozygotes. Percentage time freezing before presentation of the tone is generally lower (blue), than following exposure to the cue (red).
Figure 30 Percentage freezing during the cued trial for all GA lines tested as homozygotes. Percentage time freezing before presentation of the tone is generally lower (blue), than following exposure to the cue (red).
Fear conditioning data was analysed using linear mixed models and a likelihood ratio test. A null model and a model taking genotype as a fixed effect were calculated, both of which also included the date of experiment as a random effect. ANOVA of the two models was carried out to determine if the models were significantly different. As the difference between the two models was the inclusion/exclusion of genotype as an effect, any significant differences determined by the ANOVA must be an effect of genotype.

Data for contextual and cued conditioning were evaluated separately. For contextual conditioning, the difference in % time freezing between a pre-conditioning baseline interval and freezing to context on day 2 (i.e. percentage freezing in context – percentage freezing in baseline) for each individual mouse was analysed. For cued conditioning, the difference in % time freezing between the 180s interval prior to the conditioned stimulus (tone) and the following 180s during which the tone was sounded three times was analysed (i.e. percentage freezing post cue – percentage freezing pre cue) (Table 9).
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Table 9 Significant p-values calculated from GA lines tested in FC. p-values of ANOVA analysis comparing 2 linear models. Model one uses genotype as a fixed effect and date of experiment as random effect, model two is a null model, with the genotype effect removed. Lines with significant p values in context listed from top, lines with significant p values in cue test listed below. 5 lines had significant effects in both contextual and cued conditioning, these are indicated in bold text. All other
significant effects were seen in only one parameter, context or cue. Mean of each data set is shown, with increased means highlighted red and decreased means highlighted yellow. Right column shows FDR (false discovery rate) calculated using Benjamini-Hochberg correction, with lines that passed the FDR correction highlighted blue. Complete table of p values for all lines tested, including non-significant values, are shown in Appendix 1.

The biological significance of an increase in freezing is not clear. It is more likely to be related to an increase in anxiety rather than a learning and memory phenotype. Therefore for the remainder of analysis only lines with a decrease in freezing will be discussed.

Although Table 9 above displays every line with a p value of 0.05 or below, this threshold is not suitable for this type of data. As so many lines were assessed, some of them will have a low p-value by chance, i.e. 5% of lines will have a significant p value, equivalent to 15 lines for context and 15 lines for cue. This leads to type I errors, or incorrect rejection of a true null hypothesis, otherwise known as ‘false positives’. To try to reduce the number of false positives, a false discovery rate correction needs to be applied. In this case, the Benjamini-Hochberg correction is the most appropriate as this corrects for multiple lines assessed through the same test. When this is applied, only three lines have a significant difference which passes the false discovery correction, Cacna1b, Ccdc109b and Nisch.
Figure 31 Data for three lines with significant phenotypes in IMPC fear conditioning screen when data analysed by mixed model followed likelihood ratio test then Benjamini-Hochberg correction for false discovery rate. Nisch and Ccdc109b both have decreased response to context, whereas Cacna1b has decreased response to cue.
During the early stages of the fear conditioning screen, lines were selected for further analysis based on p values from t-tests. This selection was conducted before the full dataset was available and prior to the application of the mixed model analysis and correction for false discovery rates. Three lines were selected for further analysis Slc24a4, Aph1c and Frrs1l. These three lines were initially highlighted due to significant differences in fear conditioning results, however the decision to carry out further investigations into specific mouse mutants was based on the summation of fear conditioning results, IMPC data from other tests, and/or information from scientific literature.

**4.2.3.3 Slc24a4**

*Slc24a4* (also known as *NckX4*) is a solute linked carrier, transporting one $K^+$ and one $Ca^{2+}$ out of the cell in exchange for four $Na^+$ ([Li *et al.*, 2006]). Data from fear conditioning showed a reduced response to context, with little increase in freezing between baseline pre-shock levels and levels in the same context the following day (Figure 32).

During the initial line selection, data were analysed by t-test using the parameters ‘difference in percent time freezing for context’ and ‘difference in percent time freezing for cue’. Comparison of *Slc24a4*/* with C57BL6NTac baseline tested alongside and prior to *Slc24a4*/* shows no significant difference (p<0.07). However, *Slc24a4*/* is sub-viable, and as such was assessed alongside heterozygous *Slc24a4*/*+. T-test shows significant differences between *Slc24a4*/* and *Slc24a4*/*+, p=0.016. In addition, *Slc24a4*/* data falls into two distinct groups, with two mice showing a normal response to context and four mice exhibiting a
greatly reduced response. IMPC data has several significant phenotypes annotated: increased alkaline phosphatase, increased high density lipoprotein cholesterol, increased total cholesterol and decreased neutrophil count, and finally partial pre-weaning lethality. (http://www.mousephenotype.org/data/genes/MGI:2447362#section-associations, accessed on 12-05-2018).

![Figure 32](image)

No brain expression was detected by LACZ staining. Interestingly a literature search revealed that *Slc24a4* has been linked to Alzheimer’s disease in a recent GWAS study (Lambert *et al.*, 2013). This link to AD was further corroborated by brain methylation studies published at the time of this fear conditioning assessment (Allen *et al.*, 2015; Traynor and AE, 2015; Yu *et al.*, 2015). Other research into *Slc24a4* has been focussed on two areas, firstly in the field of amylogenesis imperfecta, an enamel defect that is linked to mutations in *Slc24a4* in humans.
(Parry et al., 2013) and secondly on the role of Slc24a4 in olfaction. Research into olfaction found that initial response of Slc24a4 knockout mice to an olfactory stimulus is normal, but that termination of the response is slower. This abnormal olfaction response may indicate a signalling deficit in Slc24a4 knockout mice, however mechanism for this response was not followed up (Stephan et al., 2011).

No other behavioural or cognitive phenotyping, nor any brain pathology, had been carried out on mouse models of Slc24a4. Due to the potential fear conditioning phenotype and the very recent and substantiated GWAS association with AD, it was decided to breed an additional cohort for further study.

Heterozygous Slc24a4+/- mice were intercrossed to generate a cohort of homozygotes, heterozygotes and wild-type mice of both sexes to assess through fear conditioning. Data from this repeated FC test shows similar results.
Figure 33 Additional cohort of Slc24a4−/− with Slc24a4+/− and Slc24a4+/+ controls. Analysis by ANOVA shows a significant effect of genotype in females (F[2,28]=5.04, p=0.0135) and males (F[2,22]=5.941, p=0.0087). Tukey’s post hoc analysis shows the significant differences to be: females; Slc24a4−/− with both Slc24a4+/− (p<0.01) and Slc24a4+/+ (p<0.001) for context percent time freezing, and between Slc24a4−/− and Slc24a4+/− (p<0.01) for cue post-tone freezing. In males differences are found between Slc24a4−/− and Slc24a4+/+ for context (p<0.05), cue pre-tone (p<0.01) and cue-post tone (p<0.001). Females; n=10 Slc24a4−/− n=11 Slc24a4+/− n=10 Slc24a4+/+, males; n=9 Slc24a4−/− n=10 Slc24a4+/− n=6 Slc24a4+/+.

When raw values are analysed, both female and male Slc24a4−/− show a significant decrease in freezing compared to wild-type litter mates in the context part of the
test \(p<0.001\) and \(p<0.05\) respectively) (Figure 33). To take into account differences in baseline and pre-cue freezing, the difference between context and baseline and between pre-cue and post-cue were analysed by ANOVA. ANOVA shows that difference in percent time freezing between baseline and context is significantly different for females \(F[2,28]=6.526, p=0.0047\), Tukey’s post hoc analysis shows difference between \(Slc24a4^{-/-}\) and \(Slc24a4^{+/+}\) \(p=0.0083\). There were no differences between pre-tone and post-tone in the cue test in females. No significant differences were found in males for difference in freezing in both context and cue.

### 4.2.3.4 Aph1c

Aph1c (anterior pharynx homolog 1c) is part of the Aph1 family of which there are two genes in humans \((APH1A\) and \(APH1B)\) with a third gene in rodents \((Aph1c)\), thought to have arisen from a duplication of Aph1b (Serneels et al., 2009). Fear conditioning data showed a significant decrease in response to context when initially analysed by t-test \((p=0.0006)\).

IMPC data from other tests shows \(Aph1c^{-/-}\) to have reduced lean mass and lac Z expression in the hippocampus. Literature search for Aph1c revealed a link to Alzheimer’s disease. One of the main features of AD is the presence of amyloid plaques in the brain. The key constituent of these plaques is Aβ, a protein cleaved
from amyloid precursor protein (APP). The protein complex that cleaves APP is γ-secretase which is made up of four subunits comprising Presenilin (1 or 2), Aph1 (a,b or c), Pen1 and Nicastrin. Different combinations of these four subunits are used to make a variety of γ-secretase units, whose differential roles are not fully understood (Serneels et al., 2005). γ-secretase is involved in many biological processes, in addition to cleaving APP. For example, deletions of Aph1a cause abnormal embryogenesis, as the variants of γ-secretase that contain Aph1a are crucial for NOTCH signalling (Jurisch-Yaksi, Sannerud and Annaert, 2013). Limited behavioural phenotyping has been done on Aph1b, Aph1c and combined Aph1b/c knockouts. A spatial working memory deficit is found in the Morris water maze test in combined Aph1b/c knockout mice but not in single Aph1c knockouts and investigation of Aβ production shows a reduction when both Aph1b and c are deleted (Serneels et al., 2009). However, Aph1c/− did not show behavioural
phenotypes when deleted alone (Dejaegere et al., 2008). Previous accounts of Aph1c expression have shown it to be limited to the testis and kidney (Jurisch-Yaksi, Sannerud and Annaert, 2013), however lacz data from IMPC shows specific expression in the CA1 region of the hippocampus (http://www.mousephenotype.org/data/imageComparator?&parameter_stable_id =IMPC_ALZ_076_001&acc=MGI:1915568, accessed on 12-05-2018).

Due to the link to Alzheimer’s disease and the presence of specific brain expression, in combination with the fear conditioning result, it was decided to generate a second cohort of Aph1c−/− for further analysis.

A new cohort of Aph1c−/− and Aph1c+/+ were bred from heterozygous intercross matings. Mice were assessed through fear conditioning, however the phenotype seen in the high-throughput screen was not recapitulated and no significant differences were found.
Figure 35 New cohort of Aph1c males and females assessed through fear conditioning. No significant differences found between $Aph1c^{-/-}$ and $Aph1c^{+/+}$ ($p=0.2$ for females and $p=0.09$ for males). n=7 $Aph1c^{+/+}$ and n=8, $Aph1c^{-/-}$ females, n=10 $Aph1c^{+/+}$ and n=10, $Aph1c^{-/-}$ males for context. Due to software issues this was reduced to n=3 $Aph1c^{+/+}$ and n=6 $Aph1c^{-/-}$ females, n=9 $Aph1c^{+/+}$ and n=8 $Aph1c^{-/-}$ males for cue.
4.2.3.5 Frrs1l

Ferric chelate reductase 1 like (Frrs1l) showed reduced freezing in cue during initial IMPC fear conditioning analysis. Frrs1l/− were assessed by t-test and compared to baseline which was tested both concurrently and prior to Frrs1l/− and Frrs1l+/−. Difference in percent time freezing for cue was significantly different (p=0.004).

![Graph showing Frrs1l expression](image)

Figure 36 Frrs1l/− and Frrs1l+/− FC data from IMPC screen, shown with baseline tested alongside and two months prior. n=7 Frrs1l/−, n=8 Frrs1l+/−, n=26 C57BL/6NTac.

IMPC phenotyping revealed that as well as abnormal fear conditioning, Frrs1l/− mice had deficits in the SHIRPA test including abnormal gait, trunk curling and limb grasping, as well as hyperactivity in open field. Frrs1l/− were found to have partial pre-weaning lethality, around 12% of mice weaned from a heterozygous intercross were homozygous, as opposed to the expected 25%. Homozygous embryos analysed by microCT at embryonic day 18.5 showed no obvious structural abnormalities. LacZ staining showed that Frrs1l is highly expressed in
the adult brain, spinal cord, trigeminal ganglion and testis. At 12.5dpc Frrs1l is expressed in the developing forebrain and spinal cord (http://www.mousephenotype.org/data/genes/MGI:2442704#section-associations, accessed on 12-05-2018)

Literature search showed that at the time the above data were collected, there was very little literature available on Frrs1l. Frrs1l was mentioned in two papers which focussed on AMPA receptors, naming it as a component of the AMPA receptor complex but not elucidating further on function (Schwenk et al., 2012, 2014). Frrs1l was also mentioned in the previously cited paper by Pandey et al. (Pandey et al., 2014) as a brain specific protein with no known function.

The fear conditioning phenotype, along with the other phenotypes found in IMPC, indicate a possible cognitive neurological disorder. These phenotypes, as well as the fact that Frrs1l is highly expressed in the brain and has very little known about its function, led us to breed a cohort for further study. Fear conditioning data is not included here as the test could not be conducted for welfare reasons. Further data and analysis of Frrs1l is continued in Chapter 5.

4.3 Discussion

4.3.1 Open field

Open field data from the secondary assessment showed high variability and did not recapitulate the reduction in activity on day two seen in the initial assessment. It has been shown that prior experience can alter behaviour in the open field (McIlwain et al., 2001; Paylor et al., 2006) and that open field behaviour is
susceptible to environmental influences (Crabbe, 1999; Lin et al., 2011; M. Simon et al., 2013). In contrast to the primary analysis that was carried out, mice in the full IMPC phenotyping pipeline had a different experience prior to the open field assessment. Previously in the baseline screen, mice were weaned, and subsequently left undisturbed, except for a weekly cage change and a daily routine checking, until 10 weeks of age when they were tested in open field. However, mice in the IMPC screen were also subjected to weight assessment every week from weaning. The IMPC cohorts therefore experienced a significant increase in the amount of handling compared to the original baseline groups. The IMPC mice were also tested by different experimenters over several batches. However, statistical analysis found that batch (and therefore experimenter, as this changed between batches) had no significant effect on the data.

Finally, the paradigm was changed to 20 minutes to assist with throughput and cause minimal disruption to the already established IMPC open field test. It was expected that this would still allow animals time to habituate as evidenced earlier (Bolivar, 2009). However, data from the secondary trial show that habituation did not occur.

The relatively low numbers in this screen and the multiple potential confounding factors make it difficult to be clear which of these changes had a significant impact on test parameters. It is likely that increased handling as well as reduction in trial time both contributed to the data inconsistencies. Despite being unable to attribute an exact cause to the change in results, it is clear that data for open field habituation is too variable within the constraints of high-throughput screening. Increased number of experimenters and handling before testing cannot be avoided when testing so many GA lines through a comprehensive pipeline. In addition,
increasing test length to thirty minutes on each day would be detrimental to throughput and logistically impossible. Open field habituation was discontinued for these reasons. However, original data indicates it may be useful for in-depth assessment, when looking in more detail at a particular GA line, providing efforts can be made to mitigate some of these confounding factors.

4.3.2 Fear conditioning

As in the baseline screen, secondary screening for FC demonstrated an increase in percent time freezing on day two when animals are placed in the same context, and an increase in freezing in the cued conditioning trial, when the tone is played in a new context. This increase in freezing indicates some memory of the conditioning events on the previous day and can be used as an assessment of non-declarative memory.

It is clear there is more variability in the response of the mice in the IMPC screen as power equation results are less favourable in terms of the magnitude of response that can be detected. Using data from the IMPC screen, mice that show only a very small increase in freezing in context can be detected, i.e. an increase in no more than 5%. Whereas in the initial screen a phenotype could still be identified as significant with a larger increase i.e. 9%. We are looking for an attenuation of the increase in freezing in the same context, therefore the smaller the increase we can measure the less sensitive the assay. Therefore the increased variability of the IMPC data means that only phenotypes with a large magnitude (very small or no increase in freezing) can be identified. This increase in variability is not unexpected due to the increased amount of handling and different tests that the mice have
been subjected to in the weeks prior to fear conditioning. However, this test is still a useful tool to identify clear phenotypes, if not sensitive enough for the detection of subtle changes. As such it was decided that this variability and loss of power was an acceptable compromise.

Downstream tests were assessed to determine whether data was affected by the inclusion of fear conditioning in the pipeline. Fructosamine, thought to be the parameter that would be most significantly affected, showed no significant change. Together, these data show that fear conditioning is a suitable test to be included in the IMPC pipeline to provide crucially missing data on cognitive function of many genes, whilst having minimal effects on other tests in the pipeline.

As discussed previously, for practical reasons, only males are available to assess through the fear conditioning paradigm. This compromise may reduce the capacity to detect phenotypes and will remove the ability to determine sexual dimorphism. Although data from B6N baseline show comparable fear response in males and females (Figure 19) there is some evidence to suggest that there may be sex differences in fear related mechanisms. Disruption of mineralocorticoid receptors had differential effects on fear conditioning between males and females, with females showing slower extinction of fear response (ter Horst et al., 2012). A study in rats showed opposing results, with females having a faster extinction of fear memory than males (Gresack et al., 2009). However both of these studies show that short term fear response and memory is normal, only the extinction over several days shows differences between males and females. Nevertheless, other studies show that female hormones such as oestrogen have an effect on response...
to fear conditioning (Gupta et al., 2001; Cover et al., 2014). Furthermore, data from the IMPC has shown that sex mediates phenotypes in approximately 15% of datasets. Despite the potential loss of interesting data that could be provided by screening both sexes, female mice were unavailable, and therefore the compromise to assess only males was necessary.

4.3.3 IMPC screen for FC phenotypes in GA lines

Since the FC screen was incorporated into the IMPC pipeline, 289 lines have been tested, a total of 4799 mice including baseline. As described above, homozygotes were tested when viable, and heterozygotes when homozygotes were non-viable. For lines where homozygotes were sub-viable, a full cohort of at least 8 heterozygous mice plus any surviving homozygotes were tested. Using general linear mixed model and likelihood ratio testing, data were analysed against baseline, with date of experiment and genotype taken into account. This analysis highlighted 68 lines where genotype had a significant effect. Of these, 5 lines expressed a significant phenotype in both the contextual and cued components of the test, Cacna1b, Commd9, Golga3, Rbpjl and Pdss2. However, four of these five lines showed an increase in one component and a decrease in the other. An increase in response to fear conditioning is not thought to be linked to learning and memory, and whilst this could relate to anxiety it was not the primary aim of the fear conditioning assessment, and therefore any increase in response was excluded from further analysis. This leaves just one gene with a response in both cue and context, Cacna1b. Cacna1b (calcium voltage-gated channel subunit alpha1 B) is a subunit of a volted gated calcium channel, primarily involved in neurotransmitter release. Cacna1b, has previously been linked to neurological disorders such as
schizophrenia in humans (Glessner et al., 2010). A mouse model with deletion of N-type calcium channels (of which Cacna1b is a subunit) shows abnormal spatial learning and impaired LTP (Jeon et al., 2007). The fear conditioning phenotype found in this screen appears to be the first annotation of a non-declarative memory phenotype for this gene and fits with previous data that shows alterations in LTP. Nisch is a cytoplasmic protein with known roles in cell migration and carcinogenesis. However recent research has found Nisch expressed in the cytoplasm of rat cortical neurons and inhibition of Nisch by shRNA seems to promote neurite growth and elongation of neurons through mechanisms that are not fully elucidated (Ding et al., 2015). No behavioural work has been carried out to understand the role of Nisch in behaviour and cognition.

Finally Ccdc109b (otherwise known as Mcub) is a mitochondrial calcium uniporter subunit which modulates calcium uptake into the mitochondria (Marchi and Pinton, 2014). Calcium uptake in mitochondria is needed for aerobic metabolism, defects in which can lead to a lack of ATP available throughout the body with wide ranging consequences. There are many diseases affecting mitochondrial function which result in cognitive disorders (Finsterer, 2012), however no research into the specific role of Ccdc109b in behaviour or cognition has been published.

All others genes screened through the IMPC fear conditioning screen expressed a significant effect in only one component, contextual or cued conditioning, in total 33 lines show a decrease in response. 33 lines with significant phenotypes from a total of 289 screened is a relatively high hit rate, 11.4%. Although, other tests used in this type of screen have shown a similar proportion of phenotypes, for example, in the European Mouse Disease Clinic (EUMODIC) screen, 313 mutant lines were analysed, of which 16.9% expressed an abnormal a phenotype in
acoustic startle or pre-pulse inhibition, and 18.2% of lines expressed abnormal phenotypes in grip strength (Ayadi et al., 2012). Analysis of IMPC data has shown that 23.4% of lines are lethal when homozygous (Dickinson et al., 2016) whereas only 2% of lines express a significant deafness phenotype (Bowl et al., 2017). Direct comparisons of hit rates across different biological functions are not necessarily useful, many processes will result from the contributions of a substantial number of genes, whereas other process may have much smaller gene networks. In other pathways there may be genes that can compensate for loss of another gene, therefore the phenotype of the knockout is masked. Consequently the proportion of genes which can cause a phenotype will vary dramatically, depending on the networks involved.

### 4.3.3.3 Statistical analysis of IMPC data

It is probable that in such a high-throughput screen with multiple lines being tested there is likely to be a high number of false positives i.e. if significant p-values of <0.05 are selected, with 289 lines tested, by chance 15 lines will be incorrectly characterised as significant. Statistical corrections can be applied to reduce the false discover rate. The Bonferroni correction is less relevant for this data set as we are only taking into account two components (context and cue). However, even this calculation approximately halves the number of significant hits. For context this means that only 13 lines show significant reductions in response, and for cue only 10 lines.

The Bonferroni correction is not the most suitable in this case, as the major factor contributing to false positives is the number of lines tested, rather than the number of tests on each line. To correct for the number of lines, the most appropriate test
is the Benjamini-Hochberg correction, which takes into account the p-value rank, the number of lines and the selected false discovery rate. After this correction, using a false discovery rate of 5%, the only lines with significant decrease in context which pass the false discovery rate test are *Nisch* and *Ccdc109b*, and in cue only *Cacna1b*. This small number of lines may still be representative of the proportion of genes involved in this specific type of cognitive response, approximately 1%, equating to around 200 genes. The hit rate is not dissimilar to the proportion of genes involved in deafness which is 2% (Bowl et al., 2017). 1% is also in line with a recent paper discussing genes involved in general cognitive ability. Davies et al, analysed data from several genome wide association studies, over 300,000 individuals, and found 148 independent loci associated with general cognitive ability, approximately 0.75% of all genes (Davies et al., 2018). General cognitive ability is assessed using a variety of tests in humans. As the study by Davies et al. was an amalgamation of several GWA studies there were differences between the studies in how the cognitive assessment was carried out. Whilst these differences could add some variability, all the methods used have shown to have some correlation in their use to estimate general cognitive function. The data from this large GWAS suggests that only a small percentage of genes (0.75%) is involved in affecting general cognitive function, which is similar to the hit rate of the IMPC cognition screen. Therefore indicative that the systems being studied may only be perturbed by deleting a fairly limited number of genes.

Statistical analysis of these high-throughput data are challenging. A p value of 0.05 clearly increases the likelihood of false positives to an unacceptably high level when considering the hundreds of lines being screened. However, using such a stringent false discovery calculation reduces the number of significant hits to such a low level
that it is likely to result in some false negatives. It is challenging to find the balance between statistically false negatives and false positives, as well as considering what is biologically relevant.

4.3.3.4 \textit{Slc24a4, Aph1c and Frrs1l}

Of the three lines that were selected for further assessment using t-test analysis earlier in the study, \textit{Frrs1l} remains significant using mixed model analysis and likelihood ratio tests, (p=0.027), \textit{Aph1c} does not reach significance (p=0.053), and \textit{Slc24a4} is not significant (0.088). In addition, none of these lines pass the correction for false discovery rate.

