



December 2005

Advance

REPORTS FROM THE MND RESEARCH INSTITUTE OF AUSTRALIA

All grants distributed by the Motor Neurone Disease Research Institute of Australia come from donations and bequests from individual supporters and contributions from the State MND Associations of Australia.

Researchers funded through the MND Research Institute of Australia are required to write reports on their work after completion of their projects.

The reports published here give a brief overview of recent projects funded by the Institute.

Dr Mark Bellingham University of Queensland

Functional differences in glutamate responses of motor neurones resistant and susceptible to death in MND

One reason why motor neurones may die selectively in MND is exposure to excessive amounts of glutamate, the main excitatory neurotransmitter released by brain synapses. Glutamate is thought to trigger motor neurone death by opening certain types of glutamate-sensitive receptors, which then allow calcium to flow into the motor neurone in excessive amounts which ultimately leads to motor neurone death. However, we do not know for certain whether motor neurones possess these glutamate receptors, and whether the presence or absence of these glutamate receptors on different motor neurones is consistent with their survival or death in MND.

The responses of glutamate receptors to glutamate are determined by the combination of several different protein subunits to make individual glutamate receptors. Each glutamate receptor subunit is coded for by a distinct gene, and the presence and relative level of genes for these subunits can be measured in very small brain tissue samples, using a technique called real time PCR. When subunit genes are present, they then control the level of subunit protein produced. Protein production can be estimated using antibodies which specifically bind to different proteins.

We have investigated one major type of glutamate receptor, the NMDA type, as opening of this receptor can allow large amounts of potentially toxic calcium to flow into the motor neurone. The relative amount of calcium moving through the NMDA receptor into the motor neurone is strongly regulated by the subunit composition of NMDA receptors.

Our measurements show that motor neurones which die during early stages of MND (hypoglossal motor neurones which activate tongue muscles) show a higher overall amount of NMDA receptors combined with higher levels of NR2B subunits, compared to levels in motor neurones which survive until very late stages of MND (oculomotor motor neurones which activate muscles moving the eyeballs). This suggests that activation of NMDA receptors

by glutamate will result in a larger flow of calcium into hypoglossal motor neurones, than into oculomotor motor neurones. This is because 1) there are more NMDA receptors to open 2) these receptors are more sensitive to glutamate, and 3) an increased amount of calcium flows through each individual NMDA receptor every time it opens.

This novel difference in NMDA receptors may constitute one factor predisposing to selective death of hypoglossal versus oculomotor neurones in MND. Drugs which directly or indirectly inhibit NMDA receptors containing NR2B subunits should be considered as possible therapies for MND.

Dr Michel Guipponi and Dr Hamish S Scott

Walter and Eliza Hall Institute of Medical Research

Gene expression profiling in mouse models of neurodegenerative diseases. Looking for common genetic pathways involved in neurodegeneration

The risk of developing Amyotrophic Lateral Sclerosis (ALS) is likely to involve a combination of a wide variety of genetic and environmental risk factors. However, around 10% of the cases are inherited (familial i.e. caused by mutation in one gene). Among these familial cases, approximately 20% are caused by mutations in superoxide dismutase (SOD1). The striking pathological and clinical similarity between familial and sporadic cases has generated enthusiasm that animal models based on mutant SOD1 might provide insight into mechanisms of both sporadic and familial disease.

We are taking advantage of the existing mouse model for ALS to identify biological processes that are dysregulated during disease progression. We have used gene expression profiling technologies to define the first "complete" gene expression signatures of spinal cord from diseased and healthy mice at different stage of disease progression. By comparing these signatures, we were able to identify genes that are statistically differentially expressed during disease progression compared to normal development.

These lists of genes have been used to identify genes that could be classified as potential genetic risk factors for developing ALS /MND. Genes exhibiting the most pronounced dysregulation will be prioritised for mutation analysis in affected patients.

