

**Investigation of RNA-mediated pathogenic pathways in a
Drosophila model of expanded repeat disease**

A thesis submitted for the degree of Doctor of Philosophy, June 2010

Clare Louise van Eyk, B.Sc. (Hons.)

School of Molecular and Biomedical Science, Discipline of Genetics

The University of Adelaide

Table of Contents

Index of Figures and Tables	VII
Declaration	XI
Acknowledgements	XIII
Abbreviations	XV
<i>Drosophila</i> nomenclature	XV
Abstract	XIX
Chapter 1: Introduction	1
1.0 Expanded repeat diseases.....	1
1.1 Translated repeat diseases	2
1.1.1 Polyglutamine diseases	2
Huntington’s disease.....	3
Spinal bulbar muscular atrophy (SBMA)	3
Dentatorubral-pallidoluysian atrophy (DRPLA)	4
The spinal cerebellar ataxias (SCAs).....	4
1.1.2 Pathogenesis and aggregate formation	7
1.1.3 Polyalanine diseases	8
1.2 Untranslated expanded repeat diseases	9
1.2.1 Dominant untranslated expanded repeat diseases	9
1.3 One pathogenic pathway or many?.....	15
1.4 A <i>Drosophila</i> model of polyglutamine and RNA toxicity	17
1.4.1 Expanded CAG and CAA-encoded polyglutamine tracts are toxic.....	19
1.4.2 Polyleucine peptides show distinct toxicity in the <i>Drosophila</i> eye	22
1.4.3 A closer look at RNA pathogenesis.....	23
Chapter 2: Materials and Methods	27
2.1 Materials.....	27
2.2 Methods	34
Chapter 3 – The RNA editing hypothesis	47
3.0 Roles for RNA as a pathogenic agent.....	47
3.1 RNA editing: roles and consequences	48
3.2 ADAR editing and disease	51
3.3 <i>Drosophila</i> Adar	52

3.4 A role for RNA editing in the dominant expanded repeat diseases?	53
3.5 Investigation of the effects of altering Adar expression in <i>Drosophila</i> expressing expanded repeat RNA	54
3.6 Investigation of the editing status of ectopically expressed CAG and CAA repeat tracts in <i>Drosophila</i>	56
3.7 Investigation of the effect of expression of CAG repeat RNA on editing of endogenous Adar editing targets in <i>Drosophila</i>	57
3.8 Summary of investigation of RNA editing as a component of CAG repeat RNA pathogenesis.....	59

Chapter 4: Identifying pathogenic pathways of expanded repeat disease by proteomic analysis 61

4.1 Identification of proteomic changes in neuronal cells expressing expanded repeat tracts	63
4.2 Identification of proteins altered in <i>Drosophila</i> expressing rCAG or rCUG repeats pan-neuronally	66
4.3 Identification of proteins altered in flies expressing rCAG or rCUG repeats compared to rCAA repeats.....	68
4.4 Evidence for involvement of nuclear transport in expanded repeat disease pathogenesis.....	70
4.5 Summary of proteomic changes elicited by expression of CAG and CUG repeats in neurons of <i>Drosophila</i>	75

Chapter 5: Identifying pathogenic pathways of expanded repeat disease by microarray analysis 77

5.1 Identification of transcriptional changes in neuronal cells expressing expanded repeat tracts: microarray experiment 1	78
5.2 Validation of cellular changes by independent microarray experiment: Microarray experiment 2	79
5.3 Comparison of microarray experiment 1 and 2	80
5.4 Gene ontology analysis of genes altered in rCAG and rCUG repeat-expressing flies compared to <i>elav>rCAA</i> flies	82
5.5 Gene ontology analysis of genes altered in rCAG and rCUG repeat-expressing flies compared to <i>elav>+</i> flies.....	86
5.6 Analysis of genes significantly altered in both microarray experiment 1 and 2 ...	88
5.7 Analysis of genes significantly altered in each microarray experiment.....	91