Further testing of \textit{Aph1c\textsuperscript{-/-}} found that the fear conditioning phenotype was not recapitulated, therefore this is likely to be a false positive, indicated by further stringent statistical analysis which included an appropriate correction for false discovery rate.

In contrast, \textit{Slc24a4\textsuperscript{-/-}} does show recapitulation of fear conditioning results, despite the initial significant difference being against heterozygous littermates rather C57BL/6NTac baseline. It is possible that this increase in significance is due to the second assessment having a smaller amount of baseline with reduced variability, it is also possible that variable penetrance of the gene had an effect. The IMPC FC data for this line showed that response to context fell into two distinct groups, two mice appeared to condition normally whilst four \textit{Slc24a4\textsuperscript{-/-}} showed almost no increase in freezing, indicating perhaps incomplete penetrance of the phenotype.

Survival data for \textit{Slc24a4} is interesting, with only half the expected number of homozygotes surviving to weaning age, an indicator of variable expressivity. It is
not clear why approximately half the homozygote mice die neonatally but it is clear that deletion of Slc24a4 has differential effects on different mice for reasons that are not yet understood. Although this fear conditioning phenotype was recapitulated, further research into this line was discontinued due to the focus moving to the Frrs1l line. Data on this fear conditioning phenotype will be made available to the scientific community to allow further follow up by other research groups.

4.3.4 Summary

In summary, several tests have been assessed for inclusion into a high-throughput pipeline, with one test proving suitable and being included in the IMPC screen. Data from the fear conditioning test has been analysed in real time over the months of data collection and lines selected for further investigation. Two of the three lines selected have produced interesting cognitive phenotypes (Slc24a4 and Frrs1l) of which one (Frrs1l) has been taken on for further study.

Statistical analysis of these data has developed through the course of the screen. Initial analysis of each line was carried out by t-test, however as the dataset grew it became clear that a more sophisticated model would be needed. Advice from statisticians indicated that a mixed model and a likelihood ratio test would be a more appropriate solution, with the inclusion of a suitable correction for false positives. Finding the correct statistical solution for this dataset has been challenging and it is not clear whether the correct balance between false positives and false negatives has been reached. However, raw data is available to the wider scientific community for the entire fear conditioning screen and further statistical analysis can be applied as appropriate. There is no doubt that these fear
conditioning data provide important information on the role of many genes in non-declarative memory, both in lines where phenotypes have been found and in lines that appear normal.
5. In-vivo phenotyping of *Frrs1l*<sup>−/−</sup>

5.1 Introduction

5.1.1 IMPC data summary

The gene ferric-chelate reductase 1 like (*Frrs1l*) was a candidate identified as part of the previously described IMPC screen for genes with learning and memory deficits (4.2.3.5).

Knock out mice for *Frrs1l* showed abnormal response in fear conditioning (figure 36). *Frrs1l*<sup>−/−</sup> baseline freezing level was significantly less than wild-types while the percentage increase in freezing in response to context was comparable to those of wild-type mice. *Frrs1l*<sup>−/−</sup> response to cue, however, was significantly less than controls (p<0.05).

5.1.2 *Frrs1l*

Up until recently, there was little information published on *Frrs1l*, otherwise known as c9orf4 or CG-6. One report linked the genomic region containing *Frrs1l* in humans to a familial dysautonomia, however on further investigation no associated genetic mutation was identified (Chadwick *et al.*, 2000). In 2014 there was an interesting study that examined literature and expression databases to find brain specific proteins with no known function. In their report, *Frrs1l* was an example of a highly abundant protein about which nothing was known (Pandey *et al.*, 2014).

At around the same time, Schwenk and colleagues published a report investigating the proteomics of AMPA receptor complexes using high resolution nanoflow liquid
chromatography tandem mass spectrometry. Twenty-one new proteins in the AMPAR complex were identified, including FRRS1L. Schwenk et al further found that FRRS1L bound exclusively with AMPAR in all brain regions, but with higher abundance in the thalamus, striatum, hippocampus and cortex (Schwenk et al., 2012).

In 2014, a follow up study showed that FRRS1L had relatively high expression compared to other AMPA receptor complex constituents (excluding the main core forming proteins GLUR1-4) in the mouse brain and that expression increases dramatically between postnatal day 14 (P14) and P28 (Schwenk et al., 2014).

FRRS1L is highly conserved throughout the animal kingdom, with pronounced sequence similarity amongst mammals as well as birds, fish and amphibians. FRRS1L sequence differs considerably in nematodes.

![Figure 37 Sequence of FRRS1L in several species of mammals, birds, fish, amphibians and nematodes.](image)

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5.1.3 Structure and function of AMPA receptors

As previously described (1.4) AMPA receptors play a critical role in synaptic transmission at glutamatergic synapses and in long term potentiation. Ultimately they are responsible for the majority of excitatory neurotransmission in the central nervous system. The core of the AMPA receptor is a heterotetrameric complex made up of pairs of homodimers containing one or two of GLUR1, GLUR2, GLUR3 or GLUR4 (alternatively known as GLUA1, GLUA2, GLUA3 and GLUA4). These core proteins make up the pore of the AMPA receptor, facilitating Na\(^+\) transport, and in the absence of a specific GLUR2 isoform, transport of Ca\(^{2+}\). AMPA receptors are predominantly postsynaptic ligand gated ion channels, in this case, the transport of ions is enabled by the binding of glutamate, which is released from the presynaptic terminal after stimulation by an action potential (Gouaux, 2004; Chater and Goda, 2014; Henley and Wilkinson, 2016).

The four GLUR subunits are expressed differentially throughout the brain. One study demonstrates that in rat brain, the major subunit in the olfactory bulb, cortex, striatum and thalamus is GLUR2. GLUR1 and GLUR2 together make up 80% of the AMPARs in the hippocampus, whereas in the brain stem and cerebellum the major constituent is GLUR4 (Schwenk et al., 2014). The relative abundance of GLURs changes little postnatally, whereas the abundance of their auxiliary proteins changes dramatically.

5.1.4 AMPA auxiliary proteins

AMPA receptor auxiliary proteins are a group of around 30 proteins that associate with the core AMPA receptor subunits depending on spatial or temporal
determinates. Of this large group of proteins, several have been investigated in more details with some elements of their function illuminated.

The TARP family are a group of proteins that make up part of the AMPA auxiliary protein pool, namely TARP-γ2, TARP-γ3, TARP-γ4, TARP-γ5, TARP-γ7 and TARP-γ8. This family was first identified in the mouse model stargazin, whose causative mutation is in Tarp-γ2, leading to abnormal gait, seizures and head bobbing activity (Tomita et al., 2003). Since then the role of the TARP’s has been shown to include alterations in deactivation and desensitisation (Milstein et al., 2007), as well as differential gating and trafficking (Tomita, Adesnik, et al., 2005; Soto et al., 2009; Kato et al., 2010; Matsuda et al., 2013) of AMPA receptors. TARPs are expressed with AMPA receptors in a cell-type specific manner and seem to affect AMPA receptor function in various ways (Bats, Farrant and Cull-Candy, 2013).

CKAMP44, cystine-knot AMPAR modulating protein, 44kDa in size, is another auxiliary protein. This has been shown to increase desensitisation and slow recovery of receptors when overexpressed in mice. Accordingly, an increase in the strength of repeated stimulations occurs when CKAMP44 is deleted in mice. These data suggest that CKAMP44 is involved in controlling the sensitisation of AMPA receptors (Engelhardt et al., 2010).

Finally, CNIH-2/3, Cornichon homologs 2 and 3, have recently been shown to slow decay of post synaptic currents, with decay happening more rapidly in cells not expressing CNIH’s. Indicating a role in the timing of synaptic transmission (Kato et al., 2010; Boudkkazi et al., 2014).
Of the approximately thirty proteins thought to be associated with AMPA receptors, only the above nine proteins have significant functional information, with the role of the remaining twenty one proteins, including FRRS1L, yet to be elucidated.

### 5.1.5 Human studies of FRRS1L

In recent years, the relevance of FRRS1L in human disease has been highlighted with the discovery of homozygous mutations in *FRRS1L* in patients with juvenile Huntington’s like disease. Affected children do not have the usual CAG triple expansion found in Huntington’s disease but do have many of the same symptoms. Patients are normal at birth and through the initial stages of development. At 18 to 24 months, patients show signs of regression, with loss of expressive speech, spasticity, impaired volitional movement, development of chorea followed by rigidity and hypokinesia, severe intellectual impairment and seizures. Whole exome sequencing of these patients revealed four homozygous mutations in *FRRS1L*, segregating within each of the four affected families (Madeo *et al.*, 2016) (Figure 38).

![Figure 38 Image from Madeo et al (2016) showing FRRS1L homozygous mutations found in human patient families.](image)

One further study found an unrelated family with similar symptoms, with the additional information that they suffered continuous spike and wave EEG activity during sleep. This family also carried the G321 mutation (Shaheen *et al.*, 2016).
As data from Schwenk et al. (Schwenk et al., 2012) show a connection between FRRS1L and AMPA receptor complexes, it was hypothesised that mutations in FRRS1L might modify AMPA receptor function. To investigate this, siRNA knockdown of FRRS1L in neuronally differentiated SH-SY5Y cells was carried out. It was found that FRRS1L knockdown led to a decrease in calcium influx and diminished AMPA-induced inward currents. Substantiating the hypothesis that FRRS1L has a role in glutamate signalling (Madeo et al., 2016).

5.2 In-vivo phenotyping results

Given the specific deficit in cued-conditioning established in the IMPC cognitive screen (4.2.3) additional cohorts of Frrs1l−/− and Frrs1l+/− were generated to investigate the extent of the behavioural/neurological deficits.

5.2.1 Increased neonatal lethality in Frrs1l−/− mice

Frrs1l−/− mice are born in normal Mendelian ratios, however, the majority of homozygous mice die 18 to 24 hours after birth. Analysis of numbers per genotype at weaning show a difference in expected ratios using chi squared analysis (p<0.0001) 11 Frrs1l−/−:187 Frrs1l+/−:83 Frrs1l+/+, whereas at P0 the ratio of genotypes is not significantly different to expected (p=0.43), 5 Frrs1l−/−:19 Frrs1l+/−:8 Frrs1l+/+ (Figure 39). The cause of this mortality is unknown. At birth mice seem normal, all pups feed, have normal skin colour and appear to be breathing. At P0 homozygous pups are indistinguishable from heterozygous or wild-type littermates. Several hours after this initial assessment, approximately 90% of the homozygous mice die. Gross pathology carried out at P0 revealed no obvious abnormalities in
44 tissues examined. This agrees with earlier data from the IMPC screening pipeline which shows that E18.5 day embryos also appear grossly normal (http://www.mousephenotype.org/data/genes/MGI:2442704#section-associations, accessed on 12-05-2018).

Due to the difficulties in breeding a suitable cohort size, further testing was carried out on females only, with a small group of males assessed in only a subset of tests. All data below refers to females only, unless otherwise specified.

Figure 39. Percentage per genotype of mice expected, born and weaned from a heterozygous intercross (birth n=32, weaning n=281). Ratio at weaning is significantly different from that expected (p<0.0001) analysis by Chi-squared test).

5.2.2  

\textit{Frrs1l}^{-/-} are smaller than littermates

Homозygous mice that survive until weaning gain less body weight with age when compared to littermate controls. \textit{Frrs1l}^{-/-} are not significantly smaller at weaning, however they gain weight at a slower rate than wild-type and heterozygote controls, with weight differences becoming significant from 5 months (Figure 40).

The difference between homozygotes and controls becomes increasingly significant as the controls continue to put on more weight with age, thus there is a progressive deficit in weight gain in \textit{Frrs1l}^{-/-}. Further analysis of IMPC data shows that \textit{Frrs1l}^{-/-}
are not only small in weight but shorter in length, with tibia length, determined at x-ray, being significantly shorter in both males (p<0.05) and females (p<0.01).

![Graph showing body weight over months for Frrs1l−/−, Frrs1l+/− and Frrs1l+/+ females.](image)

Figure 40 Mean body weight for Frrs1l−/−, Frrs1l+/− and Frrs1l+/+ females over a 12 month period. Significant differences are found between Frrs1l−/− and Frrs1l+/+ from 5 months onwards and between Frrs1l−/− and Frrs1l+/− from 7 months onwards (F[10,312]=40.19, p<0.05). Data analysed by two-way ANOVA with Tukey’s post hoc analysis. Bars show standard deviation.

5.2.3 **Motor, Behavioural and sleep phenotyping**

To elaborate on initial phenotypic findings and further investigate the suitability of the line to study the human inherited disorder, a thorough screening pipeline for *Frrs1l−/−*, along with *Frrs1l+/−* and *Frrs1l+/+* control mice was conducted.
Figure 41 Outline of phenotyping pipeline for Frrs1l+/-, Frrs1l+/+ and Frrs1l+++, carried out on females only. Age at which tests were carried out is specified on the left, and samples sizes assessed in each test are specified on the right.

Phenotyping consisted of SHIRPA, grip strength and home cage analysis, carried out three times at three monthly intervals. In addition, a single assessment of each of the following was carried out - open field, locotronic, rotarod, spontaneous alternation, home cage motor function and sleep analysis.
5.2.4 Early onset motor deficits in \textit{Frrs1l}\(^{-/-}\) mice

SHIRPA, carried out at 3, 6 and 9 months, identified abnormalities in several parameters (Figure 42). Activity in the viewing jar was increased in most \textit{Frrs1l}\(^{-/-}\) compared to heterozygous and wild-type controls. \textit{Frrs1l}\(^{-/-}\) showed an increased incidence of feet falling through the bars during observation in the viewing jar (\(p<0.001\) at three, \(p<0.05\) at six, \(p<0.001\) at nine months), coinciding with loss of balance and poor co-ordination. Gait of homozygous mice was abnormal (\(p<0.5\) at three months, \(p<0.01\) at six months, \(p<0.001\) at nine months) along with an increased incidence of limb grasping (\(p<0.01\) at all time points). At three months of age more than half of the homozygous mice slipped during negative geotaxis, by nine months this had increased to four mice falling from the grid entirely and the remaining three slipping significantly. This series of tests demonstrated abnormal co-ordination and gait in \textit{Frrs1l}\(^{-/-}\) mice, however, symptoms were already significant at three months and little further deterioration was seen over time (Figure 42).
Figure 42 Data from SHIRPA test highlights several differences between Frrs1l<sup>−/−</sup>, Frrs1l<sup>+/−</sup> and Frrs1l<sup>+/+</sup>. Frrs1l<sup>−/−</sup> have greater incidence of increased activity (A) \((p<0.05\) at three and six months, \(p<0.01\) at nine months) and feet falling through the grid in the viewing jar (B) \((p<0.001\) at three months, \(p<0.05\) at six months, \(p<0.001\) at nine months). Frrs1l<sup>−/−</sup> show some tendency to trunk curl although this is not significant (C) however they have a significant increase in limb grasping (D) \((p<0.01\) at every time point), have abnormal gait.
from an early age (E) (p<0.05 at three months, p<0.01 at six months, p<0.001 at nine months) and a reduced ability to climb down a vertical grid during the geotaxis test (F) (P<0.05 at three months, not significant at six months, p<0.001 at nine months). All statistics carried out using Fishers exact test. n=8 Frrs1+/+, n=11 Frrs1+/−, n=9 Frrs1−/−.

Figure 43 Image of homozygous mice during six month SHIRPA. Limb grasping (A) and trunk curling (B) seen in an increased number of homozygotes compared to wild-type and heterozygous controls.

Grip strength was measured at 3, 6 and 9 months of age. At each time point Frrs1+/− had a reduced grip strength compared to both Frrs1+/+ and Frrs1+/− controls. Data is highly significant at 3 months for both when normalised to body weight and raw data (p<0.001). Results remain highly significant for raw data at 6 months and 9 months, whilst normalised data becomes less clear but still statistically different as the mice age (p=0.05 at 6 and 9 months) (figure 44).
Figure 44 Grip strength data assessed at 3, 6 and 9 months (M). Data shows raw values for all four paws (A1, B1, C1) or values adjusted to body weight (A2, B2, C2) to account for body size differences in Frrs1l+/−. Data are significantly different for raw data at 3 months (F[2,23]=39.84, p<0.0001), 6 months (F[2,23]=10.9, p<0.001) and 9 months (F[2,17]=28.6, p<0.0001). Normalised data are still significant but to a less stringent level, 3 months (F[2,23]=10.15, P<0.001), 6 months (F[2,23]=3.465, p<0.05), 9 months (F[2,17]=4.499, p<0.05). Data are analysed by repeated measures ANOVA at each time point, followed by Tukey’s post hoc comparisons. Bars show standard deviation. n=8 Frrs1l+/+, n=10 Frrs1l+/−, n=8 Frrs1l−/−.
Interestingly the absolute values of the raw data for each genotype remain very similar over the 9 month period, however as the wild-type and heterozygote mice gain more weight than the homozygotes, the difference in body weight becomes more divergent, therefore reducing the significance of the data when normalised (Figure 45).

Figure 45 Data shows mean grip strength at each time point (A) and body weight taken at time of grip strength test (B). This demonstrates that grip strength values change little over time within each genotype and that change in statistical significance of normalised grip values (Figure 43 A2, B2, C2) is related to reduced gain of body weight in Frrs1l−/−. Bars show standard deviation.

Motor disabilities were further assessed using three complementary tests; rotarod, to assess the ability of the mouse to stay on an accelerating rod, locotronic ©, to assess the ability of the mouse to move along a horizontal ladder, and home cage motor function, a paradigm designed to assess standard wheel running activity as well as ability run on complex wheels requiring higher levels of co-ordination.

Frrs1l−/− have reduced latency to fall from an accelerating rotarod (p<0.05), with an average across the three trials of 37, compared to 97 and 84 for Frrs1l+/− and Frrs1l+/+ respectively (Figure 46A).
Assessment on locotronic revealed that *Frrs1*<sup>−/−</sup> had an increased error rate compared to controls, with an increased incidence of feet slipping through the bars of the ladder (Figure 46B). This correlates with the SHIRPA data which showed that *Frrs1*<sup>−/−</sup> have difficulty placing their feet on a grid without them slipping through.

![Figure 46 Latency to fall from an accelerating rotarod (A), is decreased in *Frrs1*<sup>−/−</sup> compared to *Frrs1*<sup>−/+</sup> and *Frrs1*<sup>+/+</sup>. n=8 wt, n=11 het, n=9 hom. Repeated measures ANOVA across the three rotarod trials (F[2,25]=9.266), followed by Tukey’s post-hoc analysis, shows significant differences between *Frrs1*<sup>−/−</sup> and both *Frrs1*<sup>−/+</sup> (p<0.001) and *Frrs1*<sup>+/+</sup> (p<0.05). *Frrs1*<sup>−/−</sup> also have an increased number of errors when moving along a horizontal ladder, Locotronic (B) n=8 *Frrs1*<sup>−/+</sup>, n=9 *Frrs1*<sup>−/−</sup>, n=7 *Frrs1*<sup>+/+</sup>. (F[2,21]=4.45, p<0.001) calculated by one-way ANOVA. Bars show standard deviation.

During assessment of home cage motor function, *Frrs1*<sup>−/−</sup> showed little difference in standard-wheel running during the first two weeks of the trial. However, at the start of the third week, wild-type and heterozygous mice were able to adapt to the new complex wheel but *Frrs1*<sup>−/−</sup> had difficulty, with a significant decrease in running attempts and distance run (Figure 47). Significant differences were not seen in time running, although the trend for *Frrs1*<sup>−/−</sup> is reduced.
Figure 47 Data from home cage motor function test. Parameters shown are for the first hour of the active period (1900-2000hrs) over 21 days. The standard wheel was replaced with the complex wheel on day 15, as indicated by arrows. Repeated measures ANOVA shows significant interaction between genotype and day, for distance ran (F[40,340]=1.439, p<0.05), wheel rotations (F[40,340]=1.439, p<0.05) and number of runs (F[40,340]=2.596, p<0.0001). Tukey’s post hoc multiple comparisons shows that after replacement with the complex wheel, Frrs1l/- show significantly less time running, fewer rotations and reduced number of runs compared to wild-type controls. n=6 Frrs1l+/+, n=8 Frrs1l+/-, n=5 Frrs1l-/-.

5.2.5 Frrs1l-/- mice are hyperactive

Further investigations into hyperactivity in Frrs1l-/- mice were carried out in open field tests at 10 weeks of age, to assess activity in a novel environment, and additionally in a home cage analysis set up to evaluate activity in undisturbed and non-stressful surroundings over a number of days.
Open field data shows that Frrs1l−/− are hyperactive in a novel environment, with total distance moved and velocity in the whole arena both significantly increased compared to controls (p<0.01 compared to Frrs1l+/+ controls) (Figure 48 E and F).

Figure 48 Data from open field test shows no significant difference in centre parameters; distance moved, duration and frequency (A, B and D), and a significant difference between Frrs1l−/− and Frrs1l+/−, but not Frrs1l+/+, for centre velocity (C) (p<0.05). Frrs1l−/− are significantly different for whole arena parameters; total distance moved and velocity (p<0.01 for Frrs1l+/+ and p<0.05 for Frrs1l+/−). Data is more variable for Frrs1l−/− across most parameters, due to variability, data were not normally distributed therefore all p values were calculated using Kruskal-Wallis non-parametric ANOVA. Bars show standard deviation. n=7 Frrs1l+/+, n=12 Frrs1l+/−, n=10 Frrs1l−/−.
Centre parameters, which may indicate anxiety, were only marginally altered with most showing no significant difference, and only centre velocity demonstrating a difference between \( Frrs1l^{-/-} \) and \( Frrs1l^{+/+} \) but not \( Frrs1l^{+/+} \)(Figure 48 C). This indicates that homozygotes express a purely hyperactive rather than anxiety related phenotype.

\( Frrs1l^{-/-} \) are also hyperactive in 12:12 light:dark conditions in the home cage. Mice were assessed continuously for 72 hours at 3, 6 and 9 months. Data shows that during the light (rest) phase, \( Frrs1l^{-/-} \) mice move significantly more at 3 months, but not at 6 or 9 months. During the dark (active) phase, activity of homozygotes is higher at all three time points (Figure 49).

Figure 49 Data from home cage analysis shows distance moved in the home cage in the light phase (A) or the dark phase (B). Data show the sum of distance travelled over light and dark phases over 3 days. \( Frrs1l^{-/-} \) move significantly more than \( Frrs1l^{+/+} \) and \( Frrs1l^{+/+} \) controls at 3 months \((F[2,79]=10.76, p<0.001)\) and 9 months \((F[2,79]=10.76, p<0.05)\) in the light phase. They also show increased movement at all time points during the dark phase, with highly significant differences at three months between \( Frrs1l^{-/-} \) and both heterozygous and wild-type control groups \((F[2,79]=21.99, p<0.001\) and \( p<0.0001 \) respectively), as well as differences from \( Frrs1l^{+/+} \) at 6 and 9 months \((F[2,79]=21.99, p<0.05 \) at both time points.). Data analysed by two-way ANOVA. Bars show standard deviation. \( n=8 \) \( Frrs1l^{+/+} \), \( n=13 \) \( Frrs1l^{+/+} \), \( n=9 \) \( Frrs1l^{-/-} \).
These data clearly shows that \textit{Frrs1l}^{-/-} are hyperactive in both novel and familiar environments.

Further investigation of activity data in smaller time bins clearly shows the pattern of increased activity in the homozygous mice (Figure 50). Additional analysis of these data is confounded by the fact that \textit{Frrs1l}^{-/-} were housed in cages with \textit{Frrs1l}^{+/+} and \textit{Frrs1l}^{++/}. Genotypes were mixed within cages during the randomisation process at weaning, therefore it is possible that the behaviour of the controls is altered by the hyperactive nature of the homozygotes.