We subsequently looked at the biological functions associated with these differentially expressed genes. Early disease progression was notable for the prominence of genes involved in immune response, proteolysis, and apoptosis. In contrast genes involved in metabolism, transcription and transport were less prominent. These biological functions were already known to be involved in ALS, however to better understand how they interact together to put the motor neurons at risk, we needed to determine “all” the genes involved in these biological processes. Our expression datasets contain interesting and promising genes to be involved in the pathogenesis of ALS. The identification of such genes involved in motor neuron degeneration should contribute to development of new therapeutic drugs designed to significantly slow or ideally stop disease progression of ALS and of other motor neurone diseases.

Dr Christine Hawkins and Anissa Jabbour

Murdoch Children’s Research Institute VIC

Does the spinal muscular atrophy gene SMN inhibit apoptosis?

Spinal muscular atrophy (SMA) is an inherited disease characterised by muscle wasting and weakness. We know that SMA is caused by death of a particular set of nerve cells in the spinal cord. A gene (SMN) was previously identified which, when mutated, is responsible for the disease. However the way in which mutations in this gene lead to death of the nerve cells is not known, and this ignorance complicates the development of therapies for the disease. We found that a similar gene in worms could regulate the activity of molecules that dictated the survival or death of particular cells during development of the animal. This suggested that the human SMN protein might play a role in controlling the balance between cellular survival and death. Our recent data has demonstrated that the worm SMN gene could also inhibit human proteins known to affect cell survival. We have explored molecular mechanisms that could account for the ability of the worm protein to inhibit cell death. Our data suggest that this cell death inhibition is accomplished by regulation of the production of one or more of the key cell death pathway components within the cell, rather through direct interactions between the proteins. We are now in the process of testing normal and mutant human SMN genes for their ability to interfere with cell death in our yeast system. We have also established a collaboration to test this property in the context of cell lines generated from patient material.

Dr Robert Henderson

Royal Brisbane & Women’s Hospital. QLD

Statistical motor unit number estimates

MND is a rapidly progressive muscle wasting disease due to death of motor nerves (motor units). At present there is no cure and limited treatment. One problem with finding effective treatments has been the main marker of the disease is survival and the many varied ways this uncommon disease progresses has meant the use of survival is limited in assessing the effectiveness of therapy.

At Royal Brisbane & Women’s Hospital and in collaboration with the Queensland University of Technology, School of Mathematical Sciences, we are working on a method of determining the number of motor units that supply a muscle. These methods are generally termed “Motor Unit Number Estimates”. No method has been widely applied in assessing treatments for MND.

In our method, we apply recording electrodes to the surface overlying a hand or foot muscle and stimulate the corresponding nerve to measure the responses that represent different combinations of motor units. We apply Bayesian statistics to determine the motor unit number. We studied both control subjects and MND patients, many of the latter group serially from an early stage in the disease. We have also studied the varied presentations of MND. We have been grateful and impressed with the enthusiasm of our MND patients for the project.

To date we have found that using our techniques and studying the weakest muscle, particularly in the hands, we can accurately determine the number of motor units supplying the muscle up to about 30 motor units. The methods show a decline over time in the number of motor units which may be clinically applicable. The future of the project lies in finalising a reliable method that can determine the number of motor units when a much larger number of motor units remain, and proving that it is reproducible in MND patients. This will allow the assessment of treatments that can hopefully work for this disease.

Prof Garth Nicholson ANZAC Research Institute NSW

Finding new pathogenic genes affecting motor neurones

We aim to identify new mutated genes responsible for disorders affecting motor neurones using genetic linkage studies. We have available families with a range of motor neuropathies and familial MND. Identification of gene mutations in any of these families will increase understanding of the biology of motor neurones, reveal mechanisms underlying motor neurone disorders and may provide insight into familial and sporadic forms of the disease.

Using linkage studies, we have been able to show that hereditary motor neuropathy in the largest family in our

neuropathy database is not associated with any of the 7 known chromosomal loci for hereditary motor neuropathy.

Previously, we had shown that one of our large MND families (which does not have the common SOD1 mutation) is not associated with 4 other described autosomal dominant chromosomal loci associated with familial MND. However with further expansion of this family, we found that only one locus was excluded, the data for the 3 other loci was inconclusive. We have expanded another family in our database. In this family we have excluded 4 chromosomal loci described with autosomal dominant FALS. This has shown us that a new, undescribed gene mutation is responsible for MND in this family. Our collaboration with overseas researchers in USA and UK may help locate the responsible gene.

