



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Over-Expression of SNAP-25 in Neuronal Tissues

A Thesis Submitted to the University of Dublin for the Degree of Doctor
of Philosophy

By

Alex McKee

01173111

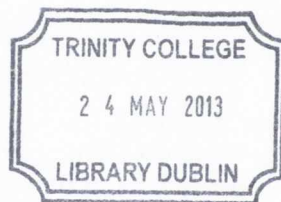
Smurfit Institute of Genetics

Trinity College Dublin

Dublin 2

Ireland

March 2011



Thesis 9850

Declaration

This thesis has not been previously submitted to this or any other university for examination for a higher degree. The work presented here is entirely my own, except where noted. This thesis may be made available for consultation within the university library. It may be photocopied or lent to other libraries for the purpose of consultation.

Acknowledgements

First, and most importantly, I would like to express my most sincere gratitude to my supervisors, Professor Jane Farrar and Professor Pete Humphries. Both have been incredibly supportive during my time in the lab and I owe my deepest thanks for their unending encouragement, understanding and patience – it would be difficult to overstate what an honour it is to have worked for them.

This thesis would not have been possible without the help and assistance of all members of the Humphries/Farrar lab both past and present, as well as all the wonderful people in the genetics department who have helped me out over the years - they are too many to mention, but in particular I would like to thank Dr Paul Kenna for always having time to help with ERGs and for teaching me the subretinal injection techniques and Dr Naomi Chadderton for invaluable help with the virus work. Dr Arpad Palfi and Dr Mary O'Reilly both deserve a special mention as postdoctoral researchers who I “adopted” as my supervisors – they gave up so much time to help me and have been fantastic mentors and friends, providing excellent advice, constructive criticism and good company.

I am grateful to the technical and administrative staff, in particular Brenda, Paul and Dave in the prep room and the staff who look after all the mice and rats.

I am especially thankful to my parents for their unending support throughout my lengthy education – they raised me, taught me, supported me and loved me through thick and thin and I'm so happy that this thesis will make them proud.

Lastly and above all, I wish to thank my wife, Rachel, who has been with me right since the very start of this long chapter of my life. For her constant love and support, for inspiring me, for making me believe in myself. I dedicate this thesis to you.

Summary

The work described within this PhD thesis represents many years work on several different projects. In brief, SNAP-25 was identified as a downregulated candidate gene of interest from a comparative microarray study in the rat hippocampus. To understand the deregulation of this genes I over-expressed it to better elucidate its function in both retina and the hippocampus.

A key method utilized in this thesis involved the generation of pure high titre recombinant adeno-associated virus (rAAV). These undoubtedly provide the most useful means of targeted gene delivery to neuronal tissues, and the proof of principle experiments described in chapters 3, 4 and 5 provide an excellent example of gene delivery to both the retina and brain. I have shown that rAAV vectors can be generated as pure, high titre preps and routinely delivered to both the retina and hippocampus, that the transduced cells can be sorted using a GFP reporter gene allowing for precise quantification of the changes in gene expression induced by the rAAV transgene.

Chapter 3 describes the generation and characterization of the viruses used in this thesis, and chapters 4 and 5 show efficient delivery to the target tissues and the resulting effects. Chapter 4 describes the effects of SNAP-25 over-expression in the retina, and the unexpected finding that it induces photoreceptor cell death. Chapter 5 provides a detailed analysis at a functional and molecular level of SNAP-25 over-

expression in the rat hippocampus. I describe the behavioural changes associated with the increase in SNAP-25 levels, in particular the significant defects in memory and learning.

Nearly every man who develops an idea works at it up to the point where it looks impossible, and then gets discouraged. That's not the place to become discouraged.

Thomas Edison

Table of Contents:

Declaration	III
Acknowledgements	IV
Summary	VI

Chapter 1 – General Introduction	page number
1.1 Introduction	3
1.2 The Eye	3
1.3 The anatomy of the retina and photoreceptor cells	5
1.3.1 The retinal pigment epithelium (RPE)	5
1.3.2 Photoreceptors	6
1.3.3 Outer nuclear layer (ONL)	7
1.3.4 Outer plexiform layer (OPL)	8
1.3.5 Inner nuclear layer (INL)	8
1.3.6 Inner plexiform layer (IPL)	9
1.3.7 Ganglion cell layer (GCL)	9
1.3.8 Nerve fibre layer	9
1.3.9 Internal limiting membrane	10
1.4 Visual transduction	10
1.5 The Hippocampus	12
1.5.1 The hippocampus and Memory	14
1.5.2 The Morris water maze	16

1.5.3 Contextual Fear Conditioning	17
1.5.4 Prepulse Inhibition of Startle (PPI)	18
1.6 Synaptosome associated protein of 25kDa (SNAP-25)	18
1.6.1 The Coloboma mouse as a model for ADHD	19
1.7 SNAP-25 in the retina	21
1.8 Adeno-associated virus (AAV)	22
1.8.1 Vectors based on adeno-associated virus	24
1.8.2 rAAV vectors for retinal gene delivery	28
1.8.3 rAAV vectors for hippocampal gene delivery	30
1.8.4 Use of rAAV vectors in animal models of neurological disorders	31
1.8.5 Safety and biodistribution of rAAV vectors	32
1.9 Objectives of this thesis	34

Chapter 2 – Materials and Methods

2.1 General materials and reagents	37
2.1.1 Reagents	37
2.1.2 Solutions	38
2.1.3 Materials and reagents for tissue culture	38
2.1.4 Materials and methods for RNA study	39

2.1.5 Animals	39
2.2 General molecular methods	40
2.2.1 Determination of DNA and RNA concentrations	
using UV spectrophotometer	40
2.2.2 Agarose Gel Electrophoresis	40
2.2.3 Phenol-Chloroform extraction and ethanol	
precipitation of nucleic acids	40
2.2.4 XL-1 Blue E.Coli competent cells preparation	41
2.2.5 Transformation of XL-1 Blue competent cells	41
2.2.6 Plasmid mini prep boiling method	41
2.2.7 Plasmid ligations	42
2.2.8 Oligonucleotide design	42
2.2.9 Polymerase chain reaction (PCR)	43
2.2.10 Restriction enzyme digestions	43
2.2.11 Maxi Preps	43
2.2.12 DNA sequencing	44
2.3 Tissue culture methods	44
2.3.1 Cell culture maintenance	44
2.3.2 Cell Passaging	44
2.3.3 Haemocytometer cell counting	45
2.3.4 Cryopreservation of cells	45
2.3.5 Cell transfection protocol using Lipofectamine2000	45

2.4 Adeno-Associated Virus (AAV)	
2.4.1 AAV production	46
2.4.2 Caesium chloride purification	47
2.4.3 Determination of genomic viral titre	48
2.5 Fluorescent activated cell sorting (FACS)	48
2.6 Electroretinogram (ERG) analysis	49
2.7 Western blot analysis	50
2.7.1 Protein purification	50
2.7.2 Protein separation by polyacrylamide gel electrophoresis (PAGE)	50
2.7.3 Transfer of separated protein lysates to nitrocellulose membrane	51
2.8 Statistical Analysis	52

Chapter 3 – Generation of recombinant Adeno-Associated Virus

3.1 Introduction	55
3.2 Methods	60
3.2.1 Plasmids	60
3.2.2 AAV production and triple transfection method	60

3.2.3 Titration of genomic AAV particles using RT-PCR	62
3.2.4 In vitro analysis of AAV	62
3.2.5 Subretinal injection	63
3.2.6 Quantitative real time RT-PCR	63
3.3 Results	64
3.3.1 Generation and purification of high titre rAAV	64
3.3.2 rAAVs transduce HEK293 cells <i>in vitro</i>	66
3.3.3 Calculating genomic titres of rAAVs	67
3.3.4 rAAVs transduce photoreceptor cells <i>in vivo</i>	68
3.3.4 Transduced photoreceptor cells overexpress SNAP-25	71
3.3.6 Optimization of stereotaxic injection into the hippocampus	72
3.3.7 Co-expression of transgenes using a mixture of rAAV vectors	74
3.4 Discussion	76
3.5 Conclusions	79

Chapter 4 – AAV mediated overexpression of SNAP-25 in the mouse retina

4.1 Introduction	83
4.2 Methods	84
4.2.1 Subretinal injection	84
4.2.2 Electroretinogram (ERG)	84

4.2.3 Fluorescent activated cell sorting (FACS) and analysis of	
gene expression in sorted cells	85
4.2.4 Histological analysis	85
4.2.5 Neurochemical analysis	86
4.3 Results	
4.3.1 AAV mediated SNAP-25 overexpression in the	
mouse retina	87
4.3.2 SNAP-25 overexpression results in impaired ERG	
responses	90
4.3.3 SNAP-25 overexpression induces photoreceptor	
cell death	90
4.3.4 SNAP-25 overexpression does not alter retinal	
neurochemistry	92
4.4 Discussion	94
4.5 Conclusions	96

Chapter 5 – AAV mediated overexpression of SNAP-25 in the rat hippocampus

5.1 Introduction	101
5.2 Methods	103
5.2.1 Animals	103

5.2.2 Site directed injection to the dorsal hippocampus	103
5.2.3 Hystological staining, immunohistochemistry & <i>In situ</i> hybridization	104
5.2.4 Fluorescent-Activated Cell Sorting (FACS) and Quantitative RT-PCR	105
5.2.5 <i>In vivo</i> microdialysis	106
5.2.6 Neurotransmitter analysis	107
5.2.7 Paired Pulse Facilitation	108
5.2.8 Open field assessment	109
5.2.9 Water maze paradigm	109
5.2.10 Passive avoidance conditioning	110
5.2.11 Pre pulse inhibition of startle	110
5.2.12 Contextual Fear Condidtioning	111
5.3 Results	
5.3.1 Site-directed injection of rAAV/SNAP-25 to the dorsal hippocampus results in enduring overexpression of SNAP-25	111
5.3.2 AAV/SNAP-25-mediated SNAP-25 overexpression results in increased glutamate release	117
5.3.3 AAV/SNAP-25-mediated SNAP-25 overexpression in the dorsal hippocampus selectively impairs	

spatial learning	118
5.3.4 Ex Vivo Electrophysiological Analyses of AAV-injected Hippocampi	124
5.4 Discussion	126
5.5 Conclusions	131
Chapter 7 – Conclusions and Future Studies	135
References	139

List of Figures and Tables:

Chapter 1

- 1.1 The Eye
- 1.2 Cellular Organisation of the retina
- 1.3 Basic Structure of Photoreceptor cells
- 1.4 Cross section of immunostained mouse retina
- 1.5 Phototransduction cascade
- 1.6 Schematic diagram of the human brain
- 1.7 Schematic diagram of the rat Brain and Hippocampus
- 1.8 Wild type AAV genome
- 1.9 Life cycle of AAV
- 1.10 rAAV production using the triple transfection procedure
- 1.11 Recombinant vectors based on different AAV serotypes

Chapter 2

2.1 pAAV-IRES-hrGFP vector map

Chapter 3

- 3.1 Wild type single stranded genome of AAV
- 3.2 Life cycles of AAV
- 3.3 Generating rAAV particles using the triple transfection protocol
- 3.4 An array of recombinant vectors based on different AAV serotypes
- 3.5 AAV constructs
- 3.6 Validation of AAV infectivity and transgene expression in HEK293 cells
- 3.7 Expression of AAV/SNAP-25 in HeLa cells
- 3.8 Calculation of genomic titre using quantitative real time RT-PCR
- 3.9 GFP expression in whole mouse retina
- 3.10 SNAP-25 subretinal injections
- 3.11 Quantification of viral SNAP-25 expression by RTPCR
- 3.12 AAV mediated eGFP expression in mouse brain 4 weeks post injection
- 3.13 Off target AAV mediated eGFP expression in mouse brain
- 3.14 Expression of DS-Red-Express2 and eGFP following co-administration of two AAV vectors.
- 3.15 Flow cytometry analysis of dissociated rat brains

Chapter 4

- 4.1 AAV constructs used in chapter 4
- 4.2 hrGFP expression in rAAV injected retinas
- 4.3 FACS sorting of hrGFP-labelled retinal cells
- 4.4 Quantitative RT-PCR analysis of SNAP-25 expression in transduced retinal cells.
- 4.5 ERG responses in AAV-injected animals
- 4.6 Retinal Histology

- 4.7 Quantifying ONL thickness in retinal sections
- 4.8 Neurochemical analysis in the retina

Chapter 5

- 5.1 Brain atlas diagram showing site of stereotaxic injection
- 5.2 Change in body weight
- 5.3 Representative fluorescent brain sections from rAAV injected rats
- 5.4 Coronal sections through the rat dorsal hippocampus
- 5.5 FACS sorting and RT-PCR analysis of GFP-labelled cells
- 5.6 Neurochemical analysis of samples collected by microdialysis
- 5.7 Spatial learning in the Morris water maze
- 5.8 Water maze 24 hour recall
- 5.9 Swim speed across the 4 training sessions
- 5.10 Contextual fear conditioning
- 5.11 Exploratory behaviour in the open field
- 5.12 Passive avoidance training
- 5.13 Prepulse inhibition of startle
- 5.14 Paired Pulse Facilitation

Chapter 1

General Introduction

1.1 Introduction

The primary purpose of this Ph.D thesis was to effectively deliver and over-express the the SNAP-25 gene in the hippocampus and the retina, and to determine its effect on memory and learning. SNAP-25 was initially identified as an interesting candidate gene for this study after being significantly down-regulated in a comparative microarray project, and my work focused on over-expressing this gene to evaluate the significance of the observed decrease in expression. In doing so, this might increase our comprehension and knowledge of the complex molecular pathways that exist in the neurons.

The purpose of this introductory chapter is to give an overview of the structure and function of the hippocampus, the retina, and SNAP-25. The clinical features and pathology of neurodegenerative disorders are also discussed here and will provide a background to the studies discussed in subsequent chapters of the thesis. An overview will also be given of the common methods used in the identification of the genes associated with both neurodegeneration, and memory and learning, and potential avenues that can be explored in the development of therapies.

1.2 The Eye

Put simply, eyes are organs that detect light. The simplest eyes in unicellular organisms detect if surroundings are light or dark, whereas in higher animals with complex retinas, light is translated into electrical and neurochemical signals, distinguishing shapes and colours and enabling vision. The first proto-eyes evolved some 540 million years ago and more advanced eyes are now found in 96% of animal species. The eye is a delicate sense organ contained within a framework of connective tissues situated in the anterior part of the orbital cavity. It is an elongated sphere consisting of three layers – the sclera, the choroid and the retina (figure 1.1). The sclera is an opaque white protective coat, continuous with the cornea, a transparent layer at the front of the eye that provides the

first refractive media which bends light onto the retina. The choroid is the middle layer containing the vasculature which supplies blood to the photoreceptors. The inner neural layer, the retina, is a complex multistructured layer lining the back of the eye. At the front of the eye the choroid thickens and forms the iris, a ring shaped ciliary body, which becomes a thin, circular muscular diaphragm regulating the opening of the eye, the pupil. The lens is attached to the ciliary body by ligaments that hold and adjust its shape to focus light onto the retina. The lens also divides the cavity of the eye into two chambers. The aqueous humour is the watery solution contained within the chamber between the cornea and the lens and the vitreous humour is a viscous gelatinous solution filling the larger cavity behind the lens. The optic nerve is situated at the back of the eye and transmits nerve impulses from the retina to the brain.

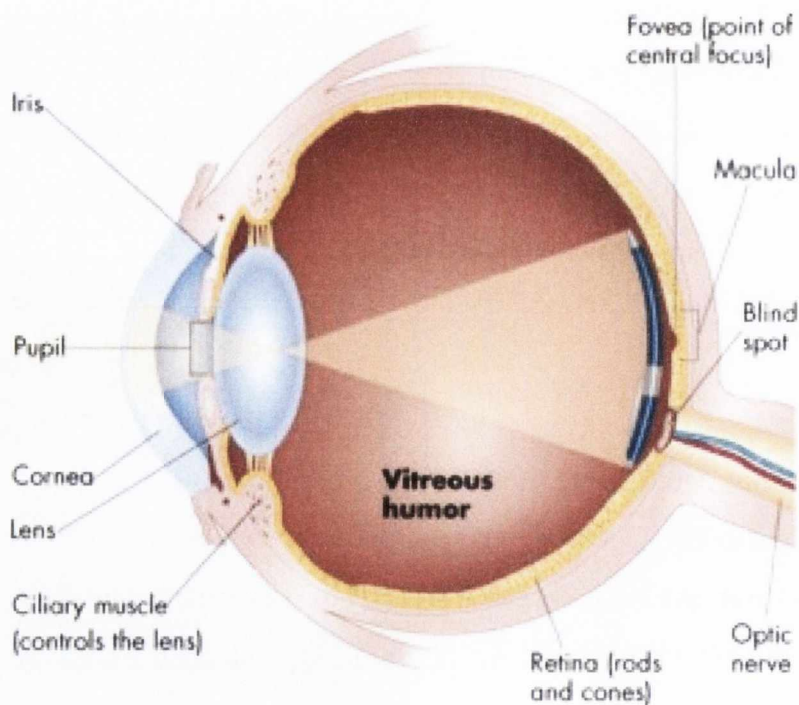


Figure 1.1: Schematic diagram of the human eye.

1.3 The anatomy of the retina and photoreceptor cells

The retina is a thin transparent neuronal layer, consisting of several layers containing many types of neural cells. The individual layers of the retina can be visualized by microscopy and include the retinal pigment epithelium (RPE), outer nuclear layer, outer pleiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fibre layer and internal limiting membrane (figure 1.2). The centre of the retina is the optic nerve head.

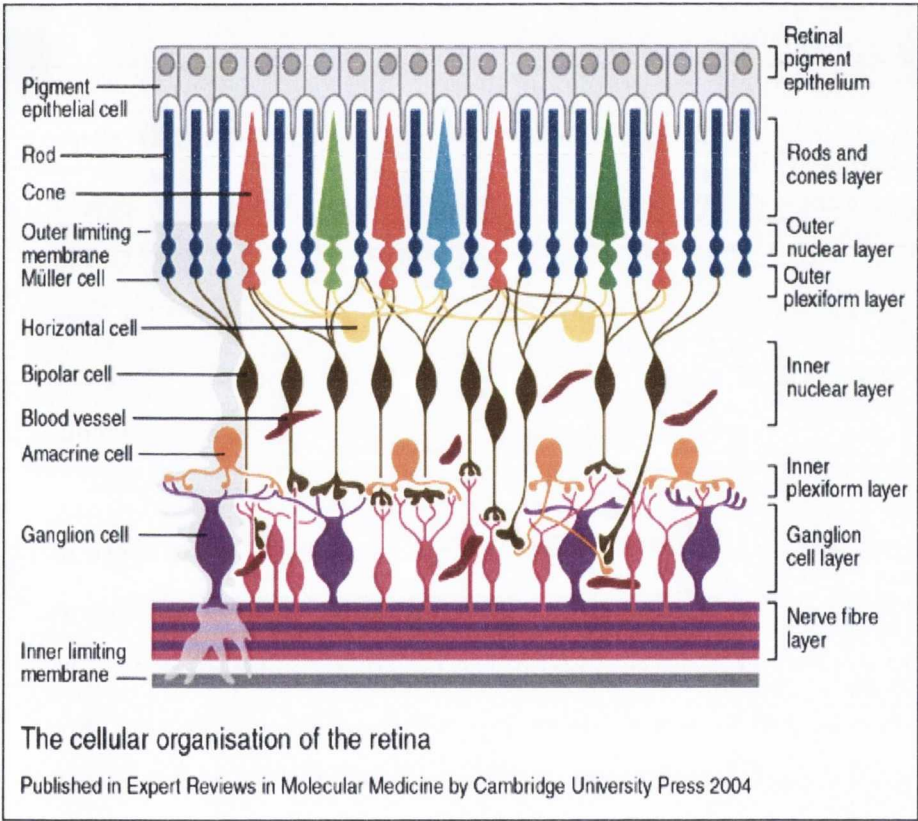


Figure 1.2: The cellular organisation of the retina

Light triggers the rod and cone cells to send an impulse to a layer of intermediate neurons. These nerve cells connect to a next layer of ganglion cells that have long fibres which join to form the optic nerve and carry the signal to the brain.

Figure adapted from WebVision (<http://webvision.med.utah.edu/imageswv/schem.jpeg>).

1.3.1 The retinal pigment epithelium (RPE)

The RPE is the outermost retinal layer consisting of a single layer of pigmented cells. It is required for normal photoreceptor outer segment development and is essential for removing unwanted substances from the choriocapillaris before entry into the retina. The RPE is the site of regeneration of the visual pigment chromophore, 11-cis-retinal, following its isomerization by light. The RPE is also involved in the phagocytosis and digestion of shed outer segment discs and the pigmented melanin granules serve to scatter light at the back of the retina.

1.3.2 Photoreceptors

The rod and cone photoreceptors are the primary neurons in the visual pathway which constitute the outer and inner segments, the outer nuclear layer and the outer plexiform layer. There are approximately 100 million rod cells and 5 million cone cells in the human retina (Curcio *et al.*, 1990). The two types of photoreceptors are arranged in a mosaic pattern with cones spaced evenly throughout the retina, surrounded by rings of rods. Variation in the density of cones and rods occurs in different regions of the retina. Cone density is highest in the fovea, while rod densities are highest at the macula (Ogden, 1989). A vertebrate photoreceptor consists of five parts (figure 1.3):

1. The outer segment which contains the photopigment responsible for converting light into a neural signal. It comprises a stack of membranous discs (~800 discs in rod cells and ~1100 in cones) enclosed within the plasmalemma of the cell. The photopigments that absorb light and trigger the phototransduction cascade are part of the major structural element in these membranous discs. As old discs are phagocytosed, they are replaced by newer discs migrating from the base of the outer segment. The membranes of rod discs contain the pigment rhodopsin, whereas cone cells have three different types of cone visual pigment corresponding to blue, green and red opsin, that are sensitive to different wavelengths of light.

2. The ciliary stalk or cilium contains motor and cytoskeletal proteins such as myosin and acts as a guiding tunnel for transferring photons to the light-sensitive pigments.
3. The inner segment, which is divided into two parts: the *ellipsoid*, which contains mitochondria providing the energy needs of the photoreceptor; and the *myoid*, located closer to the cell nucleus, which contains the other cellular organelles and where protein synthesis is concentrated.
4. The outer fibre, containing the photoreceptor nucleus.
5. The inner fibre – an axonal structure made of numerous microtubules connecting the cell body to the synaptic terminus.