![Figure 50 Raster plots of home cage analysis activity. Data recorded at 6 months of age, representative \textit{Frrs1l}^{-/-} (A) and \textit{Frrs1l}^{+/+}(B). Data is double-plotted for visualization purposes. Each vertical bar represents activity within a six minute time bin where the height of the bar denotes the amount of activity within that bin. \textit{Frrs1l}^{-/-} show increased activity compared to wild-type controls.](image)

Videos recorded during home cage analysis also revealed some unusual behaviours. \textit{Frrs1l}^{-/-} seem very agitated, sometimes performing stereotypic behaviours, such as climbing on the bars and dropping down in a set pattern, which they repeat on many occasions over a short period of time. \textit{Frrs1l}^{-/-} also demonstrated further evidence of co-ordination and grip problems, with several recorded incidences of \textit{Frrs1l}^{-/-} falling from the cage hopper or the bars on the cage
lid, as well as an inability to either judge where objects are or accurately manoeuvre around them, evidenced by homozygotes colliding into objects within the cage.

Figure 51 Video stills from home cage analysis. (A) shows loss of balance in Frrs1<sup>l<sup>−/−</sup> when walking, with the left hind foot lifted higher than normal. (B) and (C) are sequential screen shots of Frrs1<sup>l<sup>−/−</sup> showing the mouse colliding with the entrance to the tube. (D) and (E) are sequential screen shots
of Frrs1<sup>−/−</sup> feeding at the food hopper (D) before losing grip or balance and falling to the cage floor (E).

### 5.2.6 Working memory in Frrs1<sup>−/−</sup> is impaired

To assess working memory, Frrs1<sup>−/−</sup> and controls were assessed using a y-maze forced alternation paradigm. No genotype showed a preference for the novel arm in terms of duration exploring each arm, however, wild-type and heterozygous mice did show an increased frequency of entry into the novel arm, whereas Frrs1<sup>−/−</sup> did not (Figure 52). This indicates that Frrs1<sup>−/−</sup> have no memory of the maze from the first trial, as they show no preference for the novel arm in trial two.

![Y-maze diagram](attachment:Y-maze.png)

- **Figure 52** Proportion of frequency in novel arm, calculated as (frequency in novel arm / (frequency in novel arm + frequency in familiar arm)). Frrs1<sup>−/−</sup> show chance levels of frequency to enter the novel arm (50%), therefore no increase in exploration of the novel arm, in a forced alternation y-maze task. Non-parametric Mann-Whitney test shows significant difference for proportion of frequency into novel arm between Frrs1<sup>−/−</sup> and wild-type (p<0.05) but not between Frrs1<sup>−/−</sup> and Frrs1<sup>+/−</sup>. Bars show standard deviation n=7 Frrs1<sup>+/+</sup>, n=8 Frrs1<sup>+/−</sup>, n=7 Frrs1<sup>−/−</sup>.

### 5.2.7 Frrs1<sup>−/−</sup> have sleep abnormalities

Frrs1<sup>−/−</sup> were tested in two different paradigms to assess sleep in singly-housed animals. The first used video tracking to assess immobility-defined sleep, data from one day in a 12:12 light:dark cycle was analysed. The second used passive infrared
sensors that detect motion under differing light-dark conditions, comprising of 5 days in 12:12 light:dark, followed by 9 days in constant darkness, in order to assess both sleep and circadian parameters.

Using video tracked immobility defined sleep analysis we found that Frrs1l−/− spend less time in immobility defined sleep and have increased episodes of immobility, indicating sleep fragmentation (Figure 53) (p<0.05).

![Figure 53](image)

**Figure 53** Immobility defined sleep analysis of video-tracked animals shows Frrs1l−/− spend significantly less time asleep in the light phase than both Frrs1l+/− (p<0.001) and Frrs1l+/+ (p<0.05). Frrs1l−/− also have an increased number of immobile episodes in the light phase compared to Frrs1l+/− (p<0.01) and Frrs1l+/+ (p<0.05). Data analysed by Students t-test. Bars show standard deviation. n=8 Frrs1l+/+, n=9 Frrs1l+/−, n=8 Frrs1l−/−.

To further define this phenotype and to measure circadian parameters, mice were monitored using passive infrared equipment (PIR). PIR data showed that Frrs1l−/− are significantly more active in the dark phase of the light dark cycle (Figure 54A), consistent with data from both open field and home cage analysis that demonstrates hyperactivity in these mice. In support of video tracking analysis, data from PIR shows that Frrs1l−/− spend less time asleep in both the light and dark phases of the light dark cycle (Figure 54B).
Data from passive infrared (PIR) screen for activity and sleep duration. Frrs1l−/− display increased activity in the dark phase of the light dark (LD) cycle and in total light-dark combined (A). Data analysed by ANOVA, \( F[2,16]=3.727, p<0.05 \) Tukey’s post hoc analysis shows increased activity in the dark phase \( (p<0.05) \) and increased activity in total light-dark \( (p<0.01) \). Total time asleep was decreased in both the dark phase and in total light-dark \( F[2,16]=20.11, p<0.0001 \) (B). n=5 Frrs1l+/+, n=8 Frrs1l+/−, n=6 Frrs1l−/−.

Data were also analysed in 30 minutes bins to assess sleep over time. It was found that Frrs1l−/− had a significantly different sleep pattern to Frrs1l+/− and Frrs1l+/+, with reduced sleep in the dark phase (Figure 55). Data analysed by repeated measures ANOVA \( F[94,768]=1.505; p=0.002 \).
Figure 55 Time spent asleep plotted in 30 minute bins over time for 12:12 light dark cycle. Time spent asleep is reduced overall and sleep pattern shows significant differences between Frrs1l/− and other genotypes (ANOVA (F[94,768]=1.505; p=0.002). Data analysed by ANOVA. Bars show standard deviation. n=5 Frrs1l+/+, n=8 Frrs1l+/−, n=6 Frrs1l/−.
Figure 56 Sleep bout length is significantly shorter in all phases, analysed by ANOVA (F[2,16]= 13.22, p<0.001) Tukey’s post hoc analysis shows decrease in sleep bout length during the light phase (p<0.05), during the dark phase (p<0.0001) and in light-dark combined (p<0.01). Number of sleep bouts shows no significant differences between Frrs1l−/− and controls (F[2,16]=2.121, p=0.152). n=5 Frrs1l+/+, n=8 Frrs1l+/−, n=6 Frrs1l−/−.

On assessment of sleep bouts, Frrs1l−/− were found to have a significant decrease in bout length (p<0.0001) (Figure 56A). However no significant difference was seen in sleep bout number (Figure 56B).

Sleep bouts were further analysed by summing the number of bouts into bins, based on their duration, for example bin1 is number of bouts less than 1 minute in length, bin 2 is number of bouts between 1 and 2 minutes in length, with the final bin being >15 minutes (Figure 57). Analysis by repeated measures ANOVA showed a significant interaction between genotype and number of bouts within bins (p<0.0001 for light and dark phase). Post hoc analysis shows this to be due to a greater number of shorter bouts in Frrs1l−/− compared to controls in both the light and dark phase.
Figure 57 Number of sleep bouts per one minute bins up to 15 minutes, then number of sleep bouts over 15 minutes. Analysis by two way ANOVA showed that genotype had a significant effect on the number of bouts within bins (in the light phase: $F[30,240]=2.67, p<0.0001$) (A), (in the dark phase: $F[30,240]=7.25, p=0.0001$) (B). Pairwise comparisons revealed that this was due to an increased number of shorter bouts in $Frrs1^{-/-}$ ($p<0.0001$ and $p<0.001$ for bins 0-1 and 1-2 in the light phase, $p<0.0001$ and $p<0.05$ for bins 0-1 and 1-2 in the dark phase, all bins >2 minutes showed no significant differences between genotypes). $n=5$ $Frrs1^{+/+}$, $n=8$ $Frrs1^{+/+}$, $n=6$ $Frrs1^{-/-}$.

These data demonstrates that $Frrs1^{-/-}$ have significantly altered sleep, suggestive of a fragmented sleep pattern of less sleep over all, attained by a larger number of shorter bouts.
Analysis for circadian parameters was also carried out using data from PIR recordings.

No differences were found in the tau (circadian period) or alpha (length of the active phase) in constant darkness. Therefore, *Frrs1l* homozygotes appear to have an altered sleep pattern with a normal circadian rhythm.

### 5.2.8 *Frrs1l*⁻/⁻ males show similar phenotypic trends to *Frrs1l*⁻/⁻ females

Selected tests were carried out on a small group of male mice (*n*=6 *Frrs1l*⁻/⁻, *n*=5 *Frrs1l*⁺/-, *n*=7 *Frrs1l*⁺/⁺). The study was discontinued due to small sample size, diminishing further when several males were euthanized in the early phase of testing due to fight wounds. Initial data showed a similar trend for increased activity in open field (Figure 59A) and reduced grip strength at three months (Figure 59B), however data did not reach significance for either of these tests. Nonetheless, reduced latency to fall from an accelerating rotarod (Figure 59C) (*F*[2,14]=6.836, *p*<0.01), decreased grip strength at 6 months (Figure 59D)
(F[2,8]=4.978, p<0.05), and increased activity in home cage analysis during the light phase at nine months (F[2,22]=8.813, p = 0.0015) and during the dark phase at both three months and nine months (Figure 59E and F) (F[2,22]=12.1, p<0.05), were all significantly different despite the reduced sample size.

Overall, male data shows a similar trend to female data in the subset of tests for which they were both assessed.
Figure 59 Data from males shows non-significant trend towards increased activity in open field (A) and decreased grip strength at 3 months (B). Significant differences were found in latency to fall from an accelerating rotarod ($F[2,14]=6.836, p<0.01$) (C) and grip strength at six months ($F[2,8]=4.978, p<0.05$). Group housed activity in the home cage was significantly altered, with an increase in activity during the light phase at 9 months only ($F[2,22]=8.813, p = 0.0015$). During the dark phase activity was increased compared to wild-types at both three and nine months ($F[2,22]=12.1, p<0.05$), with six month data not reaching significance. Open field test analysed by Kruskal-Wallis non-parametric one way ANOVA, data rotarod analysed by repeated measures ANOVA, data for grip strength and home cage analysis analysed by two way ANOVA. $n=6$ Frrs1l$^{-/-}$, $n=5$ Frrs1l$^{+/+}$, $n=7$ Frrs1l$^{+/+}$ at 3 months.

5.2.9 Electroencephalography (EEG)

As human patients suffer from different forms of epilepsy and seizures, we evaluated whether mice lacking FRRS1L would also experience epileptic-like seizures.

Three Frrs1l$^{-/-}$ male mice and three wild-type controls (two Frrs1l$^{+/+}$ littermates and one C57BL/6NTac) were assessed for EEG using electrodes and telemetry recordings.

Preliminary analysis shows multiple seizure like events in all three Frrs1l$^{-/-}$ mice and no similar events in controls. However, in-depth analysis is on-going and the extent of the seizure behaviour in the Frrs1l$^{-/-}$ is yet to be confirmed.

Figure 60 Representative image of seizure like activity from EEG recording of Frrs1l$^{-/-}$ and Frrs1l$^{+/+}$. 

Preliminary analysis shows multiple seizure like events in all three Frrs1l$^{-/-}$ mice and no similar events in controls. However, in-depth analysis is on-going and the extent of the seizure behaviour in the Frrs1l$^{-/-}$ is yet to be confirmed.
5.3 Discussion

Frrs1l+/− mice showed a spectrum of neurological deficits, which strongly supports studies implicating loss of function of Frrs1l in a severe neurological disorder in humans. Furthermore, the similarity of deficits observed in mice would argue that this is a good model for studying the mechanisms of disease development and progression.

Body weight is reduced compared to wild-types as the mice age. This seems to be due to a reduced ability to gain weight rather than a degenerative progressive weight loss. No obvious weight loss was seen in these mice up to 14 months of age. Although IMPC data for tibia length has shown these mice to be slightly shorter, that does not account for all the difference in body weight, since data become more divergent after 5 months when there will no longer be bone growth. It is possible that the decrease in weight gain may be also due to the excessive activity in these mice. As shown in open field and home cage analysis, Frrs1l+/− are extremely active, making them likely to burn more calories and potentially gain less fat mass. However, metabolic parameters were assessed in these mice as part of the IMPC pipeline and no significant differences were found in metabolic rate when assessed at 12 weeks of age.

Unravelling the causes of neonate mortality in these mice is challenging. P0 mice have no gross abnormalities, ruling out common causes of neonate lethality such as heart or respiratory problems. However, other mouse models that effect glutamate receptor function are also neonatal lethal, including mice with a deletion of VGLUT2 (Wallen-Mackenzie, Wootz and Englund, 2010) or with a disruption of GLUR2 editing (Brusa et al., 1995). Although the mechanism for this lethality is
unknown, there is evidence that glutamate signalling is needed for proliferation, differentiation and migration of neurons during the post-natal period (Luján, Shigemoto and López-Bendito, 2005) and that there is an increase in synapses expressing AMPA receptors shortly after birth and throughout post-natal development (Petralia et al., 1999). Therefore it is conceivable that if a lack of FRRS1L causes a disruption in glutamate signalling, it could have detrimental effects on brain function in a period where AMPA receptor function is increasingly critical.

Data from SHIRPA, open field and home cage analysis shows that Frrs1l−/− are considerably hyperactive, a phenotype which has previously been associated with AMPA receptor dysfunction. For example mice with a knockout out of the gene encoding GLUR1 are hyperactive (Wiedholz et al., 2008). Conversely, unlike the consistent effects in activity seen in Frrs1l, the GLUR1 mutant increase in activity is seen only during the open field test but is not evident in home cage analysis. However, in the study by Wiedholz et al, mice were singly housed for three days and recorded for activity on the final day, whereas Frrs1l−/− mice were housed with cage mates in standard home cages.

Mutants in genes indirectly associated with AMPA receptor function show similar hyperactivity phenotypes. For example, deletion of Shank2, a key protein in the post synaptic density, causes hyperactive behaviour in mice during open field assessment (Schmeisser et al., 2012).

Further data from SHIRPA, in addition to rotarod, locotronic and home cage motor function all demonstrate a clear coordination problem in Frrs1l−/− mice. Frrs1l−/−
show an increased incidence of feet falling through the bars at SHIRPA, as well as an increased error rate in locotron. They show a reduced latency to fall from a moving rotarod and an inability to run on complex wheels. Furthermore they have a clearly distinguishable unusual gait and increased incidence of limb grasping. Mice with mutations causing Huntington’s disease, and mice with a mutation in Snap25, a protein found in the pre synaptic density, both show decreased running activity in home cage motor function assessment. Snap25 mutants show a similar incapacity to Frrs1l\textsuperscript{−/−}, with a similar running pattern in the first two weeks but an inability to learn to run on the complex wheel (Mandillo \textit{et al.}, 2014).

Other mice with mutations in proteins found in the post synaptic density have similar coordination phenotypes. For instance, the murine knockout of SHANK3, a scaffold protein in the post synaptic density, causes abnormal foot placement and reduced latency to fall on a rotarod which are both similar in Frrs1l\textsuperscript{−/−} mice. The stargazin mouse has a mutation in TARP-\textgamma 2, a member of the AMPA receptor complex, major phenotypes of these mice include ataxia, impaired co-ordination and seizures (Letts \textit{et al.}, 1998; Menuz and Nicoll, 2008).

Limb grasping and trunk curling (sometimes called ‘bat-like posture’) phenotypes have been found in mouse models of neurological disorders and neurodegeneration, such as models for Huntington’s disease (Heng \textit{et al.}, 2007), and Alzheimer’s disease (Lalonde \textit{et al.}, 2005), as well as a variety of other genetically altered mouse lines that result in disruption of normal brain function in a variety of areas, including cerebellum, basal ganglia and forebrain (Lalonde and Strazielle, 2011).
Sleep abnormalities are described in patients carrying loss of function mutations in *FRRS1L* (Shaheen et al., 2016). Homozygous mice have a fragmented sleep pattern, with less sleep overall and an increased number of shorter sleep bouts. The occurrence of sleep abnormalities alongside neurological disorders is well documented, particularly in patients with epilepsy related disorders (Crespel, Baldy-Moulinier and Coubes, 1998; Kothare and Kaleyias, 2010) but also in Huntington’s disease (Morton, 2013), Parkinson’s disease (Boeve, Silber and Ferman, 2004) and dementias (Vitielly and Prinz, 1989; Bliwise, 2004). Disturbances in sleep have been found to correlate with increased impairment of cognitive function in patients with Alzheimer’s disease (Moe et al., 1995), although it is difficult to assess whether the sleep disorder is exacerbating the disease or whether it is a consequence. In children too it has been found that fragmented sleep from 4 years of age correlates with behavioural and emotional problems in adolescence (Gregory and O’Connor, 2002). Again, the cause and consequence of these disorders are difficult to unravel, however it is clear that sleep abnormalities are correlated with neurological deficits.

Cognitive impairment in *Frrs1l*−/− is evidenced from the results of the y-maze test and from fear conditioning. The fear conditioning test carried out in IMPC could not be repeated on these mice as the hyperactivity and abnormal co-ordination appeared to be more severe in this cohort, therefore for welfare reasons it was decided not to perform such an anxiety inducing test. Unfortunately, other tests for cognition would have also been confounded by the extreme hyperactive nature of these mice. Therefore further testing for these symptoms was not carried out on this cohort.
Finally, EEG data shows that these mice suffer from recurrent short seizures, as well as some evidence of longer periods of seizure activity. Seizures are a critical symptom in epileptic disorders, which are often found in conjunction with cognitive dysfunction (Elger, Helmstaedter and Kurthen, 2004). However other diseases are associated with seizures, with an increased incidence of seizure activity in humans with Alzheimer’s disease (Palop and Mucke, 2009) as well as in commonly used Alzheimer’s mouse models such as the J20 line (Palop et al., 2007). Importantly, patients carrying mutations in FRRS1L suffer from different types of epileptic attacks and seizures; generalised tonic-clonic seizures, hemiclonic seizures, multifocal intractable seizures, and juvenile spasms evolving to Lennox-Gastaut Syndrome (Madeo et al., 2016).

Previous mouse knockouts of AMPA receptor subunit genes have shown only subtle phenotypes with differences such as spatial working memory deficits, abnormal pre-pulse inhibition and alteration in social behaviour (Sanderson et al., 2007; Wiedholz et al., 2008; Sanderson et al., 2011a). Others present variations in long term depression and excitatory synaptic transmission (Yang et al., 2011), however most do not have the overt co-ordination and mortality seen in Frrs1l⁻/⁻. These less severe changes may be due to the differential expression of GLUR’s throughout the brain, with only certain areas being affected by a reduction in one of the GLUR’s. It is also possible that there is some compensation between the different GLUR proteins (Chen et al., 2000), while the simultaneous deficits in multiple subunits may explain the more severe deficit in Frrs1l⁻/⁻.
In contrast to the milder phenotypes of the single GLUR knockouts, we do observe a greater overlap of phenotypes of the Frrs1l knockouts with mice that carry mutations causing an overall reduction in AMPA receptors. Genes involved in AMPA receptor synthesis, transport or stability, show gait abnormalities, co-ordination deficits, abnormal activity and seizures, as evidenced above for Shank2 (Schmeisser et al., 2012), Shank3 (Wang et al., 2011), Tarp-y2 (Menuz and Nicoll, 2008) and Snap-25 (Mandillo et al., 2014).

5.3.1 Correlation between human symptoms and mouse phenotypes

There is substantial overlap of phenotypes of Frrs1l-/- mice with symptoms observed in patients carrying homozygous loss of function mutations in the FRRS1L gene. Chorea, impaired volitional movement and muscle weakness are all found in human patients and have parallels in Frrs1l knockout mice. Frrs1l-/- show impaired co-ordination and very stiff gait, which is similar to the chorea seen in humans. Frrs1l homozygotes also have reduced grip strength, recapitulating the muscle weakness found in human patients (Madeo et al., 2016).

Humans have impaired cognitive function which is potentially impaired in homozygous Frrs1l mice, as demonstrated by lack of preference for the novel arm in the y-maze task and by abnormalities in cued fear conditioning (FC carried out by the IMPC).

Neonatal lethality is not recorded in humans, however FRRS1L is not currently screened for mutations in cases of still birth or miscarriage, therefore there is no evidence to either support or oppose this case. However, FRRS1L is now on the
recommended screening panel for patients with neonatal epilepsy, so it is possible that further details, and even more cases, of the human condition will emerge.

Body weight in Frrs1i/− is significantly reduced, this is not mentioned in the human diagnosis therefore any changes in human size or body weight may be subtle or not present. The fact that the number of human patients is still very low, makes that weight comparison difficult.

Interestingly Frrs1i/− mice have sleep disturbances. Whilst these patients are not described as having interrupted sleep, their electrical activity recorded during sleep is abnormal. Furthermore, patients with FRRS1L mutations have continuous spike and wave EEG activity during sleep, which is a specific type of epileptic syndrome (Shaheen et al., 2016). Interestingly, seizures have been detected in homozygous mice and some mice even expressed the spike and wave EEG activity, as observed in these patients.

Surprisingly, in the mouse model very little deterioration in condition was seen with age. Five homozygous mice were killed throughout the study as they reached previously specified humane endpoints, including; seizure without full recovery (1), self-inflicted wounds and stereotypical behaviour (2), uncoordinated gait impinging on the ability to feed (1) and breathing difficulties (1). Self-inflicted wounds became apparent when the animals were singly housed for the passive infrared (PIR) test, this type of behaviour is sometimes a consequence of increased anxiety. It is difficult to ascertain whether these deaths are a true indication of deterioration or whether normal ageing and the anxiety of phenotyping, on top of already significant symptoms is responsible.
5.3.2 Conclusion

Deletion of Frrs1l has a detrimental effect from an early age, with many of the homozygous pups dying shortly after birth and many of the motor phenotypes present from weaning. It is therefore possible that loss of Frrs1l is primarily a neurodevelopmental disorder, whose effects are far reaching into adulthood. Further studies will be needed to unravel the role of Frrs1l in neurodevelopment and disease.

The link between FRRS1L and AMPA receptors is corroborated by the similarity in phenotypes between mouse models that reduce overall AMPA receptor function in Frrs1l−/− mice.

Furthermore Frrs1l−/− mice have phenotypes which resemble many of the symptoms found in the patients carrying loss of function mutations in the same gene. These data indicate that Frrs1l−/− mice could be a useful model for the study of disease mechanism and to further define the role of AMPA receptor function in human disease.
6. Further characterisation of FRRS1L ex-vivo and in-vitro

6.1 Introduction

As described in Chapter 5, deleting Frrs1l in mice results in lethality at birth in the majority of mice, the Frrs1l/− mice that survive to adulthood suffer from a complex syndrome with many abnormalities, including smaller size, lack of motor coordination, some muscle weakness, hyperactivity, learning deficits and altered sleep patterns, as well as EEG patterns of activity that are consistent with seizures.

FRRS1L had no known role until recent years where a functional link between FRRS1L and AMPA receptors was evidenced (Schwenk et al., 2012, 2014). During the course of this project, two further papers have been published supporting the role of FRRS1L in AMPA receptor biology. AMPA receptor complexes in rat brain comprise two distinct and mutually exclusive groups. Many of the known auxiliary proteins, including the Tarp family, the CNIH’s and 10 other proteins, assemble in combinations with the pore forming GluA1-4. A second group of proteins containing FRRS1L, CPT1C, SAC1, ABHD6 and ABHD12 also assemble with GluA1-4 but in distinct complexes. Further analysis showed that AMPA receptors in complex with FRRS1L and CPT1C are restricted to the endoplasmic reticulum. In other experiments, it was shown that knock down or overexpression of Frrs1l by stereotaxic lentiviral injection in P6 rats induces changes in synaptic transmission. A decrease in amplitude of excitatory post synaptic currents (EPCS’s) was seen when Frrs1l was knocked down by shRNA, whereas an increase in EPSC amplitude was seen after overexpression of Frrs1l (Brechet et al., 2017). As a result of the above studies it was suggested that FRRS1L could be involved in AMPA receptor biogenesis: that CPT1C and FRRS1L bind with GLUR’s in the early stages of AMPA
receptor biogenesis, and that this binding primes the core of the AMPA receptor for further assembly with the remaining outer proteins. As further proteins bind, FRRS1L and CPT1C detach and the AMPAR is transported from the ER.