5.7.1 Genes changed in rCAG repeat-expressing flies compared to both <i>elav>rCAA</i> and <i>elav>+</i>	92
5.7.2 Genes changed in rCUG repeat-expressing flies compared to <i>elav>rCAA</i> and <i>elav>+</i>	94
5.7.3 Genes changed in both rCAG and rCUG repeat-expressing flies compared to <i>elav>rCAA</i>	96
5.7.4 Genes changed in both rCAG and rCUG repeat-expressing flies compared to <i>elav>+</i>	100
5.8 Summary of results from microarray analysis	103
Chapter 6: Genetic verification of candidates from microarray analysis.....	105
6.1 Modification of translated repeat phenotypes by cytoskeletal and trafficking components	108
6.2 Modification of translated repeat phenotypes by mod(mdg4), mGluRA and CG5669.....	112
6.3 Modification of translated repeat phenotypes by altering levels of <i>mbl</i> and <i>mef2</i>	115
6.4 Investigation of sequence-dependent interactions between expanded repeat RNA and Mef2, Mbl and Mod(mdg4) in <i>Drosophila</i>	117
6.5 Evidence of a role for <i>MBNL1</i> in expanded repeat disease pathogenesis.	119
6.6 Summary of results from genetic screen of microarray candidates.....	124
Chapter 7: Spinocerebellar ataxia 10: a unique untranslated repeat disease?127	
7.1 Modelling SCA10 in <i>Drosophila</i>	128
7.2 Investigation of cellular localisation of expanded rAUUCU repeats.....	130
7.3 Identification of transcriptional changes in neuronal cells resulting from expression of SCA10 repeats	132
7.4 Investigation of common transcriptional changes in flies expressing rAUUCU, rCAG and rCUG repeats.....	134
7.4.1 Common transcriptional changes in <i>Drosophila</i> expressing rCAG, rCUG and rAUUCU expanded repeats compared to <i>elav>rCAA</i>	137
7.4.2 Common transcriptional changes in <i>Drosophila</i> expressing rCAG, rCUG and rAUUCU expanded repeats compared to <i>elav>+</i>	139
7.5 Investigation of a role for the Akt/GSK3- β signalling pathway in expanded repeat disease pathogenesis	142

7.5.1 Evidence for alterations to Akt/GSK3- β signalling in the expanded repeat diseases.....	142
7.5.2 Effect of altering expression of Akt and GSK3- β in our <i>Drosophila</i> model of expanded repeat disease pathogenesis.....	145
7.6 Validation of an interaction between rAUUCU RNA and mod(mdg4), mbl and mef2 in <i>Drosophila</i>	149
7.7 Further investigation of a role for MBNL1 in expanded repeat pathogenesis....	151
7.8 Summary of <i>Drosophila</i> model for SCA10	153
Chapter 8: Discussion	155
8.1 Summary of results	155
8.2 Implications for expanded repeat disease pathogenesis.....	157
8.3 Limitations of the <i>Drosophila</i> model	158
8.4 Further Experiments.....	160
Appendices.....	163
Appendix A.....	163
Appendix B.....	166
Appendix C	209
References.....	239

Index of Figures and Tables

Chapter 1

Table 1.1: Polyglutamine diseases.....	2
Table 1.2: Dominant untranslated repeat diseases.....	10
Figure 1.1: The structure of the <i>Drosophila</i> eye.....	18
Figure 1.2: Tissue-specific expression in <i>Drosophila</i> using the <i>UAS-GAL4</i> expression system.....	19
Figure 1.3: A <i>Drosophila</i> system to investigate RNA toxicity as a component of polyglutamine pathogenesis.....	20
Figure 1.4: Investigation of the effects of expressing polyglutamine in the <i>Drosophila</i> eye.....	21
Figure 1.5: Investigation of the effects of expressing polyleucine in the <i>Drosophila</i> eye.....	23
Figure 1.6: Expression of up to four transgene insertions of rCAG and rCUG repeats does not disrupt the external structure of the <i>Drosophila</i> eye.....	25