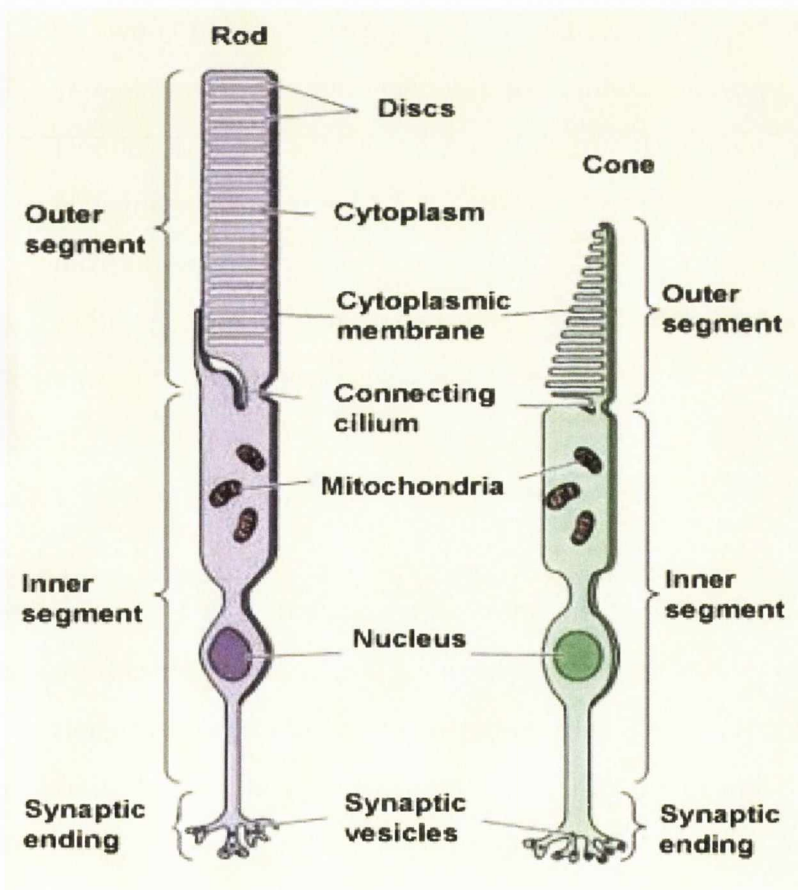


Figure 1.3: Basic structure of photoreceptor cells. Cone cells (right) are similar to rods (left), differing mainly in the shape of their outer segments.

1.3.3 Outer nuclear layer (ONL)

The ONL contains the cell bodies and nuclei of photoreceptor cells. Cone cell nuclei are slightly larger than their rod counterparts and are located in the external limiting membrane. Rod granules are much more numerous and are located at different levels throughout the ONL.

1.3.4 Outer plexiform layer (OPL)

There are two layers within the OPL: a wider external band consisting of the inner fibres of rods and cones, and a narrower band made of the dendrites of bipolar and horizontal cells. The rods synapse with bipolar and horizontal cells through round cytoplasmic expansions called *spherules*, and the cones synapse through a larger version known as *pedicles*. A horizontal cell will only synapse with a rod cell once, but each spherule may be contacted by numerous horizontal cells. Up to four bipolar cells can also connect with an individual spherule. The cone pedicle receives three different neurons arranged in a specific structural order known as a *triad*. The central axon of the triad originates from a midget bipolar cell and can connect with the same cone cell up to 25 times. The other two dendrites of the triad come from horizontal cells and only connect with the cone pedicle at one point.

1.3.5 Inner nuclear layer (INL)

The INL is probably the most complex layer of the retina and is made up of 8-12 rows of closely packed nuclei of bipolar cells, horizontal cells, amacrine cells, interplexiform cells, and supportive Muller cells. Horizontal cells modulate and transform visual signals from photoreceptor cells. Each horizontal cell has a long axon and several short dendrites with branching terminals, and unlike bipolar cells which transfer signals radially from photoreceptors, they form horizontal networks.

The bipolar cells are the main second-order neurons in the retina and their dendrites synapse with both photoreceptors and horizontal cells, while their axons synapse with

ganglion and amacrine cells. The main function of the bipolar cells is to relay information from photoreceptors to the ganglion cell layer and to receive synaptic feedback from the amacrine cells.

Muller cells are largest cells of the retina and are the major glial cells, playing a supportive role in the structure and development of the retina. Muller cells are also involved in glycogen metabolism and contribute to retinal nutrition.

Amacrine cells have a large cell body and a single process that branches extensively through the IPL. It functions mainly in carrying information horizontally, forming synapses with bipolar cells and ganglion cells, as well as the processes of interplexiform neurons.

1.3.6 Inner plexiform layer (IPL)

The IPL is the junction between the bipolar cells and ganglion cells and is mainly comprised of synaptic connections between the axons and dendrites of bipolar, ganglion, amacrine and interplexiform cells.

1.3.7 Ganglion cell layer (GCL)

The GCL is mostly comprised of ganglion cell bodies and some glial and Muller cells. The GCL generally forms a single layer of cells near the peripheral layer, although this may extend to up to 10 cells thick near the macula. Ganglion cell bodies are big, with a large nucleus and abundant cytoplasm. Approximately 1.2 million ganglion cells are present in the retina and each of these produces a single axon that converges and exits the eye at the optic nerve.

1.3.8 Nerve fibre layer (NFL)

The NFL consists of ganglion cell axons, glial cells and a rich capillary bed. It is of upmost importance because it carries the information that determines visual acuity (figure 1.4).

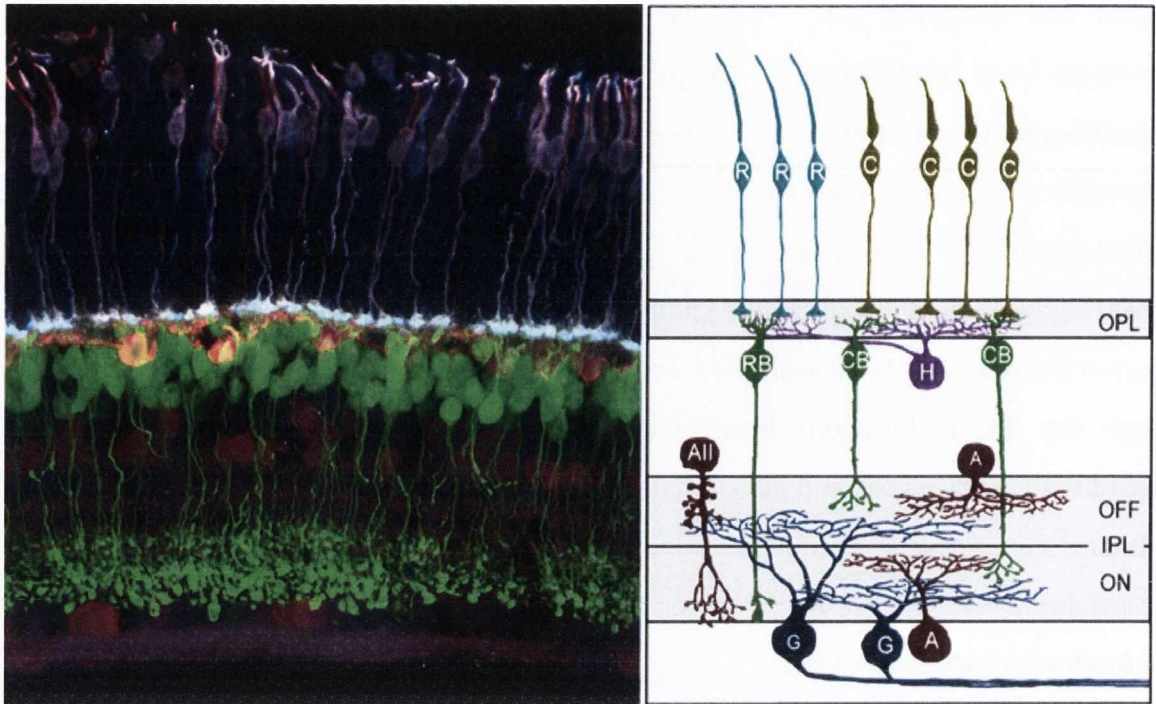


Figure 1.4: Cross-section of immunostained mature mouse retina. Photoreceptors are stained with arrestin (blue-purple), amacrine and ganglion cells are stained with calbindin (red) and bipolar cells are labelled green (left). Corresponding schematic diagram showing the major cell types of the retina and their connectivity patterns. R – rod photoreceptor; C – cone photoreceptor; OPL – outer plexiform layer; RB – Müller Glia; CB – bipolar cells; H – Horizontal cells; A – Amacrine cells (right). Reproduced from [webvision.med.utah.edu/ Wong.html](http://webvision.med.utah.edu/Wong.html)

1.3.9 Internal limiting membrane (ILM)

The ILM forms the inner most layer of the retina, next to the vitreous. It is a region of occluding junctions that serves as a tight barrier for sealing off the retina from potentially harmful materials present in the vitreous.

1.4 Visual Transduction

Visual transduction is the mechanism by which light is processed and visual information is sent to the brain via the optic nerve. The phototransduction process is initiated by the visual pigment rhodopsin. Rhodopsin is a prototypic member of the G-protein coupled receptor (GPCR) superfamily and is the main protein component of the discs located in the outer segments of photoreceptor cells. The phototransduction cascade is outlined in

figure 1.5. When light enters the eye, photons are absorbed by 11-*cis* retinal causing it to undergo isomerisation to its all-*trans* retinal form, This reaction leads to the formation of Meta II rhodopsin or R*, causing activation of the visual cascade, brought about by exposing the G protein binding sites of rhodopsin, thus permitting the binding of transducin. In photoreceptor cells, cytoplasmic levels of cGMP are controlled by the membrane bound cGMP phosphodiesterase (PDE), and the activity of this enzyme is relatively low in the dark. Upon activation of R*, the resulting conformational changes in transducin cause its alpha subunit to dissociate, and bound GDP is exchanged for GTP. GTP-bound active transducin in turn activates cGMP-PDE, which hydrolyzes cGMP to 5'-GMP.

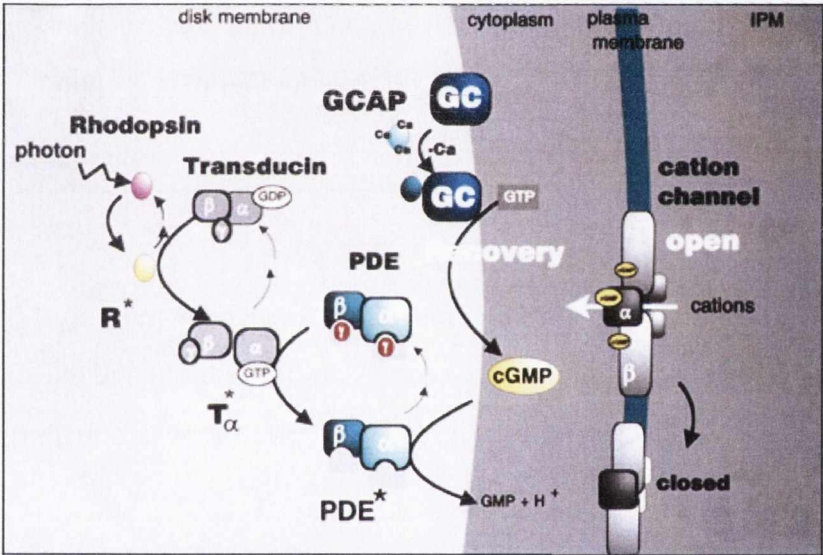


Figure 1.5: The phototransduction cascade.
Rhodopsin is activated by light, R* then activates transducin. GTP bound transducin in turn activates PDE, hydrolysing cGMP to 5'-GMP, causing the ion channels to close and hyperpolarising the cell.

Diagram reproduced from [depts.washington.edu/ opthweb/KPgraphic.html](https://depts.washington.edu/opthweb/KPgraphic.html)

In resting rod cells, high levels of cGMP keep the cyclic-GMP gated sodium channels open and the rod cells depolarized. But once cGMP is hydrolyzed to 5'-GMP, the cGMP-gated cation channels close, decreasing the inward flow of sodium and calcium ions. The blockade of positive ion flow causes the membrane to hyperpolarize (Molday *et al.*, 1998). This triggers an electrochemical impulse, releasing the excitatory

neurotransmitter glutamate at the synapse of the cell, thereby transmitting the signal through the retina and on to the brain via the optic nerve.

The effects of a single exposure to light must be short lived as the photoreceptors must quickly and reliably signal the occurrence of different photic events over time. Subsequent to photoexcitation the photoreceptor cell returns to its dark state by shutting down the visual cascade and resynthesising cGMP, mediated by a calcium feedback mechanism (Djamgoz *et al.*, 1995). Activated rhodopsin is phosphorylated by rhodopsin kinase and then bound by arrestin, preventing further interaction with transducin (Molday *et al.*, 1998). Transducin and PDE are inactivated by the hydrolysis of GTP to GDP on the transducin alpha-subunit. Guanylate cyclase, the enzyme responsible for the synthesis of cGMP from GTP, is activated by the decrease in intracellular calcium after photoexcitation. As cGMP concentrations increase again, the ion channels reopen and the photoreceptor cell is returned to its depolarized state.

1.5 The Hippocampus

The hippocampus forms part of the limbic system of the brain and is involved in memory and spatial navigation. It is located in the temporal lobe on each side of the brain and its name derives from its curved shape which resembles a seahorse (greek: hippocampus) (figure 1.6).

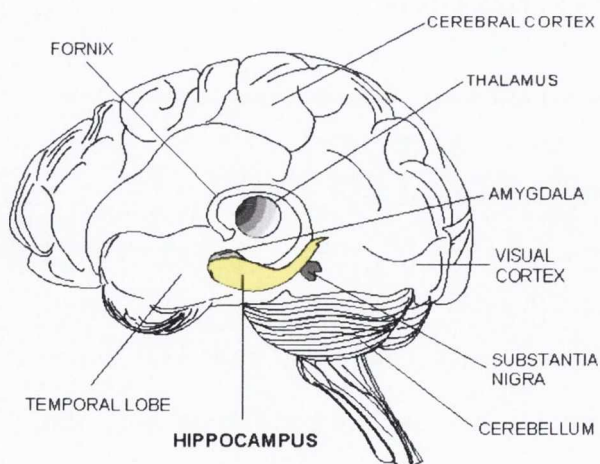


Figure 1.6: Schematic diagram of the human brain.

Yellow region highlights the hippocampus, the major area of the brain involved in memory and learning.

A remarkable feature of the hippocampus is its regularity along the longitudinal axis which forms large arrays of neurons aligned in distinct layers. In mammalian neuranatomy, the hippocampus is considered one of the phylogenetically oldest parts of the brain and is considered to be the most ordered and structured component of the nervous system. The hippocampus is comprised of the dentate gyrus, fields CA1-CA3, the CA4 or hilus (also considered to be part of the dentate gyrus), and the subiculum. The subiculum lies between the entorhinal cortex (EC) and the CA1 field and is the most inferior component of the hippocampal formation (figure 1.7).

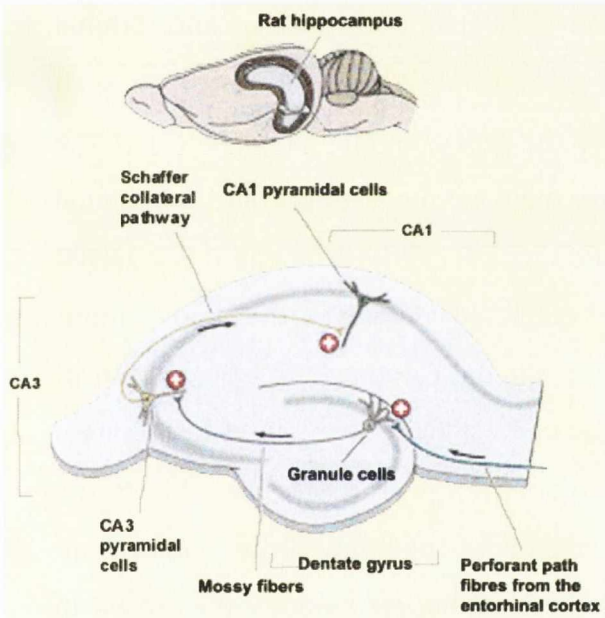


Figure 1.7: Schematic diagram of the rat brain & hippocampus.

The hippocampus proper (lower) is composed of regions with tightly packed pyramidal neurons, mainly areas CA1, CA2, and CA3. ("CA" stands for Cornu Ammonis, or Horn of Ammon). This is known as the trisynaptic circuit or trisynaptic loop of the hippocampus.

(image reproduced from <http://www.thebrain.mcgill.ca>)

The flow of information through the hippocampus proceeds from the dentate gyrus through CA3 to CA1 to the subiculum. The perforant path is the primary source of information flow, carrying input from the EC (and also from the perirhinal cortex). The primary output pathways of the hippocampus are the cingulum bundle (a collection of

white matter fibres) and the fimbria/fornix (a C-shaped bundle of fibres), which arise from the CA1 and subiculum (figure 1.7). While it is widely accepted that each of these regions plays a unique and vital role in the processing of information through the hippocampus, the specific contribution of each component is still not yet fully understood.

1.5.1 The Hippocampus and Memory

Clinical evidence from several patients, coupled with experimental studies on animals suggest a lesion or damage to any area of the hippocampus can have a significant effect on memory and learning. Damage to the parahypocampal, perirhinal, or entorhinal cortices (and not the hippocampus) produce a greater deficit in memory storage, such as object recognition, than selective lesions of the hippocampus alone (and not the overlying cortex). However, the hippocampus may be more important for spatial representation. Hippocampal lesions in mice and rats interfere with memories involved with space and context and it is thought that single cells alone in the hippocampus encode specific spatial information. Functional magnetic resonance imaging (fMRI) of normal human brains show more intense hippocampal activity in the right hemisphere involved with spatial memories, whereas memories for words, people, objects etc involve greater activity in the left hemisphere. Taken together, these findings are consistent with clinical data suggesting that lesions of the left hippocampus result in deficits in verbal memory, whereas lesions of the right hippocampus give rise to problems with spatial orientation. It is important to note that patients with amnesia arising as a result of hippocampal lesions can remember their childhood and other factual knowledge acquired before the injury. This suggests that the hippocampus is only involved in processing, not storing the information – long-term storage of episodic and semantic knowledge occurs in the cerebral cortex.

The hippocampus has three major pathways: (1) the *perforant pathway* (from the EC to the granule cells); (2) the *mossy fibre pathway* (from the granule cells to the pyramidal cells in the CA3 region); and (3) the *Schaffer collateral pathway* (from the CA3 to the

CA1 pyramidal cells (figure 1.7). Timothy Bliss and Terje Lomo (1973) showed that each of these pathways is sensitive to a stimulus that may have occurred several hours previously. Using microelectrodes in rabbits, a brief stimulus to any of the three pathways was shown to increase the amplitude of the excitatory postsynaptic potentials in the targeted hippocampal neurons. This is known as *long-term potentiation* (LTP). LTP can be studied *in vivo*, where it can last for days or even weeks, or in hippocampal slices *in vitro*, where it can last for several hours.

LTP is an artificially induced change in synaptic strength produced by electrical stimulation but does this synaptic plasticity occur naturally and how does it affect information processing in the hippocampus? O'Keefe and Dostrovsky discovered in 1971 that the hippocampus actually contains a cognitive map of the spatial environment in which an animal moves. They showed that the firing pattern of individual pyramidal cells encodes the location of the animal in a particular space. A mouse's hippocampus has around one million pyramidal cells, each of which is a potential *place cell*, encoding a position in space. Different place cells fire as the animal moves through space and the animal is thought to form a "place field" or an internal representation of the space it occupies. Animals introduced into new environments were shown to form new place fields within minutes, which were stable for weeks or even months. The same pyramidal cells can be used in more than one spatial map and the rapid formation of these fields and their persistence provides a useful experimental model for the study of LTP. Defects in LTP should interfere with spatial memory processing. The spatial memory test used in our experiments is the Morris water maze task where the animal must find a submerged platform hidden underwater in a small pool. When released at random locations in the pool, the mouse or rat must use spatial cues in the room (light, markings on walls etc) to find its bearings and locate the platform. Richard Morris demonstrated that when animals were injected with NMDA receptor antagonists, they cannot navigate to the submerged platform, despite being able to swim directly to a visible platform in control experiments. This suggested that a mechanism involving NMDA in the hippocampus is involved in spatial learning (Morris *et al.*, 1989).

Coupled with the Morris water maze as a test for spatial memory, we have also used a passive avoidance box as a measure of non-spatial memory. In these experiments, the rats were placed in a small box with two compartments. One side consists of a small, darkened box, linked to the other side that is considerably larger and brighter. Rats naturally prefer to be in small dark spaces but entry into the black compartment is punished with a mild but inescapable electric shock to the feet. After one or more such conditioning sessions, the rats will learn to avoid the dark compartment. Passive avoidance working conditions basically involves timing transitions, ie: the time it takes for the animal to move from the bright to the dark compartment after a conditioning session.

1.5.2 The Morris water maze in the study of memory and learning

The Morris water maze (MWM) was developed 30 years ago to investigate spatial learning in the laboratory rat (Morris *et al.*, 1982). It has since become one of the most frequently used tools in behavioural neuroscience, with the original paper describing its use being cited in the scientific literature over 2500 times. One of the reasons for its success as a model is its relative simplicity, the device consists of a large circular pool filled with opaque water in which a small escape platform is hidden. During a number of training trials, rats learn to find the platform and escape the pool and the latency time for the animal to reach the platform is recorded. Although this basic procedure is simple, the MWM has been used in some of the most sophisticated experiments in the study of the neurobiology and neuropharmacology of memory and learning. It has also been used to validate animal models of neurocognitive disorders and has helped develop significant insights into various areas of neuroscience. Many factors need to be properly controlled when carrying out MWM studies. Factors such as body weight, circadian rhythms, species of animal, age of animal and sex of animal can all affect performance. Animals that are sick, stressed or aged have severely impaired spatial learning and therefore it is important to carry out work on a cohort of healthy age-matched, male rats. The MWM was initially developed as an instrument with particular

sensitivity to the effects of hippocampal lesions in rats. Interestingly, the spatial learning impairment of hippocampal-lesioned rats is related to the volume of damaged hippocampal tissue, and dorsal hippocampal lesions have been reported to have more profound effects than ventral hippocampal lesions (Moser *et al.*, 1993; 1995). The spatial learning deficits of hippocampal-lesioned rats did not appear to be due to a discrimination learning impairment, and rats with lesions can still acquire a nonspatial task (Gallagher *et al.*, 1992) or a taste aversion-based discrimination task (Skinner *et al.*, 1994), and contextual fear conditioning is also still possible in these animals (Cho *et al.*, 1999).

The current hypothesis is that the hippocampus is permanently involved with tasks in which memories have to be acquired and retrieved during performance (“on-line memory performance”) and is also involved in memory consolidation.

1.5.3 Contextual Fear Conditioning

Fear conditioning is a method by which animals learn to fear a new stimuli and is a form of learning associated with a particular neutral context (ie: a room or box) or a neutral stimulus (ie: a sound). The neutral stimulus is paired with an aversive stimulus (ie: shock) and eventually, the neutral stimulus alone can elicit a state of fear. In the vocabulary of classical conditioning, the fear is the “conditioned response”. In rats, the conditioned response is measured by freezing – rats will normally investigate and move freely around a neutral space, but if afraid, they will freeze. While the amygdala is known to be involved in fear conditioning, the hippocampus is essential for contextual fear conditioning.

In the context of the research described in this PhD thesis, training and testing took place in a rodent observation cage (30 x 37 x 25cm), the side walls of which were constructed of stainless steel, and the back walls and doors constructed of clear Plexiglass. The floor consisted of 20 steel rods through which a scrambled shock could be delivered. During training, rats receive three 1 second shocks with an interval of 1 minute between shocks. During recall testing, rats were placed back into the same

chamber as used in conditioning, in the absence of a foot shock, for an 8 minute context test. The time spent by each rat “freezing” was measured – freezing is defined as behavioural immobility except for movement needed for respiration.

1.5.4 Pre-Pulse Inhibition of Startle (PPI)

Prepulse inhibition is a neurological phenomenon in which a weaker prestimulus (prepulse) inhibits the reaction of an animal to a subsequent stronger startling stimulus. The reduction in the amplitude of the startle response reflects the ability of the nervous system to adapt to a strong sensory stimulus (in this case a loud acoustic noise) when a preceding weaker signal is given. PPI can be detected in numerous species ranging from mouse to human. Defects or deficits in PPI are linked to aberrant sensorimotor gating and typically manifest in an inability to filter out the unnecessary information. Such defects are noted in patients suffering from Alzheimer’s disease and schizophrenia.