Data from the second paper corroborates some of those conclusions using different experimental paradigms, but also finds some contradictory results. Overexpression of Frrs1l in hippocampal neurons reveals co-localisation with a proportion of the AMPA receptors, in agreement with Brechet et al. Conversely it was found that overexpression of Frrs1l in hippocampal neurons did not affect synaptic transmission, as measured by EPSCs. However, the discrepancy is due to experimental design inconsistencies. Both papers, however, show that the reduction of Frrs1l by either viral knockdown in P6 rats or CRISPR/Cas9 mediated deletion in neurons, caused a decrease in EPSC's, as well as a decrease in surface GLUR1 (Brechet et al., 2017; Han et al., 2017). Interestingly, conflicting data on FRRS1L localisation were evidenced in the two papers, with Han et al showing FRRS1L throughout the cytoplasm and at the cell surface with surface GLUR’s, inconsistent with the findings from Brechet et al who found FRRS1L only in the ER.

Finally, Han et al, shows that surface to total GLUR1 ratio is decreased in cells lacking FRRS1L, which is suggested to be due to reduced trafficking but which could also fit with the theory from Brechet et al that FRRS1L is involved in AMPA receptor biogenesis, since both mechanisms would lead to a reduction in GLUR1 at the surface.

Interestingly, three more families have been diagnosed with mutations in FRRS1L in addition to the families found by Madeo et al and Shaheen et al, two novel mutations and a reoccurrence on the G321 mutation (Madeo et al., 2016; Shaheen
et al., 2016; Brechet et al., 2017). G321 and V195E mutations cause a similar phenotype to the previously reported mutations, however, K155E seems to be less severe, with children having some ability to move, delayed speech and increased mental age compared to other affected families. This reduced severity of phenotype correlates with the immunoblot analysis that shows other mutations are unable to assemble with GLUR1 whereas K155E has only reduced assembly.

Together these data describe intriguing evidence for the role of FRRS1L in AMPA receptor biogenesis and trafficking. Further work on the mouse KO can provide useful data to expand on that mechanistic link and perhaps provide novel additional functional information.

6.2 Results

6.2.1 Confirmation of deleted allele

Firstly, it was essential to confirm whether the deletion of Frrs1l in the Frrs1l<sup>tm1b/tm1b</sup> (Frrs1l<sup>−/−</sup>) mouse resulted in the complete ablation of gene expression. To ascertain this we analysed gene expression by quantitative PCR (qPCR) in P0 brain and adult cerebellum. qPCR primers were designed to cover all five exons of the Frrs1l gene. The results confirmed that no Frrs1l exon products were expressed in Frrs1l<sup>−/−</sup> mice, with close to zero expression for two of the amplicons and some residual expression of the third amplicon which amounted to a sixteen fold reduction. This third amplicon spanned the boundary of exon 4-5 of the Frrs1l gene and does not include a known splice variant.
Figure 61 qPCR carried out on P0 brain and cerebellum from 14 month old adult mice. Data showed no expression of exons 1-3, and minimal levels of expression of exon 4-5 at both ages in Frrs1l−/− mice when normalised to expression in Frrs1l+/+ (n=5 Frrs1l−/−, n = 5 Frrs1l+/+ at P0, n=4 Frrs1l−/−, n = 4 Frrs1l+/+ for adults)

6.2.2 Effect of lack of FRRS1L on AMPA receptors expression

The striking neurological alterations observed in the mice, together with the seizures and the potential link with AMPA receptors, prompted us to assess the level of expression for the four core AMPA receptor subunits, GLUR1-4 in these mice. We found no difference in gene expression levels in any of the core AMPA receptor genes (encoded by genes Gria1-4) in P0 brain, however there was a small but significant reduction in Gria3 and Gria4 in adults (p<0.05 and p<0.001 respectively).

These results indicate that the deletion of FRRS1L has little effect on the regulation of gene expression of GLURs.
Figure 62 qPCR for genes encoding GLUR1-4, the core pore forming proteins of the AMPA receptor complex. Data shows no changes in Gria expression between Frrs1l\(^{-/-}\) and Frrs1l\(^{+/+}\) at P0, and a small but significant reduction in Gria3 and Gria4 in adults (F[1,24]=35.19, p=0.0018, two way ANOVA, Sidak’s multiple comparisons showed significance of p<0.05 for Gria3 and p<0.001 for Gria4) (n=5 Frrs1l\(^{-/-}\), n = 5 Frrs1l\(^{+/+}\) at P0, n=4 Frrs1l\(^{-/-}\), n = 4 Frrs1l\(^{+/+}\) for adults).

6.2.3 AMPA receptor protein levels in Frrs1l\(^{-/-}\)

Previous studies have found that CRISPR mediated deletion of Frrs1l results in reduced GLUR1 protein levels at the cell surface as well as intracellularly in primary neurons (Han et al., 2017). Moreover, the same mechanisms might apply to all AMPA receptor biogenesis (Brechet et al., 2017).

To assess whether this is the case in Frrs1l\(^{-/-}\) mice, we performed western blots of P0 brain and adult cerebellum using antibodies against GLUR1, GLUR2 and GLUR4.
Figure 63 Immunoblot analysis of GLUR1, GLUR2 and GLUR4 in P0 brain shows significantly decreased levels of GLUR1 (p<0.01). GLUR2 separated into two distinct bands in the wild-type mice, however in homozygotes the lower band is much more prominent (quantification of top band shows a significant difference between genotypes p<0.001), however total GLUR2 levels across both bands together are not significantly different. GLUR4 levels are the same in both Frrs1l<sup>-/-</sup> and Frrs1l<sup>+/+</sup>. Synaptic proteins PSD-95, SNAP25 and CAMKII are also unchanged between Frrs1l<sup>-/-</sup> and Frrs1l<sup>+/+</sup>. Data analysed by Students t-test. All protein levels normalised to ACTIN (n=5 Frrs1l<sup>-/-</sup>, n = 5 Frrs1l<sup>+/+</sup> for all except GLUR2 and GLUR4, where n=4 Frrs1l<sup>-/-</sup>, n = 4 Frrs1l<sup>+/+</sup>)
Starting with the analysis of levels in P0 brain, GLUR1 levels significantly reduced in homozygous mice \( (P<0.01) \) (Figure 63A). Total GLUR2 levels are not significantly different between homozygotes and wild-types, however, two distinct bands are present in the wild-types, with a dramatic reduction in the upper band in \( Frrs1l^{+/} \) \( (p<0.001) \) (Figure 63B). Quantification of GLUR4 levels showed that there was no significant difference between \( Frrs1l^{+/} \) and wild-types, however there is a slight electrophoretic shift in band position in the homozygotes (Figure 63B). To assess whether changes in the synapse were AMPA receptor specific or affected other proteins, three abundant synaptic proteins were quantified, PSD-95, SNAP25 and CAMKII. No significant differences were seen between \( Frrs1l^{+/} \) and wild-types in any of these protein levels (Figure 63C and D).

In adult cerebellum, the differences are more prominent, showing that total protein levels of GLUR1, GLUR2 and GLUR4 are markedly reduced in homozygotes compared to \( Frrs1l^{+/+} \) controls \( (P<0.0001, P<0.01 \) and \( P<0.01 \) respectively)\( ) \) (Figure 64A and B). Moreover, as observed in neonate brain, there is a similar change in electrophoretic mobility of GLUR2 and GLUR4 immunoreactive bands in homozygous adult brain.

Again, assessment of levels of other synaptic protein markers was carried out. Pre synaptic protein SNAP25, and post synaptic proteins PSD-95, CAMKII and GRIN1 (a major component of NMDA receptors) were all detected at similar levels in \( Frrs1l^{+/-} \) and \( Frrs1l^{+/+} \). These data suggest that FRRS1L is having an impact specifically on AMPA receptor levels, with other synaptic proteins remaining unaffected.
Figure 64 Immunoblot analysis of adult cerebellum. GLUR1, GLUR2 (A) and GLUR4 (B) levels are all significantly reduced in Frrs1+/− (p<0.001, p<0.01, p<0.001 respectively). Immunoreactive band mobility is modified in Frrs1+/− compared to Frrs1+/+ for GLUR2 and GLUR4, with a thin band for GLUR2 in Frrs1+/− as opposed to a diffuse band in Frrs1+/+, as well as a small change in position of

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GLUR4. As in P0 brain, PSD-95, SNAP25 and CAMKII remain unchanged, as does NMDA receptor component GRIN1. Data analysed by Students t-test. All protein levels normalised to ACTIN (n=4 Frrs1l−/−, n = 4 Frrs1l+/+)

6.2.4 Excitatory synapses

As levels of synaptic proteins, other than AMPA, are not altered between Frrs1l−/− and Frrs1l+/+ this suggests no overall decrease in synapse number. In order to confirm this, we counted the proportion of excitatory synapses in the hippocampus and cerebellum and found no differences in excitatory synapse number between wild-type and Frrs1l−/− brains (Figure 65).

Figure 65 Formalin fixed wax embedded sections of whole brain from 16 week old mice were stained with VGLUT1 to mark excitatory synapses. Synapses were counted in the CA1 region of the hippocampus, total number compared between three Frrs1l−/− and three C57BL/6NTac controls. Representative confocal images of the stratum oriens and stratum radiatum of the CA1 region of the hippocampus for Frrs1l−/− and wild-type controls (C57BL/6NTac) stained with VGLUT1 (green). Plots show the VGLUT1 positive puncta quantification. No significant differences found by t-test.
6.2.5 AMPA glycosylation

A possible explanation for the differences observed in the protein band mobility in GLUR2 and GLUR4 could be caused by alterations in their post translational modification. AMPA receptors go through a series of glycosylation steps before they are transported to the synapse. This maturation by glycosylation is more prominent in GLUR2 and GLUR4 subunits. N-glycosylation of GLUR2 and GLUR4 occurs in the lumen of the endoplasmic reticulum after biosynthesis of the core subunits (Tucholski et al., 2013, 2014). This immature glycosylation contains a large amount of high-mannose sugars. AMPA receptors are then transported to the Golgi apparatus, where glycosylation is modified to remove high-mannose sugars and increase the complexity of the glycosylation.

To investigate whether any of these processes are altered in the absence of FRRS1L, we performed molecular mass shift assays using two enzymes: endoglycosidase H (Endo-H), which digests high-mannose containing sugars, and peptide-N-glycosidase F (PNGase F) which removes all glycosylation. Assays were performed on adult cerebellum and resultant digests quantified by western blotting.

After the digestion with PNGase F, which removes all forms of glycosylation, GLUR2 and GLUR4 electrophoretic band mobility’s shifted for both genotypes, indicating both wild-type and Frrs1l<sup>-/-</sup> are glycosylated. However, samples treated with Endo-H showed some crucial differences between Frrs1l<sup>-/-</sup> and wild-type controls (Figure 66A). In wild-type mice two bands were present after digestion, a lower band, containing approximately 18% of the total GLUR2 and GLUR4, was shifted relative to the undigested band, indicating around 18% of the AMPA receptors in wild-type cerebellum are imm maturely glycosylated. The second band did not shift from the
undigested position, demonstrating that the remaining 82% of AMPA receptors in wild-types were insensitive to Endo-H and therefore maturely glycosylated (Figure 66B and C). Conversely in Frrs1l\(^{-/-}\) we observed almost the opposite proportions. Again, two bands were present in the same location as for wild-types, however in the Frrs1l\(^{-/-}\) samples, the lower band contained an increased proportion of protein, 65% for GLUR2 and 45% for GLUR4. This indicates that in Frrs1l\(^{-/-}\) more than double the proportion of AMPA receptors are in the immature phase of glycosylation.

![Figure 66](image)

**Figure 66** Molecular mass shift assay to assess glycosylation state of GLUR2 and GLUR4 in Frrs1l\(^{-/-}\) and Frrs1l\(^{+/+}\). Both Frrs1l\(^{-/-}\) and Frrs1l\(^{+/+}\) show a shift in band position when treated with PNGase, indicating both are glycosylated (A). However, Frrs1l\(^{-/-}\) have a larger proportion of GLUR2 and GLUR4 protein digested by ENDO-H (A and B), showing that a greater proportion of the total GLUR2 and GLUR4 is in the immaturely glycosylated state (p<0.0001 and p=0.0001 respectively) (n=3 Frrs1l\(^{-/-}\), n = 3 Frrs1l\(^{+/+}\)) (C). Data analysed by ANOVA with Sidak’s multiple comparisons.
6.2.6  AMPA localisation by synaptic fractionation

It has been shown that immaturely glycosylated AMPA receptors are not trafficked to the synaptic membrane (Dingledine et al., 1999; Traynelis et al., 2010). To assess whether there is any abnormal trafficking in Frrs1l−/− mice, we carried out synaptic fractionation using adult forebrain samples. This allowed us to measure the relative proportions of GLUR2 and GLUR4 in the cytoplasmic fraction and in the post-synaptic membrane of mutants and controls. As previously observed with whole brain homogenates, there is less GLUR2 and GLUR4 in the post-synaptic membranes of the homozygotes. Interestingly, the proportion of GLUR2 and GLUR4 in the cytoplasmic fraction of homozygote brains is higher than in the wild-type controls (Figure 67A and B). GLUR1 levels are proportionally reduced in both subcellular fractions, indicating that this subunit is not retained in the cytoplasm, but its levels are dramatically low in both the cytoplasmic and synaptic fractions. The levels of GRIN1 (NMDA receptor maker) is not affected in the Frrs1l−/− post-synaptic membranes.

In conclusion, there is a greater proportion of AMPA receptors containing GLUR2 and GLUR4 retained the cytoplasmic fraction in Frrs1l−/− than in wild-type controls. Indicating that in Frrs1l−/− mice, not only is there a reduction in the total amount of all AMPA receptors, but also that the remaining GLUR2 and GLUR4 AMPA receptor subunits are immaturely glycosylated and retained in the cytoplasmic fraction to a greater extent than in wild-types.
Figure 67 Synaptic fractionation to assess localisation of GLUR1, GLUR2 and GLUR4 in Frrs1l\(^{-/-}\) and Frrs1l\(^{+/+}\) forebrain. Ratio of GLUR1 in the cytoplasmic and the synaptic fractions is unchanged across genotypes. Ratio of GLUR2 and GLUR4 is significantly different between Frrs1l\(^{-/-}\) and Frrs1l\(^{+/+}\) with a larger proportion of GLUR2 and GLUR4 containing AMPA receptors found in the cytoplasmic fraction (\(p<0.05\) and \(p<0.001\) respectively) (A and B). GRIN1 shows no significant difference between Frrs1l\(^{-/-}\) and Frrs1l\(^{+/+}\) in the post synaptic fraction (C) (\(n=3\) Frrs1l\(^{-/-}\), \(n = 3\) Frrs1l\(^{+/+}\)) Data analysed by students t-test.

6.2.7 Gross pathology

In order to evaluate whether any of these molecular alterations had related major brain structural alterations, brain sections of Frrs1l\(^{-/-}\) and controls were assessed at 16 weeks and 14 months by staining with haematoxylin and eosin (H&E). No
gross abnormalities in structure were found in the cortex, hippocampus or cerebellum of the homozygous mice.

Figure 68 Representative sections of Frrs1L 

6.2.8 FRRS1L – cellular localisation

Any work on subcellular localisation of FRRS1L has been hampered by the lack of a suitable specific commercial antibody. To circumvent this, FRRS1L was cloned into two mammalian expression vectors, a C terminally V5 His tagged pcDNA™3.1/V5-His (Thermo Fisher Scientific Inc.) and an N terminally GFP tagged Gateway™ pcDNA™-DEST53 vector.
Neuro2A cells transfected with V5His tagged Frrs1l were co-stained for GLUR1 to assess whether over expression of Frrs1l altered the trafficking or abundance of GLUR1 containing AMPA receptors.

Preliminary data for co-staining with GLUR1 showed no discernible difference between GLUR1 expression or localisation between transfected and non-transfected Neuro2a cells. Further work on quantification of GLUR1 would be needed to clarify this result.

FRRS1l appears to be localised in the perinuclear cytoplasm, overlapping with but not exclusively localised to the ER.

Site directed mutagenesis was carried out on the N-terminally GFP tagged clone to introduce one of the mutations found to be detrimental in humans, and present in three separately identified families, G321X. This was then transfected alongside the wild-type Frrs1l GFP tagged clone to assess whether this truncated form was associated with any differences in localisation or changes in protein abundance. Surprisingly, no differences were found in localisation between G321X and wild-type FRRS1L in these preliminary data. Co-staining was carried out to assess more definitively if there is mis-localisation of the mutant FRRS1L-G321X, however cross reaction between the ER marker and the plasmid confounded interpretation of the results (data not shown). Further work would need to be carried out to confirm whether there are any localisation differences between the wild-type and mutant FRRS1L proteins.
Neuro2a cells transfected with GFP tagged wild-type FRRS1L and GFP tagged mutant FRRS1L carrying a point mutation causing disease in humans. Stained with antibodies to GFP (red) and mounted with DAPI (blue). No clear differences in localisation were seen between wild-type and mutant FRRS1L in these preliminary data. Scale bar is 20um.

### 6.2.9 Primary neuron Sholl analysis

In order to evaluate if molecular alterations in the AMPA receptor availability would affect the structure of the neurons, Sholl analysis was carried out on primary
cortical neurons. Primary cortical neurons were prepared from 15.5dpc embryonic forebrain generated from heterozygous intercrosses. Neurons were cultured for 6 days before being fixed and stained with tubulin along with GFAP to distinguish neurons and glia. Isolated neurons were selected for Sholl analysis (n=17 Frrs1l/− from three embryos, n=13 Frrs1l+/+ from two embryos) in order to determine whether there were any differences in neuronal branching architecture.

![Sholl analysis](image)

Figure 71 Sholl analysis of primary neurons shows that neuron length (A), neuron branching and pattern of branching (B) are not significantly different between Frrs1l−/− and Frrs1l+/+. Data analysed by t-test (A) and general linear mixed model with poisson distribution to account for replicates within animals (B). n=17 Frrs1l−/− from three embryos, n=13 Frrs1l+/+ from two embryos.
Figure 72 Representative images of Sholl analysis for one Frrs1−/− (A,B,C) and one Frrs1+/+ (D,E,F) primary neuron. Sholl images (A) and (D) indicate number of branches in each shell by colour. Image of the representative traced neuron overlayed with colours from Sholl analysis (B) and (E). Sholl plot profile showing number of branches and distance from axon hillock (C) and (F).
No differences were found in neuron length, number of branches or pattern of branching between \textit{Frrs1l}^{-/-} and \textit{Frrs1l}^{+/+}.

6.3 Discussion

6.3.1 Knockout validation

Firstly it was critical to confirm that the genetic alteration induced in these mice caused a complete absence of FRRS1L protein. qPCR primers were designed to cover all five exons of the Frrs1l gene, including the start of exon 3 where the Frrs1l knockout cassette was inserted. cDNA generated from \textit{Frrs1l}^{-/-} and \textit{Frrs1l}^{+/+} shows that there is no expression of exons 1-3 and greatly reduced expression of exons 4-5. Data from Ensembl shows only two splice variants of \textit{Frrs1l}, a full length variant containing all five exons and a shorter version which skips exon two and is thought to undergo nonsense mediated decay (\textit{Ensemble.org}, 2018; Zerbino \textit{et al.}, 2018). A shorter version containing only exons four and five is not expected, and is likely to be non-functional.

6.3.2 AMPA receptor levels

As discussed above (6.1), FRRS1L has been linked with assembly of AMPA receptor complexes (Schwenk \textit{et al.}, 2012, 2014; Brechet \textit{et al.}, 2017; Han \textit{et al.}, 2017). Our data shows that expression of core AMPA receptor genes is unaltered at P0 for all four Gria genes. In adults \textit{Gria3} and \textit{Gria4} are slightly reduced, whilst \textit{Gria1} and \textit{Gria2} remain unaltered.

Although transcription is relatively similar between \textit{Frrs1l}^{-/-} and wild-type controls, receptor protein levels are dramatically reduced in \textit{Frrs1l}^{-/-}. At P0 we see a significant reduction in the amount of GLUR1, which is not apparent for GLUR2 and...
GLUR4. This could be due to the relevant abundance of the subunits at birth. GLUR1 is the most highly expressed, with GLUR2 expression significantly lower and GLUR4 present in only relatively small amounts until later in development (Luján, Shigemoto and López-Bendito, 2005). In adults, immunoblots show that all three proteins GLUR1, GLUR2 and GLUR4 are significantly reduced. These data fit with the hypothesis that FRRS1L is acting post translationally on AMPA receptors as the expression of core AMPA receptor genes is unaltered but total protein levels are dramatically reduced.

### 6.3.3 AMPA receptor processing

In addition to changes in protein levels, we found electrophoretic shifts in GLUR2 and GLUR4 protein bands for both adult and P0 proteins, which could indicate alterations in the post translational mechanisms of maturation. Previous work by other groups evidenced that GLUR2 and GLUR4 undergo glycosylation in order to function as mature receptors (Tucholski et al., 2014). Here, glycosylation was assessed using molecular mass shift assays and an increased proportion of immaturesly glycosylated AMPA receptors was found in Frrs1l−/− samples. This is further corroborated by synaptic fractionation experiments showing that an increased proportion of GLUR2 and GLUR4 containing AMPA receptors are found in the cytoplasmic fraction in Frrs1l−/− compared to Frrs1l+/+ controls.

Immature glycosylation occurs in the ER while conversion from immature to mature glycosylated forms occurs in the Golgi apparatus. The data from both molecular mass shift assays and synaptic fractionation studies suggest that, in the absence
of FRRS1L, AMPA receptor complexes are either retained in the ER, are not processed past the initial phase of the Golgi apparatus or are not trafficked efficiently between the two organelles. Therefore it is possible that the absence of FRRS1L may lead to incomplete biogenesis of AMPA receptors. Interestingly, Han et al found that FRRS1L is localised in dynein but not kinesin vesicles. Dynein vesicles, transport proteins from the ER to the Golgi apparatus (Murshid and Presley, 2004). Therefore, another possibility is that FRRS1L acts as a chaperone for this process, and that the absence of FRRS1L impairs this transition to the Golgi thus impairing full maturation and subsequent trafficking of AMPA receptors to the plasma membrane.

Interestingly other components of the synapse appear to be unaltered in mutants. Levels of several proteins were assessed, both pre-synaptic (SNAP25) and post synaptic (PSD-95 and CAMKII), as well as the post synaptic NMDA receptor protein (GRIN1). All of these levels remained unaltered between homozygotes and wild-types. In addition, primary neurons appear normal, Sholl analysis shows no significant differences and synaptic counts in adult brain are similar across genotypes. These data all indicate a specific alteration in AMPA receptors that does not seem to affect neuronal structure itself or change the abundance of the other major ionotropic glutamate receptor, NMDA.