Dr Roger Pamphlett University of Sydney
Gene environment interactions in MND

Many people think that sporadic MND is due to a genetic susceptibility to some environmental toxin. One group of toxins, heavy metals, have been suspected since they enter motor neurons in preference to other cells. The body has developed efficient ways of dealing with heavy metals, in particular by using a group of proteins known as metallothioneins that detoxify the metals when they enter the body. One of this group, the protein metallothionein 3, is present in the nervous system only. Using DNA from the Australian MND DNA Bank we therefore looked at this gene in a number of people with MND as well as people without the disease. There were no changes in this gene to suggest that it was making people more susceptible to heavy metals and therefore to MND. We are continuing to look at other members of this gene family to see if one of them could be responsible for susceptibility to MND.

Dr William D. Phillips University of Sydney
Role of the acetylcholine receptor-associated protein, rapsyn, in the mature nervous system

This grant-in-aid was requested to allow us to progress towards the generation of transgenic mice in which it is possible to inactivate the gene for the acetylcholine receptor-associated protein, rapsyn, in a tissue- and time-specific manner (rapsyn conditional mice). The ultimate aims of the project are to define the role of rapsyn in maintaining healthy synapses between motor neurons and muscle fibres.

Generation of the rapsyn conditional mice involves the following steps:

1. Design and generation of the targeting construct (plasmid) and associated probes
2. Electroporation of targeting construct into embryonic stem (ES) cells and selection of homologous recombinant ES cell clones

3. Derivation of germ-line chimeric mice by microinjection of ES cells into blastocysts
4. Deletion of the Neomycin resistance cassette by breeding with FLP recombinase transgenic mice.
5. Breeding to homozygosity and preliminary experiments on the conditional knockout

In collaboration with Dr Peter Noakes and Mary White at the University of Queensland we have now completed step 2 in the protocol above. Southern blotting identified embryonic stem cell clones with the intended homologous recombination of the rapsyn. We intend to complete steps 3 and 4 during 2005. We are currently preparing a proposal to NH&MRC for 2006 Project Grant funding that will include studies with the rapsyn conditional mouse. We are thankful to the MND Research Institute for allowing us to move forward with the rapsyn conditional mouse project that will help us to define the role of rapsyn in maintaining functional neuromuscular synapses.

Dr Rodney Rietze Queensland Brain Institute
Stimulation of endogenous stem cells to replace lost motor neurones

It has been convincingly demonstrated in all mammals, including man, that the adult brain and spinal cord contain a discrete population of precursor cells that continue to generate new nerve cells throughout adult life. More recent studies have extended these findings to show that the resident precursor cells can be activated following brain injury, causing them to migrate to the damaged area, replace the lost population, and finally reintegrate into the existing circuitry of the brain and spinal cord. Together, these studies highlight the possibility that resident precursor cells can be activated to replace those populations lost during the degenerative process associated with MND.

The challenge now is to determine precisely which precursor cell population(s) are being recruited and how to direct these cells to produce mature cell types. Until recently, progress was impeded because it was impossible to distinguish between different types of precursor cells. However, we have now developed a one step kit, which enables us to determine whether stem cells, or more committed progenitor cells are activated. Using this kit, we have discovered that the infusion of some factors directly into the brain increases stem cell numbers, while others only increase progenitor cell numbers. Knowing this, we can now focus on two distinct (stem and progenitor) cell therapies for the replenishment of lost motor neurones.

Dr Bryce Vissel Garvan Institute of Medical Research
Adeno-associated virus mediated delivery of genes to study and treat spinal cord disorders

The aim of this project is to develop the technologies, techniques and systems to deliver therapeutic genes to the

central nervous system. Recent work by our collaborators Dr. Brian Kaspar and Professor Fred Gage in San Diego revealed that viral mediated delivery of the neurotrophic factor IGF-I greatly reduced the onset of the symptoms in a mouse model of MND. This provides great hope that it will be possible to develop gene therapy approaches for MND.