1.6 Synaptosome-associated protein of 25kDa (SNAP-25)

SNAP-25 is a presynaptic exocytotic protein that plays a role in a number of neuronal functions including axonal growth, dendrite formation, fusion of synaptic vesicles with the cell membrane and the expression of long-term potentiation (LTP). SNAP-25 has been loosely implicated in the aetiology of attention-deficit hyperactivity disorder (ADHD) (Thapar *et al.*, 2005), schizophrenia (Thompson *et al.*, 1998, 2003; Young *et al.*, 1998; Sokolov *et al.*, 2000; Fatemi *et al.*, 2001; Halim *et al.*, 2003) and is also involved in memory consolidation (Roberts *et al.*, 1998; Hou *et al.*, 2004).

Disruption of SNAP-25 function by antibody microinjection in lamprey meticulospinal synapses resulted in inhibition of neurotransmitter release although numbers of synaptic vesicles in contact with the presynaptic plasma membrane appeared to remain unaffected. These results support a critical role SNAP-25 in membrane fusion but not in vesicle targeting to the presynaptic membrane (Low *et al.*, 1999).

The first suggestion that SNAP-25 plays a role in memory/learning was reported when selective inhibition using SNAP-25 antisense oligonucleotides in rat cortical neurons *in vitro* was shown to prevent neurite elongation (Osen-Sand *et al.*, 1993). An increase in SNAP-25 mRNA levels in granule cells of the hippocampus was observed by *in situ* hybridization 2 hours after the induction of long term potentiation (LTP), again suggesting a role for SNAP-25 in synaptic plasticity (Roberts *et al.*, 1998).

Using learning/memory behavior screening, Hou and colleagues (2004) identified SNAP-25 as one of the differentially expressed genes in the rat hippocampus upon behavioral training. They then showed that inhibition of SNAP-25 function with antisense oligonucleotides resulted in a deficit in long-term memory for step-down inhibitory avoidance and interfered with the LTP in the CA1 region (Hou *et al.*, 2004). Interestingly, this paper's results contradict the findings of our collaborators in the ANRG. Microarray work carried out at both UCD and Wyeth has shown that SNAP-25 expression is reduced post-training. This finding was then verified by RT-PCR analysis and western blot, and indeed, all methods confirmed a significant decrease in SNAP-25 at 12-24 hours post-training.

1.6.1 The *coloboma* mouse as a model for ADHD

Mice heterozygous for the semi-dominant mutation coloboma (Cm) exhibit profound spontaneous hyperactivity, head bobbing, and ocular dysmorphology (Hess *et al.*, 1992). Mutant mice were generated by neutron irradiation resulting in a 1-2cM deletion on mouse chromosome 2 encompassing the phospholipase C isoform β -1 and -4, jagged-1 and SNAP-25 genes. Normally a chromosomal deletion of this size would result in the loss of up to, or even more than 30 genes, so it is interesting to note that there are currently only 4 known genes in this region which appear to be deleted.

The hyperactivity of Cm mice averages three times (and can even exceed 10 times) the activity of control littermates. The variability in hyperactivity suggests a loss of control of activity rather than a simple increase in basal motor behaviour. Despite this extreme

hyperactivity phenotype and large genomic deletion, the *coloboma* CNS appears relatively normal with no obvious neuropathology.

However, whereas low levels of SNAP-25 are clearly required for the *coloboma* phenotype, the absence of another gene in the deleted gene must also contribute to the hyperactivity phenotype because paradoxically, pure (ie: gene targeted) SNAP-25 knockout mice are not hyperactive (Washbourne *et al.*, 2002). This unexpected finding, together with the observation that hyperactivity in *Cm* littermates is variable suggests other factors must be involved.

Evidence suggests that hyperactivity in the *coloboma* mouse results from a deficiency of dopamine release in the striatum caused by haploinsufficiency of SNAP-25 (Raber *et al.*, 1997). As SNAP-25 is involved in synaptic vesicle docking and fusion required for normal synaptic transmission, the decreased expression of this gene may also be linked to behavioural deficits in learning and aberrant hippocampal physiology, which also correspond to features of ADHD.

To examine the contribution of decreased SNAP-25 mRNA and protein levels in *Cm* mice, a transgenic mouse over-expressing a SNAP-25 minigene was generated (Hess *et al.*, 1996). This transgenic mouse was then bred with mice heterozygous for the *Cm* deletion and the resulting progeny were examined for rescue of the hyperactivity phenotype. While the transgene had no apparent affect on wild-type offspring, mice carrying the *Cm*/+ mutation and also bearing the two copies of the SNAP-25 minigene were restored to normal activity. Mice with one copy of the minigene and heterozygous for *Cm* (*mini*/+ *Cm*/+) showed reduced locomotor activity (although not as low) which suggested a dose-dependent rescue by the minigene. Administration of *D*-amphetamine, which usually restores normal locomotor activity in *Cm* mice was also used to test this dose-dependency (Hess *et al.*, 1996). Whereas this transgenic rescue of the hyperactivity component of the *coloboma* phenotype strongly suggests the involvement of SNAP-25, the other phenotypes of the *Cm* mice (headbobbing and eye dysmorphology) were not rescued by the SNAP-25 minigene which suggests they are most likely caused by the deletion of the other neighbouring genes.

Interestingly, although not all brain regions of the SNAP-25 minigene transgenic *Cm/+* mice showed near normal levels of SNAP-25 expression in rescued mice, the striatum and cerebellum (regions implicated in locomotor activity) and the hippocampus did show high levels of expression, again suggesting that SNAP-25 is important for normal locomotor function.

1.7 SNAP-25 in the retina

SNAP-25 protein has been detected in developing and mature photoreceptors in the Brazilian opossum, *Monodelphis domestica* (West Greenlee *et al.*, 1998), the rat (Chen *et al.*, 1999; West Greenlee *et al.*, 2001), ferret (Karne *et al.*, 1997) and mouse. In the adult retina, immunohistochemistry revealed significant levels of SNAP-25 are localized in the synaptic regions and inner segments of the photoreceptors (Chen *et al.*, 1999; West Greenlee *et al.*, 2001). SNAP-25 clearly has a role in the synaptic function of photoreceptors but is also thought to play a role the fusion of rhodopsin-containing vesicles with the inner segment membrane as SNAP-25 has been shown to co-localise with the golgi apparatus in photoreceptor cells (Morgans and Brandstatter, 2000).

Studies of retinas of SNAP-25 heterozygote knockout mice (ie: expressing 50% of normal SNAP-25 levels) have shown that mice have an average of 33% more recoverin positive cells than WT littermates at embryonic stages (unpublished results, review by West Greenlee *et al.*, 2002). This finding suggests that mice with lower levels of SNAP-25 have increased numbers of photoreceptors during early stages of retinal development. The review also mentions research in progress which showed an increase in cone function and suggests that lowered SNAP-25 levels somehow favour cone development perhaps due to a decrease in vesicular release of an unknown factor which would suppress differentiation of progenitor cells into photoreceptors during retinal development. While the review summarizes this work, the citation is for “manuscript in preparation” and unfortunately no such work has since been published. However, based on this and the other findings referred to above it was of interest to utilize the SNAP-25 AAV I have

generated to investigate the effects of SNAP-25 over-expression on photoreceptor development, retinal function, and also on outer segment disc formation.

1.8 Adeno-Associated Virus (AAV)

Adeno-Associated Virus is a *Dependovirus* of the *Parvovirinae* subfamily of Parvoviruses. It was originally isolated as a contaminant of adenoviral cultures, hence the name adeno-associated virus (Berns *et al.*, 1996). AAV is similar to other parvoviruses: it is non-enveloped, has icosahedral symmetry, and a diameter of 18-26nm. At its core, AAV has a beta barrel motif made up of two anti-parallel beta sheets (Rabinowitz and Samulski, 2002; Xie *et al.*, 2002). The AAV genome is 4.7kb in length made up of single stranded DNA. It comprises just two genes, which encode four replication (*rep*) proteins and three structural (*cap*) proteins (figure 1.8).

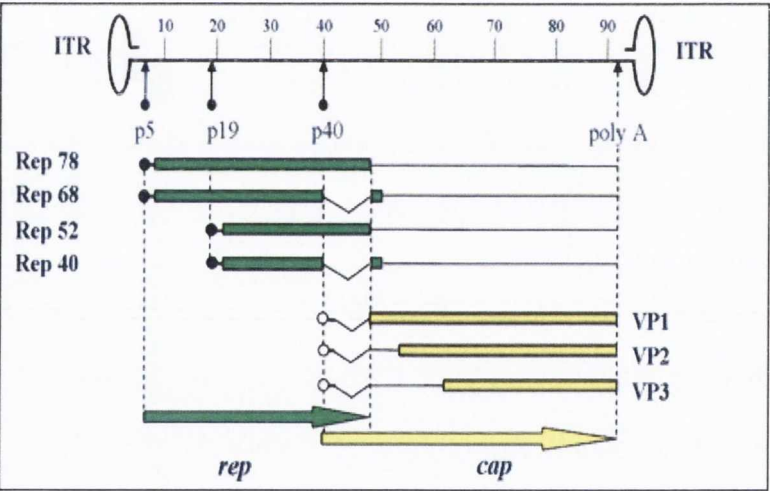


Figure 1.8: Wild type AAV genome.

The AAV genome contains two ORFs, that direct the synthesis of 6 major RNA species, due to the presence of 3 different transcription promoters and alternative splicing signals. The *rep* ORF encodes four regulatory proteins that are involved in AAV DNA replication, gene expression and packaging. The *cap* gene encodes three structural proteins, VP1, VP2 and VP3, that constitute the capsid.

(Image reproduced from <http://hvd.ens-lyon.fr>)

Flanking the *rep* and *cap* genes at either end of the single stranded genome, are two 145 nucleotide open reading frames known as inverted terminal repeats (ITRs) which typically form a T-shaped hairpin secondary structure. ITRs are essential for viral

packaging into the AAV capsid and also for expression and replication of the viral genome. ITRs are also necessary for the conversion of single-stranded DNA into the double-stranded form from which AAV gene expression can occur - it is this double stranded form which persists episomally or which integrates into the host genome (Weitzman *et al.*, 1994; Wang *et al.*, 2005; Zhou & Muzyczka, 1998).

A unique feature of AAV is that it requires a helper virus to complete its own life cycle (latent to lytic phase) in infected cells. The helper virus, typically adenovirus or a member of the herpes virus family provides protein or RNA stimuli, which trigger the AAV promoters causing increased AAV gene expression, enhance the transport and splicing of the AAV pre-mRNAs, and in the case of herpes simplex virus-1, support replication of the AAV genome (Weindler and Heilbronn, 1991). A detailed description of the AAV life-cycle is outlined in figure 1.9. In the absense of helper virus, the AAV genome integrates into the host cell DNA and enters a latent phase. In humans, integration occurs at a specific locus on chromosome 19q13.3-qter, known as the AAVS1 site (Kotin *et al.*, 1992).

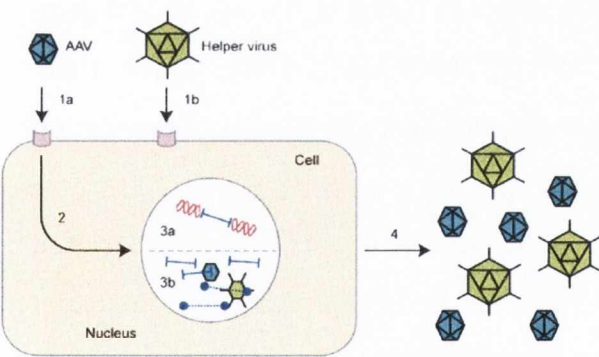


Figure 1.9: Life Cycle of AAV

Stages shown are: (1) virus binding and entry into the cell; (2) intracellular trafficking; (3) intracellular replication and virus production; and (4) release of intracellular viral particles. Stages 1 and 3 include the absence (1a, 3a) or presence (1b, 3b) of helper virus; in the absence of helper virus, the AAV genome integrates into the host cell DNA in order to replicate. (image adapted from Blechacz & Russell, 2004).

1.8.1 Vectors based on adeno-associated virus

Recombinant vectors derived from AAV can be generated relatively easily. This is due to the fact that AAV ITRs are the only piece of the AAV genome required *in-cis* for efficient viral packaging. In other words, to produce a recombinant AAV (rAAV) vector, the *rep* and *cap* genes are simply replaced with a transgene and its associated regulatory sequences (intron and polyA tail). The total size of the resulting recombinant AAV cassette including the transgene, regulatory sequences and the two ITRs cannot greatly exceed the size of the wild-type AAV genome (4.7kb) to ensure efficient viral packaging. This size constraint is considered by many to be the greatest limitation of rAAV technology, although recent findings by Auricchio and colleagues have shown that up to 8.9kb can be efficiently packaged when using AAV2/5, which greatly expands the therapeutic potential of this vector system (Alloca *et al.*, 2008).

For efficient encapsulation of the recombinant transgene into viral capsids, we typically transfect the recombinant plasmid into human embryonic kidney (HEK293T) cells in culture, together with two other plasmids: an AAV helper plasmid encoding the *rep* and *cap* genes *in trans*; and an adenoviral helper plasmid, supplying the adenoviral gene products (E1a, E1b, E2b, E4 & VA RNA). This is commonly known as the triple transfection technique (figure 1.10), and importantly, ensures there is no contamination from wild-type adenovirus or from wild-type AAV.

To date, 14 different AAV serotypes (AAV1-12, AAV-mo.1, AAV-ra.1) have been isolated and cloned, with approximately 9 of these being available commercially (Maramatsu *et al.*, 1996; Chiorini *et al.*, 1997; Rutledge *et al.*, 1998; Chiorini *et al.*, 1999; Xiao *et al.*, 1999; Gao *et al.*, 2002a, Mori *et al.*, 2004; Schmidt *et al.*, 2006; Lochrie *et al.*, 2006) (Figure 1.12). AAV-2 was the first recombinant AAV and has been used as for successful gene transfer in a number of different animal models (Snyder *et al.*, 1999; Monahan & Samulski, 2000, , Buch *et al.*, 2008). rAAV-2 vectors have also been shown to be capable of mediating efficient and sustained transgene expression in a variety of cell types and tissues and have also been used to deliver therapeutic genes to correct for gene defects

in various animal models of human disorders (Eliger *et al.*, Xiao *et al.*, 2000; Bosch *et al.*, 2000; Jung *et al.*, 2001). In total, AAV-2 has been used in over 20 different clinical trials ranging from haemophilia B to retinoblastoma (Kay *et al.*, 2000; Hurwitz, 2000; Manno *et al.*, 2002). Fascinating trials are currently underway by Robin Ali and colleagues (UCL), Jean Bennett and colleagues (U.Penn), and William Hausworth and colleagues (U. Florida) using rAAV-2/2 to deliver a therapeutic RPE65 gene to the retinas of young adult LCA patients, the preliminary results of which showed great promise (Bainbridge *et al.*, 2008). Although none of the recipients showed a significant change in retinal responses measured by ERG, one patient had an improvement in visual function measured by microperimetry and also showed improvement in a subjective test of visual mobility. In a follow up study, each patient showed a modest improvement in measures of retinal function, and although normal vision was not achieved, these results were encouraging (Maguire *et al.*, 2008). By increasing the dose of the transgene using more powerful promoters a further improvement was demonstrated, with the greatest improvement noted in children, indicating that for this disorder (and perhaps others), the therapy should be targeted as early as possible (Maguire *et al.*, 2009). The potential of rAAV vectors for retinal gene therapy is significant and at the time of writing (2011) there are several encouraging trials in phase II and III.

Of the other AAV serotypes, most were isolated as contaminants of adenoviral cultures, although AAV-7 and -8 were isolated from rhesus monkeys (Gao *et al.*, 2002b). Serotype-5 is considered to be the most divergent AAV sequence and was isolated from a condylomatous lesion (genital warts caused by human papilloma virus infection) (Bantel-Schaal & Zur Hausen, 1984).

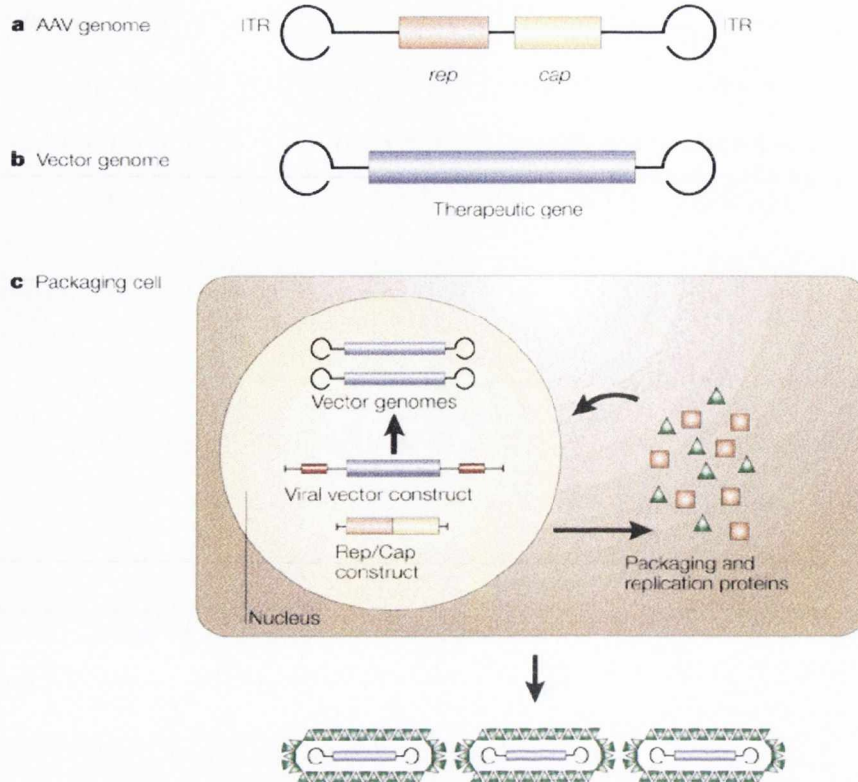


Figure 1.10: rAAV production using the Triple Transfection procedure.

a) The adeno-associated virus (AAV) genome contains the sequences essential for transduction — the inverted terminal repeats (ITRs) and the genes *rep* and *cap*. **b)** In the vector genome, *rep* and *cap* are replaced by the therapeutic gene. **c)** The Rep and Cap gene proteins provided by the packaging constructs are required to produce single-stranded DNA genomes that are encapsulated by the coat proteins. AAV — a non-enveloped virus — is assembled in the nucleus. (Image reproduced from Somia & Verma, 2000).

One of the main advantages of working with rAAV vectors is that the packaging plasmid can be interchanged between different AAV serotypes, resulting in the assembly of hybrid AAV vectors containing the genome of one serotype (ie AAV-2), but with different capsids (Duan *et al.*, 2001; Hildinger *et al.*, 2001). These hybrid or pseudotyped AAV vectors are described using two numbers, for example, AAV-2/5, where the first number indicates the origin of the ITRs, and the second number is the capsid (figure 1.11).

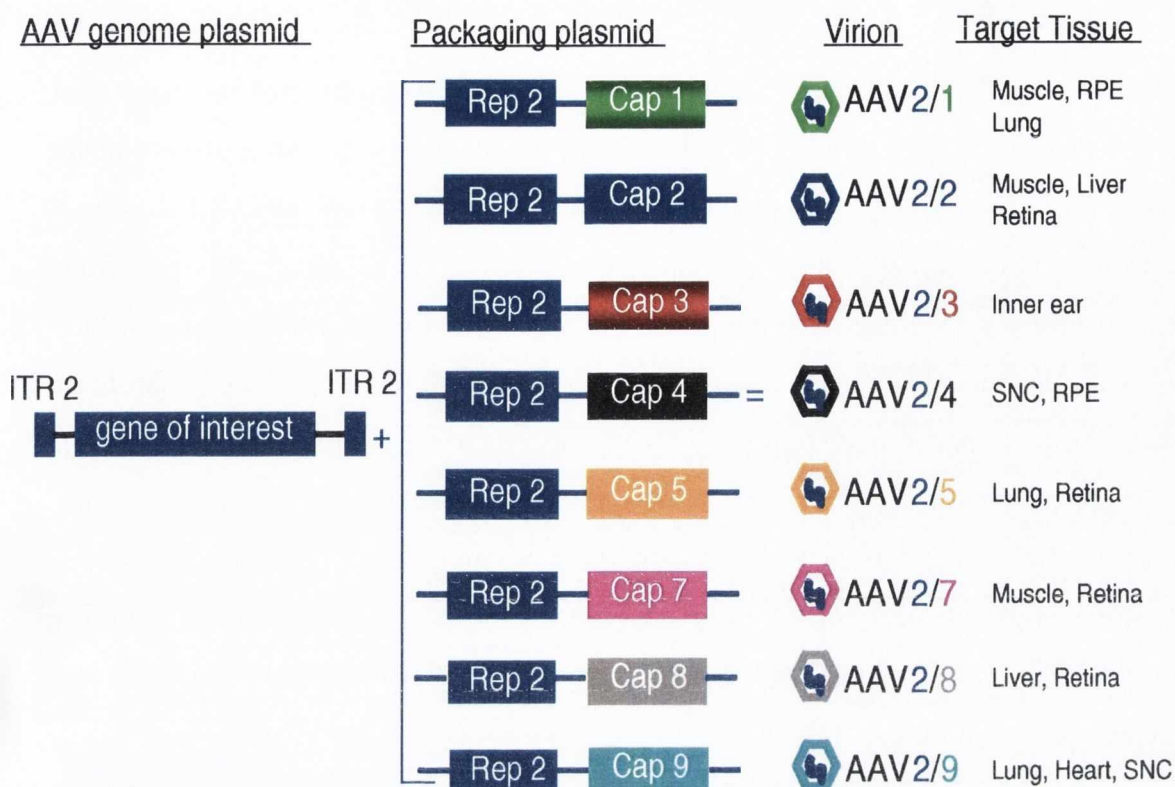


Figure 1.11: An array of recombinant vectors based on different AAV serotypes. Transfection in the packaging cells of the plasmids containing: the AAV genome (with the therapeutic gene contained between the viral inverted terminal repeats, ITRs) and the packaging sequences (the *cap* genes encoding for the capsids and the *rep* open reading frames belonging to serotype 2 to allow the most efficient packaging of a viral genome with AAV2 ITRs) results in production of recombinant virions. The hybrid virions containing the genome of one serotype (i.e. AAV2) and a different capsid (AAV2/1–9) have tropism dependent on the capsid sequence. (reproduced from Auricchio *et al.*, 2008).

The capsid protein sequences are significantly varied between different serotypes and are the main contributing factor in determining which tissues the virus preferentially infects. The main benefit of pseudotyping is that the resulting rAAVs display the unique tropisms and serological properties of their capsid serotype, which are different from those of AAV-2. AAV-2 capsid proteins interact with a receptor complex on the surface of target cells including heparin sulphate (Summerford & Samulski, 1998), fibroblast growth factor receptor 1 (Qing *et al.*, 1999) and integrin (Summerford *et al.*, 1999). These receptor complexes are absent from the luminal surface of airway epithelia, therefore rendering it useless for transduction of lung tissue. In contrast, AAV-5 receptor complexes including O-linked sialic acid is located on these airway epithelia (Walters *et al.*, 2001). Thus, AAV-2 virus, pseudotyped with the AAV-5 capsid has allowed efficient infection of the lung *in vivo* (Zabner *et al.*, 2000).