6.3.4 Brain morphology

Brain morphology of Frrs1l−/− appears grossly normal at 16 weeks and 14 months. Initially this was surprising as in humans there is some cortical and cerebellar atrophy. However, this seems to be limited in patients and early brain scans were
normal, even though patients were already severely affected. It could be that in mice the length of time necessary to observe atrophy or degeneration is not the same. However it is possible that pathology in mice will not develop in the same way as in humans. There is some evidence from mouse models where gene function appears similar in human and murine systems, however genetic mutations have diverse effects. For example the Duchene muscular dystrophy mouse model shows similar initial pathology in the form of muscle wastage in early life, however in humans this wastage continues and leads to lethality, whereas in mice the period of muscle wastage is followed by muscle regeneration (Stedman et al., 1991; Pastoret and Sebille, 1995). Similarly in Alzheimer’s Disease (AD) recapitulating the human pathology has been challenging. Humans with mutations in APP develop full AD pathology, amyloid plaques and neurofibrillary tangles. However, mice which overexpress mutated human APP show only amyloid plaques and do not develop neurofibrillary tangles (McGowan, Eriksen and Hutton, 2006; Morrissette et al., 2009). Other AD models have suffered with similar problems, with only subsets of the pathology being recapitulated in mouse models (Lewis et al., 2000; Duff and Suleman, 2004; Duyckaerts, Potier and Delatour, 2007).

In the case of Frrs1I, the lack of morphological changes also fits with the finding that other synaptic proteins, and indeed synapse number, appear no different. This implies that gross structure is normal, but that a specific defect in AMPA receptors is seen at the synapse. It is not known whether other synaptic proteins are altered in humans, and whether synapse number is consistent between affected and healthy individuals, as there is no human post mortem data as yet.
6.3.5 Carnitine palmitoyltransferase 1c (Cpt1c)

No previous work has been carried out on mouse models of Frrs1l modulation, however, there has been some investigation into the function of Cpt1c, the protein suggested to share some functionality with FRRS1L during AMPA receptor biogenesis (Brechet et al., 2017). Mice with deletions of Cpt1c show some similarities to the phenotypes observed in Frrs1l−/− mice. Cpt1c KO mice have motor deficiencies such as decreased latency to fall from a rotarod and reduced muscle strength (Carrasco et al., 2013) as well as deficits in learning and memory in the Morris water maze (Carrasco et al., 2012), similar to findings in Frrs1l−/−. Activity in Cpt1c KO mice is altered, however they show hypo activity, both during overnight assessment and in the SHIRPA test, as opposed to Frrs1l−/− which are extremely hyperactive in both environments. These data are difficult to compare since the overnight assessment in Cpt1c KO’s was carried out in singly-housed animals, as opposed to the group housed Frrs1l. A difference in housing is likely to significantly alter behaviour, however, comments from the SHIRPA assessment suggest Cpt1c KO mice appear slow, whereas this is not the case in Frrs1l−/−. Data from Carrasco et al. (2013) also shows that the motor impairment seems to be less severe than Frrs1l−/− at younger ages but that it deteriorates with age. Similar deterioration is not apparent in Frrs1l−/− mice, however the co-ordination deficits are severe even from a young age. qPCR and immunoblot analysis of GLUR1 and GLUR2 in Cpt1c KO mice shows results that correlate with those found in Frrs1l−/− in that expression of Gria1 and Gria2 are not altered but total protein levels of GLUR1 and GLUR2 are reduced.

Cpt1c primary hippocampal neurons show reduced spine maturation, a parameter that was not measured in Frrs1l−/− mice.
The phenotypic overlap with *Cpt1c* KO mice as well as the inconsistencies are intriguing. There are some clear similarities in the coordination, muscle strength and memory phenotypes, as well as the unaltered expression of the core AMPA genes and reduction in protein levels of AMPA receptors. However there are also dissimilarities in phenotype, *Frrs1l*−/− are hyperactive, and in general deficits seem more severe in *Frrs1l*−/−, especially in terms of lethality, which is not mentioned in any of the CPT1C papers and therefore assumed to be normal. It seems possible that CPT1C and FRRS1L are both involved in the AMPA receptor biogenesis process as they seem to affect AMPA receptor levels in the same way, however they may have other unknown roles in neurons that lead to these differential phenotypic effects.

**6.3.6 Summary**

In summary, absence of FRRS1L causes a general reduction in AMPA receptor levels, with levels of other ionotropic glutamate receptors and other post synaptic proteins remaining unchanged. Evidence of immaturity glycosylated AMPA receptors indicates that FRRS1L is needed for post translational processing of AMPA receptors.

Involvement in AMPA receptor processing could provide a conceivable mechanism for the phenotypes seen in the *Frrs1l*−/− mice. Neonatal lethality, co-ordination, muscle strength, hyperactivity, seizures and sleep disturbances may be consequences of alterations in glutamate signalling caused by changes in AMPA receptor number. Indeed, as discussed above (5.3), several models that modulate glutamate signalling can result in phenotypes such as neonatal lethality, abnormal
behaviour and seizures (Osten and Stern-Bach, 2006; Christie et al., 2010; Wang et al., 2011)
7.0 Conditional deletion of *Frrs1l*

7.1 Introduction

*Frrs1l*⁻/⁻ mice present a variety of significant phenotypes including smaller size, coordination problems, hyperactivity, seizures and high rate of neonatal lethality. Some of these phenotypes, particularly the hyperactivity, are extreme and could be masking some more subtle changes such as those indicating aspects of cognitive function. Another important complication when studying these mice is the high level of neonatal mortality, as very few *Frrs1l*⁻/⁻ survive past postnatal day one. This high level of mortality means large numbers of mice have to be bred in order to produce a suitable cohort, which has ethical as well as financial implications.

It is clear from the mortality data that loss of *Frrs1l* has neurodevelopmental effects, however it is not clear how much of the adult phenotype is a consequence of these developmental changes or whether FRRS1L deletion in adults has sustained consequences.

To address the confounding effects of the complete knockout and to delineate the neurodevelopmental effects from the role of FRRS1L in adulthood, a conditional allele was generated to study the effect of deletion of *Frrs1l* in a temporal manner.

7.1.1 Breeding for conditional deletion – controlling genetic background

The *Frrs1l* knockout first (tm1a) cassette was utilized to create a conditional allele. First by crossing *Frrs1l*⁻/⁻ to a flp expressing line, then by breeding *Frrs1l*⁻⁻⁻ to a tamoxifen inducible ubiquitously expressing CRE line. Steps were taken to control genetic background as much as possible as *Frrs1l*⁻/⁻ and the flp
recombinase line \((\text{Gt}(\text{ROSA})26\text{Sor}^{\text{tm2}}(\text{CAG-flp0}, \text{EYFP})^Ics)\) are both co-isogenic on C57BL/6N, however, the CRE line \((\text{B6.Cg-Tg(UBC-cre.ESR1)}^1\text{Ejb/J})\) mice are congeneric on a C57BL/6J background. There are several known differences between C57BL/6N and C57BL/6J, illustrated in Chapter 3 above and in Simon et al (M. M. Simon et al., 2013), as well as more potential phenotypic differences that have not yet been highlighted. Some of these known differences are found in tests such as rotarod, and in the general activity level of the two strains. As these are phenotypes of interest in this study, and because of potential additional background effects, it is critical to control the genetic background of the experimental cohort as much as possible. Due to time constraints, backcrossing all lines onto the same background was not feasible, therefore we designed a breeding scheme that produced controlled heterogeneity. \(\text{Frrs1}^{\text{tm1c/}}\), cre positive males are an F1 of C57BL/6N and C57BL/6J. These F1’s were backcrossed onto C57BL/6N using co-isogenic C57BL/6N \(\text{Frrs1}^{\text{tm1c/}}\) females (Figure 73). Therefore the genetic background of the phenotyping cohort comprises 75% C57BL/6N and 25% C57BL/6J.

Given the limits’ of using test cohorts of mixed genetic background the genetic mix is still the same across all groups including controls. Therefore genetic background should not skew the data in favour of one group, and any phenotype that is detected may be more robust than those identified in isogenic cohorts.
Figure 73 Breeding scheme showing conversion of $Frrs1^{tm1a/+}$ to $Frrs1^{tm1c/+}$ using flop recombinase. Followed by breeding of experimental cohort incorporating globally expressed tamoxifen inducible cre, Ubiq-cre. Bars show proportion of background genome in each mouse (blue indicates C57BL/6N, red indicates C57BL/6J). Experimental cohorts are a mix of 25% C57BL/6J and 75% C57BL/6N.

7.1.2 Experimental controls

Several additional variables need to be controlled for in addition to the genetic background alone. Cre lines often contain transgenic insertions of DNA not found in the murine genome, any alterations to DNA have the potential to alter gene function, either by disrupting a gene they have been inserted in or by having upstream or downstream effects on nearby genes. For example, Nestin$^{cre}$, which is a tissue specific cre used to delete neuronal expression of a gene, has been shown to lead to phenotypes including weight loss and alterations in conditioned fear response (Giusti et al., 2014). Therefore it is critical to have control groups to
confirm the phenotype seen is not a result of the cre line alone. As the cre line used in this study is tamoxifen inducible, further controls need to be included to discount the possibility that tamoxifen is leading to any phenotype. Low doses of tamoxifen should have little effect, however it is known that tamoxifen affects oestrogen signalling and bone mass (Starnes et al., 2007) and it is possible that the stress of repeated dosing may itself have an effect on behaviour, regardless of the substance being administered. Therefore tamoxifen dosed controls must also be included.

A full list of controls used in this study is detailed in table 10.

<table>
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<tr>
<th>Genotype</th>
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<tr>
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<td>Experimental group</td>
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<td>Control for effect of cre and tamoxifen on wild-types</td>
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<tr>
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<td>Control for effect of cre alone in wild-types</td>
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<tr>
<td>Frrs1&lt;sup&gt;+/+&lt;/sup&gt;, cre neg, tamoxifen</td>
<td>Control for effect of tamoxifen or dosing regime in wild-types</td>
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<tr>
<td>Frrs1&lt;sup&gt;+/+&lt;/sup&gt;, cre neg, no dose</td>
<td>Wild-type control</td>
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Table 10 All groups used in conditional deletion study and reasons for inclusion.
7.1.3 Phenotyping pipeline

Mice were dosed by oral gavage at 5 weeks of age with 5 daily doses of tamoxifen dissolved in corn oil, a total dose of 500ug/kg. Previous data has shown this dose to be effective in activating recombination with this particular cre line (McMurray et al., 2013).

Mice were then left for two weeks following dosing before testing began. This was to allow time for FRRS1L protein to be cleared, and also to allow mice to recover from any stress induced by the dosing.

We hypothesized that FRRS1L protein is necessary for the correct biogenesis and maturation of AMPA receptors and their location at the synapses, therefore we expect that by deleting Frrs1l in adulthood, there would be a great reduction in the de novo AMPA receptors in the cells, and so the levels of AMPA receptor will be eventually reduced similar to previous Frrs1l−/− mice. Therefore it was also necessary to allow sufficient time for AMPA degredation to take place. The half-life of AMPA receptors in PC12 cells or in hippocampal neurons is 41 and 52 hours respectively (Horikawa and Nawa, 1998). Whereas hippocampal neurons treated with cycloheximide show a 50% reduction in AMPA receptors within 2 hours, followed by a slower rate of degradation, reaching 40% after 24 hours (Fadó et al., 2015). These data indicate that AMPA receptor levels under normal conditions will halve approximately every two days. However, this is based on in vitro data, and there may be differences in in vivo degradation rate.

Following dosing and rest period, the Frrs1ltm1c/tm1c cre positive, tamoxifen dosed mice and controls were tested through the following phenotyping pipeline:
Figure 74 Pipeline for phenotyping of conditional Frrs1l knockout mice, showing phenotyping tests and age at test.

Mice were assessed through a phenotyping pipeline, both recapitulating some of the tests carried out on Frrs1l/− mice to determine similarities or differences in phenotype, and also including more focus on cognitive tests.

All mice to be tested through the phenotyping pipeline were randomized and blinded at weaning, then housed in cages of three.
7.2 Results

For ease of visualization, limited control groups are displayed in the figures. However, full statistical analysis was carried out on all seven control groups, results of which can be found in Appendix 2.

7.2.1 Body weight

No differences were seen in body weight between Frrs1floxed/floxed cre positive, tamoxifen dosed mice and all other control groups (Figure 75). Sample size is too small to delineate any significant effects on body weight, however data for the conditionally deleted groups appears comparable to control groups.

![Figure 75](Image)

Figure 75 Body weight of female (A) and male (B) Frrs1floxed/floxed cre pos tamoxifen dosed mice and control groups. No differences were seen between the experimental group and any of the control groups for either sex. Data analysed by ANOVA.

7.2.2 Open field

Frrs1floxed/floxed cre positive, tamoxifen dosed mice and controls were assessed using an open field habituation protocol in order to assess both activity and spatial memory.
The current dataset shows no indication of hyperactivity in *Frrs1<sup>tm1c/tm1c</sup>* cre positive, tamoxifen dosed mice. Genotype effect on each parameter was assessed for both trial 1 and trial 2 independently. Each of seven control groups was compared to the experimental group, significant differences were seen in run 1 for centre total distance moved in males only between, *Frrs1<sup>tm1c/tm1c</sup>*, cre pos, no dose and *Frrs1<sup>+/+</sup>*, cre pos, no dose compared to *Frrs1<sup>tm1c/tm1c</sup>* cre pos, tamoxifen.

**Open field - individual trials**

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<th>Parameter</th>
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<th>Females - Trial 2</th>
<th>Males - Trial 1</th>
<th>Adjusted p value</th>
<th>Males - Trial 2</th>
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<td>No significant effects</td>
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</tr>
<tr>
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<td>No significant effects</td>
<td>F(7,68, 1.829, p=0.0957)</td>
<td><em>Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;</em>, cre pos, tamoxifen vs. <em>Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;</em>, cre neg, no dose</td>
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<td><em>Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;</em>, cre pos, tamoxifen vs. <em>Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;</em>, cre pos, no dose</td>
<td>0.0216</td>
</tr>
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<td>Centre frequency</td>
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<td>No significant effects</td>
<td>No significant effects</td>
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<tr>
<td>Centre latency</td>
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<td>No significant effects</td>
<td>No significant effects</td>
<td>No significant effects</td>
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</tr>
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</table>
Table 11 Genotype comparison of open field parameters, with individual trials analysed separately. Most parameters showed no significant differences between experimental groups in either trial. Significant differences were seen in males only, for parameters centre distance moved and centre duration. Data analysed by ANOVA, followed by Dunnett’s multiple comparisons test comparing Frrs1lm1c/tm1c, cre pos, tamoxifen, to each control group.

Similarly, for centre duration, significant differences were only seen in trial one in males only between Frrs1lm1c/tm1c, cre neg, no dose and Frrs1lm1c/tm1c, cre pos, no dose when compared to Frrs1lm1c/tm1c, cre pos, tamoxifen. Open field centre latency, centre velocity, whole arena distance moved and whole arena velocity showed no significant differences between Frrs1lm1c/tm1c, cre pos, tamoxifen and any other genotypes for either sex in either trial (Table 11 and Figures 76 and 77).

Overall, although there are some differences between the Frrs1lm1c/tm1c, cre pos, tamoxifen dosed mice and some of the controls, the majority of the control groups were not significantly different, indicating there is no hyperactivity in the conditionally deleted mice.

Habituation, indicated by a change in behaviour in trial two, is apparent across several parameters; in males, all groups except Frrs1+/+, cre pos, tamoxifen showed an effect of habituation in at least one parameter. In females the effect is less significant, with only three of the eight groups showing habituation in one
parameter or more (Table 3). With the current sample size, effects of habituation may be difficult to establish. Nevertheless early indications suggest that most groups habituate, including $\text{Frrs1}^{tm1c/tm1c}$, cre pos, tamoxifen dosed mice.

Figure 76 Open field habituation of $\text{Frrs1}^{tm1c/tm1c}$, cre pos, tamoxifen dosed females and three control groups, data shows results of two 30 minute trials carried out on consecutive days. Open field parameters plotted separately, total distance moved in the centre of the arena (A), duration in the
centre (B), frequency in the centre (C), velocity in the centre (D), distance moved in the whole arena (E), velocity in the whole arena (F). No differences in overall activity were found in any parameter. n=4 Frrs1tm1c/tm1c, cre pos, no dose, n=5 Frrs1tm1c/tm1c, cre pos, tamoxifen, n=1 Frrs1+/+, cre neg, no dose, n=4 Frrs1+/+, cre pos, tamoxifen.

Figure 77 Open field habituation of Frrs1tm1c/tm1c, cre pos, tamoxifen dosed males and three control groups. Data shows results of two 30 minute trials carried out on consecutive days. Open field parameters plotted separately, total distance moved in the centre of the arena (A), duration in the centre (B), frequency in the centre (C), velocity in the centre (D), distance moved in the whole arena (E), velocity in the whole arena (F).
(E), velocity in the whole arena (F). No differences were found in any parameter. n=4 \textit{Frrs1}^{tm1c/tm1c}, cre pos, no dose, n=6 \textit{Frrs1}^{tm1c/tm1c}, cre pos, tamoxifen, n=5 \textit{Frrs1}^{+/+}, cre neg, no dose, n=6 \textit{Frrs1}^{+/+}, cre pos, tamoxifen.

Table 12 Comparison of open field data between trial 1 and trial 2 within each experimental group. Significant differences indicate a change in behaviour between trials and therefore a habituation effect for that group. Seven out of eight groups in males show significant differences in at least one parameter \((p<0.05)\) (green) with \textit{Frrs1}^{+/+}, cre neg, no dose the only group with no habituation observed. In females, only three of eight groups show an effect of habituation (orange). Data analysed by one way ANOVA with Sidak multiple comparisons.

7.2.3 Home cage analysis

Home cage analysis was used to assess hyperactivity in a familiar environment and to compare to data on \textit{Frrs1}^{+/+} which exhibited hyperactivity in this test.
Figure 78 All groups were assessed in home cage analysis at 10 weeks of age. No significant differences between Frrs1tm1c/tm1c, cre pos, tamoxifen dosed mice and any other genotype were found at any time point, with the exception of Frrs1+/+, cre pos, no dose, which had significantly more activity in the light than Frrs1tm1c/tm1c, cre pos, tamoxifen females (F[7,29]=2.518, p = 0.0375). Total distance moved during the light phase over a three day period for females (A) and males (B). Total distance moved during the dark phase over a three day period for females (C) and males (D). Data analysed by ANOVA with post hoc Dunnett’s multiple comparisons test. n=4 Frrs1tm1c/tm1c, cre pos, no dose, n=5 Frrs1tm1c/tm1c, cre pos, tamoxifen, n=4 Frrs1+/+, cre neg, no dose, n=6 Frrs1+/+, cre pos, tamoxifen for females, n=4 Frrs1tm1c/tm1c, cre pos, no dose, n=8 Frrs1tm1c/tm1c, cre pos, tamoxifen, n=4 Frrs1+/+, cre neg, no dose, n=6 Frrs1+/+, cre pos, tamoxifen for males.

Activity data from home cage analysis shows no hyperactivity in Frrs1tm1c/tm1c, cre pos, tamoxifen dosed mice. Only one significant difference was found between
Frrs1<sup>tm1c/tm1c</sup>, cre pos, tamoxifen and the control groups, with the experimental group showing significantly less movement than Frrs1<sup>+/+</sup>, cre pos, no dose mice during the day, in females only.

### 7.2.4 Locotronic

All mice were assessed using the locotronic horizontal ladder to determine any coordination deficits. Analysis by ANOVA shows that genotype and dose have no effect on the number of errors.

![Locotronic](image)

Figure 79 Data from locotronic shows no differences in number of errors between any groups, both female (A) and male (B), data analysed by ANOVA with Dunnett’s multiple comparisons. n=5 Frrs1<sup>tm1c/tm1c</sup>, cre pos, no dose, n=7 Frrs1<sup>tm1c/tm1c</sup>, cre pos, tamoxifen, n=5 Frrs1<sup>+/+</sup>, cre neg, no dose, n=8 Frrs1<sup>+/+</sup>, cre pos, tamoxifen for females, n=8 Frrs1<sup>tm1c/tm1c</sup>, cre pos, no dose, n=8 Frrs1<sup>tm1c/tm1c</sup>, cre pos, tamoxifen for males.

### 7.2.5 Rotarod

Rotarod was used as a further test to assess motor co-ordination and to allow data correlation with Frrs1<sup>+/</sup>. Analysis by repeated measures ANOVA shows significantly
reduced latency to fall in both male and female \textit{Frrs1}^{tm1c/tm1c}, cre pos, tamoxifen dosed mice. In males, the experimental group is significantly different to all control groups, however in females significant differences were only present in three of the seven control groups (Table 13).

Figure 80 \textit{Frrs1}^{tm1c/tm1c}, cre pos, tamoxifen females have reduced latency to fall from an accelerating rotarod, when compared to 3/7 controls groups (A). \textit{Frrs1}^{tm1c/tm1c}, cre pos, tamoxifen dosed male mice have significantly reduced latency to fall compared to all control groups (B). n=5 \textit{Frrs1}^{tm1c/tm1c}, cre pos, no dose, n=7 \textit{Frrs1}^{tm1c/tm1c}, cre pos, tamoxifen, n=5 \textit{Frrs1}^{+/+}, cre neg, no dose, n=7 \textit{Frrs1}^{+/+}, cre pos, tamoxifen for females, n=8 \textit{Frrs1}^{tm1c/tm1c}, cre pos, no dose, n=8 \textit{Frrs1}^{tm1c/tm1c}, cre pos, tamoxifen, n=7 \textit{Frrs1}^{+/+}, cre neg, no dose, n=8 \textit{Frrs1}^{+/+}, cre pos, tamoxifen for males.

<table>
<thead>
<tr>
<th>\textit{Frrs1}^{tm1c/tm1c}, Cre pos, tamoxifen vs.</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Frrs1}^{tm1c/tm1c}, Cre pos, no dose</td>
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<td>0.0436</td>
</tr>
<tr>
<td>\textit{Frrs1}^{tm1c/tm1c}, Cre neg, tamoxifen</td>
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<td>0.0005</td>
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<tr>
<td>\textit{Frrs1}^{tm1c/tm1c}, Cre pos, no dose</td>
<td>0.1025</td>
<td>0.0221</td>
</tr>
<tr>
<td>\textit{Frrs1}^{+/+}, Cre neg, no dose</td>
<td>0.7146</td>
<td>0.0181</td>
</tr>
</tbody>
</table>
Table 13 Analysis of rotarod data by ANOVA followed by Dunnett’s multiple comparisons post hoc test shows significant differences between *Frrs1*tm1c/tm1c, Cre pos, tamoxifen dosed mice and all control groups in males, and three of seven control groups in females. Data shown as adjusted p values from Dunnett’s post hoc analysis.

These results indicate that abolishing *Frrs1* in adulthood also causes some motor coordination defects.

### 7.2.6 Y-maze

Conditional knockout mice and controls were tested in a forced alternation y-maze task to assess spatial working memory. No significant differences were found in either duration or frequency in the novel arm between *Frrs1*tm1c/tm1c Cre pos, tamoxifen dosed mice and any other groups.
Figure 8.1 Ratio of time spent (A) and (B) and ratio of frequency (C) and (D) in novel arm for females and males respectively. Ratio calculated as \(\frac{\text{novel arm}}{\text{novel arm} + \text{familiar arm}}\). No significant differences were found between Frrs1tm1c/tm1c, cre pos, tamoxifen and any other groups by ANOVA with Dunnett’s multiple comparisons test. \(n=4\) Frrs1tm1c/tm1c, cre pos, no dose, \(n=4\) Frrs1tm1c/tm1c, cre pos, tamoxifen, \(n=7\) Frrs1+/+, cre pos, tamoxifen for females, \(n=6\) Frrs1tm1c/tm1c, cre pos, no dose, \(n=9\) Frrs1tm1c/tm1c, cre pos, tamoxifen, \(n=9\) Frrs1+/+, cre pos, tamoxifen for males.