Chapter 2

Table 2.1: Lines to drive expression using the <i>UAS-GAL4</i> system.....	33
Table 2.2: Candidate gene lines used in this study.....	33

Chapter 3

Figure 3.1: Proposed outcomes of site-specific and promiscuous RNA editing.....	50
Figure 3.2: Effect of expression of rCAA, rCUG or rCAG repeats in a heterozygous Adar null background.....	55
Figure 3.3: Effect of reducing Adar levels on <i>Drosophila</i> eye phenotypes elicited by expression of CAG or CAA-encoded polyglutamine or CUG-encoded polyleucine...	56
Figure 3.4: There is no detectable editing of pure CAG or CAA repeats expressed pan-neuronally in <i>Drosophila</i>	57
Figure 3.5: There is no detectable decrease in editing of normal Adar targets when rCAG repeat transcripts are expressed throughout the nervous system of <i>Drosophila</i>	58

Chapter 4

Figure 4.1: Obtaining <i>Drosophila</i> expressing untranslated repeats pan-neuronally for microarray and proteomic analysis.....	62
Figure 4.2: Rationale for use of this <i>Drosophila</i> model for investigation of early changes in expanded repeat disease.....	62
Figure 4.3: Overview of 2D-DIGE experiment procedure and analyses performed. .	64
Figure 4.4: Summary of changes in protein abundance detected in flies expressing rCAG, rCUG and rCAA RNA when compared to <i>elav</i> >+ control flies.....	65
Figure 4.5: Modification of phenotypes resulting from expression of translated CUG, CAG and CAA repeats by insertion of a Minos element upstream of DPxr2540-1 or knocking down expression of Alcohol dehydrogenase.....	68
Figure 4.6: Summary of changes in protein abundance detected when rCAG and rCUG repeat expressing flies were compared directly to <i>elav</i> >rCAA flies.....	69
Figure 4.7: Co-expression of an RNAi construct targeting Nup62 enhances CAG and CUG but not CAA eye phenotypes.....	72
Figure 4.8: Overexpression of Nup62 in the <i>Drosophila</i> eye suppresses both polyglutamine and polyleucine eye phenotypes.....	74

Chapter 5

Figure 5.1: Overview of microarray experiments.....	79
Figure 5.2: comparison of the two <i>elav</i> -GAL4 driver lines used in this study.....	80
Figure 5.3: Overview of total number of genes significantly altered in each microarray experiment and genes which were significantly altered in both experiments.....	81
Figure 5.4: Gene ontology analysis of genes which were significantly altered in <i>Drosophila</i> expressing rCAG or rCUG RNA pan-neuronally.....	84
Table 5.1: Common changes for <i>elav</i> >rCAG compared to <i>elav</i> >rCAA in experiment 1 and 2.....	88
Table 5.2: Common changes for <i>elav</i> >rCUG compared to <i>elav</i> >rCAA in experiment 1 and 2.....	89
Table 5.3: Common changes for <i>elav</i> >rCAG compared to <i>elav</i> >+ in experiment 1 and 2.....	90
Table 5.4: Common changes for <i>elav</i> >rCUG compared to <i>elav</i> >+ in experiment 1 and 2.....	90
Table 5.5: Genes of particular interest for <i>elav</i> >rCAG identified in microarray experiment 1.	93

Table 5.6: Genes of particular interest for <i>elav>rCAG</i> identified in microarray experiment 2.	93
Table 5.7: Genes of particular interest for <i>elav>rCUG</i> identified in microarray experiment 1.	94
Table 5.8: Common transcriptional changes in <i>elav>rCAG</i> and <i>elav>rCUG</i> compared to <i>elav>rCAA</i> in experiment 1.	97
Table 5.9: Common transcriptional changes in <i>elav>rCAG</i> and <i>elav>rCUG</i> flies compared to <i>elav>rCAA</i> in experiment 2.....	99
Table 5.10: Common transcriptional changes in <i>elav>rCAG</i> and <i>elav>rCUG</i> compared to <i>elav>+</i> in experiment 1.....	102
Table 5.11: Common changes for <i>elav>rCAG</i> and <i>elav>rCUG</i> compared to <i>elav>+</i> in experiment 2..	102