In addition to cellular tropism, capsid proteins can also influence levels of gene expression. Studies have shown that AAV vectors with the same genome but with different capsids show different transduction efficiency and temporal transgene expression changes in retina and muscle (Aurichio *et al.*, 2001b; Chao *et al.*, 2000). Taken together these studies all show that the benefits of pseudotyping can largely expand the range of target tissues for AAV-mediated gene transfer.

1.8.2 rAAV vectors for retinal gene delivery

The retina has several unique properties making it particularly suitable for AAV-mediated gene transfer. The eye is relatively small and enclosed, which limits the amount of virus needed to achieve a therapeutic effect and limits the exposure of other tissues to the virus. The eye is a thin laminar structure, making it accessible via multiple routes and different areas of the eye and the various cell types within can be infected depending on the method of delivery. Another useful feature is that treatment dosage can easily be extrapolated from animal research up to human trials (Bennett, 2000). There are also several physical barriers within the eye that further spatially limit the

spread of the virus. The subretinal space is separated from the blood supply by the retinal pigment epithelium (RPE), and the blood-retinal barrier (BRB) prevents widespread diffusion of the virus to systemic circulation. The retina is considered to be an “accessible” part of the nervous system and there are a number of sophisticated non-invasive methods to evaluate the efficacy of treatment over time. Retinal function can be monitored by ophthalmoscopy, electroretinography (ERG), optical coherence tomography (OCT) and the measurement of afferent papillary responses and visual evoked potentials (VEPs).

Since the first demonstration of subretinal delivery of rAAV encoding a LacZ reporter gene (Ali *et al.*, 1996), many advances in further understanding AAV biology have been made. In mouse retina, subretinal injection of both AAV-2 and AAV-5 have been shown to transduce both RPE and photoreceptor cells (Ali *et al.*, 1996; Ali *et al.*, 1998; Yang *et al.*, 2002). AAV-5 appears to have a much greater transduction efficiency than that of AAV-2, so much so that at 5 weeks post injection, the ratio of transgene expressing cells in AAV-5 infected retinas was approximately 1000 times greater than AAV-2 (Yang *et al.*, 2002). The onset of gene expression was also faster, and the viral genome copy number per eye were both greater in AAV-5 vectors. AAV-1 and -6 were found to predominantly infect the RPE, being approximately 15 times more efficient than AAV-2 vectors (Xiao *et al.*, 1998; Auricchio *et al.*, 2001b; Yang *et al.*, 2002). Similarly, AAV-4 exclusively transduces the RPE, whereas AAV-3 vectors only infect retinal cells with low efficiencies (Yang *et al.*, 2002; Weber *et al.*, 2003). Intravitreal injection of AAV-2 leads to efficient transduction of ganglion cells, the trabecular meshwork, and various cell types within the inner nuclear layer (Ali *et al.*, 1996; 1998; Auricchio *et al.*, 2001b; Liang *et al.*, 2001; Martin *et al.*, 2002). Intravitreal injection has also been shown to transduce the optic nerve (Guy *et al.*, 1999; Dudus *et al.*, 1999) and this discovery led to the subsequent treatment of a form of optic nerve neuritis in rodents and a form of glaucoma in rats (Guy *et al.*, 1998; McKinnon *et al.*, 2002).

Subretinal delivery of rAAV has also been carried out in larger animal models such as dogs and non-human primates, which better mimic the human eye as they have both

cone and rod photoreceptors. As in murine models, AAV-2 and AAV-5 vectors both efficiently transduce RPE and photoreceptor cells, and AAV-5 appears to be more efficient at infecting photoreceptors (Bennett *et al.*, 1999; Lotery *et al.*, 2003).

There have also been many reports in the literature which describe the use of chimaeric AAV serotypes in which the vector is flanked by AAV-2 ITRs, but encapsidated in AAV-1,-2,-3,-4, or -5 capsids. Subretinal injection of AAV-2/1 expressing eGFP has shown early onset of eGFP expression after 3-4 days, but with transgene expression limited to the RPE. AAV-2/2 and -2/5 vectors have been shown to transduce both RPE and photoreceptor cells efficiently (Aurichio *et al.*, 2001b), but onset of transgene expression occurs at 2-4 weeks post-injection for both vectors. Interestingly, there was a marked absence of transduced cells after intravitreal injection, and taken together, these results emphasize the effects of both modes of injection, and the effect of serotype on cell type transduction. A separate study examined the transduction efficiencies of AAV-2/1-5 in a variety of cell types. In the retina, AAV types -2/5 and -2/4 were the most efficient, followed by -2/1, establishing a hierarchy for efficient serotype-specific vector transduction depending on the target tissue (Rabinowitz *et al.*, 2002).

Another important factor in determining cell-specific transduction of rAAVs is the selection of a promoter.

1.8.3 rAAV vectors for hippocampal gene delivery

A particularly useful approach for studying the contribution of proteins in specific neuronal circuits is to introduce transgenes into adult rodent brains by using viral vectors. Recombinant AAV vectors are non pathogenic and result in long term transgene expression – they have been shown to readily persist for months or years in slowly dividing or non-dividing cells, as is the case for most adult brain cells (Gao *et al.*, 2005). Of the approximately 35 known serotypes of AAV identified to date, at least 14 have been engineered into recombinant viral vectors (Burger *et al.*, 2005; Buning *et al.*, 2008). The prototype AAV-2 vector is relatively inefficient at transducing cells in the CNS, and, as many humans are seropositive for AAV-2, this limits its use in clinical applications.

Cross packaging with capsid proteins from other AAV serotypes therefore can greatly improve gene transfer (Davidson *et al.*, 2000; Passini *et al.*, 2003; Burger *et al.*, 2004, 2005; Cearley & Wolfe, 2006; Taymans *et al.*, 2007). For instance, the serotype-5 capsid allows for increased spread of the recombinant vector when compared with serotype-2 capsid (Davidson *et al.*, 2000; Burger *et al.*, 2004; Paterna *et al.*, 2004). rAAV-2 preferentially targets the dentate gyrus of the hippocampus, with only weak transduction in the CA1 and CA3 fields (Passini *et al.*, 2002, 2003; Ahmed *et al.*, 2004, Burger *et al.*, 2004), whereas rAAV-1 and -5 transduce the entire hippocampus including the pyramidal neurons in the CA1-CA3 region (DiPasquale *et al.*, 2003; Burger *et al.*, 2004; Richichi *et al.*, 2004; Klugmann *et al.*, 2005).

1.8.4 Use of AAV vectors in animal models of neurological disorders

rAAV-2 containing a brain-derived neurotrophic factor (BDNF) transgene has been shown to persistently express in atrophic neurons for up to 18 months after injury and the BDNF resulted in a counteraction of atrophy in both acute and chronic stages after injury (Ruitenber *et al.*, 2004). rAAV-2 vectors expressing tyrosine hydroxylase (TH), aromatic-L-amino acid decarboxylase (AADC), and guanosine triphosphate cyclohydrolase (GCH) successfully increased dopamine levels in the striatum and improved motor function, persisting for at least 12 months after intrastriatal injection (Mandel *et al.*, 1999; Shen *et al.*, 2000; Kirik *et al.*, 2002). rAAV-2 vectors expressing glial cell-line derived neurotrophic factor (GDNF) has also shown to be effective in animal models of Parkinson's disease (Kirik *et al.*, 1998; Bjorklund *et al.*, 2000; Eslamboli *et al.*, 2003, 2005).

It is also important to note that there have been some studies investigating the effects of transgene over-expression in the hippocampus on memory and learning (in wild type rats). Long-lasting forms of memory are known to be mediated by changes in gene expression and protein synthesis. Therefore, over-expression of genes in the hippocampus should impact on behavioural plasticity. Over-expression of vascular endothelial growth factor (VEGF) resulted in a ~2 fold increase in neurogenesis

associated with improved cognition, whereas over-expression of placental growth factor had a negative effect on neurogenesis and inhibited learning (Cao *et al.*, 2004). Klugmann *et al* (2005) used an AAV1/2 vector to achieve robust expression of Homer proteins. They found opposing effects on learning behaviour using both long and short isoforms of the gene, impairing memory with Homer1a, but slightly enhancing memory with Homer 1c and 1g (Klugmann *et al.*, 2005). Hippocampal Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), a gene implicated in spatial learning, neuronal plasticity, epilepsy, and cerebral ischemia, was knocked down using RNAi delivered from a small hairpin cassette, delivered by an AAV vector. This suppression of CaMKII caused an impairment in spatial memory (Babcock *et al.*, 2005). The same group then investigated the effects of CaMKII over-expression and found that it actually caused an improved performance in the water maze (although, the results were not as convincing) (Poulsen *et al.*, 2007). Taken together, these findings suggest that the use of viral vectors to transduce cells efficiently *in vivo* can be used effectively to extend our understanding of hippocampal plasticity, learning and memory.

1.8.5 Safety and Biodistribution of AAV vectors

AAV is regarded as one of the safest of all known viral-based vector systems and it has never been associated with any human malignancy. A recent study by Stilwell and Samulski (2004) used a microarray approach to compare genes modulated during AAV or adenoviral infection in cell culture. Interestingly, AAV infection did not induce pathogenic gene expression whereas adenoviral infection induced immune and stress response genes (Stilwell & Samulski, 2004). As AAV vectors retain only the ITRs and are stripped of all other viral genes, they are “gutless” by design and generally evade cytosolic immune responses in the transduced cells. For safe and successful gene therapy using AAV and especially for clinical work, it is imperative to evaluate an accurate assessment of vector biodistribution within the target tissue. A number of studies have evaluated the biodistribution following various routes of administration. The majority of these have shown AAV infection is limited to the target tissue, with

multiple cell types effectively transduced depending on mode of administration and the virus serotype. However, some studies have shown viral spread into systemic circulation. For example, in non-human primate studies, delivery of rAAV2/2 to the lung (Conrad *et al.*, 1996) or muscle (Favre *et al.*, 2001) resulted in detectable vector DNA in the serum at 6 days (muscle) or 14 days (lung) post-infection. 18 months after intramuscular injection rAAV was detectable in the liver and peripheral blood mononuclear cells (PBMC) and certain lymph node sites (Favre *et al.*, 2001).

The mammalian retina was thought to be relatively safe from excess viral spread as it has physical barriers such as the sclera and the blood-retina barrier. However, a report by Weber *et al.*, (2003) has shown that viral vector DNA is detectable in the serum following subretinal delivery into dogs and non-human primates as early as 15 minutes (and for up to 25 days) after administration. Another report using intravitreal administration of an rAAV2/2 vector expressing GFP showed GFP expression persisting for up to 6 months in the optic nerves and brains of both mice and dogs (Guy *et al.*, 1999). A more recent report has shown that following either subretinal or intravitreal injection, rAAV sequences were detectable in the PBMC at early time points after vector administration, although nothing was detectable in the liver or gonads. Interestingly, vector sequences were detected in the brain after intravitreal injection suggesting that AAV2/2 has the ability to be transported trans-neuronally (Rolling *et al.*, 2004).

Long-term gene expression is apparently not accompanied by any significant toxicity in animals with low transduction rates and using relatively weak promoters (Mandel *et al.*, 1997, 1998). Even when using an rAAV vector containing a CMV-driven GFP transgene, there is no significant toxic effect up to 12 months post-injection (Daniels *et al.*, 2003). However, there is some evidence of rAAV vectors causing immune responses, especially when animals have been preexposed to AAV-2 (Peden *et al.*, Sanftner *et al.*, 2004).

1.9 Objectives of this thesis

The primary aim of this thesis was to over-express SNAP-25 in neuronal tissues. The basic principles involved, the structure and function of the target tissues, and a general overview of the current research in these fields has been provided in chapter 1. Chapter 2 describes the general methods used in this thesis project. In chapter 3 I describe the generation and characterization of recombinant adeno-associated virus (rAAV) vectors, as well as demonstrating efficient delivery techniques into the mouse retina and brain, and the rat brain. Chapters 4 and 5 describe the use of an rAAV containing a SNAP-25 transgene in both the retina, and hippocampus respectively, and demonstrate that over-expression of this gene causes a severe degeneration of photoreceptor cells in the retina, and in the hippocampus, SNAP-25 over-expression causes altered glutamate release, leading to impaired spatial memory acquisition.

Chapter 2

Materials and Methods

2.1 General materials and reagents

2.1.1 Reagents

- [i] Agarose gel: Agarose powder w/v (Sigma Genosys, Pampisford, Cambridgeshire, UK) was weighed depending on percentage gel required and dissolved in 1x Tris-Acetate-EDTA (TAE) buffer.
- [ii] Chemicals and Antibiotics: All chemical reagents, growth media and antibiotics (unless stated) were obtained from 1) BDH Laboratory Supplies, Poole, UK; 2) Calbiochem, Nottingham, UK; 3) GIBCO, Paisley, UK; or 4) Sigma-Aldrich, Stenheim, Germany.
- [iii] Enzymes and Buffers: Restriction enzymes and their appropriate buffers were obtained from New England Biolabs, Frankfurt, Germany. Taq DNA polymerase was obtained from Promega, Madison, Wisconsin, USA; Pfu Turbo Taq Polymerase, T4 DNA ligase were obtained from Stratagene, La Jolla, California, USA. Lysozyme was obtained from Sigma Aldrich.
- [iv] Molecular weight standards: 100bp and 1kb DNA ladders were obtained from New England Biolabs.
- [v] DNA Kits: EndoFree Plasmid Maxi-Prep Kit, Qiaquick Gel Extraction Kit, PCR Purification Kit, RNeasy Mini Kit were obtained from Qiagen, Hilden, Germany.
- [vi] DNA Sequencing: Big Dye Terminator v3.1 Cycle Sequencing Kits were purchased from Applied Biosystems, Foster City, California, USA.
- [vii] Tissue Culture: Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 medium, foetal calf serum (FCS), L-Glutamine, taurine, B-27 supplement, trypsin-EDTA, Phosphate Buffered Saline (PBS, Ph7.2), Hank's Balanced Salt Solution (HBSS) were obtained from Invitrogen, Paisley, UK.

- [viii] DNA Oligonucleotides were obtained from Sigma-Genosys, (Pamford, UK), and resuspended in nuclease free water to a final concentration of 100 μ M.

2.1.2 Solutions

- [i] Luria-Bertani medium (LB medium): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1%(w/v) NaCl. To prepare LB agar plates 1.5% (w/v) grade A agar was added to the medium. LB broth and agar were autoclaved at 15p.s.i for 20 minutes. E.Coli cultures were selected using 50 μ g/ml ampicillin and 25 μ g/ml tetracycline.
- [ii] Gel Loading Dye: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol and 30% (w/v) glycerol in double distilled water (ddH₂O) makes a 6x solution.
- [iii] M-STET buffer: 5% Triton X-100, 50mM EDTA pH8.0, 100mM Tris pH5.0, 5% sucrose.
- [iv] dNTP mix: 10 μ l of dGTP, dATP, dTTP, and dCTP were diluted in 160 μ l ddH₂O to give a final concentration of 10mM. This was then added to a PCR reaction mix to yield a final concentration of 1.25mM.
- [v] 1xTris-Acetate-EDTA (TAE) buffer (pH8.0): 0.04M Tris-Acetate, 0.01M EDTA.

2.1.3 Materials and reagents for Tissue Culture

- [i] All plates and pipettes used for tissue culture were obtained from Sarstedt Ltd, Numbrecht, Germany.
- [ii] DNase enzyme was obtained from Promega, Madison, Wisconsin, USA.
- [iii] Tissue Culture Medium: Complete DMEM: DMEM, 10% (w/v) FCS, 2mM sodium pyruvate.
- [iv] Lipofectamine 2000 was obtained from Invitrogen, Paisley, UK.

- [v] Freezing medium: 2ml Fetal Calf Serum (FCS), 1ml dimethyl sulfoxide (DMSO), 2ml DMEM.

2.1.4 Materials and Methods for RNA study

- [i] RNeasy Mini Kit was obtained from Qiagen, Hilden, Germany; and RNase Away was obtained from Invitrogen, Paisley, UK. Trizol reagent was supplied by Molecular Research Centre, Cincinnati, Ohio, USA
- [ii] Quantitech Sybr Green RT-PCR reaction kit was obtained from Qiagen, Hilden, Germany.

2.1.5 Animals

All animal experiments were conducted in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement regarding the use of animals in ophthalmic and vision research. Two strains of wild-type mice were used C57BL/6J mice and 129/Sv mice, both supplied by Harlan Laboratories, Oxfordshire, UK. One mutant mouse strain was used in this study – the Rhodopsin knockout (*rho*^{-/-}) mouse, which was generated in this lab in collaboration with Mario Cappecchi, University of Utah, USA. All rat experiments used male Wistar-CRL rats obtained from the colony in the Biomedical Facility in University College Dublin or from the Bioresources Unit, TCD. All rats were injected at postnatal day 49, with subsequent testing, behavioural and microdialysis, beginning at postnatal days p80-84. Water and food were provided to all animals *ad libitum*. All procedures described were conducted in accordance with the regulations of the Animal Research Ethics Committee of University College Dublin, and Trinity College Dublin and were carried out by individuals (myself included) holding the appropriate licenses issued by the Department of Health.

2.2 General Molecular Methods

2.2.1 Determination of DNA and RNA concentrations using UV spectrophotometer.

A Nanodrop TD-1000 spectrophotometer (Thermo-Fischer Scientific, Waltham, MA, USA) was used to determine concentrations of DNA and RNA. The spectrophotometer was zeroed using ddH₂O as a blank and absorbance was measured for each sample at 260nm. DNA and RNA concentrations were calculated by multiplying the absorbance at 260nm by 50, or 40, respectively, to give a concentration in micrograms per microlitre ($\mu\text{g}/\mu\text{l}$).

2.2.2 Agarose gel electrophoresis.

Agarose powder was weighed out at 0.8%-2.0% (w/v) and dissolved in 1xTAE buffer. This mixture was then heated until a gel had formed, and ethidium bromide was added to a final concentration of 0.5 $\mu\text{g}/\text{ml}$. The gel was allowed to set with a comb inserted and DNA samples (containing loading dye) were loaded into the wells accompanied by a DNA size marker. The gel was electrophoresed at a constant voltage of $\sim 100\text{V}$. DNA was visualized with a UV transilluminator (Hoeffer) and images were captured using Gene Genius Bioimaging system (Syngene, Cambridge, UK).

2.2.3 Phenol-Chloroform extraction and ethanol precipitation of nucleic acids.

Nucleic acids were mixed with an equal volume of buffer-saturated phenol (pH 7.5), vortexed and left standing on ice for 1 minute. The samples were then centrifuged at 13,000rpm in a Micromax centrifuge (Thermo Fischer Scientific, Waltham, MA, USA) at 4°C for 15 minutes. The upper aqueous phase containing the nucleic acids was transferred to a 1.5ml eppendorf tube. An equal volume of 1:1 buffer-saturated phenol and chloroform mix was added to the sample, and this was vortexed and centrifuged as before. The upper phase was again carefully removed and added to a new tube for ethanol precipitation. Nucleic acids were precipitated by adding one tenth volume of

3M sodium acetate and 3x volume of 100% ethanol. The samples were incubated at -20°C for 1-2hrs and centrifuged as before. The supernatant was removed and the remaining pellet was washed with 400µl of 70% ethanol and centrifuged again at 13,000 rpm for 15 minutes. The ethanol was then removed, the pellet air dried, and resuspended in 20-50µl ddH₂O.

2.2.4 XL1-Blue *E.coli* competent cells preparation

A loopful of XL1-blue *E.coli* was cultured in 10ml LB broth containing 25µg/ml of tetracycline with constant shaking in an orbital shaker at 37°C overnight. 250µl of the overnight culture was then used to inoculate 50ml LB broth containing 25µg/ml tetracycline and this was cultured until an OD₆₀₀ of 0.3-0.5 was reached (~2hours). Cells were then centrifuged at 3,000rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 50ml of chilled 50mM CaCl₂. The cell suspension was incubated on ice for 20 minutes and then centrifuged at 3,000rpm for 10 minutes. The cells were suspended in 5ml chilled 50mM CaCl₂ and kept on ice overnight for maximum competency.

2.2.5 Transformation of XL1-Blue competent cells.

20-50ng of DNA (or ~5µl transformation mix) was added to 100µl competent XL1-blue cells and left on ice for >20 minutes. The cells were then subjected to a heat shock at 42°C for ~90 seconds and returned to ice for 2 minutes. 1ml of LB was added to the cells and this was incubated for 1hr at 37°C. 200µl of the transformed cells were inoculated on LB-tet plates and incubated overnight at 37°C overnight. Transformants were selected by vectors ability to resist designated antibiotics. Suitable single colonies could then be picked and used to inoculate 10ml LB cultures for miniprepping.

2.2.6 Plasmid Mini-Prep Boiling Method.

Single transformed colonies were picked and inoculated overnight in 10ml LB selective medium with agitation in an orbital shaker at 37°C. Cultures were centrifuged at

3,000rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 60µl M-STET containing 20µl lysozyme (section 2.1.1). The cell suspension was transferred to a 1.5ml eppendorf tube and incubated on ice for 15 minutes. The cell suspension was then placed in a boiling water bath for 90 seconds, and centrifuged at 13,000rpm at 4°C for 15 minutes. The coagulants were scooped out with a tooth-pick and discarded and 5µl RNaseIT (10mg/ml) was added to each sample and incubated at 42°C for 15 minutes. 400µl phenol and 100µl chloroform were added to each sample and centrifuged at 13,000rpm for 15 minutes. The upper phase was then transferred to a new clean tube and an equal volume of isopropanol was added – this was then left at room temperature for 10 minutes. The samples were again centrifuged for 13,000rpm for 10 minutes, the supernatant discarded, and cell pellets were washed with 75% ethanol. After centrifugation, ethanol was discarded and the pellet air-dried, then resuspended in 20µl ddH₂O.

2.2.7 DNA Ligations.

Ligations were carried out in a total volume of 15µl at various ratios of vector:insert. Appropriate digest, ligase, and insert controls were included for each reaction. Each reaction also contained 1.5µl 10x ligase buffer and 1 unit of ligase enzyme per µg of DNA plasmid. Reactions were incubated overnight at 16-18°C.

2.2.8 Oligonucleotide Design

Primer sequences were designed using Primer3 software (free online at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Typically, primers were designed to be 21 nucleotides in length, have little or no secondary structure or primer dimers, and an approximate GC content of 50-60%. All primers were resuspended in nuclease-free ddH₂O at a concentration of 100pmol/µl or 100µM.

2.2.9 Polymerase Chain Reaction (PCR).

Standard PCR reactions were carried out in a 25µl reaction volume containing: 1 unit Taq polymerase, 10pmoles each primer, 0.5mM dNTP mix, 20-100ng DNA template, 10-20mM MgCl₂ buffer. Thermal cycling was carried out according to manufacturers instructions, using an annealing temperature typically 5 degrees lower than the melting temperature of the primers.

2.2.10 Restriction Enzyme Digestions

Enzyme digestions were carried out in 20-100µl reaction volumes containing 1 unit of restriction enzyme per µg of DNA with the appropriate buffer at 1x concentration. Restriction digests were typically incubated for 1-2 hours at 37°C and then DNA fragments were analysed for digestion using gel electrophoresis.