7.2.7 Grip strength

Mice were assessed for grip strength and data analysed by ANOVA. No significant differences were found between Frrs1tm1c/tm1c, cre pos, tamoxifen and any control group for both males and females.
Figure 82 Grip strength assessment showed no differences between any genotypes for females (A) and males (B), data analysed by repeated measures ANOVA with Dunnett’s multiple comparisons post hoc analysis. n=4 Frrs1<sup>tm1c/tm1c</sup>, cre pos, no dose, n=5 Frrs1<sup>tm1c/tm1c</sup>, cre pos, tamoxifen, n=3 Frrs1<sup>+/+</sup>, cre neg, no dose, n=3 Frrs1<sup>+/+</sup>, cre pos, tamoxifen for females, n=6 Frrs1<sup>tm1c/tm1c</sup>, cre pos, no dose, n=7 Frrs1<sup>tm1c/tm1c</sup>, cre pos, tamoxifen, n=4 Frrs1<sup>+/+</sup>, cre neg, no dose, n=8 Frrs1<sup>+/+</sup>, cre pos, tamoxifen for males.

### 7.2.8 Fear conditioning

Mice were assessed through a fear conditioning paradigm to determine any differences in non-declarative memory and to compare to the IMPC data for Frrs1<sup>+/−</sup> which showed abnormal response in the cue trial. Data showed normal response to context in Frrs1<sup>tm1c/tm1c</sup>, cre pos, tamoxifen dosed animals, with a significant increase in freezing in the same context the day after the shock was administered for males (p<0.05). However, the increase in freezing during the context test did
not reach significance in $\text{Frrs1}^{\text{tm1c/tm1c}}$, cre pos, tamoxifen dosed females, although this is likely to be due to a small sample size rather than a learning and memory deficit ($p=0.051$).

Figure 83 Fear conditioning data shows a normal response for $\text{Frrs1}^{\text{tm1c/tm1c}}$, cre pos, tamoxifen dosed mice during the context assessment (A – females, B – males). During the cue test, overall freezing is low before the tone is sounded compared to 5/7 control groups in females (C) and 6/7 control groups in males (D). During the post cue phase $\text{Frrs1}^{\text{tm1c/tm1c}}$, cre pos, tamoxifen show significantly reduced freezing compared to all control groups. n=5 $\text{Frrs1}^{\text{tm1c/tm1c}}$, cre pos, no dose, n=7 $\text{Frrs1}^{\text{tm1c/tm1c}}$, cre pos, tamoxifen, n=3 $\text{Frrs1}^{+/+}$, cre neg, no dose, n=7 $\text{Frrs1}^{+/+}$, cre pos, tamoxifen for females, n=8 $\text{Frrs1}^{\text{tm1c/tm1c}}$, cre pos, no dose, n=9 $\text{Frrs1}^{\text{tm1c/tm1c}}$, cre pos, tamoxifen, n=7 $\text{Frrs1}^{+/+}$, cre neg, no dose, n=9 $\text{Frrs1}^{+/+}$, cre pos, tamoxifen for males.
In the cue trial there is a clear increase in freezing in response to the tone in all groups (p<0.01 for all groups and both sexes). However, overall time freezing in Frrs1\textsuperscript{tm1c/tm1c}, cre pos, tamoxifen dosed mice is reduced in comparison with control groups. Frrs1\textsuperscript{tm1c/tm1c}, cre pos, tamoxifen dosed mice have significantly reduced freezing pre cue compared to 5/7 control groups in females and 6/7 control groups in males. During post cue freezing, significant differences are seen in comparison to all control groups for both sexes.

<table>
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<td>Frrs1\textsuperscript{tm1c/tm1c}, cre pos, tamoxifen vs.</td>
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<tr>
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<tr>
<td>Frrs1&lt;sup&gt;+/+&lt;/sup&gt;, cre neg, no dose</td>
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</tr>
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<td><strong>Males</strong></td>
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<tr>
<td>Frrs1&lt;sup&gt;+/+&lt;/sup&gt;, cre neg, no dose</td>
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<td>Frrs1&lt;sup&gt;+/+&lt;/sup&gt;, cre neg, tamoxifen</td>
<td>0.0001</td>
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<td>0.0001</td>
</tr>
<tr>
<td>Frrs1&lt;sup&gt;+/+&lt;/sup&gt;, cre pos, tamoxifen</td>
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</table>
Table 14 Statistical analysis by ANOVA, followed by Dunnett’s multiple comparisons test shows that total percent time freezing in the cue test is significantly different between \(Frss1^{tm1c/tm1c}, \text{cre pos}, \text{tamoxifen}\) and all other groups in both males and females during the post cue phase. In the pre-cue phase, the \(Frss1^{tm1c/tm1c}, \text{cre pos}, \text{tamoxifen}\) group is significantly different to 5/7 control groups in females and 6/7 control groups in males.

7.2.9 Startle and Pre-pulse inhibition

All mice were assessed for response to a startle and for pre-pulse inhibition, which is an indication of sensorimotor gating (Geyer, Mcilwain and Paylor, 2002; Powell, Zhou and Geyer, 2009). Data shows no significant differences between \(Frss1^{tm1c/tm1c}, \text{cre pos}, \text{tamoxifen}\) dosed mice and any of the control groups, however this lack of significance is likely to be due to high variability and small sample size. Initial data appears to show a reduced response in \(Frss1^{tm1c/tm1c}, \text{cre pos}, \text{tamoxifen}\) dosed mice for the startle and all pre-pulse tones.

![Figure 84](image_url)

Figure 84 Startle and pre-pulse inhibition amplitude for females (A) and males (B). No significant differences were found between any groups by ANOVA. n=4 \(Frss1^{tm1c/tm1c}, \text{cre pos}, \text{no dose}\), n=6 \(Frss1^{tm1c/tm1c}, \text{cre pos}, \text{tamoxifen}\), n=2 \(Frss1^{+/+}, \text{cre neg}, \text{no dose}\), n=6 \(Frss1^{+/+}, \text{cre pos}, \text{tamoxifen}\) for females, n=6 \(Frss1^{tm1c/tm1c}, \text{cre pos}, \text{no dose}\), n=8 \(Frss1^{tm1c/tm1c}, \text{cre pos}, \text{tamoxifen}\), n=4 \(Frss1^{+/+}, \text{cre neg}, \text{no dose}\), n=7 \(Frss1^{+/+}, \text{cre pos}, \text{tamoxifen}\) for males.
### 7.2.10 Sleep assessment

Conditionally deleted Frrs1l mice and controls were assessed through PIR to ascertain whether there were any differences in sleep parameters, as Frrs1l\(^{-/-}\) had reduced and fragmented sleep (5.2.7). No significant differences were found, however sample size for this test is small, as a proportion of the mice are not at the correct age to test. Although there are no significant differences there is a possible trend for a mild reduction in sleep time with shorter sleep bouts, however this will need to be confirmed by increasing the sample size.

![Preliminary analysis of sleep data for conditional deletion of Frrs1l and controls. ANOVA shows no significant difference between Frrs1l\(^{tm1c/tm1c}\), cre pos, tamoxifen dosed mice and controls for sleep duration or sleep bout length. n=2 Frrs1l\(^{tm1c/tm1c}\), cre pos, no dose, n=4 Frrs1l\(^{tm1c/tm1c}\), cre pos, tamoxifen, n=2 Frrs1l\(^{+/+}\), cre neg, no dose, n=4 Frrs1l\(^{+/+}\), cre pos, tamoxifen for females.](image-url)
7.2.11 Longitudinal SHIRPA data

Mice were assessed through a SHIRPA protocol to determine whether gait, coordination and grip strength phenotypes seen in \( Frrs1^{l/-} \) were also present in the conditional deletion. No distinct abnormalities were seen during SHIRPA assessment at 10 weeks of age. All mice showed normal co-ordination, with normal feet placement on bars. Gait abnormalities were noticed in 2/6 female \( Frrs1^{tm1c/tm1c} \), cre pos, tamoxifen dosed, whilst all controls were normal. In males, only 1/8 had an abnormal gait, although two of the control mice also displayed abnormalities. These differences were not significant. No trunk curling or limb grasping was seen in any of the groups. 3/8 males slipped during negative geotaxis, whilst all controls and females were normal (Tables 15 and 16). However, behaviour during geotaxis was unusual, with the majority of conditionally deleted mice displaying abnormal tail movements (4/6 females and 6/8 males).

<table>
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<tr>
<th></th>
<th>Total mice assessed</th>
<th>Tremor</th>
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<th>Abnormal gait</th>
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<tr>
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<tr>
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<td></td>
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<tr>
<td>Gender</td>
<td>Genotype</td>
<td>Treatment</td>
<td>Values</td>
<td>Probability</td>
</tr>
<tr>
<td>--------</td>
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Table 15 Data from SHIRPA carried out at 3 months and 9 months showing incidence of tremors, feet falling through the bars and abnormal gait. Increase in incidence between 3 and 9 months is indicated by purple highlighting. Sample size shown in columns 3 and 4, note sample size for 9 month cohort is smaller as only a subset of mice had reached this age at the time of writing. Frrs1\textsuperscript{tm1c/tm1c}, cre pos, tamoxifen dosed mice show an increase in the incidence of tremors, feet falling through the bars and abnormal gait. Only one wild-type group shows a similar increase in one of these parameters, with Frrs1\textsuperscript{+/+}, cre pos, tamoxifen dosed mice showing an increase in abnormal gait. Sample size is too small to assess significance of these increases.
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<tr>
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Table 16 Data from SHIRPA carried out at 3 months and 9 months showing incidence of elevated tail, lack of startle response occurrence of slipping during negative geotaxis. Increase in incidence between 3 and 9 months indicated by purple highlighting. Sample size shown in columns 3 and 4, note sample size for 9 month cohort is smaller. Frrs1tm1c/tm1c, cre pos, tamoxifen dosed mice show elevated tails at 9 months which is not present at 3 months and not seen in any of the control groups. Frrs1tm1c/tm1c, cre pos, tamoxifen dose mice have decreased response to startle which also occurs in several control groups. Finally Frrs1tm1c/tm1c, cre pos, tamoxifen show an increase in slips during negative geotaxis. Sample size of this preliminary data is too small to assess statistical significance at this point.

At the time of writing, a subset from each cohort had reached the nine month timepoint and a second SHIRPA assessment completed. The purpose of this second assessment is to highlight any deterioration in condition over time following Frrs1l deletion in young adults. Sample size at nine months is currently insufficient to assess statistical significance however there is evidence of increased incidence of tremors, feet falling through the bars, abnormal gait, elevated tails and slipping during negative geotaxis. At three months, the majority of Frrs1l\textsuperscript{tm1c/tm1c}, cre pos, tamoxifen dosed mice had unusual tail movements during geotaxis, however by nine months this was apparent in all Frrs1l\textsuperscript{tm1c/tm1c}, cre pos, tamoxifen dosed mice assessed so far, whilst all controls show normal tail movement.

Startle response is absent in a sub-set of mice across several groups, including Frrs1l\textsuperscript{tm1c/tm1c}, cre pos, tamoxifen dosed mice.
7.3 Discussion

7.3.1 Some \textit{Frrs1}^-/- phenotypes are not recapitulated in \textit{Frrs1} conditional knockout mice

Open field, home cage analysis, grip strength and locotronic show little difference between \textit{Frrs1}\textsuperscript{tm1c/tm1c}, cre pos, tamoxifen dosed mice and all other control groups, demonstrating that the extensive hyperactivity and co-ordination phenotypes seen in \textit{Frrs1}^-/- mice are not present in the conditional knockout. It is important to determine whether phenotypes are caused by a neurodevelopmental deficit, which also leads to reduced function in adults, or whether the same phenotypes are found in adults that have normal development but reduced AMPA receptor level in adulthood.

As \textit{Frrs1} is deleted at a later stage, neurons will have a normal pool of AMPA receptors at the time of tamoxifen dosing, which are likely to gradually decrease over time. This is in contrast to the constitutive knockout where the levels of AMPA receptors are already diminished at birth. As \textit{Frrs1}\textsuperscript{tm1c/tm1c} have a higher basal level of AMPA receptors, even after tamoxifen dosing, they may show a less severe phenotype initially, which may become more pronounced as the AMPA receptor levels continue to diminish over time.

In order to ascertain which of the phenotypic differences are due to development effects of loss of function in adulthood, it is essential to measure AMPA receptor protein levels and associate them with phenotype severity. If the AMPA receptor levels in the conditional deletion are comparable to the levels in the constitutive knockout, then the alterations in phenotype severity are likely to be due to developmental effects. However, if AMPA receptor levels in the conditional deletion
remain higher than in the constitutive knockout, it will be difficult to disentangle the neurodevelopmental consequences from those caused by acute loss of AMPA receptor biogenesis in adulthood.

7.3.2 Potential degenerative phenotypes are seen in mice with *Frss1l* conditionally deleted

Preliminary data from SHIRPA testing at nine months of age indicates some degeneration in several parameters. An increased incidence of, abnormal gait, feet falling through a grid floor, slipping during negative geotaxis and elevated tails, is seen in *Frrs1l*<sup>tm1c/tm1c</sup>, cre pos, tamoxifen dosed mice. However, an insufficient number of mice have reached this time point and although phenotypes appear to be deteriorating it is not yet clear if these data will show a statistically significant change in comparison to data collected at 3 months of age.

It should also be noted that there is a decrease in mice exhibiting a normal startle response (after presentation of click box) in both the *Frrs1l*<sup>tm1c/tm1c</sup>, cre pos, tamoxifen dosed mice and a proportion of the control groups. This is likely to be due to the age related hearing loss seen in C57BL/6NTac mice (Mianné et al., 2016) and it is not yet evident that there is an increase in hearing loss in mice with the conditional deletion of *Frrs1l*.

7.3.3 Co-ordination, cognitive and sleep differences are present in both the global and conditional knockout

Despite the lack of recapitulation of some phenotypes, there are others that are clearly evidenced in the *Frrs1l*<sup>tm1c/tm1c</sup>, cre pos, tamoxifen dosed mice. Rotarod is
significantly different between the conditionally deleted male group and all other controls. Rotarod differences in females are less pronounced, with differences only significant between the conditionally deleted mice and 3/7 control groups, however further analysis shows this to be due to one individual that is less affected than others. That there is one mouse that appears to be less affected could be due to normal variability in the phenotype, although it is also possible that the deletion is not complete in this animal and therefore AMPA receptor levels are higher. This needs to be investigated post mortem by qPCR analysis of Frrs1l expression throughout different areas of the brain.

Data from y-maze is not significant, although there is a trend for the conditionally deleted mice to show chance levels of alternation (means are 52% and 53% for females and males respectively), whilst other genotypes show a slight preference for the novel arm (mean >60% alternation). The control groups do not show a robust preference for the novel arm, therefore it is possible that even if a lack of preference exists in the conditional allele, it may be indistinguishable from control performance. An increase in sample size is needed in order to properly interpret these data.

Fear conditioning data shows that Frrs1l<sup>tm1c/tm1c</sup>, cre pos, tamoxifen dosed mice appear to learn in both context and cue paradigms.

Cue data is interesting in that all mice appear to associate the sound of the tone with an aversive memory, evidenced by the increase in freezing, however overall freezing levels are much lower in Frrs1l<sup>tm1c/tm1c</sup>, cre pos, tamoxifen dosed mice than in any of the control groups.
Freezing pre-tone is reduced in $Frrs1^{tm1c/tm1c}$, cre pos, tamoxifen dosed mice compared to 5/7 control groups in the females and 6/7 control groups in the males, whereas freezing post-tone is significantly reduced compared to all control groups for both sexes. The reason for this difference is unclear, overall activity levels are normal in open field and home cage analysis, and freezing levels in the context component of the test are comparable to controls. A similar response in cued fear conditioning was seen in $Frrs1^{-/-}$ mice, however the reduction in freezing was thought to be due to hyperactivity. It now appears that that may not be the case, since the phenotype is very similar in the conditionally deleted mice which show no evidence of hyperactivity.

Finally, no significant differences were seen in sleep analysis. However, there is a trend for $Frrs1^{tm1c/tm1c}$, cre pos, tamoxifen dosed mice to show decreased overall sleep as well as shorter sleep bouts. Similar abnormalities in sleep were found in $Frrs1^{-/-}$ mice but with a greater magnitude. Again, greater samples sizes will help to delineate whether this is a true phenotype in the conditional knockout.

### 7.3.4 Summary

Data for conditional knockout mice is preliminary and greater sample sizes are needed for all groups to determine statistically significant changes. However, initial analysis of these data show that the phenotype of $Frrs1^{tm1c/tm1c}$, cre pos, tamoxifen dosed mice is less extreme than in $Frrs1^{-/-}$. Early developmental effects of $Frrs1$ deletion cause high levels of neonatal mortality through mechanisms that are not yet understood indicating that $Frrs1$ plays a crucial role in early postnatal
development (5.2.1). In contrast, *Frrs1l* deletion after this critical developmental period appears to have no effect on mortality to date.

AMPA receptor levels in *Frrs1l*/* mice are low from birth, which may lead to a reduced capability to learn from an early age. Therefore, learned behaviours and motor skills, such as gait, may never be fully acquired. In contrast, in *Frrs1l*tm1c/tm1c synapse development and LTP should be normal during development and the ability to learn behaviours will be unaffected prior to tamoxifen dosing. However, there may be detrimental effects on learning new tasks which are introduced after deletion has taken place.

Data from these conditionally deleted mice indicate processes that need continual input from *Frrs1l*, and consequently AMPA receptors, throughout life, rather than only during development. Phenotypes such as lack of co-ordination, sleep and unusual behaviour in fear conditioning, demonstrate that *Frrs1l* is needed for normal behaviour in adults. Further analysis on conditionally deleted mice will help to delineate any changes in cognition as well as characterise any degeneration. Post-mortem assessment of *Frrs1l* expression is essential to confirm deletion. In addition, quantification of levels of AMPA receptor core proteins in specific brain regions is crucial to confirm that the phenotypes seen are a consequence of altered AMPA receptor levels, and by how much the protein levels are reduced in the conditional mice compared to the constitutive deletion.
8.0 Discussion

8.1 Using mice to model human disease – future directions

The major aim of this work was to find new mouse models to expand our knowledge and understanding of abnormal cognition and behaviour. Using mice to model cognitive function in humans has always been a challenge. Human cognition is complex and cannot be recapitulated fully in mice. However, many studies have shown some of the underlying mechanisms of cognition to be similar, and mutations or genetic alterations in corresponding genes in mice and humans can have comparable phenotypic outcomes. Bedell et al review some of these models, discussing one hundred human genes with disease causing mutations which have corresponding mouse models with mutations in the murine homologue (Bedell et al., 1997). Twenty two of the one hundred genes in this review display neurological phenotypes in mice that are similar to the human condition. In addition, models have been generated for Huntington’s disease (Giralt et al., 2012), x-linked intellectual disability (Van Dam et al., 2000) and Rett syndrome (Moretti et al., 2005) amongst many others, where mouse phenotypes recapitulate many of the human symptoms. Therefore there is clearly insight to be gained about the human condition by studying mouse models.

There have been several papers in recent years proclaiming that mouse studies are not reproducible and that their use in finding suitable drugs to treat human disease is limited (Bracken, 2009; Pedro-roig and Emmerich, 2017). However, there are many possible reasons for these problems, it is not necessarily the case that the organism is being studied is not appropriate to answer the pertinent scientific questions (Kafkafi et al., 2018). By defining the sources of the variability and
Inconsistency there is an opportunity to address the problems. Genetic background is likely to be a key factor in the lack of reproducibility, many papers include incorrect strain nomenclature or incomplete descriptions of strains, partly due to a common misconception that sub-strains are similar in phenotype and do not need to be distinguished (Podhorna and Brown, 2002; Bardgett et al., 2003). However it is clear that sub-strains can have very different phenotypes (Velez et al., 2010; M. M. Simon et al., 2013) and therefore may differentially affect the consequences of specific genetic alterations. In addition, advances in genome sequencing have allowed us to have a better understanding of genetic drift, highlighting how quickly spontaneous mutations can become fixed in colonies (Frazer et al., 2007; Lynch, 2010). Lynch et al. estimate the mutation rate in the male germline to be approximately 103 snp’s per generation, therefore an inbred strain kept for ten generations in one facility will be significantly different to the same strain kept for ten generations in another.

Publication bias is another major factor in the misinterpretation of mouse studies. The majority of studies are only published when the findings are positive, as such, if a drug is tested and fails to produce the required effect, that result may not be made available publically. Thus when interpreting the available literature, results look more favourable for a particular drug than would be the case if all experimental results were accessible (Sena et al., 2010). In recent years there have been moves to address this problem, with initiatives from groups such as the National Centre for the 3R’s (reduction, replacement and refinement) to publish negative results and some journals more willing to accept such papers. F1000 is a recently developed open research publishing platform that will accept all papers that meet the criteria, which includes negative findings (F1000 Research, Open for Science,
Nevertheless, while most funding still relies on papers being published in high-impact factor journals, the reporting of negative results may always be a lower priority for researchers (Schooler, 2011; Eisen, Ganley and Maccallum, 2014).

Experimental design, such as testing at the relevant age (Jackson et al., 2017), using appropriate controls, appropriate sample sizes based on statistical calculations, amongst many other variables also potentially have a significant impact on the reproducibility of a study (Kilkenny et al., 2010). Many mouse models have provided us with crucial information about biological systems and pathological mechanisms, and, although there are some concerns with reproducibility, many studies have been reproduced and have provided valuable insight. However, there is now an opportunity to improve the way mice are used to model human disease. Detailed genome sequencing has highlighted the importance of background genetics, and in-depth studies into large datasets have detailed the importance of metadata and appropriate experimental design. By addressing some of these areas we can strive towards making better animals models that continue to further our understanding of basic biology as well as being clinically relevant to human disease.

Whilst working to improve reproducibility in data gained from mouse models there are still some important factors to take into consideration. For example, lifespan is very different, around 2 years (+/- 6 months) for laboratory mice (Yuan et al., 2009), whereas for humans in many western countries the life expectancy is over 80 for males, and over 85 for females (Kontis et al., 2017). Therefore the changes in humans that are a result of many years carrying a genetic mutation, may not be modelled well in a mouse that only lives a fraction of the time (Jackson et al., 2017). Examples of this are apparent in neuromuscular disorders, where mouse
models do not degenerate in the same way as humans with similar disease. In fact some mutations that cause long term degeneration in humans only show limited degeneration in mice before a process of muscle regeneration takes over (Stedman et al., 1991; Pastoret and Sebille, 1995). Alzheimer’s models also present challenges in terms or recapitulating human pathology. Mutations in APP in humans cause early onset Alzheimer’s disease, with typical plaque and tangle morphology, however the same mutations in mice only result in amyloid plaques, with absence of neurofibrillary tangles (McGowan, Eriksen and Hutton, 2006). The mechanisms for these differences are not well understood. It is important that the differences and similarities between mouse and humans are always considered during experimental design and that the expectations of biological significance are realistic. It may be that certain aspects of disease cannot be recapitulated in mice, nevertheless we can still draw important conclusions from systems that are similar, and can even use the differences to try and further understand disease processes. The dissimilarities between mice and humans need not negatively affect experiments providing any limitations are taken into account in both experimental design and interpretation of results.

Another difference between mice and humans is that specific gene sequences, and their corresponding regulatory elements, may be different. It is possible to ‘humanise’ sequences that differ between species in order to make the murine protein and regulation more akin to the human version. Altering mouse genes to make them more similar to human genes can be technically difficult, although this has been accomplished in some systems. For example the Kymouse contains part of a humanised immune system, with the entire human immunoglobulin variable gene repertoire inserted into ES cells and subsequently into mice (Lee et al., 2014).
In the case of the Kymouse, the system altered is well defined and contains a relatively small number of genes, it may be more difficult to do this in another biological context, particularly one that is less well understood. In recent years CRISPR/Cas9 gene editing has made gene humanisation more achievable, especially in examples where the difference between human and mouse genes is only a few nucleotides, these can be modified comparatively easily using the CRISPR/Cas9 system. However there are still many circumstances where the human sequence is significantly different to the mouse, therefore more complex genome editing technologies may need to be used. Humanising genes has the potential to generate mouse strains that may be more relevant for pre-clinical studies, however relatively few genes have been modified to date and the consequences of replacing mouse genes with the human version is likely to be gene and system dependent.