Chapter 6

Figure 6.1: Method to generate <i>Drosophila</i> expressing polyglutamine or polyleucine in the eye along with an RNAi construct targeting a candidate gene.	106
Table 6.1: Overview of genes and alleles tested for genetic interaction with expanded repeats.	107
Figure 6.2: Effect of altering levels of cytoskeletal and trafficking components on polyglutamine and polyleucine eye phenotypes in <i>Drosophila</i>	111
Figure 6.3: Modification of polyglutamine and polyleucine eye phenotypes by altering levels of Mod(mdg4), mGluRA and CG5669.	114
Figure 6.4: Modification of polyglutamine and polyleucine eye phenotypes in <i>Drosophila</i> by altering levels of Mbl and Mef2.....	117
Figure 6.5: Mod(mdg4) and Mef2 show a sequence-dependent interaction with CUG repeat RNA in <i>Drosophila</i>	119
Figure 6.6: Co-expression of CUG-encoded polyleucine enhances the MBNL1 eye phenotype.	121
Figure 6.7: Overexpression of MBNL1 enhances both CAA and CAG-encoded polyglutamine eye phenotypes in <i>Drosophila</i>	122
Figure 6.8: Expression of expanded untranslated CAG, CUG and CAA repeats in <i>Drosophila</i> overexpressing MBNL1.....	123
Table 6.2 Summary of results from genetic screen of microarray candidates.....	126

Chapter 7

Figure 7.1: Constructs generated to model SCA10 pathogenesis in <i>Drosophila</i>	129
Table 7.1: rAUUCU repeat constructs injected into <i>Drosophila</i>	129
Figure 7.2: Expression of up to four transgene insertions of an expanded ATTCT repeat does not alter the exterior appearance of the <i>Drosophila</i> eye.....	130
Figure 7.3: rAUUCU repeat-containing transcripts form aggregates in a sub-set of <i>Drosophila</i> cells.....	132
Figure 7.4: Gene ontology analysis of transcripts altered in <i>Drosophila</i> expressing rAUUCU RNA compared to both <i>elav>+</i> and <i>elav>rCAA</i>	135
Table 7.2: Percent of transcripts commonly altered in <i>Drosophila</i> expressing rCAG or rCUG repeats and rAUUCU repeats pan-neuronally.....	135
Figure 7.5: Comparison of transcripts altered in <i>Drosophila</i> expressing rCAG, rCUG and rAUUCU repeats pan-neuronally.....	136
Table 7.3: Changes common to <i>elav>rAUUCU</i> , <i>elav>rCAG</i> and <i>elav>rCUG</i> flies compared to <i>elav>rCAA</i>	139
Table 7.4: Changes common to <i>elav>rAUUCU</i> , <i>elav>rCUG</i> and <i>elav>rCAG</i> flies compared to <i>elav>+</i>	141
Figure 7.6: Alteration to activity of the Akt/GSK3- β signalling pathway can explain a number of the changes observed in microarray analysis of flies expressing rCAG, rCUG and rAUUCU repeats in the nervous system.	143
Figure 7.7: Investigation of a role for the Akt/GSK3- β signalling pathway in pathogenesis in poly-leucine and poly-glutamine-expressing <i>Drosophila</i>	147
Figure 7.8: Investigation of a role for the Akt/GSK3- β signalling pathway in RNA-mediated pathogenesis in <i>Drosophila</i>	149
Figure 7.9: Genetic validation of candidates from microarray analysis of <i>Drosophila</i> expressing rAUUCU RNA.	151
Figure 7.10: Interaction of rAUUCU repeats with MBNL1 in the <i>Drosophila</i> eye.....	152