2.2.11 Maxi Preps

Subsequent to verifying that the recombinant clones contained the correct DNA sequences, large-scale DNA preps were made using Qiagen Maxi-prep kits according to the manufacturer's instructions. In brief, a single colony was picked and grown for 8 hours in selective LB at 37°C with constant shaking. 1ml of this starter culture was then used to inoculate 500ml selective LB and this was then grown for ~16 hours with constant shaking at 37°C. Bacterial cells were then collected by centrifugation at 3,000rpm. Pellets were resuspended in 10ml buffer P1, and 10ml buffer P2 was then added and mixed. After 5 minutes, 10ml of chilled buffer P3 was added and the cell lysate was transferred to an injection syringe and incubated for 10 minutes. During this time, a High Speed Maxi Tip was equilibrated by adding 10ml of buffer QBT and allowing it to elute under gravity flow. The cell lysate was then poured into the Maxi Tip, and the lysate eluted under gravity flow (the DNA binds to the matrix in the Maxi Tip). The Maxi Tip was then washed with 60ml buffer QC. DNA was then eluted with 15ml buffer QF and collected in a clean 50ml tube. DNA was then precipitated by adding 10.5ml of

isopropanol, incubated at room temperature for 5 minutes. A Qia-precipitator maxi module was then fixed onto the end of a syringe and the precipitate was flushed through under constant pressure. The Qia-precipitator was then washed with 70% ethanol and DNA was then eluted with 1ml buffer TE.

2.2.12 DNA Sequencing

Sequencing was carried out using the ABI310 Genetic Analyser (Applied Biosystems, Santa Clara, CA, USA). Sequencing reactions were carried out in a final volume of 10µl containing ~500ng DNA, 2µl Big Dye v3.1 enzyme, 2µl 5x reaction buffer, and 5pmol primer. Thermal cycling was then carried out 26 times under the following conditions: 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Samples were then precipitated using 4.5µl 2M Sodium Acetate (NaOAc), 62.5µl Ethanol (EtOH), and 23µl ddH₂O for 15 minutes, then centrifuged at 13,000rpm for 20 minutes. After carefully removing the supernatant, the pellet was washed with 70% EtOH, airdried, and resuspended in 25µl deionized formamide. Samples were then denatured at 95°C for 5 minutes and cooled on ice before loading into the sequencer. DNA sequences were collected using the ABI Prism 310 data collection software.

2.3 Tissue Culture Methods

2.3.1 Cell culture maintenance.

All mammalian cell cultures (American Type Culture Collection, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) in a 5% CO₂ incubator at 37°C.

2.3.2 Cell Passaging.

Cells were passaged routinely to maintain exponential cell growth and minimize cell death due to excess confluency. DMEM media was aspirated, and cells were washed twice with phosphate buffered saline (PBS) (pH7.2). PBS was then aspirated and 2ml trypsin-EDTA was added to dissociate cells from cultured plates. Plates were then incubated at 37°C for ~5 minutes to enhance dissociation of cells. The action of trypsin

was inhibited by the addition of 10mls of DMEM per ml of trypsin used. Cells were then centrifuged at 1,000rpm for 5 minutes in a swing bucket centrifuge. Supernatant was discarded and the cell pellet was resuspended in 2ml DMEM and plated at a concentration of 6×10^6 cells/ml per 10cm plate.

2.3.3 Haemocytometer cell counting

Cells were trypsinised as described above and an aliquot was diluted 1:10 in DMEM. A clean glass coverslip was placed on top of the haemocytometer and $\sim 5\text{--}10\mu\text{l}$ of the diluted sample was added to the edge of the haemocytometer to allow dispersion through the counting grid by capillary action. Cells present within the four 16-square areas were counted and the total number of cells present in the original cell stock was calculated using the following equation: avg number of cells from the four 16-square areas $\times 10$ (dilution) = cells $\times 10^4$ /ml.

2.3.4 Cryopreservation of cells.

In order to maintain a healthy cell stock, cells were frozen down during their exponential growth phase or at low passage numbers. Pre-chilling freezing ampoules were labeled with the date of the freezing, cell type and passage number. Subsequent to trypsinisation, cells were resuspended in 500 μl DMEM at a concentration of 5×10^6 – 1×10^7 cells/ml. Equal volumes of cells and freezing medium (500 μl each) were added to the pre-chilled ampoules and stored at -80°C or in liquid nitrogen. To regenerate a frozen stock, cells were thawed at room temperature for 10 minutes. The freezing ampoules were then placed in sterilized tubes and centrifuged at 1,000rpm for 5 minutes. Freezing medium was then aspirated and the cell pellet resuspended in 10ml DMEM.

2.3.5 General transfection protocol using Lipofectamine 2000

Mammalian cells were trypsinised, counted, and plated at the appropriate cell density in DMEM so that the cells would be 80-90% confluent at the time of transfection. The

appropriate amount of DNA was then diluted in Opti-MEM (serum free medium). The required amount of Lipofectamine-2000 (Invitrogen) was also diluted in Opti-MEM and incubated at room temperature for 5 minutes. The diluted lipofectamine-2000 was then mixed with the DNA, allowing complexes to form at room temperature for 25 minutes. Suitable amounts of the mixture was then added to each well and incubated at 37°C for 24-72 hours.

2.4 Adeno-associated Virus (AAV)

2.4.1 AAV production

All AAV vectors used in this study were generated in this laboratory with the help of Dr. Naomi Chadderton. Recombinant AAVs were generated using the helper virus free, triple transfection method described by Xiao *et al* (1998). The SNAP-25 cDNA was cloned into the pAAV-IRES-hrGFP vector (Stratagene, Figure 2.1), flanked by the inverted terminal repeats (ITR) of AAV2. The adenoviral helper plasmid, pHelper (Stratagene) and a second helper plasmid pRep2/Cap5 containing AAV-2 Rep and serotype capsid 5 proteins (Stratagene) were used to generate AAV2/5.

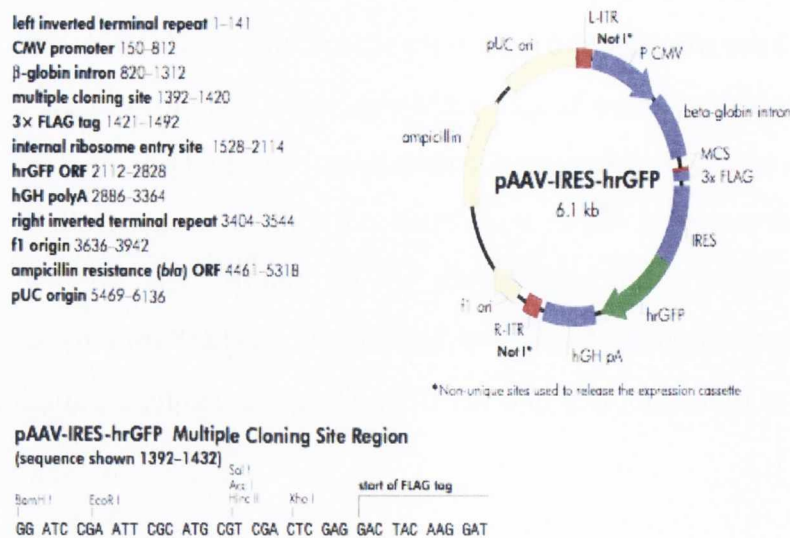


Figure 2.1: Vector map of pAAV-IRES-hrGFP (Stratagene). Key restriction sites, IRES-linked hrGFP, and CMV promoter are clearly illustrated.

HEK293 cells (ATCC accession number CRL-1573) were seeded in fifty 150mm plates at a cell density of 5×10^6 cells/ml and grown to confluency in 20ml DMEM containing 10% FCS. For each plate, 12.5µg pAAV-SNA25-IRES-hrGFP (or pAAV-IRES-hrGFP & pCMV-GFP for control viruses), 12.5µg pRep2/Cap5, and 25µg pHelper (a ratio of 1:1:2) were prepared in 1ml volume of 150mM NaCl, pH5. 1ml of polyethylenimine (0.1g/L 150mM NaCl, pH5.0) (Reed *et al.*, 2006) was then added to the mixture and was left incubating at room temperature for 8 minutes before adding to the HEK293 cells. 7 hours post-transfection, the media was replaced with 20ml of fresh DMEM containing 10% FCS.

2.4.2 Caesium chloride purification method.

Caesium chloride purification of virus was carried out as described previously by Auricchio *et al* (2001a). 48 hours post-transfection, infected HEK293 cells were collected by scraping and centrifugation at 1000g for 30 minutes. Cell pellets were then resuspended in 10ml PBS and subjected to 3 rounds of freeze thawing (from -80°C to 37°C). 10mg RNaseA and 10,000 units of DNaseI were added to the cell suspension and incubated at 37°C for 30 minutes. The cell suspension was then centrifuged for 15 minutes at 1,000rpm and the supernatant transferred to a new tube. 0.5% (final concentration) of deoxycholic acid was added to the supernatant and incubated for 30 minutes at 37°C. Caesium chloride (CsCl_2) 0.454g/ml was added to the supernatant (4.45g to 10ml supernatant) and incubated on ice for 5 minutes. A density gradient was then prepared in polyallomer Beckmann tubes (89mm height, 24mm diameter) by layering 9ml of 1.4 density CsCl_2 (548.3mg/ml) over 9ml of 1.6 density CsCl_2 . The supernatant containing CsCl_2 was then applied to the top of the gradient. The tubes were equilibrated and centrifuged at 25,000rpm in a Beckman SW28 rotor for 18 hours at 4°C. 1ml fractions were collected using a 16G syringe needle and the refractive index was calculated using a refractometer (Thermo Fischer Scientific, Waltham, MA, USA). Fractions within the range of 1.373-1.368 were collected and pooled in a polyallomer Beckman tube (6ml volume, 13mm height, 51mm diameter) equilibrated with the 1.4 density CsCl_2 and centrifuged at 59,000rpm in a Beckman fixed angle rotor VTi65 for 18

hours at 4°C. 0.5ml fractions were collected and those possessing a refractive index from 1.373-1.368 were again pooled into a single Beckman polyallomer tube and centrifuged as before. 0.5ml fractions with a refractive index of 1.373-1.368 were collected and dialysed in Slide-a-Lysers (Pierce, Rockford, IL, USA).

2.4.3 Determination of genomic viral titre.

AAV genomic titres were established post-dialysis using quantitative real time RT-PCR according to the method described by Rohr *et al*, (2002). A 1µl sample was treated before measurement with 7U DNaseI (30 minutes at 37°C, 10 minutes at 70°C, cool to 4°C) and 7µg Proteinase-K (60 minutes at 50°C, 20 minutes at 95°C). Quantitative real time RT-PCR was then carried out on the DNaseI and proteinase-K treated samples with primers specific for hrGFP using the following parameters: 10 minutes pre-incubation at 95°C, then 36 cycles of: 15s at 95°C, 5s at 67°C, 10s at 72°C) (7300 Q-PCR, Applied Biosystems). To determine copy number of the AAV DNA, a standard curve was generated using a five log spanning serial dilution (10^4 - 10^8) of the vector plasmid containing the transgene and hrGFP. Genomic titres, ie: viral particles per ml (vp/ml) were determined using the following calculation: Copy number in quantitative real time RT-PCR x Dilution (100) x dilution of template (50) x factor to yield genomic particles/ml (222).

2.5 Fluorescent Activated Cell Sorting (FACS)

Retinas were FACS sorted and RNA was extracted from AAV-infected cells using the RNeasy Mini Kit (Qiagen) (n=6). For FACS sorting, retinas were dissociated by trypsin digest into single cells. Retinal cells expressing hrGFP were isolated (10,000-50,000 per session) using a Beckman-Coulter Ultra fluorescence-activated cell sorter (Beckman-Coulter Inc., Fullerton, CA). Gene-specific primers for RT-PCR were designed in exonic sequence and so that the product also spanned an intron, using the Primer3 algorithm (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). RNA was extracted from sorted retinal cells using the RNeasy Mini Kit (Qiagen) and Quantitative PCR was

performed on an ABI 7300 Real Time PCR System (Applied Biosystems) using the Quantitech PCR kit according to manufacturer's instructions (Qiagen). Serial dilutions of RNA were used to generate standard curves of crossing cycle number versus the logarithmus of concentration for each gene of interest. A linear regression line calculated from the standard curves allowed relative transcript levels in each RNA sample to be determined. Values were normalized to the relative amounts of RDS present in the same RNA preparations and a melting curve was also carried out after each amplification step, which provides confirmation that specific PCR products were formed in the reaction. Expression of SNAP-25 in injected eyes was expressed as an average increase with endogenous values used as the baseline.

2.6 Electroretinogram (ERG) analysis

Animals were dark adapted overnight and were prepared under dim red light. Pupils were dilated with 1% cyclopentolate and 2.5% phenylephrine. Animals were anesthetized with ketamine and xylazine (16 and 1.6 $\mu\text{g}/10\text{g}$ body weight, respectively) injected intraperitoneally. Standardized flashes of light were presented to the mouse in a Ganzfield bowl. ERG responses from both eyes were recorded simultaneously by means of contact lens electrodes (Medical Workshop), with use of 1% amehocaine as topical anesthesia. Reference and ground electrodes were positioned subcutaneously, $\sim 1\text{mm}$ from the temporal canthus and anterior to the tail, respectively. Responses were analyzed using a Retiscan Retiport electrophysiology unit (Roland Consulting). The protocol was based on that approved by the International Clinical Standards Committee for human ERG. Rod-Isolated responses were recorded using a dim white flash (~ 25 maximal intensity, where maximal flash intensity was $3 \text{ candelas}/\text{m}^2/\text{s}$) presented in the dark adapted state. Maximal combined rod-cone response to the maximal intensity flash was then recorded. After a 10-min light adaptation to a background illumination of $30 \text{ candelas}/\text{m}^2$, cone isolated responses were recorded to the maximal intensity flash, presented initially as a single flash and subsequently as 10Hz flickers. a-Waves were measured from the baseline to the trough and b-waves from the baseline (in the case of

rod-isolated responses) or from a-wave to trough.

2.7 Western Blot Analysis

2.7.1 Protein Purification

Retinas of wild type 129sv (n=2) and Rho-/- (n=2) mice were homogenised with a 21-gauge needle (The homogenate was passed through the needle approximately 20 times), in boiling buffer containing 62.5 mM Tris, 2 % SDS, 10 mM dithiothreitol (DTT), 10 µl protease inhibitor cocktail/100ml (Sigma Aldrich, Ireland). The homogenate was centrifuged at 10,000 g for 20 min @ 4°C, and the supernatant was removed for occludin analysis. The supernatant was stored @ -20°C for future work.

Protein concentrations were determined using the Micro-BCA[®] (Pierce, MSC, Dublin, Ireland) assay and a BSA standard curve, and equal amounts of protein were loaded onto SDS-PAGE gels.

2.7.2 Protein separation by polyacrylamide gel electrophoresis (PAGE)

Whole retina lysate samples were analysed by electrophoresis through 10 % and 7.5 % (w/v) polyacrylamide gels as described by Laemmli UK (1970). An Atto dual slab mini vertical electrophoresis system (9 x 8 cm) gel apparatus was employed for the procedure. Typically, a 10% (w/v) resolving gel was prepared using 1.66 ml 30 % acrylamide (37.5:1, acrylamide: bisacrylamide), 1.25 ml 1.5 M Tris-Cl pH 8.8, 50 µl 10 % SDS, 25 µl 10 % APS (ammonium persulphate) and 7.5 µl TEMED (N, N, N', N'-tetramethylethylenediamine) made up to a final volume of 5 ml with distilled H₂O. The gel was polymerised between two glass plates supplied with the gel apparatus. A 4 % stacking gel was prepared using 320µl 30 % acrylamide, 625 µl 0.5 M TrisCl (pH 6.8), 25µl 10 % SDS, 12.5µl 10 % APS and 7.5µl TEMED made up to a final volume of 2.5 ml with distilled H₂O. The stacking gel was poured on top of the polymerised resolving gel

and a comb was placed into it to allow for the formation of sample wells. Once polymerised, the comb was removed and the gel was placed vertically into the electrophoresis tank containing approximately 500 ml 1 X tris glycine running buffer (0.025 M Tris, 0.25 M Glycine, 0.1 % (w/v) SDS).

Protein samples were resuspended in Laemmli sample loading buffer [1 X buffer is made of 50 mM TrisCl pH 6.8, 10 % glycerol (v/v), 2 % SDS (w/v), 0.1 % bromophenol blue (w/v) and 5 % β -2-mercaptoethanol (v/v)]. The samples were boiled for 2-5 min to ensure complete denaturation, centrifuged for 2 min at 13,000 rpm and loaded into the sample wells. Electrophoresis was carried out at 15 mA (per gel) until the bromophenol blue dye component of the sample buffer had reached the bottom of the gel (approx. 1 hour 20 min).

Gels were subsequently stained in a filtered solution of 0.25 % (w/v) coomassie brilliant blue R250, 42 % methanol and 17 % acetic acid for 20 min. De-staining was performed in 30 % methanol 10 % acetic acid and bands could be seen after 30 min. For western blot analysis, gels were transferred to nitrocellulose membrane as described below.

2.7.3 Transfer of separated protein lysates to nitrocellulose membrane

Protein samples were electrophoresed on 10 % SDS-PAGE as described above. The gel was then transferred using a semi-dry electro-blotter (Bio-Rad, Ireland). Eight pieces of Whatmann paper and one piece of nitrocellulose membrane were cut to the exact size of the gel. These were soaked briefly in transfer buffer (3.03 g tris-base, 14.42 g glycine and 200 ml methanol, made up to 1 L and pH adjusted to 8.3). Four pieces of Whatmann paper were placed on the anode and the nitrocellulose membrane was placed on top. The resolving gel was placed on top of this, followed by the remaining four pieces of Whatmann. The cathode was placed over this and the semi-dry electroblot apparatus was run at 0.9 mA per cm² of gel for 1 hour 30 min. Due to the presence of SDS in the acrylamide gel, proteins are conferred with a net negative charge and are attracted to the positive charge of the anode. This causes the protein to move from the gel onto the nitrocellulose membrane.

Non-specific binding sites were blocked by incubating the membrane at room temperature with 5 % non-fat dry skimmed milk in TBS (0.05 M Tris, 150 mM NaCl, pH 7.5) for 2 hours. Membranes were briefly washed with TBS, and incubated with polyclonal goat anti-A-20 (Santa Cruz Biotech) overnight at 4°C. The membranes were washed with TBS, and incubated with a secondary anti-goat (IgG) antibody with a HRP conjugate (1:2500), for 3 hours at room temperature. Immune complexes were detected using SuperSignal® West Pico (Pierce).

2.8 Statistical Analyses

For all experiments presented in this study, mean values, standard deviations (SD), standard error of the mean (SEM), and paired student *t*-tests were calculated using DataDesk 6.0 PPC (Data Description Inc., New York, NY, USA) or Microsoft Excell (Microsoft Corp, USA). Analysis of variance (ANOVA) post-hoc tests for multiple comparisons were calculated using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant at P-values <0.05.

Chapter 3

Generation of Recombinant Adeno-Associated Virus

3.1 Introduction

Efficient and targeted gene delivery is pivotal in attaining a successful therapy and is therefore a critical factor in achieving the aims of this project. Significant efforts have been focused on the development of vectors for gene delivery as outlined in Chapter 1. To this end, development and optimization of a desirable delivery system was a primary goal of this study We therefore decided to concentrate our efforts on optimizing gene delivery using AAV vectors. Such vectors may be utilized to deliver nucleotide-based tools to a target tissue and modulate gene expression thereby providing a powerful means for in vivo exploration of gene function within that tissue.

As briefly discussed in Chapter 1, AAV belongs to the *Parvoviridae* family and it is a non-pathogenic virus. AAVs contain a linear, single-stranded genome containing two genes, *rep* and *cap* required for viral genome replication and structural proteins, respectively (Rabinowitz and Samulski, 2002). The 4.7kb genome is enclosed within two symmetric T shaped palindromic terminal sequences called inverted terminal repeats (ITRs) (figure 3.1).

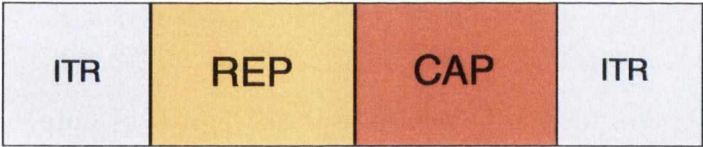


Figure 3.1: Wild type single stranded genome of AAV. There are two genes, *rep* and *cap*. *Rep* codes for proteins involved in viral replication, structural gene expression and integration into the host genome. *Cap* codes for capsid structural proteins. At both ends of the genome there is a 145bp inverted terminal repeat (ITR)

AAV was originally isolated as a contaminant from adenoviral stocks in the 1960s and is considered a dependovirus because it requires the co-infection of a “helper” virus to replicate. In the absence of co-infection by adenovirus (or herpes simplex virus or human papilloma virus), AAV remains latent in the host cell (figure 3.2).

Recombinant AAV (rAAV) has demonstrated significant promise as a DNA delivery vector and could prove to be particularly useful in gene therapy applications to treat human disease (Somia & Verma., 2000). Clinical studies using rAAV vectors to treat a wide array of disorders such as alpha-1-antitrypsin deficiency, Alzheimer's disease, arthritis, Batten's disease, Canavan's disease, cystic fibrosis, haemophilia B, HIV infection, Parkinson's disease, Lebers' congenital amaurosis, muscular dystrophy, prostate cancer and melanoma are underway (Carter *et al.*, 2005; Warrington *et al.*, 2006; Fiandaca *et al.*, 2008).

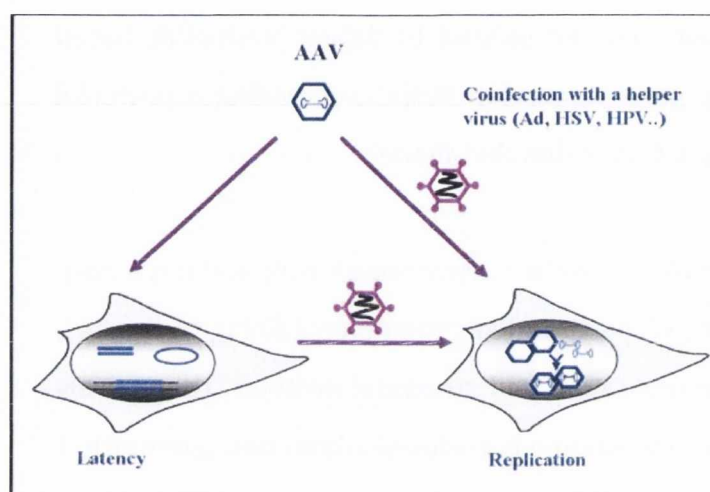


Figure 3.2: Life cycle of wild type AAV. AAV is a dependovirus and once it has infected the host cell it enters a latency phase (left). It requires co-infection of either adenovirus (Ad), herpes simplex virus (HSV) or human papilloma virus (HPV) for AAV genome replication and for packaging into infectious particles.