In summary, there is a large body of evidence supporting the use of mice to model human disease and understand basic biology, however there are some experimental design and interpretation caveats that should be considered in order to make the best use of each mouse model.

### 8.2 Discovering new mouse models of cognition and behaviour – future directions

#### 8.2.1 Gene editing

The last few years have seen tremendous changes in the way mouse models are generated, with RNA guided endonucleases such as CRISPR/Cas9, becoming an
increasingly popular method for mutant generation. Screening methods are also changing, with forward genetics chemical mutagenesis screens seeing a decline in popularity. Whilst the accessibility of the genome and increased efficiency in generating specific mutations is driving research into reverse genetics. Large scale population GWAS, whole genome sequencing (WGS) and whole exome sequencing (WES) have revolutionised the diagnosis of human disease, with new causative mutations being discovered both in known and novel genes. Finding the mutation, however, does not necessarily suggest or decipher a disease mechanism, especially in genes for which there is relatively little research data. Studying null alleles is an extremely useful step in illuminating mechanisms and pathways in which the deleted gene is involved. Creating a global deletion is a very effective starting point to begin delineation of gene function. Furthermore, conditional technologies such as the knockout first allele, provide an additional advantage of creating both a constitutive knockout and a conditional knockout without having to carry out further transgenesis. As demonstrated for Frrs1I, the conditional deletion is key to defining phenotypes and highlighting specific roles of the gene in either a tissue or time dependent manner. Conditional alleles provide a route to study gene function in adult tissues when the developmental role of that gene leads to reduced viability or study of function in a specific tissue without the confounding effects of phenotypes in other systems. As a result of these significant advantages, the use of conditional alleles is likely to be widespread in the future for genes where the function cannot be studied with constitutive deletion models.

Since the advent of CRISPR/Cas9 it has become easier to recapitulate in mice the homologous mutations found in human disease. Whilst knockout alleles provide important insight into gene function, and conditional knockout alleles help to
understand the temporal and spatial functions of null alleles, they do not necessarily recapitulate the mutations seen in humans. Single nucleotide changes many generate a range of different alleles allowing examination of hypomorphs, hypermorphs, antimorphs and neomorphs rather than only null alleles. These different types of mutation are likely to have differential affects compared to a complete knockout and will provide additional insight into disease mechanisms. Over and above null alleles and point mutations there are other ways in which the genome can be modified and studied, copy number variants are of increasing interest, with studies showing that additional copies of unmutated genes can have detrimental effects (McCarroll and Altshuler, 2007; Marshall et al., 2017). In some cases multiple genes need to be modified in one animal to help understand certain pathways or recapitulate certain pathologies. This leads to some mice carrying many different mutations, such as the triple transgenic model of Alzheimer’s disease (Oddo et al., 2003). It is likely that the study of specific disease causing point mutations as well as other structural variants and more complex models carrying multiple mutations may increase in the future, leading to a new era of mouse models for human cognitive diseases.

8.2.2 Phenotyping

Classical tests for cognition and behaviour have been enormously useful in finding and understanding animal models with cognitive or behavioural deficits. However, with advances in technology, new and more complex tests are being developed which may allow further insight into the intricacies of cognitive function. Touch screen tests developed over the last decade provide the ability to test cognition without the aversive environment of a water maze or the administration of a
footshock, although many protocols do require food restriction (Romberg, Alexa E Horner, et al., 2013). Touchscreen tests can be made more complex or used to delineate specific behaviours, for example some studies suggest that the touchscreen system is able to assess attention and behavioural flexibility (Romberg, Alexa E. Horner, et al., 2013; Richter et al., 2014). Touchscreen tests are unique in that paradigms can be very similar in mice and humans. In classical cognitive tests, mice may explore a maze or search for a submerged platform, these data would then be correlated with human tests for cognition which involve different paradigms but allow approximation of a similar response. Recent advances in touchscreen testing have started to use identical paradigms for mice and humans, and whilst the interpretation may not be identical between the two species, some of the bias between different tests is removed (Nithianantharajah et al., 2015).

Assessment of the mouse in its home environment is another area of growth in the mouse phenotyping that is providing some novel and useful information. Most behaviour and cognitive tests require the mouse to be handled by a human and placed in an unfamiliar environment. Mazes and touchscreen chambers are not the mouse’s normal environment and so might modify the normal response, for example if a mouse has heightened stress levels it may not perform well in memory tasks (Conrad et al., 2004; Benoit, Rakic and Frick, 2015). Assessing behaviour in the home cage environment allows the mouse to behave without input from humans, in an environment that is as normal as possible in the laboratory setting. Several systems that assess homecage behaviour have been produced, although many of them involve moving the mouse into a different environment that then becomes its home cage. Other systems assess the behaviour of mice in the cage
they have been maintained in from weaning alongside cage mates (Bains et al., 2016). This true home cage environment provides the unique opportunity to assess normal behaviour, motor function and social interactions in an undisturbed setting. With further development it may be possible to use home cage systems to assess memory in the form of novel object recognition, or perhaps in the future to incorporate some of the more complex tests such as touch screen assays in the home cage.

The touch screen test and the home cage monitoring system are just two examples of how advances in technology are impacting behavioural and cognitive research. Whilst neither of these systems is suitable for high-throughput phenotyping in their current form, it is likely that with further development, similar tests may be used to screen for novel mouse models in the future.

### 8.3 IMPC screening for fear conditioning phenotypes

The IMPC screen was designed to be an open resource with several key purposes - firstly to try and produce better animal models by performing extensive QC and controlled breeding on a co-isogenic genetic background, secondly by collecting and publishing extensive metadata on all tests, and finally by making all data, positive or negative, available on freely accessed web portals. The intention was to make better mouse models of genes already being studied, for example by generating true null mutants using a standard, carefully planned format. More importantly a key objective was to generate new GA lines and screen for new mutant phenotypes that display characteristics of human disease.

The IMPC is an excellent resource, providing a wealth of data and the opportunity to assess the function of many genes, however at its outset it lacked a reliable test
which could assess cognition. Many popular tests for cognition need large sample sizes or long training periods, therefore it was not apparent whether there was a cognitive test available that was sufficiently robust and suitable for high-throughput. A primary aim of this work was to find such a test, one that could access cognitive function whilst withstanding the rigours of high-throughput testing. Various cognition tests were assessed in order to choose a suitable paradigm to include in the high-throughput IMPC screen. After a period of baseline testing, a fear conditioning protocol was developed and selected to be incorporated in the phenotyping pipeline. The standard operating procedure developed was disseminated across the consortium and the details made available on the web portal for anyone wishing to check the procedure or to repeat the test using the same experimental set up.

Over the course of five years almost 300 genetically altered lines have been successfully screened through the fear conditioning test. Statistical analysis has highlighted several lines that may warrant further study including Cacna1b, Nisch and Ccdc109b. These lines are the only three that pass the test for false discovery rate. However, as discussed, the most appropriate statistical test to determine true positives is challenging to select. A compromise has to be made between an over representation of false positives or of false negatives. It may be that selecting lines with true phenotypes cannot be decided purely using statistical calculations, but that statistics can be used, along with data from other tests, to help distinguish a biologically relevant phenotype. Using the added knowledge of pleiotropic effects has been invaluable in the interpretation of fear conditioning data. For example, a line such as Frrs1l was statistically significant but failed the test for false discovery rate, however other phenotypes found as part of IMPC indicated a neurological
disorder, and therefore it was prudent to investigate this line. *Slc24a4* is another example where other IMPC phenotypes added essential information. The viability information on *Slc24a4* was a useful factor in the decision to take this line for further testing, since the sub-viability could imply variable expressivity and there was a clear variability in the fear conditioning response which may be explained by incomplete penetrance. This variability resulted in the data not being significant when compared to baseline, however the evidence of possible incomplete penetrance, the statistical difference to heterozygous littermates and the interesting GWAS link to Alzheimer’s disease convinced us that it was a suitable line for further analysis. The FC phenotype was recapitulated and the phenotyping data is now publically-available. The *Slc24a4*−/− mice are archived in a public repository providing open access to a potentially interesting new model that may be valuable in Alzheimer’s disease research.

The statistical tests carried out to select lines for additional phenotyping in the early stages of this screen were less sophisticated than the final analysis. Retrospective analysis, along with an increase in the volume of baseline data, has altered the statistical significance for some lines that have been assessed. As large data sets from high-throughput screens increase in volume, careful thought needs to be given to the statistical analysis. New methods may need to be developed in order to deal with these type of data high-throughput data. Appropriate statistics are critical to ensure researchers are provided with useful, biologically-relevant results from which they can draw appropriate conclusions about gene function.
8.4 Frrs1l

The Frrs1l line was selected for further study primarily based on the fear conditioning data but other phenotypes found in the IMPC screen were also factors in this decision and the fact that this gene had no known function but was highly expressed in the brain made it a reasonable prospect for further study. Soon after our decision to focus on this line, we were made aware of a clinical research group studying patients suffering from a juvenile Huntington’s like disease which seemed to be caused by mutations in FRRS1L. There is a high correlation between the phenotypes identified in the mice and the symptoms in humans. Symptoms such as abnormal gait, muscle weakness, sleep abnormalities and seizures are found in both humans and the Frrs1l mouse model.

Examining the constitutive deletion came with some complications, both in terms of welfare and interpretation of results. It was decided not to repeat the fear conditioning on the constitutive knockout as the second cohort appeared very agitated and possibly anxious, making a stress inducing test such as one involving a foot shock unacceptable in terms of animal welfare. Other cognitive tests were carried out, however the confounding factor of hyperactivity was difficult to mitigate. Finally, Frrs1l mice had high levels of neonatal lethality, so much so that not enough males could be generated to produce a statistically relevant cohort. Despite these challenges, studying Frrs1l has produced some extremely interesting and important data on the biological function of Frrs1l and its potential role in human disease. In-vivo data on AMPA receptor levels and processing has produced novel information that deletion of Frrs1l in-vivo greatly reduces the number of AMPA receptors in the brain. We have also shown that, in the absence of Frrs1l, post translational modification of AMPA receptors is incomplete, resulting
in reduced levels of receptors at the synapse. This correlates with human data that shows patients with *FRRS1L* mutations have modified AMPA inward currents (Madeo *et al.*, 2016). Critically these data from *Frrs1l<sup>−/−</sup>* add to the diagnosis in humans by indicating that these changes in currents are likely due to a lack of AMPA receptors at the synapse, most likely through a reduction in the production of mature AMPA receptors.

The hyperactivity phenotype of the constitutive knockout rendered the delineation of more subtle phenotypes unattainable. Therefore to access the intricacies of cognitive function another approach was needed. A cohort of conditionally deleted mice, in which *Frrs1l* was removed at five weeks of age, was assessed through various phenotyping tests. Deletion in young adults would allow us to delineate whether the phenotypes were rooted in developmental alterations or whether *Frrs1l* is needed for sustained adult function. This proved a fruitful experiment, and, although data is preliminary, it appears that a subset of the phenotypes are present in adult knockouts. No lethality has been seen in the conditional deletion so far, indicating that immediately post birth may be an essential time for AMPA receptor function. The extreme hyperactivity and very obvious gait abnormalities are not present in the conditional deletion, making it possible to carry out further cognitive testing. Y-maze data is not significantly different, although further mice need to be tested for this dataset to have sufficient power and it is possible that the trend towards random arm choice in the conditional deletion may become significant. However, fear conditioning shows a very similar phenotype to the original IMPC screen data, with reduced freezing in response to cue. The fear conditioning result requires some further analysis as freezing levels are low at both
pre and post cue time points. This low freezing before the tone is also true of the IMPC screen data, though at the time was attributed to hyperactivity.

More interestingly, there appears to be some deterioration in performance in the conditionally deleted mice, something not detected in the constitutively deleted animals. SHIRPA data shows very few abnormal phenotypes at three months of age with an increasing number of co-ordination problems developing as the mice age.

Taking advantage of the conditional approach has allowed us to disentangle some of the developmental phenotypes and importantly to show that Frrs1l has a role to play in adult behaviour and cognition that is not linked to neurodevelopmental processes.

### 8.4.1 Future work on Frrs1l

There is a great deal of work to be carried out to gain more insight into the mechanism and to fully characterise the phenotype caused by deletion of Frrs1l in mice. Firstly, for the constitutive deletion, Frrs1l\(^{-/-}\), seizure activity need to be fully characterised in terms of type and frequency. This will inform us on the similarity between mouse and humans seizures and establish how closely the mouse model recapitulates the human condition.

The conditionally deleted mice are only part way through phenotyping assessment, which includes further cognitive function assessment and longitudinal analysis of phenotype development as the mice age. On termination of the conditional studies, levels of Frrs1l expression and AMPA receptor protein need to be assessed in order
to delineate whether AMPA receptors are reduced in a similar way to the constitutive deletion and whether the extent of the reduction is the same.

Further ex-vivo work has to be carried out, such as localisation of the tagged wildtype and mutated \textit{FRRS1L} protein with appropriate markers to determine subcellular location.

The mechanism of how Frrs1l deletion leads to disease in mice and humans is still in the early stages of elucidation. However, this work has highlighted some areas that are of clinical relevance and could be investigated in terms of treatment. AMPA receptors as drug targets have been considered in the past with limited success. Although in many cases drugs were sought to reduce AMPA excitability, as they are over active in some forms of ALS and epilepsy (Rogawski, 2011; Chang, Verbich and Mckinney, 2012), whereas Frrs1l deletion appears to make them under active. Interestingly AMPA receptor levels are also reduced in Alzheimer’s disease, leading to potential similarities between \textit{FRRS1L} deletion and AD. There is ongoing research into modulating AMPA receptor function through auxiliary proteins in order to combat the reduction seen in AD (Swanson, 2009; Chang, Verbich and Mckinney, 2012). If this upregulation of AMPA receptors proves fruitful it may be possible to use the same drugs to alleviate some symptoms in patients with \textit{FRRS1L} mutations. As shown from the mouse model, it is possible that much of the severe phenotype develops during neurodevelopment, therefore it is probable that the most effective therapies will be those given at an early age. Despite the complete lack of Frrs1l, some AMPA receptors are formed and seem to be functioning in the synapse, indicating another mechanism or compensation from another protein. Further
investigation may illuminate how this occurs and may highlight potential drug targets that can be upregulated to increase AMPA receptor levels.

8.5 Overall summary

In summary, over the course of this project cognitive tests have been assessed, modified for high-throughput screening and included in a large scale phenotyping project. Almost 300 lines have been screened and several phenotypes of interest highlighted, while informative data, both negative and positive, has been disseminated.

Of the mutant lines characterised, at least one serves as a novel model for a human neurological disorder. Upon further investigation, the Frrs1l model has provided insight into previously unknown AMPA receptor mechanisms and has gone some way towards illuminating the causes behind a devastating human disease. Future GA line characterisations should continue to provide enlightenment into the study of human cognition and the disease processes whereby this is affected.
Appendix 1

Table of p-values and means for every line tested through the IMPC fear conditioning screen.

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Table 17: All p-values calculated from GA lines tested in FC. p-values of ANOVA analysis comparing 2 linear models, one model with genotype as a fixed effect and one without genotype as a fixed effect. Lines with p values below 0.05 highlighted in green, for each of the significant p-values, the direction of change in indicated with yellow showing a decrease in freezing and red showing an increase.
### Appendix 2

<p>| Genotype and parameter | Females | Males | | | | | | | | | |
|------------------------|---------|-------|------------------|------------------|---------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|                        | Sample size | ANOVA F-Statistic and p value | Adjusted p value | Sample size | ANOVA F-Statistic and p value | Adjusted p value | | | | | | | | | | | |
| <strong>Open field - centre distance moved</strong> | | | | | | | | | | | | | | | | | | |
| <strong>Trial 1</strong> | | | | | | | | | | | | | | | | | | |
| Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre neg, no dose | 2 | 0.593 | 1 | | | | | | | | | | | | | | | |
| Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre neg, tamoxifen | 5 | 0.686 | 6 | | | | | | | | | | | | | | | |
| Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre pos, no dose | 4 | 0.999 | 9 | | | | | | | | | | | | | | | |
| Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre pos, tamoxifen | 5 | N/A | 6 | | | | | | | | | | | | | | | |
| Frrs1&lt;sup&gt;+&lt;/sup&gt;/+, cre neg, no dose | 1 | 0.073 | 3 | | | | | | | | | | | | | | | |
| Frrs1&lt;sup&gt;+&lt;/sup&gt;/+, cre neg, tamoxifen | 7 | 0.999 | 6 | | | | | | | | | | | | | | | F(7,68)=2.357, p=0.0323 |
| Frrs1&lt;sup&gt;+&lt;/sup&gt;/+, cre pos, no dose | 4 | F(7,48)=3.876, p=0.002 | 7 | | | | | | | | | | | | | | | 0.0176 |
| Frrs1&lt;sup&gt;+&lt;/sup&gt;/+, cre pos, tamoxifen | 4 | 0.191 | 7 | | | | | | | | | | | | | | | 0.7729 |
| <strong>Trial 2</strong> | | | | | | | | | | | | | | | | | | |
| Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre neg, no dose | 2 | 0.997 | 8 | | | | | | | | | | | | | | | 0.9996 |
| Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre neg, tamoxifen | 5 | 0.957 | 2 | | | | | | | | | | | | | | | | 0.9947 |
| Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre pos, no dose | 4 | 0.978 | 5 | | | | | | | | | | | | | | | | 0.4741 |
| Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre pos, tamoxifen | 5 | N/A | 6 | | | | | | | | | | | | | | | N/A |</p>
<table>
<thead>
<tr>
<th></th>
<th>Frrs1+/+, cre neg, no dose</th>
<th>Frrs1+/+, cre neg, tamoxifen</th>
<th>Frrs1+/+, cre pos, no dose</th>
<th>Frrs1+/+, cre pos, tamoxifen</th>
</tr>
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<tr>
<td></td>
<td>1</td>
<td>7</td>
<td>4</td>
<td>4</td>
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<tr>
<td></td>
<td>0.877 1</td>
<td>0.999 9</td>
<td>0.082 2</td>
<td>0.090 9</td>
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<tr>
<td>Open field - centre duration</td>
<td></td>
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<tr>
<td>Trial 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frrs1\textsuperscript{tm1c/tm1c}, cre neg, no dose</td>
<td>2</td>
<td>0.651 5</td>
<td>0.999 6</td>
<td>0.999 7</td>
</tr>
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<td>Frrs1\textsuperscript{tm1c/tm1c}, cre neg, tamoxifen</td>
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<td>0.779 9</td>
<td>0.999 4</td>
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<td>Frrs1\textsuperscript{tm1c/tm1c}, cre pos, no dose</td>
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<td>0.999 5</td>
<td>0.999 7</td>
<td>0.999 7</td>
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<tr>
<td>Frrs1\textsuperscript{tm1c/tm1c}, cre pos, tamoxifen</td>
<td>5</td>
<td>N/A</td>
<td>0.999 4</td>
<td>N/A</td>
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<tr>
<td>Trial 2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Frrs1\textsuperscript{tm1c/tm1c}, cre neg, no dose</td>
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<td>0.999 6</td>
<td>0.999 7</td>
<td>0.999 7</td>
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<td>0.878 5</td>
<td>0.999 7</td>
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<tr>
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<td>4</td>
<td>0.999 7</td>
<td>0.999 7</td>
<td>N/A</td>
</tr>
<tr>
<td>Frrs1\textsuperscript{tm1c/tm1c}, cre pos, tamoxifen</td>
<td>5</td>
<td>N/A</td>
<td>0.999 7</td>
<td>N/A</td>
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</tbody>
</table>

\(F(7,48) = 3.318, p = 0.0058\)

\(F(7,68) = 1.829, p = 0.0957\)
| Frrs1*+/+, cre neg, no dose | 1 | 0.518 | 5 | 0.9972 |
| Frrs1*+/+, cre neg, tamoxifen | 7 | 0.999 | 7 | 0.9994 |
| Frrs1*+/+, cre pos, no dose | 4 | 0.769 | 7 | 0.9995 |
| Frrs1*+/+, cre pos, tamoxifen | 4 | 0.111 | 6 | 0.99 |

**Open field - centre frequency**

<table>
<thead>
<tr>
<th><strong>Trial 1</strong></th>
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<tbody>
<tr>
<td>Frrs1*+/+, cre neg, no dose</td>
</tr>
<tr>
<td>Frrs1*+/+, cre neg, tamoxifen</td>
</tr>
<tr>
<td>Frrs1*+/+, cre pos, no dose</td>
</tr>
<tr>
<td>Frrs1*+/+, cre pos, tamoxifen</td>
</tr>
<tr>
<td>Frrs1*+/+, cre neg, no dose</td>
</tr>
<tr>
<td>Frrs1*+/+, cre neg, tamoxifen</td>
</tr>
<tr>
<td>Frrs1*+/+, cre pos, no dose</td>
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<td>Frrs1*+/+, cre pos, tamoxifen</td>
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<table>
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<tr>
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<tr>
<td>Frrs1*+/+, cre neg, tamoxifen</td>
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<tr>
<td>Frrs1*+/+, cre pos, no dose</td>
</tr>
<tr>
<td>Frrs1*+/+, cre pos, tamoxifen</td>
</tr>
<tr>
<td>Trial 1</td>
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<tr>
<td>---------</td>
</tr>
<tr>
<td><strong>Open field - centre latency to first entry</strong></td>
</tr>
<tr>
<td><strong>Frrs1tm1c/tm1c, cre neg, no dose</strong></td>
</tr>
<tr>
<td><strong>Frrs1tm1c/tm1c, cre neg, tamoxifen</strong></td>
</tr>
<tr>
<td><strong>Frrs1tm1c/tm1c, cre pos, no dose</strong></td>
</tr>
<tr>
<td><strong>Frrs1tm1c/tm1c, cre pos, tamoxifen</strong></td>
</tr>
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**Trial 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
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<tbody>
<tr>
<td>Frrs1tm1c/tm1c, cre neg, no dose</td>
<td>0.913 2</td>
<td>0.905 3</td>
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<td>Frrs1tm1c/tm1c, cre neg, tamoxifen</td>
<td>0.999 9</td>
<td>0.321 1</td>
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<td>0.049 1</td>
<td>0.999 7</td>
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<td>Frrs1tm1c/tm1c, cre pos, tamoxifen</td>
<td>0.049 1</td>
<td>N/A</td>
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**Trial 2**

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<th>Trial 2</th>
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<tbody>
<tr>
<td>Frrs1tm1c/tm1c, cre neg, no dose</td>
<td>0.999 7</td>
<td>0.999 7</td>
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<tr>
<td>Frrs1tm1c/tm1c, cre neg, tamoxifen</td>
<td>0.999 7</td>
<td>0.999 7</td>
</tr>
<tr>
<td>Frrs1tm1c/tm1c, cre pos, no dose</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Frrs1tm1c/tm1c, cre pos, tamoxifen</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Strain</td>
<td>Condition</td>
<td>Sample Size</td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>-------------</td>
</tr>
<tr>
<td>Frrs1&lt;sup&gt;+/−&lt;/sup&gt;, cre neg, no dose</td>
<td>1</td>
<td>0.804</td>
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<tr>
<td>Frrs1&lt;sup&gt;+/−&lt;/sup&gt;, cre neg, tamoxifen</td>
<td>7</td>
<td>0.999</td>
</tr>
<tr>
<td>Frrs1&lt;sup&gt;+/−&lt;/sup&gt;, cre pos, no dose</td>
<td>4</td>
<td>0.928</td>
</tr>
<tr>
<td>Frrs1&lt;sup&gt;+/−&lt;/sup&gt;, cre pos, tamoxifen</td>
<td>4</td>
<td>0.089</td>
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**Open field - centre velocity**