We are therefore keen to establish the technique of viral mediated gene delivery in the nervous system that we used in the USA. Not only does this technique offer the promise of developing gene therapy, but it also provides us with the technology to investigate the role of a variety of genes in spinal cord and brain function.

The work is directed to establishing the technologies in our lab to underpin future research work. The first step towards this goal was to bring a new colony of mice into Australia that carry a gene which leads to a syndrome in mice similar to that seen in human MND. Once the mice are breeding well at the Garvan we will be able to use them in our own studies and also make them available to anyone in Australia who needs them.

The second aspect of our work has been to develop the viral mediated gene delivery ('gene therapy') techniques in our lab. The major approach that we proposed was to generate and use adeno-associated virus (AAV) mediated gene delivery. AAV is a very effective virus for gene therapy because it seems to deliver 'therapeutic genes' effectively to neurons in the central nervous system without eliciting side effects. We have successfully generated AAV in the lab. We have made the first batch of virus to inject in the central nervous system of mice. Now that has been achieved, we will test the ability of this virus to infect nerve cells in the central nervous system. In particular we will test whether the AAV we have made is capable of delivering a gene called GFP. This is the first step towards establishing the proof-of-principle needed before we commence experiments directed towards gene therapy.

More recently, we also started to generate another recombinant virus called lentivirus. We believe lentivirus holds more promise for gene therapy applications than AAV. Like AAV, the lentivirus can infect the nervous system and deliver therapeutic genes in the central nervous system without eliciting undesirable effects. However, the lentivirus has the strong advantage that it can carry larger therapeutic genes. We have had a little more trouble generating lentivirus at a high enough infectivity ('titre') and we are working vigorously for this goal.

The support of the Institute is an investment. It is establishing the foundations for a great deal of future work.

Prof Jeffrey Zajac University of Melbourne
The neuroprotective capacity of IGF-I in Kennedy's disease

Kennedy's disease, or spinobulbar muscular atrophy

(SBMA), is an inherited form of MND affecting adult males. The cause is a particular type of mutation within the gene for the androgen receptor, a protein that is central to mediating the effects of male sex hormones (androgens). The mutation is an expansion of a 'triplet repeat' (CAG) sequence, which encodes a polyglutamine tract in the translated protein. Research conducted in our laboratory is endeavouring to understand the biological basis for degeneration of motor neurones. The main aim of the current research is to assess insulin-like growth factor I (IGF-I) as a potential neuroprotective agent against degeneration in Kennedy's disease.

Our research is taking two main approaches to answer central questions regarding the potential of IGF-I to protect against Kennedy's disease-induced neurodegeneration. Firstly, we aim to test out the in vivo effect of IGF-I in our transgenic mouse model of Kennedy's disease.

The second approach that we have employed involves the use of in vitro cell expression models. We have stably-transfected cell lines into which we have introduced the androgen receptor (both normal and Kennedy's disease mutant). Our initial investigations have concentrated on interactions between the IGF system and the androgen receptor using transactivation assays. The reporter for this system is a luciferase vector that is highly responsive to androgen treatment in the presence of receptor. Using this system we have found that androgen receptor transactivation is influenced by the IGF-I receptor in both ligand-dependant and -independent fashion. We have tested this effect in different cell types and data would indicate that this effect is also cell-type dependent, although this requires further characterisation. Transactivational ability conferred by the Kennedy's disease-mutant versus normal is reduced. We have preliminary data that demonstrates that the Kennedy's disease mutant receptor loses the potentiation observed with the normal receptor, however, again, this remains to be further characterised. Current research is attempting to confirm and further understand these effects. The next steps of our research will be to investigate further the effect of IGF-I on androgen receptor transactivation to establish the possibility that IGF-I signalling may be compromised in Kennedy's disease. Furthermore, current research is studying gene regulation in cells transfected with normal and Kennedy's disease mutant cells so that we can identify genes that may be involved in motor neurone degeneration.

Lastly, I would like to acknowledge the support of the MND Research Institute for the financial support that we have been granted. It is predominantly due to this generous support that we are able to continue our research into Kennedy's disease. We believe that understanding the role of IGF-I is crucial to this area of research which may eventually translate into a viable therapy for individuals affected by Kennedy's disease.