While rAAV represents an attractive option for gene delivery to the CNS, it is of note that AAV titres need to be very high ($<10^{12}$ vp/ml) for both retinal and hippocampal injections as the volume to be delivered is limited in rodents (3µl in the retina and only 1µl in the hippocampus). Therefore we first sought to devise the best AAV production protocol. We chose to utilize the plasmid triple-transfection protocol developed by Xiao *et al* (1998) which eliminates the risk of contaminating virus particles associated with herpes simplex virus and adenovirus systems (figure 3.3). This approach was then applied to the development of an rAAV vector containing the SNAP-25 cDNA. The recombinant expression plasmid (containing the SNAP-25 cDNA cloned into the plasmid

multiple cloning sequence (MCS)) is co-transfected into HEK293 cells with pHelper (carrying adenovirus-derived genes) and pAAV-RC (carrying AAV-2 replication and AAV-5 capsid genes) which together supply all of the *trans* acting factors necessary for AAV replication and packaging. Recombinant AAV particles can then be prepared from the infected HEK293 cells and used to infect a wide variety of mammalian cell types.

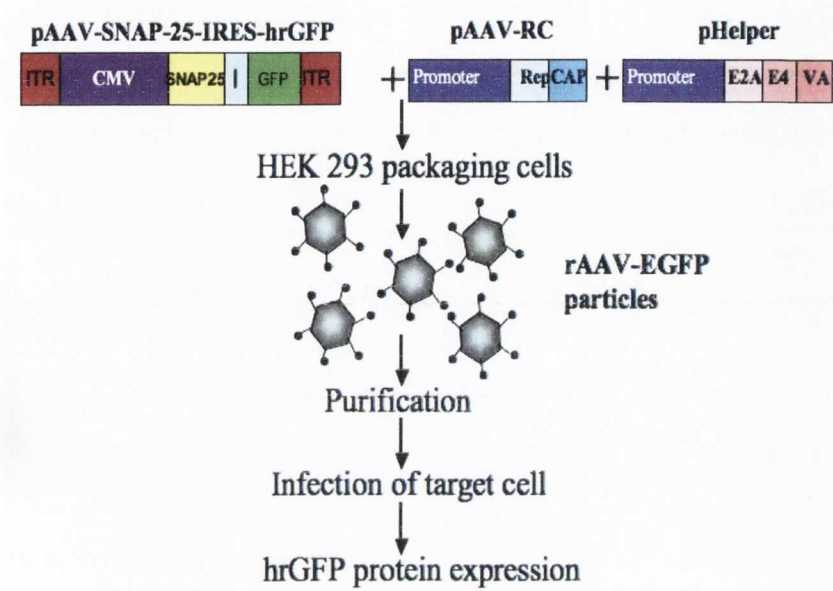


Figure 3.3: generating rAAV particles using the triple transfection protocol. initially developed by Xiao *et al*, (1998). Recombinant AAV expression cassettes are co-transfected into HEK293 cells together with pAAV-RC (also called pRep2/Cap5), containing the AAV-2 *rep* and AAV-5 *cap* genes, and pHelper, which provides most of the adenovirus gene products required for production of infectious rAAV particles, ie: E2A, E4, and VA RNA genes. rAAV particles can then be harvested from cells and purified by CsCL₂ gradient centrifugation.

Since the generation of the first recominant AAV-2 vector (Hermonat & Muzyczka, 1984; Tratschin *et al.*, 1984), 108 novel capsid sequences have been isolated (55 from human and 53 from non-human primates) about 9 of these have been cloned into the commonly used vectors for gene transfer (Surace & Auricchio, 2008). rAAV vectors can be generated using both the capsid proteins and genomes from the same serotype, or the vector genome from AAV-2 can be used in conjunction with a capsid gene from another serotype, as discussed in Chapter 1. As the capsid protein confers specific cellular tropism, vector pseudotyping allows for targeting different cell types (figure

3.4). AAV-2/5 has been repeatedly shown to be the most efficient at transducing both photoreceptor cells of the retina (Surace & Auricchio, 2008) and the pyramidal and granule cells of the hippocampus, and was therefore the AAV pseudotype used in these studies.

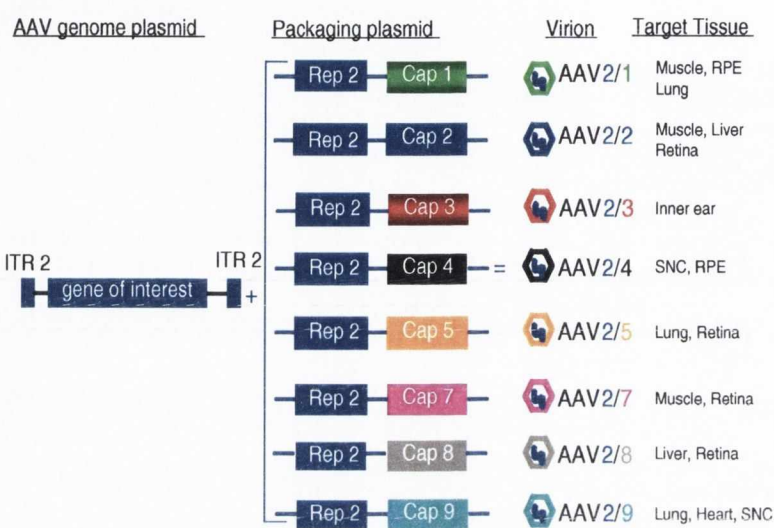


Figure 3.4: An array of recombinant vectors based on different AAV serotypes.

Transfection in the packaging cells of the plasmids containing: the AAV genome (with the therapeutic gene contained between the viral inverted terminal repeats, ITRs) and the packaging sequences (the *cap* genes encoding for the capsids and the *rep* open reading frames belonging to serotype 2 to allow the most efficient packaging of a viral genome with AAV2 ITRs) results in production of recombinant virions. The hybrid virions containing the genome of one serotype (i.e. AAV2) and a different capsid (AAV2/1–9) have tropism dependent on the capsid sequence. (reproduced from Auricchio *et al.*, 2008).

However, recent evidence has suggested that AAV-2/7 and -2/8 are more efficient than -2/5 at transducing cells in the hippocampus (Taymans *et al.*, 2007). More recent evidence has shown that AAV9 is particularly efficient at transfecting neuronal cells in vivo (Cearney *et al.*, 2008), with intravenous/systemic administration of self-complimentary rAAV2/9 showing particularly high transfection rates of neonatal neurons and adult astrocytes (Faust *et al.*, 2009; Duque *et al.*, 2009).

Once high titre viruses (between $5-8 \times 10^{12}$) were generated they were transfected into HEK293 cells to test for virus viability and transgene expression and then injected into mouse retina to further evaluate expression levels and check for immunofluorescence. In an additional set of experiments we also investigated the efficiency of AAV2/5 to transfect neuronal cells in the hippocampus and to optimize our stereotaxic injection procedure into brain. Furthermore we sought to perfect a method to overcome a major limitation of AAV vectors, namely the limited packaging capacity (~4.7kb). Packaging capacity for rAAV is a critical issue that may determine how rAAV vectors can be applied for human gene therapy, as many target and therapeutic genes will be greater than 4.7kb in size. By increasing the wild type AAV genome it was suggested that 5.3kb was the maximum limit for efficient packaging of AAV (Hermonat *et al.*, 1997). More recently it has been reported that large vectors up to 8.9kb could be packaged into rAAVs using suitable AAV serotypes (Alloca *et al.*, 2008). While much ongoing research has focused on increasing the size limit, co-administration of two AAV vectors may provide an effective and simple alternative solution in certain situations (without affecting viral titre). For example, when delivering two or more transgenes, in theory, it is possible to administer the components using two separate vectors, thereby potentially “doubling” packaging capacity (providing both vectors transfect the same cell). Therefore we sought to investigate this possibility *in vivo* in the rat brain, using two rAAV2/5 vectors carrying CMV-driven eGFP or DS-Red-Express2 reporter genes. A mixture of the two vectors was co-administered by stereotaxic injection into the rat dorsal hippocampus. 5-6 weeks post-injection, animals were sacrificed and marker gene expression was observed using fluorescence microscopy and confirmed by flow cytometry. I found that significant co-transduction with DS-Red-Express2 and eGFP was detected using fluorescent microscopy, indicating that co-administration of two rAAV vectors could deliver approximately 10kb if the cargo consists of two or more genes which could be divided between the two vectors. This approach could be particularly useful for delivering gene therapies consisting of two components, such as therapeutic gene and reporter gene, or for suppression/replacement therapies.

3.2 Methods

3.2.1 Plasmids

Recombinant AAVs were generated using the helper virus free, triple transfection method of Xiao *et al* (1998). SNAP-25 was amplified from a cDNA library generated from hippocampal RNA (a gift from Keith Murphy, UCD), using pfu turbo taq polymerase (Stratagene) and primers containing a BamHI restriction site (forward) CGGGGATCCATGGCCGAGGACGCAGA; and a XhoI restriction site (reverse) CCGCTCGAGTTAACCACTTCCCAGCATCTT. The SNAP-25 cDNA was cloned into the pAAV-IRES-hrGFP plasmid (Stratagene), flanked by the inverted terminal repeats of AAV2. The adenoviral helper plasmid pHelper (Stratagene) and a second helper expressing plasmid AAV-2 Rep and serotype 5 capsid proteins (pRep/Cap5), (Hildinger *et al.*, 2001) were used to generate rAAV 2/5. To generate AAV/Control and AAV/hrGFP rAAVs, the pAAV-eGFP and pAAV-IRES-hrGFP (Stratagene) vectors were used.

3.2.2 AAV Production & Triple Transfection method

AAV HEK293 cells were seeded in 50X150mm plates (un-coated) at 5×10^6 cells/plate and grown to confluence in 20ml DMEM containing 10% FCS.

For each plate, 1ml 150mM NaCl, pH5 containing 12.5µg pAAV-SNAP-25-IRES-hrGFP, 12.5µg pRep/Cap5 and 25µg pHelper (quantified by spectrophotometer OD 260/280 readings in TE solution) was mixed with 1ml polyethylenimine (0.1g/litre 150mM NaCl, pH5) and left to stand at room temperature for 8mins before adding to the cells (10% final volume – 2mls added to 18mls). Seven hours post transfection the media was replaced with 20mls DMEM containing 10% FCS and the cultures incubated for 48hrs. Vector-containing cells were dislodged by scrapping and collected by centrifugation at 1000g for 30mins.

The cell pellet was resuspended in 10mls PBS and subjected to 3 rounds of freeze (-80°C) and thaw (37°C) and incubated with 10mg RNaseA and DNase I for 30min at 37°C. Following 15min centrifugation at 1000g, the 10 ml supernatant was transferred to a

new tube and incubated with 0.5% (final concentration) deoxycholic acid for 30min at 37°C.

Samples were then purified by caesium chloride gradient. Briefly, 0.454g CsCl₂/ml was added to the sample (4.54g CsCl to the 12ml supernatant) and incubated on ice for 5min. Meanwhile a density gradient was prepared in polyallomer Beckman tubes (89mm height x25mm diameter, order no. 326823); 12ml of 1.4 density CsCl₂ (548.3mg/ml) with 12ml of 1.6 density CsCl₂ (816.5mg/ml) pipetted below. The sample was then applied to the top of the gradient. The tubes equilibrated and spun at 25,000rpm in a Beckman SW28 rotor for 18hrs at 4°C. 1ml fractions were collected by 16 gauge syringe needle and the refractive index read on a refractometer. Fractions within the range 1.373-1.368 were collected and pooled in polyallomer Beckman tubes (8.9ml volume, 60mm height x16mm diameter, order no. 361623), equilibrated with the 1.4 density CsCl₂ and spun at 59,000rpm in a Beckman fixed angle Ti70.1 rotor for 18hrs at 4°C. 0.5ml fractions were collected and those within the refractive index (1.373-1.368) were pooled into a single polyallomer Beckman tube (6 ml volume, 13mm height x51mm diameter, order no. 342412), equilibrated with the 1.4 density CsCl₂ and spun at 59,000rpm in a Beckman fixed angle rotor VTi65 for 18hrs at 4°C. Following collection, 0.5ml fractions from within the range 1.373-1.368 were dialysed against 10litres of PBS over 40hrs (1litre/4hrs) using 0.1-0.5ml Slide-a-Lyzers (Pierce, order no. 66383). [for general preps, e.g. AAV.CMV.EGFP, fractions 1.372-1.369 equating to 3x0.5ml were dialysed, as above, using 0.5ml-3ml Slide-a-Lyzers (Pierce, order no. 66380) and concentrated using a Millipore Centricon filter/concentrator (YM-30, 30,000 MWCO, order no. 4242AM).

3.2.3 Titration of genomic AAV particles using Quantitative Real-Time PCR

The genomic viral titre of each of the rAAV vectors generated as part of this study (AAV/SNAP-25, AAV/Control and AAV/hrGFP) were determined using quantitative real time RT-PCR to amplify a sequence of the GFP (either hrGFP or eGFP, depending on the virus being titred) with reference to a standard curve generated from serially diluting a known concentration of plasmid vector according to the method of Rohr *et al.*, (2002) (figure 3.8). Each sample was treated before measurement with 7U DnaseI (30min at 37°C, 10min at 70°C, cool to 4°C), and 7µg proteinase-K (60min at 50°C, 20min at 95°C). RT-PCR was carried out on an ABI 7300 real time PCR system (Applied Biosciences, Foster City, CA, USA) using the following parameters: 10mins preincubation at 95°C, followed by 36 cycles of 15sec at 95°C, 5sec at 67°C and 10sec at 72°C. To determine copy number of the transgene containing viral DNA, a standard curve was generated using a five log spanning serial dilution (10^4 - 10^8) of the plasmid vector containing the transgene (pAAV-IRES-hrGFP, or pCMV-GFP). Genomic titres, ie: viral particles (vp/ml) were determined using the following calculation – Copy number in quantitative real time RT-PCR x Dilution (100) x Dilution of template (50) factor to yield genomic particles/ml (222).

3.2.4 *In vitro* analysis of AAV

Viral vectors were then used to transduce cells *in vitro*, in order to establish viral viability, confirm overexpression of the transgene and to determine the percentage of transfected cells. HEK293 cells were seeded in 2ml DMEM containing 10% FCS at 1×10^6 cells per well in a 6 well plate and incubated for at least 3 hours at 37°C. Media was then aspirated and the cells were washed twice with PBS. AAV/SNAP-25, AAV/hrGFP, or AAV/Control vectors were then added to wells, at an MOI of 250-2500, in 250µl PBS. After incubation for 1 hour at 37°C, 1ml of media was added, and the cells were incubated overnight. Cells were then washed again with PBS, fixed with 4%

paraformaldehyde and counterstained with DAPI for fluorescence microscopy. To assess cell death, approximately 300 live cells and apoptotic nuclei per sample were counted from 3 fields of view, and subsequent mean values and standard errors calculated. Significance was assessed using an unpaired *t*-test.

3.2.5 Subretinal Injection

Mice were anesthetized by intraperitoneal injection of medetomidine and ketamine (10 and 750µg/10g body weight, respectively). Pupils were dilated with 1% cyclopentolate and 2.5% phenylephrine, and, under local analgesia (Amethocaine), a small puncture was made in the sclera. 3µl of rAAV/SNAP-25, rAAV/Control, or rAAV/hrGFP was injected subretinally into the right eyes of adult C57 mice (n=2) and male adult CD1 mice (n=9) using a 33 gauge blunt hamilton syringe (figure 3.8) [NB: 2 different wild-type mouse strains were used, due to availability of mice in the Bioresources Unit, TCD]. Following subretinal injection, a reversing agent (100 µg/10 g body weight [Atipamezole Hydrochloride] was delivered by intraperitoneal injection. Body temperature was maintained using a homeothermic heating device. After four weeks, mice were sacrificed and eyes were either embedded in agar (n=3) and cut into 50-100µm sections on a vibrotome (Leica VT 1000S) for immunohistological analysis, or RNA was extracted using the RNeasy Mini Kit (Qiagen) (n=6).

3.2.5.1 Stereotaxic injection – see 5.2.2 Site directed injection to the dorsal hippocampus

3.2.6 Quantitative RTPCR to analyse SNAP-25 expression

Gene-specific primers for RT-PCR were designed in exonic sequence and so that the product also spanned an intron, using the Primer3 algorithm (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 WWW.cgi>). RNA was extracted from dissected retinas using the RNeasy Mini Kit (Qiagen) (n=5) and Quantitative PCR was

performed on an ABI 7300 Real Time PCR System (Applied Biosystems) using the Quantitech PCR kit according to manufacturer's instructions (Qiagen). Serial dilutions of RNA were used to generate standard curves of crossing cycle number versus the logarithmus of concentration for each gene of interest. A linear regression line calculated from the standard curves allowed relative transcript levels in each RNA sample to be determined. Values were normalized to the relative amounts of RDS present in the same RNA preparations and a melting curve was also carried out after each amplification step, which provides confirmation that specific PCR products were formed in the reaction. Expression of SNAP-25 in injected eyes was expressed as an average increase with wild-type values used as the baseline.

3.3 Results

3.3.1 Generation and purification of High Titre rAAV

To generate an AAV vector for SNAP-25 delivery, the AAV helper-virus free system (Xiao *et al.*, 1998) was used to allow for the production of infectious recombinant human AAV-2 virions without the need for helper virus (Figure 3.3). The *rep* and *cap* genes are absent from the viral vector (pAAV-IRES-hrGFP) and are provided in-trans on the pRep2/Cap5 plasmid (Hildinger *et al.*, 2001). A second pHelper plasmid provides the adenovirus gene products (E2A, E4 & VA RNA genes) required for the production and packaging of infectious rAAV particles (figure 3.3). The remaining adenoviral gene product is provided by the HEK293 cells which stably express the adenoviral E1 gene. Upon infection of the host cell, the single stranded virus must become double stranded for gene expression to occur. The virus relies on cellular (ie host) replication factors for double stranded synthesis of the complimentary strand to occur and this conversion is considered to be the rate limiting step in expression of the recombinant transgene. This can be accelerated using adenoviral superinfection or etoposides like camptothecin or sodium butyrate. However, agents to accelerate gene expression are toxic to the target cells and can kill target cells if left on the cells. The use of etoposides is therefore only

recommended for short-term use or in obtaining viral titers. Typically, the AAV genome will form high molecular weight concatemers that are responsible for stable gene expression in cells.

A recombinant AAV-2/5 vector was designed to express both the SNAP-25 gene and the hrGFP gene (AAV/SNAP-25) under the control of the cytomegalovirus (CMV) promoter, allowing for the direct identification of cells expressing the viral transgene both *in vitro* and *in vivo*. As SNAP-25 is expressed endogenously in the retina and brain, it was necessary to have a GFP tag to aid identification of transduced cells. The GFP tag was also very useful for sorting the transduced cells using FACS. Two control viruses were also generated using empty plasmid vectors (ie: backbone plasmids from Stratagene with no transgene cloned into the MCS) – these were pAAV-IRES-hrGFP and pAAV-eGFP.

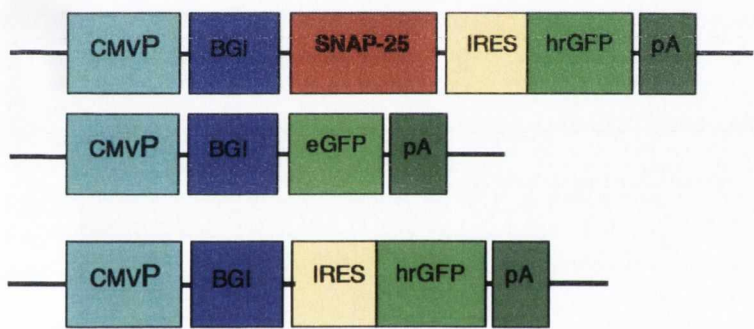


Figure 3.5: AAV constructs.

Top: AAV/SNAP-25; middle: AAV/Control; bottom: AAV/hrGFP. Viral transcripts were expressed under the control of the CMV immediate early promoter. The β -globin intron (BGI) is located at the 5' end of the transcript and the human growth hormone polyadenylation signal (pA) is located at the 3' end of the transcript. A humanized recombinant green fluorescent protein reporter gene (hrGFP) linked by a internal ribosome entry site (IRES) was used, allowing both SNAP-25 and hrGFP proteins to be translated from the same transcript. In this way, hrGFP expression may be used to ascertain the infection efficiency for the desired target cell type and also serves as a useful expression marker for the SNAP-25 protein. The AAV/Control virus uses an eGFP marker (enhanced green fluorescent protein).

All three vectors were prepared using the same techniques. Once the SNAP-25 cDNA had been inserted into the pAAV-IRES-hrGFP plasmid, the presence of the SNAP-25 insert was confirmed using sequence analysis, PCR - using one vector specific primer and one SNAP-25 specific primer, and also by digestion with restriction enzymes. Each

recombinant AAV expression plasmid was then grown in sufficient quantities for subsequent transfection into HEK293 cells. Each transfection requires 12.5µg of both the recombinant expression vector and the pRep2/cap5 plasmid, as well as 25µg of the pHelper vector and this DNA must be of high quality and purity. Viruses were harvested and purified using a CsCl₂ gradient and genomic titres were calculated using quantitative RT-PCR (figure 3.8).

3.3.2 rAAVs transduce HEK293 cells *in vitro*

As an initial step to evaluate the viability of the rAAV constructs, each virus was transfected into HEK293 cells *in vitro*. Cells were fixed, counterstained with DAPI and analysed by fluorescence microscopy 24-48 hours post-AAV infection. Green cells were clearly visible demonstrating efficient transduction of the virus and expression of the transgene cassette (Figure 3.6).

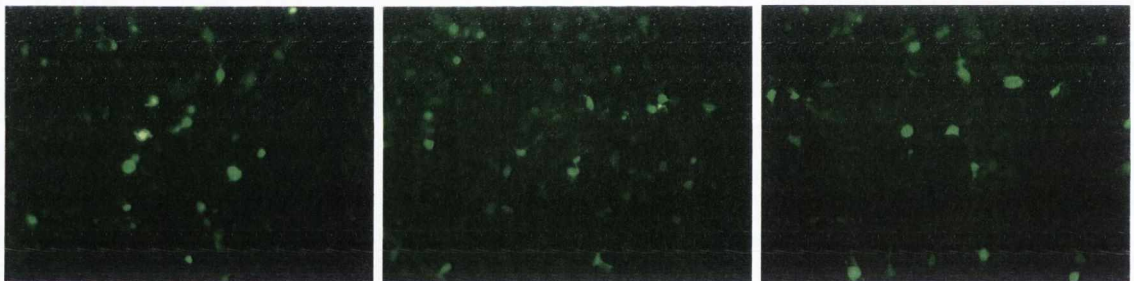


Figure 3.6: Validation of AAV infectivity and transgene expression in HEK293 cells. AAV/SNAP-25 (left), AAV/Control (middle), and AAV/hrGFP (right) transduced HEK293 cells were fixed and analysed for GFP expression 48 hours post-infection by fluorescent microscopy.