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Clare van Eyk

Acknowledgements

I would like to thank my supervisor, Rob Richards, for giving me support and advice as well as plenty of independence throughout this project. I am also very grateful to my co-supervisor, Louise O'Keefe, for the many helpful conversations in the fly room and for encouraging me to stay positive. Thanks also to the various people who have assisted in aspects of this project; particularly to Jo Milverton for performing microinjections and to Gareth Price for assistance with the microarray studies.

To all Richards lab and Genetics Discipline members, past and present, thanks for making the lab and the building such a fun and rewarding working environment. Special thanks to Saumya Samaraweera and Amanda Choo, for distracting me when I needed it most, and to Sonia Dayan for always taking the time to chat, even when you had a million things on the go.

I am also grateful to all of my family and friends who have given me love and support in too many ways to mention. In particular, I would like to thank my parents, Helen and Bernie van Eyk, who have always encouraged us to aim high in whatever we choose to do. Also to Simon Wells: for intellectual debate (and occasionally admitting that I'm right).

Abbreviations

°C: degrees Celsius

%: percentage

μA: microamps

μg: micrograms

μL: microlitre

A: Adenosine

ADAR: Adenosine deaminase acting on RNA

ADD1: Adducin 1

ALS: Amyotrophic lateral sclerosis

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionateglutamate

AR: Androgen receptor

ATP: adenosine triphosphate

BDNF: Brain-derived neurotrophic factor

bp: base pairs

C: cytosine

cDNA: complementary DNA

CLCN-1: Chloride channel 1

CNS: central nervous system

CUG-BP: CUG binding protein

Cy: cyanine

CYFIP2: Cytoplasmic FMR1 interacting protein 2

da: daughterless

DAPI: 4'-6-Diamidino-2-phenylindole

DEPC: diethyl pyrocarbonate

DIGE: differential in-gel electrophoresis

DM: Myotonic dystrophy

DMF: dimethyl formamide

DMPK: Dystrophia myotonica protein kinase

DNA: deoxyribonucleic acid

DRPLA: Dentatorubral-pallidoluysian atrophy

dsRNA: double-stranded RNA

DTT: dithiothreitol

EDTA: ethylene diamine tetra-acetic acid

elav: embryonic lethal abnormal vision

emPAI: exponentially modified protein abundance index
ESI: electro-spray ionisation
FA: formic acid
FMR1: Fragile X mental retardation 1
FMRP: Fragile X mental retardation protein
FXTAS: Fragile X tremor-ataxia syndrome
G: guanosine
GABA: gamma-aminobutyric acid
GFP: green fluorescent protein
GluCl- α : Glutamate-gated chloride channel α
GluR-B: AMPA receptor subunit B
GMR: glass multimer reporter
GSK3: Glycogen synthase kinase 3
HD: Huntington's disease
HDL-2: Huntington's disease-like-2
hnRNP: Heterogenous ribonucleoprotein
Hr38: Hormone receptor-like in 38
Hts: Hu-li tai shao
HTT: Huntingtin
I: inosine
Insc: Inscuteable
IPTG: isopropyl β -D-1-thiogalactopyranoside
IR: Insulin receptor
JPH3: Junctophilin-3
kb: kilobase
kDa: kilodalton
KLHL1: Kelch-like 1
LB: Luria broth
M: Molar
Mbl: Muscleblind (*Drosophila*)
MBNL: Muscleblind-like
MEF: Myocyte enhancing factor
mg: milligrams
mGluRA: metabotropic glutamate receptor A
miRNA: microRNA