**Trial 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Condition</th>
<th>Sample Size</th>
<th>p</th>
<th>F (Trial 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frrs1&lt;sup-tm1c/tm1c&lt;/sup&gt;, cre neg, no dose</td>
<td>2</td>
<td>0.999</td>
<td>9</td>
<td>0.9468</td>
</tr>
<tr>
<td>Frrs1&lt;sup-tm1c/tm1c&lt;/sup&gt;, cre neg, tamoxifen</td>
<td>5</td>
<td>0.999</td>
<td>9</td>
<td>0.9996</td>
</tr>
<tr>
<td>Frrs1&lt;sup-tm1c/tm1c&lt;/sup&gt;, cre pos, no dose</td>
<td>4</td>
<td>0.602</td>
<td>3</td>
<td>0.9996</td>
</tr>
<tr>
<td>Frrs1&lt;sup-tm1c/tm1c&lt;/sup&gt;, cre pos, tamoxifen</td>
<td>5</td>
<td>N/A</td>
<td>6</td>
<td>N/A</td>
</tr>
<tr>
<td>Frrs1&lt;sup&gt;+/−&lt;/sup&gt;, cre neg, no dose</td>
<td>1</td>
<td>0.992</td>
<td>3</td>
<td>0.9999</td>
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<tr>
<td>Frrs1&lt;sup&gt;+/−&lt;/sup&gt;, cre neg, tamoxifen</td>
<td>7</td>
<td>0.975</td>
<td>6</td>
<td>0.9977</td>
</tr>
<tr>
<td>Frrs1&lt;sup&gt;+/−&lt;/sup&gt;, cre pos, no dose</td>
<td>4</td>
<td>F(7,48)=1.389, p=0.2317</td>
<td>0.987</td>
<td>7</td>
</tr>
<tr>
<td>Frrs1&lt;sup&gt;+/−&lt;/sup&gt;, cre pos, tamoxifen</td>
<td>4</td>
<td>0.999</td>
<td>7</td>
<td>0.397</td>
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**Trial 2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Condition</th>
<th>Sample Size</th>
<th>p</th>
<th>F (Trial 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frrs1&lt;sup-tm1c/tm1c&lt;/sup&gt;, cre neg, no dose</td>
<td>2</td>
<td>0.928</td>
<td>9</td>
<td>0.9871</td>
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<tr>
<td>Frrs1&lt;sup-tm1c/tm1c&lt;/sup&gt;, cre neg, tamoxifen</td>
<td>5</td>
<td>0.410</td>
<td>9</td>
<td>0.7989</td>
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<tr>
<td>Frrs1&lt;sup-tm1c/tm1c&lt;/sup&gt;, cre pos, no dose</td>
<td>4</td>
<td>0.999</td>
<td>4</td>
<td>0.3531</td>
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<tr>
<td>Frrs1&lt;sup-tm1c/tm1c&lt;/sup&gt;, cre pos, tamoxifen</td>
<td>5</td>
<td>N/A</td>
<td>6</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Frrs11</strong>/+, cre neg, no dose</td>
<td>1</td>
<td>0.298</td>
<td>5</td>
<td>0.6086</td>
</tr>
<tr>
<td>-----------------------------</td>
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<td>--------</td>
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<tr>
<td><strong>Frrs11</strong>/+, cre neg, tamoxifen</td>
<td>7</td>
<td>0.984</td>
<td>6</td>
<td>0.388</td>
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<tr>
<td><strong>Frrs11</strong>/+, cre pos, no dose</td>
<td>4</td>
<td>0.979</td>
<td>7</td>
<td>0.6223</td>
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<tr>
<td><strong>Frrs11</strong>/+, cre pos, tamoxifen</td>
<td>4</td>
<td>0.981</td>
<td>2</td>
<td>0.186</td>
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<table>
<thead>
<tr>
<th><strong>Open field - whole arena total distance moved</strong></th>
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**Trial 1**

<table>
<thead>
<tr>
<th><strong>Frrs11</strong>tm1c/tm1c, cre neg, no dose</th>
<th>2</th>
<th>0.375</th>
<th>3</th>
<th>0.9698</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frrs11</strong>tm1c/tm1c, cre neg, tamoxifen</td>
<td>5</td>
<td>0.347</td>
<td>2</td>
<td>0.9677</td>
</tr>
<tr>
<td><strong>Frrs11</strong>tm1c/tm1c, cre pos, no dose</td>
<td>4</td>
<td>0.299</td>
<td>1</td>
<td>0.313</td>
</tr>
<tr>
<td><strong>Frrs11</strong>tm1c/tm1c, cre pos, tamoxifen</td>
<td>5</td>
<td>N/A</td>
<td>6</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Frrs11</strong>/+, cre neg, no dose</td>
<td>1</td>
<td>0.056</td>
<td>8</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>Frrs11</strong>/+, cre neg, tamoxifen</td>
<td>7</td>
<td>0.951</td>
<td>6</td>
<td>0.9999</td>
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<tr>
<td><strong>Frrs11</strong>/+, cre pos, no dose</td>
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<td>0.583</td>
<td>7</td>
<td>0.2006</td>
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<tr>
<td><strong>Frrs11</strong>/+, cre pos, tamoxifen</td>
<td>4</td>
<td>0.251</td>
<td>6</td>
<td>0.9411</td>
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</table>

F(7,48)=2.942, p=0.0120  
F(7,68)=2.438, p=0.0273

**Trial 2**

<table>
<thead>
<tr>
<th><strong>Frrs11</strong>tm1c/tm1c, cre neg, no dose</th>
<th>2</th>
<th>0.983</th>
<th>1</th>
<th>0.9999</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frrs11</strong>tm1c/tm1c, cre neg, tamoxifen</td>
<td>5</td>
<td>0.999</td>
<td>6</td>
<td>0.8729</td>
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<tr>
<td><strong>Frrs11</strong>tm1c/tm1c, cre pos, no dose</td>
<td>4</td>
<td>0.597</td>
<td>1</td>
<td>0.3079</td>
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<tr>
<td><strong>Frrs11</strong>tm1c/tm1c, cre pos, tamoxifen</td>
<td>5</td>
<td>N/A</td>
<td>6</td>
<td>N/A</td>
</tr>
<tr>
<td>Strain</td>
<td>Treatment</td>
<td>OC</td>
<td>Sample Size</td>
<td>Mean</td>
</tr>
<tr>
<td>--------</td>
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<td>---</td>
<td>-------------</td>
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<tr>
<td>Frrs1+/+, cre neg, no dose</td>
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<td>0.9926</td>
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<td>0.999</td>
<td>4</td>
<td>0.9999</td>
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<td>7</td>
<td>0.1946</td>
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<td>0.253</td>
<td>7</td>
<td>0.2597</td>
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**Open field - whole arena velocity**

**Trial 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>OC</th>
<th>Sample Size</th>
<th>Mean</th>
<th>SD</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Frrs1^m1c/tm1c^, cre neg, no dose</td>
<td>2</td>
<td>0.470</td>
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<tr>
<td>Frrs1^m1c/tm1c^, cre neg, tamoxifen</td>
<td>5</td>
<td>0.483</td>
<td>6</td>
<td>0.9677</td>
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<td></td>
</tr>
<tr>
<td>Frrs1^m1c/tm1c^, cre pos, no dose</td>
<td>4</td>
<td>0.425</td>
<td>6</td>
<td>0.313</td>
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</tr>
<tr>
<td>Frrs1^m1c/tm1c^, cre pos, tamoxifen</td>
<td>5</td>
<td>N/A</td>
<td>6</td>
<td>N/A</td>
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<tr>
<td>Frrs1^+/+, cre neg, no dose</td>
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<td>3</td>
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<td>Frrs1^+/+, cre neg, tamoxifen</td>
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<td>0.955</td>
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<td>7</td>
<td>0.2006</td>
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<td>Frrs1^+/+, cre pos, tamoxifen</td>
<td>4</td>
<td>0.358</td>
<td>6</td>
<td>0.9411</td>
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**Trial 2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>OC</th>
<th>Sample Size</th>
<th>Mean</th>
<th>SD</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Frrs1^m1c/tm1c^, cre neg, no dose</td>
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<td>6</td>
<td>0.8729</td>
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<td>N/A</td>
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<tr>
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<td>0.997</td>
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<td>0.030</td>
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<td>0.192</td>
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**Home cage analysis - distance moved during light phase**

<p>| | | | |</p>
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<thead>
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</thead>
<tbody>
<tr>
<td><strong>Frrs1</strong>tm1c/tm1c, cre neg, no dose</td>
<td>3</td>
<td>0.826</td>
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<tr>
<td><strong>Frrs1</strong>tm1c/tm1c, cre neg, tamoxifen</td>
<td>4</td>
<td>0.083</td>
<td>5</td>
</tr>
<tr>
<td><strong>Frrs1</strong>tm1c/tm1c, cre pos, no dose</td>
<td>4</td>
<td>0.083</td>
<td>5</td>
</tr>
<tr>
<td><strong>Frrs1</strong>tm1c/tm1c, cre pos, tamoxifen</td>
<td>5</td>
<td>N/A</td>
<td>8</td>
</tr>
</tbody>
</table>

**F(7,29)=2.518, p=0.0375**  
**F(7,34)=0.6056, p=0.7470**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td><strong>Frrs1</strong>+/+, cre neg, no dose</td>
<td>4</td>
<td>0.296</td>
<td>4</td>
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<tr>
<td><strong>Frrs1</strong>+/+, cre neg, tamoxifen</td>
<td>7</td>
<td>0.821</td>
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<td><strong>Frrs1</strong>+/+, cre pos, no dose</td>
<td>4</td>
<td>0.012</td>
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<td><strong>Frrs1</strong>+/+, cre pos, tamoxifen</td>
<td>6</td>
<td>0.223</td>
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**Home cage analysis - distance moved during dark phase**

<p>| | | | |</p>
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<td><strong>Frrs1</strong>tm1c/tm1c, cre neg, no dose</td>
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<td><strong>Frrs1</strong>tm1c/tm1c, cre neg, tamoxifen</td>
<td>4</td>
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<td><strong>Frrs1</strong>tm1c/tm1c, cre pos, no dose</td>
<td>4</td>
<td>0.999</td>
<td>5</td>
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<td>5</td>
<td>N/A</td>
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</table>

**F(7,29)=0.3331, p=0.9323**  
**F(7,34)=0.6787, p=0.6888**
<table>
<thead>
<tr>
<th>Frrs1+/+, cre neg, no dose</th>
<th>4</th>
<th>0.054</th>
<th>4</th>
<th>0.9996</th>
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</thead>
<tbody>
<tr>
<td>Frrs1+/+, cre neg, tamoxifen</td>
<td>7</td>
<td>0.902</td>
<td>7</td>
<td>0.971</td>
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<tr>
<td>Frrs1+/+, cre pos, no dose</td>
<td>4</td>
<td>0.159</td>
<td>4</td>
<td>0.769</td>
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<tr>
<td>Frrs1+/+, cre pos, tamoxifen</td>
<td>6</td>
<td>0.959</td>
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<td>0.6474</td>
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**Locotronic - total number of errors**

<table>
<thead>
<tr>
<th>Frrs1tm1c/tm1c, cre neg, no dose</th>
<th>5</th>
<th>0.628</th>
<th>4</th>
<th>0.7224</th>
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<tr>
<td>Frrs1tm1c/tm1c, cre neg, tamoxifen</td>
<td>7</td>
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<td>7</td>
<td>0.9791</td>
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<td>Frrs1tm1c/tm1c, cre pos, no dose</td>
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<td>0.8909</td>
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<tr>
<td>Frrs1tm1c/tm1c, cre pos, tamoxifen</td>
<td>7</td>
<td>F(7,43)=0.6825, p=0.6820</td>
<td>N/A</td>
<td>8</td>
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<tr>
<td>Frrs1+/+, cre neg, no dose</td>
<td>5</td>
<td>0.874</td>
<td>7</td>
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<td>Frrs1+/+, cre neg, tamoxifen</td>
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<td>0.917</td>
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<td>Frrs1+/+, cre pos, no dose</td>
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<tr>
<td>Frrs1+/+, cre pos, tamoxifen</td>
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<td>0.825</td>
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<td>0.9975</td>
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**Rotarod latency to fall**

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<tr>
<th>Frrs1tm1c/tm1c, cre neg, no dose</th>
<th>6</th>
<th>0.004</th>
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<th>0.0436</th>
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<td>0.164</td>
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<td>Frrs1tm1c/tm1c, cre pos, no dose</td>
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<td>F(7,44)=2.505, p=0.0295</td>
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<td>Frrs1tm1c/tm1c, cre pos, tamoxifen</td>
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<td>N/A</td>
<td>9</td>
<td>N/A</td>
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<tr>
<td>Frrs1+/+, cre neg, no dose</td>
<td>5</td>
<td>0.714</td>
<td>6</td>
<td>0.0181</td>
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<tr>
<td>Group</td>
<td>n</td>
<td>Y-maze duration</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----</td>
<td>-----------------</td>
<td>---------</td>
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<tr>
<td></td>
<td></td>
<td>Familiar arm</td>
<td></td>
<td></td>
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<tr>
<td>Frrs1+/-, cre neg, tamoxifen</td>
<td>8</td>
<td>0.130 9</td>
<td>0.0001</td>
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<tr>
<td>Frrs1+/-, cre pos, no dose</td>
<td>7</td>
<td>0.025 2</td>
<td>0.0002</td>
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<tr>
<td>Frrs1+/-, cre pos, tamoxifen</td>
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<td>0.038 2</td>
<td>0.0027</td>
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<td>Novel arm</td>
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<tr>
<td>Frrs1tm1c/tm1c, cre neg, tamoxifen</td>
<td>7</td>
<td>0.660 4</td>
<td>0.9851</td>
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<tr>
<td>Frrs1tm1c/tm1c, cre pos, no dose</td>
<td>3</td>
<td>0.671 2</td>
<td>0.9017</td>
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<td>4</td>
<td>N/A 9</td>
<td>N/A</td>
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<tr>
<td>Frrs1+/-, cre neg, tamoxifen</td>
<td>7</td>
<td>0.506 8</td>
<td>0.1899</td>
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<tr>
<td>Frrs1+/-, cre pos, no dose</td>
<td>3</td>
<td>0.785 7</td>
<td>0.4309</td>
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<td>Frrs1+/-, cre pos, tamoxifen</td>
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<td>0.446 8</td>
<td>0.2747</td>
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<td>Frrs1tm1c/tm1c, cre neg, tamoxifen</td>
<td>7</td>
<td>F(5,78)=0.1213</td>
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<td>Frrs1tm1c/tm1c, cre pos, no dose</td>
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<td>F(5,111)=0.0598</td>
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<td>Frrs1tm1c/tm1c, cre pos, tamoxifen</td>
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<tr>
<td>Frrs1+/-, cre neg, tamoxifen</td>
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<tr>
<td>Frrs1+/-, cre pos, no dose</td>
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<tr>
<td>Frrs1+/-, cre pos, tamoxifen</td>
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<tr>
<td>Genotype</td>
<td>CRE Status</td>
<td>Treatment</td>
<td>n</td>
<td>Y-maze Frequency in Arm</td>
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<tr>
<td>-------------------------------</td>
<td>------------</td>
<td>-----------</td>
<td>---</td>
<td>-------------------------</td>
</tr>
<tr>
<td><em>Frrs1</em>^tm1c/tm1c*, cre</td>
<td>neg</td>
<td>tamoxifen</td>
<td>7</td>
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<tr>
<td><em>Frrs1</em>^tm1c/tm1c*, cre</td>
<td>pos</td>
<td>no dose</td>
<td>3</td>
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<tr>
<td><em>Frrs1</em>^tm1c/tm1c*, cre</td>
<td>pos</td>
<td>tamoxifen</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Frrs1</em>^+/+, cre</td>
<td>neg</td>
<td>tamoxifen</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td><em>Frrs1</em>^+/+, cre</td>
<td>pos</td>
<td>no dose</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><em>Frrs1</em>^+/+, cre</td>
<td>pos</td>
<td>tamoxifen</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td><em>Frrs1</em>^+/+, cre</td>
<td>neg</td>
<td>tamoxifen</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td><em>Frrs1</em>^+/+, cre</td>
<td>pos</td>
<td>no dose</td>
<td>3</td>
<td></td>
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<tr>
<td><em>Frrs1</em>^+/+, cre</td>
<td>pos</td>
<td>tamoxifen</td>
<td>7</td>
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**Familiar Arm**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CRE Status</th>
<th>Treatment</th>
<th>n</th>
<th>Y-maze Frequency in Arm</th>
<th>p-value 1</th>
<th>p-value 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Frrs1</em>^tm1c/tm1c*, cre</td>
<td>neg</td>
<td>tamoxifen</td>
<td>7</td>
<td></td>
<td>0.189</td>
<td>0.1774</td>
</tr>
<tr>
<td><em>Frrs1</em>^tm1c/tm1c*, cre</td>
<td>pos</td>
<td>no dose</td>
<td>3</td>
<td></td>
<td>0.005</td>
<td>0.0789</td>
</tr>
<tr>
<td><em>Frrs1</em>^tm1c/tm1c*, cre</td>
<td>pos</td>
<td>tamoxifen</td>
<td>4</td>
<td></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Frrs1</em>^+/+, cre</td>
<td>neg</td>
<td>tamoxifen</td>
<td>7</td>
<td></td>
<td>0.005</td>
<td>0.1435</td>
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<tr>
<td><em>Frrs1</em>^+/+, cre</td>
<td>pos</td>
<td>no dose</td>
<td>3</td>
<td></td>
<td>0.125</td>
<td>0.0161</td>
</tr>
<tr>
<td><em>Frrs1</em>^+/+, cre</td>
<td>pos</td>
<td>tamoxifen</td>
<td>7</td>
<td></td>
<td>0.055</td>
<td>0.0315</td>
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</table>

**Novel Arm**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CRE Status</th>
<th>Treatment</th>
<th>n</th>
<th>Y-maze Frequency in Arm</th>
<th>p-value 1</th>
<th>p-value 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Frrs1</em>^tm1c/tm1c*, cre</td>
<td>neg</td>
<td>tamoxifen</td>
<td>7</td>
<td></td>
<td>0.801</td>
<td>0.3531</td>
</tr>
<tr>
<td><em>Frrs1</em>^tm1c/tm1c*, cre</td>
<td>pos</td>
<td>no dose</td>
<td>3</td>
<td></td>
<td>0.489</td>
<td>0.3855</td>
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<tr>
<td><em>Frrs1</em>^tm1c/tm1c*, cre</td>
<td>pos</td>
<td>tamoxifen</td>
<td>4</td>
<td></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Frrs1</em>^+/+, cre</td>
<td>neg</td>
<td>tamoxifen</td>
<td>7</td>
<td></td>
<td>0.083</td>
<td>0.4829</td>
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</table>

Y-maze frequency in arm is measured as the number of times the animal entered each arm. The results are shown with the sample size (n) and the p-value for the statistical tests performed.

For the familiar arm, the results are as follows:
- *Frrs1*^tm1c/tm1c*, cre neg, tamoxifen: n=7, p-value=0.999
- *Frrs1*^tm1c/tm1c*, cre pos, no dose: n=6, p-value=0.998
- *Frrs1*^tm1c/tm1c*, cre pos, tamoxifen: n=9, p-value=N/A
- *Frrs1*^+/+, cre neg, tamoxifen: n=7, p-value=0.011
- *Frrs1*^+/+, cre pos, no dose: n=5, p-value=0.939
- *Frrs1*^+/+, cre pos, tamoxifen: n=9, p-value=0.818

For the novel arm, the results are as follows:
- *Frrs1*^tm1c/tm1c*, cre neg, tamoxifen: n=1, p-value=0.801
- *Frrs1*^tm1c/tm1c*, cre pos, no dose: n=4, p-value=0.489
- *Frrs1*^tm1c/tm1c*, cre pos, tamoxifen: n=9, p-value=N/A
- *Frrs1*^+/+, cre neg, tamoxifen: n=5, p-value=0.083

Additionally, a one-way ANOVA was conducted on the familiar arm data with the following results:
- For the familiar arm, the F(5,78) = 7.415, p < 0.0001, indicating a significant difference among the groups.
- For the novel arm, the F(5,111) = 3.461, p = 0.0061, indicating a significant difference among the groups.
<table>
<thead>
<tr>
<th></th>
<th>Start arm</th>
<th>Grip strength</th>
<th>Fear conditioning - context</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frrs1</strong>+/+, cre pos, no dose</td>
<td>3</td>
<td>0.836</td>
<td>5</td>
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<tr>
<td><strong>Frrs1</strong>+/+, cre pos, tamoxifen</td>
<td>7</td>
<td>0.755</td>
<td>6</td>
</tr>
<tr>
<td><strong>Start arm</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>Frrs1</strong>tm1c/tm1c, cre neg, tamoxifen</td>
<td>7</td>
<td>0.137</td>
<td>4</td>
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<td>0.004</td>
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<td><strong>Frrs1</strong>tm1c/tm1c, cre pos, tamoxifen</td>
<td>4</td>
<td>N/A</td>
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<tr>
<td><strong>Frrs1</strong>+/+, cre neg, tamoxifen</td>
<td>7</td>
<td>0.001</td>
<td>4</td>
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<tr>
<td><strong>Frrs1</strong>+/+, cre pos, no dose</td>
<td>3</td>
<td>0.007</td>
<td>5</td>
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<tr>
<td><strong>Frrs1</strong>+/+, cre pos, tamoxifen</td>
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<td>0.052</td>
<td>9</td>
</tr>
<tr>
<td><strong>Grip strength</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>Frrs1</strong>tm1c/tm1c, cre neg, no dose</td>
<td>3</td>
<td>0.999</td>
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<tr>
<td><strong>Frrs1</strong>tm1c/tm1c, cre neg, tamoxifen</td>
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<td>N/A</td>
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<tr>
<td><strong>Frrs1</strong>+/+, cre neg, no dose</td>
<td>3</td>
<td>0.997</td>
<td>5</td>
</tr>
<tr>
<td><strong>Frrs1</strong>+/+, cre neg, tamoxifen</td>
<td>7</td>
<td>0.910</td>
<td>7</td>
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<td><strong>Frrs1</strong>+/+, cre pos, no dose</td>
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<tr>
<td><strong>Frrs1</strong>+/+, cre pos, tamoxifen</td>
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<td>0.923</td>
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</table>

F(7,29)=0.935, p=0.4950

F(7,39)=0.3009, p=0.9492
<table>
<thead>
<tr>
<th></th>
<th>Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre neg, no dose</th>
<th>Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre neg, tamoxifen</th>
<th>Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre pos, no dose</th>
<th>Frrs1&lt;sup&gt;tm1c/tm1c&lt;/sup&gt;, cre pos, tamoxifen</th>
<th>Frrs1&lt;sup&gt;+/+&lt;/sup&gt;, cre neg, no dose</th>
<th>Frrs1&lt;sup&gt;+/+&lt;/sup&gt;, cre neg, tamoxifen</th>
<th>Frrs1&lt;sup&gt;+/+&lt;/sup&gt;, cre pos, no dose</th>
<th>Frrs1&lt;sup&gt;+/+&lt;/sup&gt;, cre pos, tamoxifen</th>
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<tbody>
<tr>
<td></td>
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<td>3</td>
<td>8</td>
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<td></td>
<td>F(7,74)=0.3652, p=0.9196</td>
<td>F(7,98)=2.132, p=0.0471</td>
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**Fear conditioning - cue**
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Table 18: Table showing complete statistical analysis for conditional deletion line against all controls. Females data on left, male data on right, sample size for each genotype in each test is indicated. F statistic and p-value from ANOVA shown, along with p values calculated with Dunnett’s post hoc analysis.
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