A common problem encountered when using vectors containing IRES elements is that the gene downstream of the IRES often suffers from much lower expression than when using other vectors (some reports suggest it may be 10-50 fold lower). It is important to note that the gene upstream of the IRES (in this case, SNAP-25) is not effected. We estimated that hrGFP expression (ie: fluorescence) was routinely ~10-fold lower than that observed when compared with fluorescence from AAV/Control (eGFP-expressing) transduced cells. To counteract this problem and to better visualise the hrGFP expression in transduced cells, we used a multiplicity of infection (MOI) 10 fold greater when using AAV vectors containing the IRES. 48 hours post infection, significant

numbers of HEK293 cells were green under fluorescent microscopy, indicating efficient expression of the recombinant viral transgenes (figure 3.6).

rAAV/SNAP-25 was also transfected into HeLa cells to confirm viral mediated transgene expression (figure 3.7)

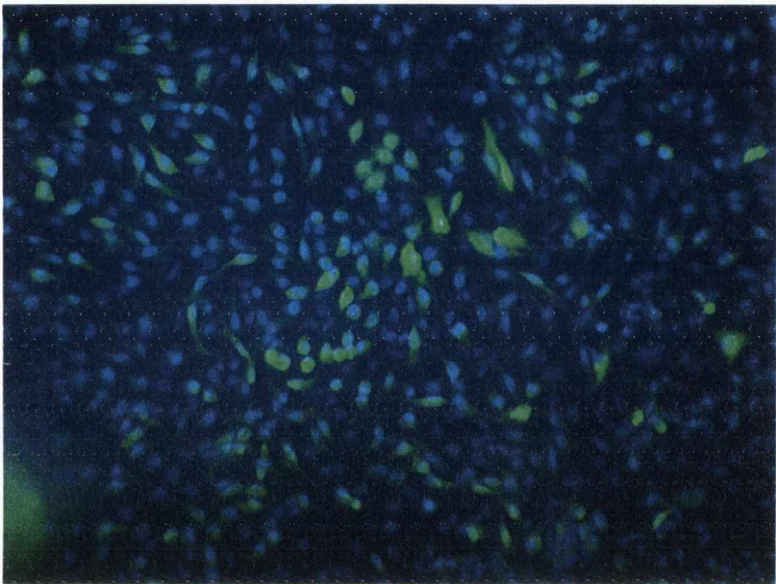


Figure 3.7: Expression of AAV/SNAP-25 in HeLa cells.
AAV/SNAP-25 at an MOI of 2500 was added to HeLa cells and cultured overnight. Fluorescence microscopy clearly demonstrates efficient expression of the viral transgene cassette.

3.3.3 Calculating genomic viral titres of rAAVs

Viral titres were calculated using the method described by Rohr *et al.*, (2002), using quantitative real time RT-PCR to amplify a sequence from the GFP tag of each viral construct, with reference to a standard curve generated by serially diluting (10^4 - 10^8) a known concentration of vector (figure 3.8). Viral copies were then calculated using the following calculation: Copy number in quantitative real time RT-PCR x Dilution (100) x Dilution of template (50) factor to yield genomic particles/ml (222).

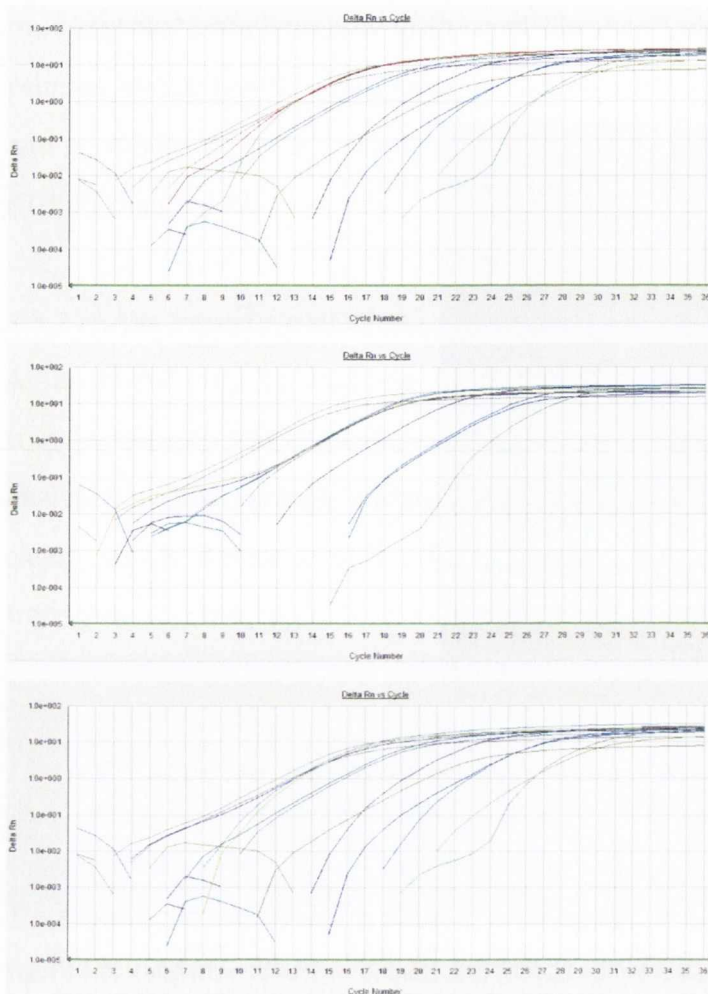


Figure 3.8: Calculating AAV genomic titres using quantitative RT-PCR.

Amplification curves for GFP quantification obtained from ABI 7300 real time PCR software. Standard curves were calculated using 4 serial dilutions and these served to quantify RNA in unknown samples. Standards for AAV/SNAP-25 (top) and AAV/hrGFP (bottom) = 2.17×10^6 ; standard for AAV/Control (middle) = 5.99×10^6 .

3.3.4 rAAVs efficiently transduce photoreceptor cells *in vivo*

To further evaluate the efficiency of the AAV/SNAP-25 virus and to investigate its ability to transduce neuronal cells *in vivo*, I investigated if subretinal injection into adult mice resulted in robust expression of the transgene cassette. 3 μ l of AAV/SNAP-25 was injected subretinally into the right eyes of adult mice, using the left eye as an uninjected control. Animals were allowed to recover for 4 weeks, perfusion fixed and then the injected retinas analysed for hrGFP expression using fluorescent microscopy. A 4-5 week

recovery period was used to allow for sufficient accumulation of the hrGFP protein. Eyes were enucleated and the retinas dissected and after a ~4 hour post fixation period in 4% paraformaldehyde, whole retinas were immediately examined for fluorescence, simply by flat-mounting the retina on a microscope slide. When the photoreceptor side of the retina was viewed, large numbers of green cells could clearly be seen, covering approximately 30-50% of the retina (Figure 3.9).

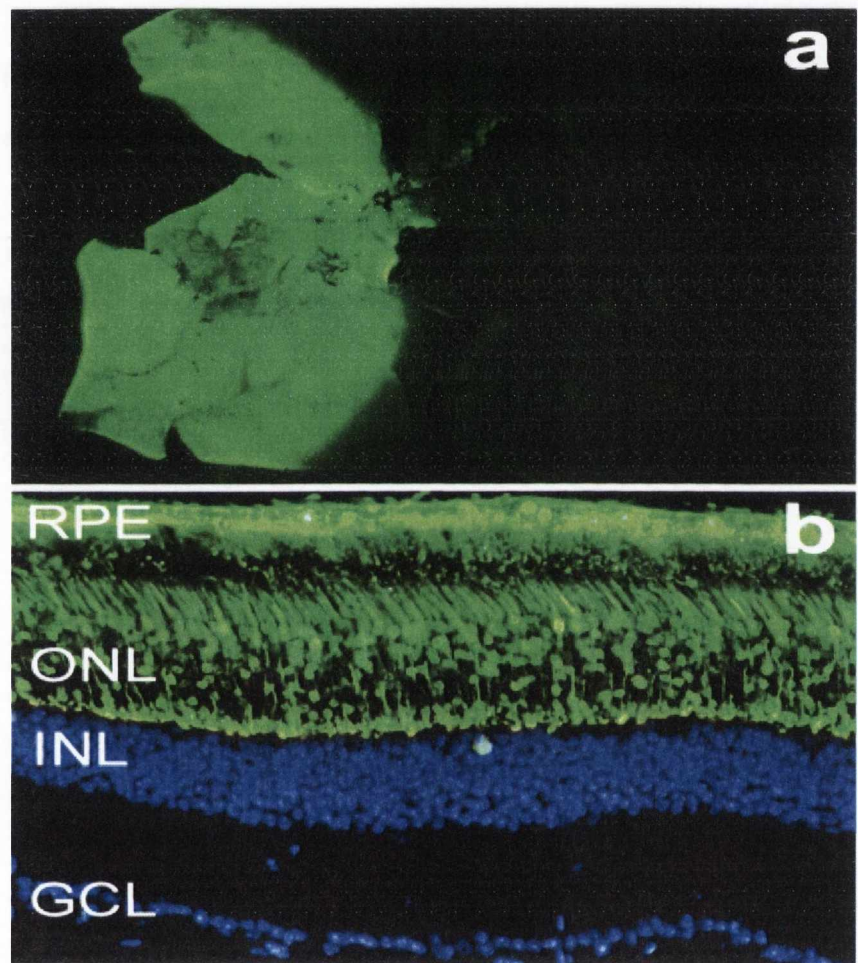


Figure 3.9: GFP expression in whole mouse retina.
a: Image of whole dissected flat mounted retina prior to embedding and sectioning from an adult CD1 mouse 5 weeks post-injection with eGFP-expressing AAV. eGFP fluorescence was detected over a significant proportion (approximately 30-50%) of the retina. **b:** retinas were then sectioned and analysed by fluorescence microscopy, showing high transfection efficiency in the photoreceptor cell layer. Green = GFP expression; Blue = DAPI counterstain; RPE = retinal pigment epithelium; ONL = outer nuclear layer; INL = inner nuclear layer; GCL = ganglion cell layer.

Retinas were then embedded in 4% agarose and cut into 50µm sections for fluorescent microscopy analysis. Cross sections of AAV/SNAP-25 retinas show robust expression of the hrGFP protein in the photoreceptors of the outer nuclear layer (ONL), showing that the virus efficiently expresses the transgene cassette (Figure 3.10, A-C). Sections were counterstained with DAPI and also with an anti-rhodopsin antibody to visualise the retinal nuclei and also the photoreceptors (Fig 3.10, D&E).

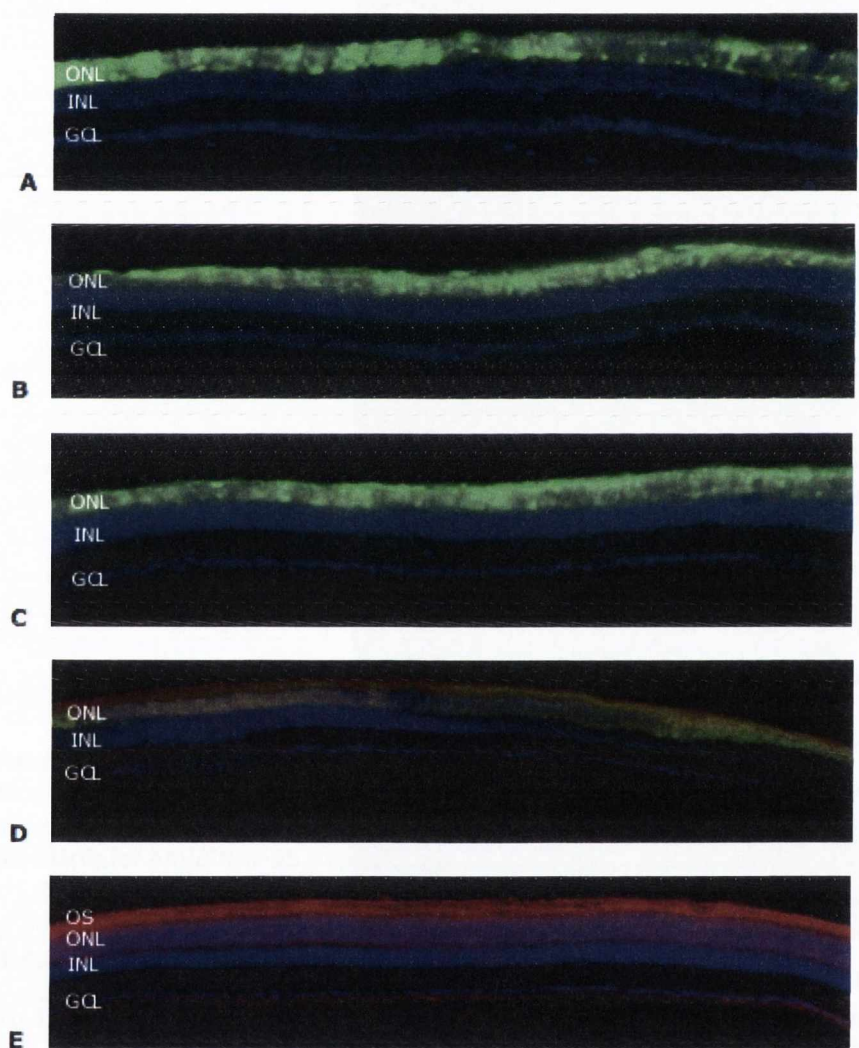


Figure 3.10: SNAP-25 AAV subretinal injections.

50µm vibratome sections of mouse retina 5 weeks post injection. hrGFP (linked to SNAP-25 by IRES) and driven by CMV promoter is expressed predominantly in photoreceptor cells of the retina. A-C show viral gene expression from each injected mouse eye (n=3). hrGFP expression was seen to cover approx 30-50% of each injected eye (See figure 3.9). D&E show costaining with rhodopsin, D=injected; E=uninjected control (20X magnification)

It is interesting to note that although the cell morphology and histology is not clearly visible (because the section is so thick, ~50µm) the sections from AAV/SNAP-25 injected retinas (A-D) are clearly thinner than the uninjected retinas (E), particularly in the ONL. This unusual finding led us to further investigate if overexpression of SNAP-25 induces photoreceptor degeneration (See Chapter 4).

3.3.5 Transduced photoreceptor cells overexpress SNAP-25

In a separate cohort of mice, 3µl of AAV/SNAP-25 was injected into the right eye, with the left eye untreated, as before (n=5). After 4 weeks, animals were killed, the retinas dissected, and RNA extracted for quantitative real time RT-PCR analysis. RNA from AAV/SNAP-25 injected eyes was compared with RNA from uninjected eyes. As SNAP-25 is expressed endogenously in the murine retina, levels of RDS/peripherin were also quantified and SNAP-25 levels were normalized accordingly. SNAP-25 expression was increased by 176% in the retinas of AAV/SNAP-25 injected eyes compared to control (Figure 3.11).

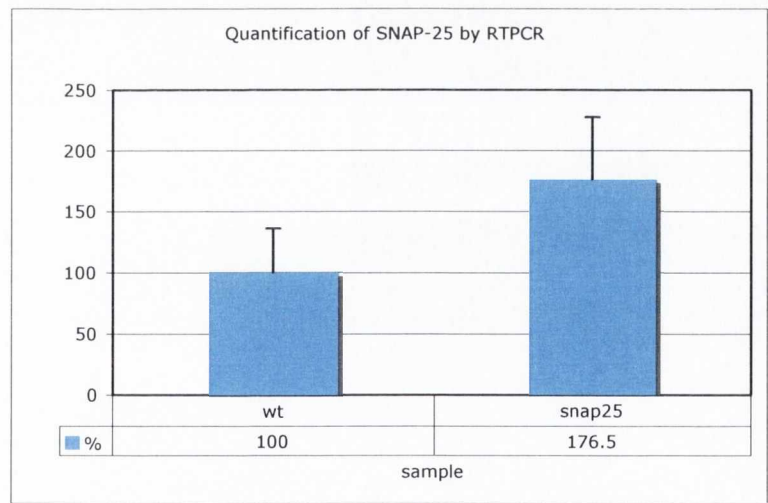


Figure 3.11: Quantification of viral SNAP-25 expression by RTPCR. Comparative expression of SNAP-25 in uninjected control and retinas injected with SNAP-25 AAV. SNAP-25 is endogenously expressed in murine retina, so to determine levels of viral SNAP-25 expression, RNA from retinas injected with 3ul of SNAP-25 AAV prep with a viral titre of 8×10^{12} genomic particles/ml (n=5) was compared to RNA from the uninjected control eyes. As an internal control RDS/peripherin levels were also quantified and SNAP-25 values were normalized to these accordingly. (control=100% $\pm$ 35.6, injected=176% $\pm$ 51.4 p value = <0.01)

3.3.6 Optimization of stereotaxic injection into mouse hippocampus

A vital technique for infusing rAAV vectors into the hippocampus is stereotaxic injection (this technique is discussed further and in more detail in chapter 5, including methods – “5.2.2 Site directed injection to the dorsal hippocampus”). We first investigated the feasibility of this technique in mice. In brief, mice were anaesthetised, as above, then placed in a stereotaxic frame. The skull was exposed, the relevant stereotaxic coordinates were used to target the dorsal hippocampus. A small hole was drilled in each hemisphere, and the infusion needle was lowered slowly using the relevant stereotaxic coordinates to target the dorsal hippocampus in both sides (ie bilateral injection).

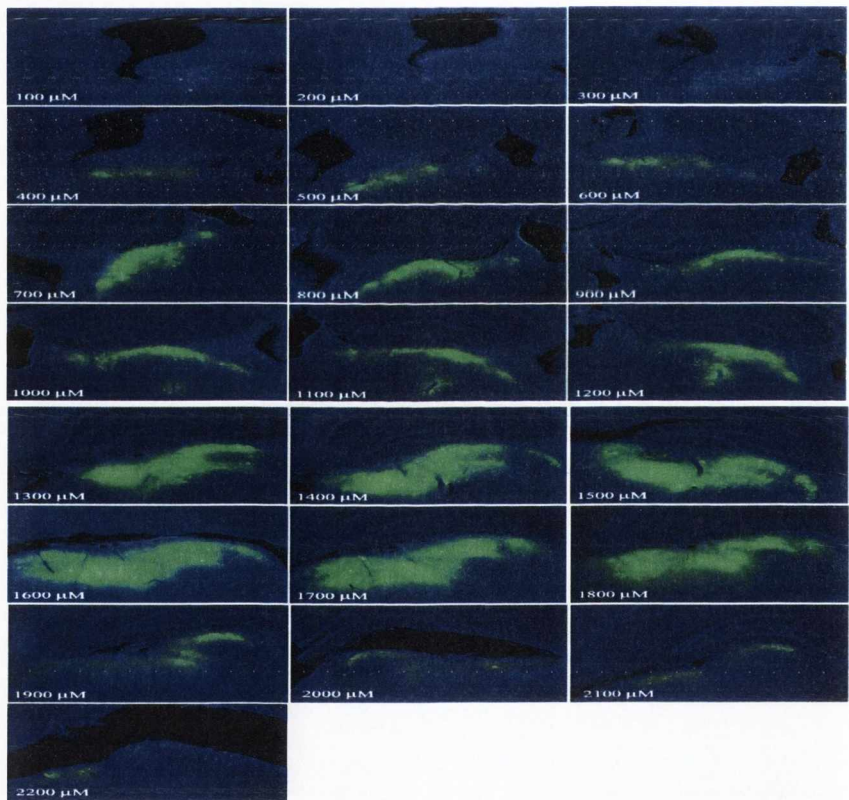


Figure 3.12: AAV mediated eGFP expression in mouse brain 4 weeks post stereotaxic injection. Mice were injected with 3μl AAV/eGFP, cut into 100μM thick sections using a vibrotome, and counterstained using DAPI (blue). We attempted to target the dorsal hippocampus but unfortunately due to the tiny size of the target tissue, the injection needle missed and virus was instead perfused into the thalamus. This was a recurring problem, however this figure was included as it shows excellent spread of viral gene expression over 21 serial sections, demonstrating that virus spreads for ~2mm from the injection site.

Approximately 1µl of AAV/eGFP was infused into the tissue over 10 minutes. Mice (n=3) were sacrificed 4-5 weeks post-injection, the brains removed, and sectioned. Figure 3.12 shows significant viral mediated eGFP expression over 19 consecutive 100µm thick serial sections. This shows that 1µl of rAAV diffuses approximately 1mm from the injection site into hippocampal tissue, and that transgene expression can be detected at significant levels in the surrounding areas. However, unfortunately – despite repeated attempts, it was very difficult to target the mouse dorsal hippocampus.

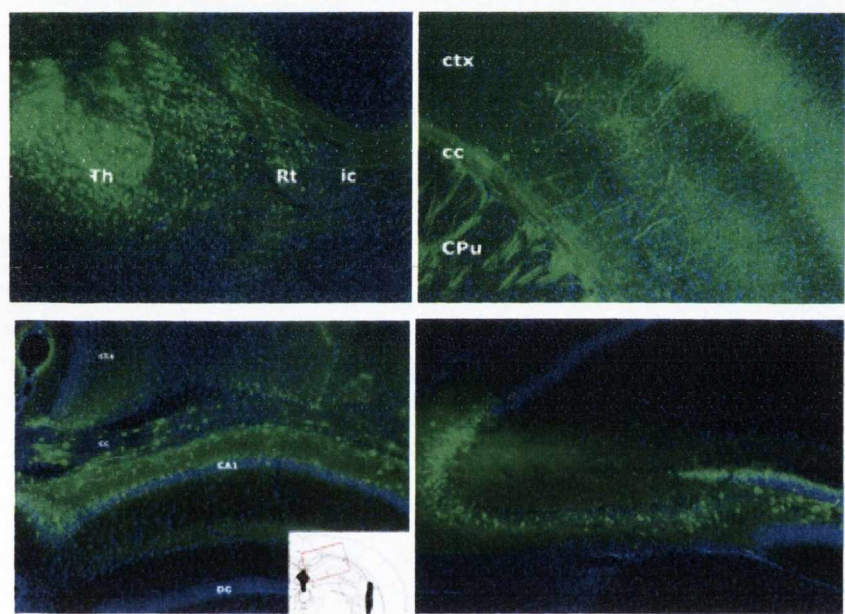


Figure 3.13: Off target expression of eGFP in mouse brain
Shown here are further representative brain sections showing significant expression of eGFP in mouse brain 5 weeks post stereotaxic injection. However, the target tissue in both injections was in fact the hippocampus – the top two panels show expression in surrounding brain tissues, again highlighting how difficult it is to routinely target the hippocampus. Th = thalamus; Rt = reticular thalamic nucleus; ctx= cortex; cc = corpus callosum; Cpu = caudate putamen. Although the lower panels demonstrate hippocampal eGFP expression, the pattern of expression varied from mouse to mouse, sometimes showing reporter gene marking the CA1 (lower left panel), whereas in other injections show expression in the dentate gyrus or CA3 neurons (lower right panel). The inset in the bottom left panel represents the mouse brain atlas used for determining stereotaxic coordinates.

We repeatedly found off-target viral-mediated transgene expression as a result of variation in the injections – essentially, the mouse hippocampus is too small a tissue to efficiently target and generate reproducible results, even with a stereotaxic frame (figure 3.13).

3.3.7 Co-expression of transgenes using a mixture of rAAV vectors.

In order to analyse the applicability of dual transduction with AAV two marker AAVs were used – AAV/eGFP and AAV/dsRed. In vivo neuronal co-transduction with the two marker AAVs was explored in the hippocampus. The dentate gyri in adult wild type rats were stereotaxically injected (coordinates: A/P -3.8, M/L -1.5 and D/V -3.5) with a mixture of 1.5×10^9 vp AAV-DsR and 1.5×10^9 vp AAV-GFP (n=5). Four weeks post-injection brains were fixed and processed for histology. Fluorescent microscope imaging of a representative section from the injected area is depicted in figure 3.15. The overlay of the DsR and GFP signals illustrates that a significant portion of the cells co-express the two marker genes. Notably, the co-labeled cells show yellow or orange colours in the overlaid images (Figure 3.14).