MJD: Machado Joseph disease
mL: millilitres
mM: millimolar
MQ: MilliQ™ purified water
mRNA: messenger RNA
MS: mass spectrometry
MS/MS: tandem mass spectrometry
MTMR1: Myotubularin-related protein 1
TOR: target of rapamycin
ng: nanograms
NGF: Nerve growth factor
NL IPG: non-linear immobilised pH gradient
NMDAR: N-methyl-D-aspartate receptor
NPC: Nuclear pore complex
dNTP: deoxyribonucleoside triphosphate
NUP: nucleoporin
NUR77: Nuclear receptor 77
OPMD: Oculopharyngeal muscular dystrophy
para: paralytic sodium channel
PBS: phosphate buffered saline
PBST: PBS + Tween
PKR: RNA regulated protein kinase
pmol: picomole
polyQ: polyglutamine
polyL: polyleucine
PP2A: Protein phosphatase 2A
PP2R2B: PP2A regulatory subunit 2B
PSF: Poly-pyrimidine-tract associated splicing factor
Q: glutamine
RISC: RNA-induced silencing complex
RNA: ribonucleic acid
RNAi: RNA interference
ROS: reactive oxygen species
Rp49: Ribosomal protein 49
rpm: revolutions per minute

RyR: Ryanodine receptor
SAP: Shrimp alkaline phosphatase
SBMA: Spinal bulbar muscular atrophy
SCA: Spinocerebellar ataxia
SDS: sodium dodecyl sulphate
SERCA: Sarcoplasmic/endoplasmic reticulum calcium ATPase
Sgg: Shaggy
siRNA: small interfering RNA
SOC: super-optimal broth with catabolite repression
SSC: saline sodium citrate
T: thymine
TAE: tris-acetate EDTA
TBE: tris-borate EDTA
TBP: TATA-box binding protein
TNNT: Troponin T
TudorSN: Tudor Staphylococcal nuclease
U: uracil
UAS: upstream activation sequence
UTR: untranslated region
UV: ultraviolet
V: Volts
VDRC: Vienna *Drosophila* RNAi Centre
X-gal: 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
YAC: yeast artificial chromosome

***Drosophila* nomenclature**

Throughout this thesis, *Drosophila* genes are represented by italicised lower-case text (for example “*htt*”), RNAs are represented by lower-case non-italicised text (for example “htt”) and proteins are represented by non-italicised text with a capital first letter (for example “Htt”).

Abstract

Expansion of a repeat sequence beyond a pathogenic range has been identified as the cause of a group of neurodegenerative diseases known as the expanded repeat diseases. Disease-associated repeat tracts have been found both within the coding region of genes, such as the CAG repeat coding for polyglutamine, or within non-coding regions. Despite the identification of the mutation involved in these diseases, the mechanism by which this type of mutation leads to cell death remains unclear. There is a substantial amount of evidence to suggest that RNA-mediated toxicity plays a role in pathogenesis of both the polyglutamine diseases and the untranslated dominant expanded repeat diseases. A common feature of the expanded repeats involved in each of these diseases is the ability of the repeat-containing RNA to form a hairpin secondary structure and therefore it has been predicted that similar mechanisms may be responsible for initiating cellular dysfunction and death in each case. This study uses a *Drosophila* model to investigate the intrinsic, RNA-mediated toxicity of three repeat sequences (CUG, CAG and AUUCU) associated with degeneration in human disease. Using a combination of hypothesis-driven and non-biased approaches, early changes elicited in response to neuronal expression of these expanded repeat tracts have been investigated. A hypothesis of a role for RNA editing in CAG repeat pathogenesis was explored using this *Drosophila* model. Microarray and proteomic approaches were also utilised to identify pathways which are perturbed by the expression of these repeat sequences. The results described in this thesis demonstrate a degree of sequence- and context-independent toxicity of expanded repeat RNA in this model, suggesting that this kind of effect may also be a component of pathogenesis in the disease situation. Pathways commonly perturbed in response to expression of these RNA species may represent particularly valuable therapeutic targets, since preventing this type of effect could provide positive outcomes in a number of diseases.