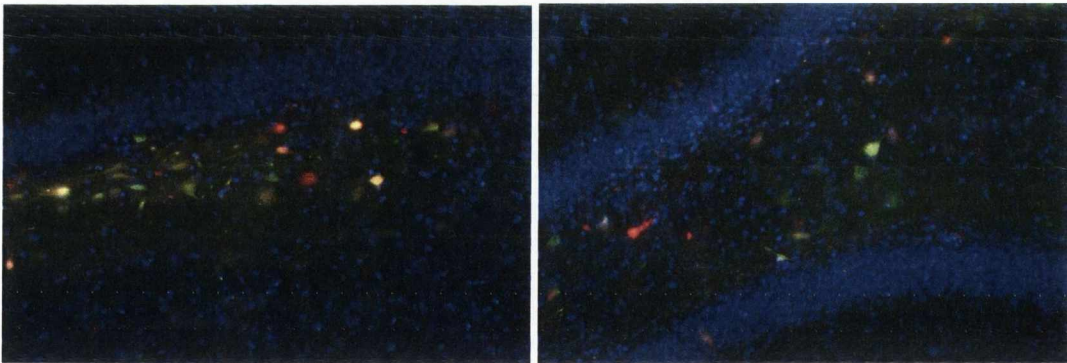


Figure 3.14: Expression of DS-Red-Express2 and eGFP following co-administration of two AAV vectors. Representative fluorescence microscopy sections of rat brain 5 weeks post injection of 1×10^{12} viral particles of both AAV/eGFP and AAV/dsRed, co-stained with DAPI (blue). Both red and green reporter gene expression is visible, however, several yellow cells indicate that it is possible to co-express 2 viral transgenes from 2 separate vectors.

We next sought to isolate and sort these cells for quantification using flow cytometry. A separate cohort of rats (n=6) were injected, as before. However figure 3.15 shows that not enough viable cells were isolated to generate significant cell counts for analyses. This is most likely due to a low transfection rate as a result of the relatively low viral titre.

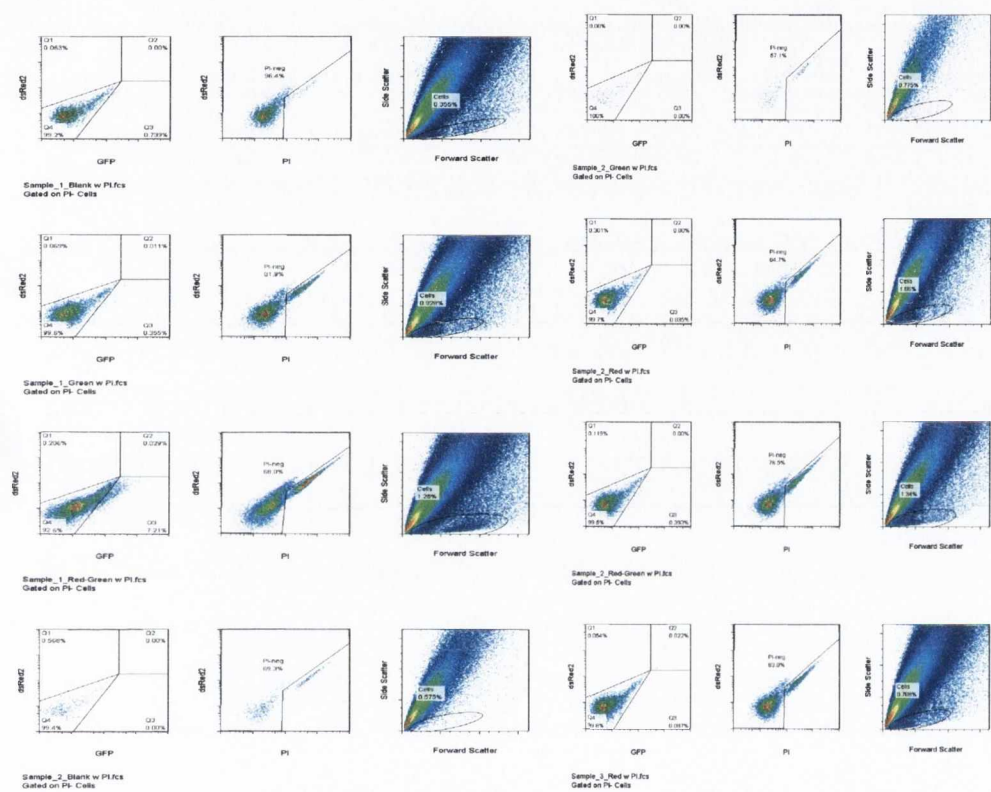


Figure 3.15: Flow Cytometry analysis of dissociated rat brains, 5 weeks post injection. Rat brains were removed 5 weeks post injection and hippocampus was dissected and dissociated. The hippocampal cell suspension was then analysed by flow cytometry to determine the exact degree of co-expression. 10 samples were analysed for co-expression of both marker genes. 6 control samples were also analysed to set gate parameters (2x uninjected controls; 2 AAV/dsRed-only controls; and 2 AAV/eGFP-only controls). Unfortunately, transfection efficiency was not at suitable levels to sort significant numbers of fluorescent cells and these results are inconclusive.

3.4 Discussion

The main aim of this chapter of my thesis was to generate and characterize high-titre recombinant AAV vectors. AAV vectors were chosen for this study based its stable characteristics *in vivo*, its low immunogenicity, and because it is non-pathogenic. Using a “gutless” AAV system ensures that AAV vectors display a very low frequency of integration into the host genome. In addition, the property of AAV to persist extrachromasomally in infected cells allows for stable and strong expression *in vivo*. Numerous reports have demonstrated a prolonged expression for over 18 months in both rodents and larger animal models (Synder *et al.*, 1999; Mount *et al.*, 2002). An important limitation of vector delivery when using rAAV for gene therapy applications is to deliver sufficient amount of the virus to the target tissue or cell type without causing an immune response which could destroy the transduced cells. However, the subretinal space is an immune-privileged site, so by first generating pure viral preps of sufficiently high titres ($<10^{12}$ vp/ml), and then delivering the virus by subretinal injection, immune responses can be avoided (Bennett *et al.*, 1996), and large numbers of cells can be efficiently transduced. Taken together this makes gene delivery by subretinal injection of rAAV a favourable avenue for gene therapy. For this chapter of my thesis however, subretinal injection of viral preps was used merely as a relatively quick and easy way to evaluate if the rAAV transduced neuronal cells *in vivo* and efficiently expressed the viral transgene.

rAAVs were first used to transduce HEK293 cells *in vitro*, using fluorescence microscopy to evaluate transduction efficiency and transgene expression. One limitation of using IRES-linked hrGFP as a fluorescent marker appears to be the relatively lower levels of expression, and therefore fluorescence of the hrGFP (humanised renilla

green fluorescent protein) when compared to eGFP (enhanced green fluorescent protein) linked to a CMV promoter with no IRES. Using an IRES results in significantly lower expression of the downstream gene. When used in cell culture, AAV/SNAP-25 and AAV/hrGFP infected cells appear significantly less fluorescent than the AAV/Control (containing eGFP) infected cells. For this reason we used an MOI 10

times greater when infecting with the AAV/SNAP-25 and AAV/hrGFP for the *in vitro* experiments. While this appears to be a limitation, it should be noted that the gene upstream of the IRES element is not subject to the poor expression levels reported for the hrGFP protein. It is also worth noting that the GFP protein appears to accumulate – if the virus was left on the HEK293 cells for 48 hours instead of 24 hours, there appeared to be greater fluorescence in the transduced cells, even though the cells had become over-confluent due to the extra 24 hours of growth. Indeed, in the mouse brain, there are some reports that GFP expression is best observed at 9 months post-injection (Beverly Davidson, personal communication).

We next sought to evaluate AAV/SNAP-25 in neuronal cells *in vivo*, using subretinal injection in mice to deliver the virus to the retina. To allow for good viral infection, full recovery of the retina, and to ensure sufficient accumulation of the GFP protein, animals were analysed 4-5 weeks post-injection. Photoreceptor cells of the outer nuclear layer (ONL) were effectively transduced (Figures 3.9 & 3.10). Subretinal injection of ~3µl creates a retinal detachment, and a bubble at the back of the eye can clearly be seen when performing the surgery. This detachment gradually settles back onto the RPE and the retina can fully recover. The size and shape of the retinal detachment is often a measure of how successful each injection is. It should be noted that subretinal injection is a complicated procedure, requiring a steady hand, and for this reason a great deal of variability between injections can sometimes be observed. However, once the technique is mastered, good subretinal injections can become routine, and because each injection can clearly be evaluated any problems such as haemorrhaging or retinal scarring can be noted during the surgery and taken into account when later assessing the retina. Using this technique I was routinely able to achieve good retinal detachments, usually observing approximately 30-50% of the retina forming a bubble subsequent to each injection, and indeed, this could be seen when whole mount retinas were examined for fluorescence, with approximately 30-50% of the photoreceptor surface covered with green cells (Figure 3.9). Some reports have demonstrated that repeated injection of AAV allows for wide-spread transduction of the retina and that this technique does not have an adverse effects or increased surgical trauma (Lotery *et al.*, 2003), suggesting that it could be possible to transduce a much greater population of cells. However, in the

case of this experiment, repeated injections were not necessary, although it is worth noting that this could be useful for further studies.

To evaluate the approximate increase in SNAP-25 expression levels in AAV/SNAP-25 injected retinas, compared to uninjected controls, whole retinas were dissected and RNA was extracted for real time RT-PCR analysis. SNAP-25 is a synaptic protein and is therefore endogenously expressed in the retina. Therefore, RDS/peripherin was used as a control housekeeping gene and values for each RNA sample were normalised, and the normalised expression levels of SNAP-25 in AAV-injected eyes were compared to uninjected. A 176% increase was observed (Figure 3.11). It should be noted that this increase corresponds to the increase in SNAP-25 in the whole retina and not in the transduced cells. As the photoreceptor layer makes up a fraction of the total retina, and because only a fraction of photoreceptors are transduced, it is probable that the actual increase in expression in the transduced cells is much higher. To investigate this further I utilized fluorescent activated cell sorting (FACS) technology to efficiently sort green cells and compare levels of SNAP-25 expression in AAV/SNAP-25 compared to AAV/hrGFP transduced cells. These findings are discussed in Chapter 4.

One interesting finding was that the retinas of AAV/SNAP-25 injected mice appeared to be thinner than the uninjected controls, with an apparent photoreceptor degeneration visible at 4 weeks post-injection (Figure 3.10). Although the basic retinal morphology in these sections is visible, detailed histological examination is not possible because the vibrotome-cut sections are so thick (~50µm). Finer detail would be seen when using OCT embedded sections cut on a cryostat but unfortunately we have consistently found that the hrGFP protein does not cryopreserve well. However, vibrotome sections can be analysed on a confocal microscope, where individual transduced photoreceptor cells are clearly visible, along with a significantly thinner ONL than an uninjected eye. This observation was investigated further and the findings discussed in chapter 4.

In this chapter of my thesis I also carried out work to optimize the stereotaxic injection procedure to mouse brain, to demonstrate efficient targetting of our AAV2/5 constructs to hippocampal neurons (figures 3.13). For this optimization work AAV/eGFP was used. Despite showing efficient expression of the viral reporter

transgenes by fluorescent microscopy analysis of brain sections 4-5 weeks post injection (figures 3.13), achieving routine targeting of the dentate gyrus proved difficult. This is because of the small size of the target tissue. Based on these results we decided to use wistar rats for all future brain injections.

In a separate set of experiments I next investigated if two viral transgenes could be co-expressed *in vivo* following co-administration of a mixture of 2 separate rAAV vectors, one containing a “red” marker gene (DS-Red-Express2) and the other containing a “green” (eGFP) marker gene. This work forms part of a methods paper (Palfi *et al.*, 2012), as an attempt to reproduce interesting recent results from the laboratory carried out in mouse retina. In these studies, members of our group found that subretinal injection of both viruses resulted in significant co-expression of both red and green reporter genes, resulting in large numbers of “yellow” cells (approx 70% of retinal cells contained both DS-Red-Express2, as confirmed by flow cytometry, Palfi *et al.*, 2012). 5 weeks post stereotaxic injection of 1×10^{12} viral particles of each rAAV, animals were sacrificed and brains sectioned. Fluorescence microscopy analysis of these sections clearly shows not only ds-red and eGFP expressing cells, but also several “yellow” cells expressing both transgenes (figure 3.14), demonstrating that co-administration of 2 rAAVs provides a useful tool for delivering different transgenes into the same cell, potentially overcoming the 4.7kb capacity limitation of AAV. However, unfortunately, as we delivered a relatively low volume (1ul) of virus at only 1×10^{12} (compared with 8×10^{12} in other hippocampal injections, see chapter 5), we were unable to achieve a significantly high proportion of transduced cells, resulting in poor flow cytometry results (figure 3.14). It would be interesting to further investigate this finding using rAAV with other serotypes (2/8 or 2/9), and with larger injection volumes.

3.5 Conclusions

Collectively, the central results of this chapter of my thesis are that high-titre viruses (one containing a SNAP-25 transgene and two different GFP controls) were generated and characterized. The plasmids were cloned, purified, transfected into cells, virus was then harvested, purified, titred, and transduced into cells both in culture and *in vivo*. Viruses were shown to effectively express their fluorescent GFP

tags in cell culture, and when subretinally injected into mice, a significant proportion of the retina was transduced. My results show large numbers of transduced photoreceptor cells, providing further evidence that rAAV-2/5 preferentially targets the ONL of the retina.

Experiments described in this chapter of my thesis also focused on optimizing the stereotaxic injection procedure for delivery of rAAV to the hippocampus.

Generating pure, high-titre virus is an important hurdle to cross for effective gene therapy, especially for clinical application. Indeed, we generated several different AAV/SNAP-25 viral preps before we finally obtained a suitably pure and high titre prep and even now our viral prep protocol is being improved. The cell culture process itself can cause impurities, including residual host cell proteins and DNA, and helper plasmid DNA, and the CsCl₂ purification step can also sometimes lead to residual caesium in the viral prep. Whereas these impurities might be acceptable for animal research, now that AAV technology is moving to the clinic, a major challenge for the translation of promising research to clinical development is the manufacture and certification of AAV vectors for clinical use (Carter *et al.*, 2005; Wright, 2008). An important strategy to ensure adequate translation to the clinic will therefore be to determine and implement standard Good Manufacturing Processes, analytical methods, and quality systems.

Chapter 4

AAV-mediated Overexpression of SNAP-25 in the Mouse Retina

4.1 Introduction

The main aim of this chapter of my thesis was to inject the high-titre AAV/SNAP-25 virus into the mouse retina and investigate its effects at a functional and molecular level.

In photoreceptor cells, light-induced signal cascades cause glutamate to be released and in the opposing post-synaptic cell, glutamate receptors bind the glutamate causing depolarization and its consequent functions. Although glutamate is the most abundant neurotransmitter in the CNS and it is the main excitatory neurotransmitter in the retina, excess glutamate is toxic (Romano *et al.*, 1998). "Excitotoxicity" occurs in several neurodegenerative diseases when glutamate accumulates outside cells causing calcium ions to enter via NMDA receptor channels, resulting in dysfunction of downstream signaling systems, in turn leading to neuronal damage and eventually, cell death. Mechanisms of excitotoxicity include damage to mitochondria by high concentrations of intracellular calcium ions, Ca^{2+} activation of proteases and endonucleases, as well as glutamate/ Ca^{2+} -mediated activation of pro-apoptotic transcription factors (or down-regulation of anti-apoptotic genes).

Using a recombinant adeno-associated virus (AAV) vector we have shown that SNAP-25 overexpression in photoreceptor cells induces a retinal degeneration. As the virus contained a GFP marker, it was possible to sort transduced cells using FACS. Real time RT-PCR analyses of RNA extracted from GFP-positive cells demonstrated an approximate 30-fold increase in SNAP-25 expression in the transduced cells. Electroretinogram (ERG) analysis demonstrated a significant decrease in rod responses and Immunohistological analysis and resin sectioning showed a severe retinal degeneration, with significant photoreceptor cell death. Levels of glutamate and other neurotransmitters were analysed, however, no significant changes were observed at 4 weeks post injection. However, it is still possible that this phenomenon may be as a result of glutamate excitotoxicity and could represent a novel mechanism of photoreceptor degeneration.

To investigate if this phenomenon occurs in the retina and to examine the possible role of SNAP-25 in glutamate toxicity, we utilized a recombinant AAV2/5 vector to over-express SNAP-25 in the photoreceptor cells of mice. In this regard it is notable

that SNAP-25 is normally expressed in the photoreceptor and bipolar cells of the retina (Brandstatter *et al.*, 1996). SNAP-25 overexpression induces a degeneration of the photoreceptor cells. This finding could implicate SNAP-25 as a potential candidate gene for a variety of retinopathies and it is interesting to note that SNAP-25 is located on chromosome 20p12.2(10,258,333-10,280,051), which maps to the GLCK1 locus, a candidate region for primary open-angle glaucoma (Wiggs *et al.*, 2004).

4.2 Methods

4.2.1 Subretinal Injection

3µl of AAV-SNAP25-IRES-hrGFP or AAV-IRES-hrGFP was injected subretinally into adult male adult CD1 mice (n=12) using a 33 gauge blunt hamilton syringe, as described previously in chapter 3. Electroretinogram analysis was carried out 4 weeks post injection and mice were sacrificed for RNA and histological analysis.

4.2.2 Electroretinogram (ERG)

Animals were dark adapted overnight and were prepared under dim red light. Pupils were dilated with 1% cyclopentolate and 2.5% phenylephrine. Animals were anesthetized with ketamine and xylazine (16 and 1.6 µg/10g body weight, respectively) injected intraperitoneally. Standardized flashes of light were presented to the mouse in a Ganzfield bowl. ERG responses from both eyes were recorded simultaneously by means of contact lens electrodes (Medical Workshop), with use of 1% amehocaine as topical anesthesia. Reference and ground electrodes were positioned subcutaneously, ~1mm from the temporal canthus and anterior to the tail, respectively. Responses were analysed using a Retiscan Retiport electrophysiology unit (Roland Consulting). The protocol was based on that approved by the International Clinical Standards Committee for human ERG. Rod-Isolated responses were recorded using a dim white flash (-25 maximal intensity, where maximal flash intensity was 3 candelas/m²/s) presented in the dark adapted state. Maximal combined rod-cone response to the maximal intensityflash was then recorded. After a 10-min light adaptation to a background illumination of 30 candelas/m², cone isolated responses were recorded to the maximal intensity flash,

presented initially as a single flash and subsequently as 10Hz flickers. a-Waves were measured from the baseline to the trough and b-waves from the baseline (in the case of rod-isolated responses) or from a-wave to trough.

4.2.3 Fluorescent activated cell sorting (FACS) and analysis of gene expression in sorted cells

Retinas were FACS sorted and RNA was extracted from AAV-infected cells using the RNeasy Mini Kit (Qiagen) (n=6). For FACS sorting, retinas were dissociated by trypsin digest into single cells. Retinal cells expressing hrGFP were isolated (10,000-50,000 per session) using a Beckman-Coulter Altra fluorescence-activated cell sorter (Beckman- Coulter Inc., Fullerton, CA). Gene-specific primers for RT-PCR were designed in exonic sequence and so that the product also spanned an intron, using the Primer3 algorithm (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). RNA was extracted from dissected retinas using the RNeasy Mini Kit (Qiagen) (n=6) and Quantitative PCR was performed on an ABI 7300 Real Time PCR System (Applied Biosystems) using the Quantitech PCR kit according to manufacturer's instructions (Qiagen). Serial dilutions of RNA were used to generate standard curves of crossing cycle number versus the logarithmus of concentration for each gene of interest. A linear regression line calculated from the standard curves allowed relative transcript levels in each RNA sample to be determined. Values were normalized to the relative amounts of RDS present in the same RNA preparations and a melting curve was also carried out after each amplification step, which provides confirmation that specific PCR products were formed in the reaction. Expression of SNAP-25 in injected eyes was expressed as an average increase with endogenous values used as the baseline.

4.2.4 Histological analysis

Animals were euthanased under CO₂. The eyes were gently proptosed by placing a curved forceps behind each eye. A small cautery mark was made on the cornea of each eye at the 12 o'clock position using a Solan low temperature cautery (Xomed surgical instruments Inc. Jacksonville, USA). Each eye was fixed in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde overnight overnight at 4°C. Following

overnight fixation eyes were trimmed of extraocular muscles and bisected through the optic nerve and the cautery mark. A wedge shaped incision was made in the cornea of each hemi-globe, in the region of the cautery mark so that the superior-inferior orientation of the subsequent sections could be confidently identified. The hemi-cups were washed 6 times for 10 minutes in 0.1M phosphate buffered saline (PBS) pH7.2. The tissue was then soaked in Osmium Tetroxide in 0.1M PBS pH7.2 for 30 minutes. Serial washes for 1 hour in 50%, 75%, 95% ethanol were followed by three washes in 100% ethanol for 30, 45 and 45 minutes respectively. The tissue was then washed three times in propylene oxide for 30, 45 and 45 minutes respectively. The eye cups were then soaked in a solution containing 50% propylene oxide and 50% Agar 100 resin (Agar Scientific Ltd. Cambridge, UK) for 2 hours. This was followed by incubation in full strength resin for 4 hours. The hemi cup was then mounted in a BEEM capsule with the conical end removed, the cut surface of the specimen being positioned flush with the cap. The capsule was then filled with fresh resin that was subsequently polymerized at 65°C for 24 hours. 0.5-1.0µm sections were cut using a Reichert-Jung Ultracut microtome. Sections were stained with Toluidine Blue solution (1% sodium borate (aqueous), 1% sodium azure, 1% Toluidine Blue).

4.2.5 Neurochemical analysis

Adult C57B/6 Mice were subretinally injected as before with 3µl of either AAV/SNAP-25 (right eye) or AAV/hrGFP (left eye). 5 weeks post-injection, animals were euthanased with CO₂. Eyes were enucleated and the retinas dissected and placed immediately in ice cold Ringer's intravenous infusion solution (compound sodium chloride) (Baxter Healthcare Ltd., Norfolk, UK). Samples were centrifuged for 30 minutes at 15,000rpm at 4°C and the supernatant was frozen for HPLC analysis. Glutamate was determined by precolumn derivatization of a 10 µl dialysate sample with o-phthaldialdehyde/mercaptoethanol reagent and separation by reversed-phase HPLC on a Biophase ODS 5 µM particle column (Knauer, Berlin, Germany). The mobile phase contained 0.1 M sodium acetate, 6.25% methanol, 1.5% tetrahydrofurane, pH 6.95 and was perfused at a flow rate of 1 ml/min. A linear gradient system was used to clean the column after elution of glutamate. This

involved switching to 100% methanol for 2 min before switching back to the original acetate buffer. The excitation wavelength in the fluorescence detector (Hitachi F1000, Tokyo, Japan) was set at 370 nm and the emission cut off filter was set at 450 nm. The limit of detection was 0.5 pmol/sample for glutamate (Morari *et al.*, 1994). The GABA assay employed was based on precolumn derivatization of a 10 µl sample with o-phthaldialdehyde/tbutylthiol reagent and separation by reversed-phase HPLC on a Nucleosil 3 C18 column perfused under isocratic conditions at the flow rate of 0.8 ml/min. The mobile phase was 0.15 M sodium acetate, 1 mM EDTA, 50% acetonitrile, pH 5.4. The BAS LC4B electrochemical detector (Bioanalytical Systems, West Lafayette, IN) was set at + 0.75 V. The limit of detection was 50 fmol/sample (Morari *et al.*, 1994).

4.3 Results

4.3.1 AAV mediated SNAP-25 overexpression in the mouse retina

High titre ($5-8 \times 10^{12}$ vp/ml) rAAV2/5 vectors expressing SNAP-25 and hrGFP (AAV/SNAP25), or hrGFP alone (AAV/hrGFP) were used in this study (Figure 4.1).

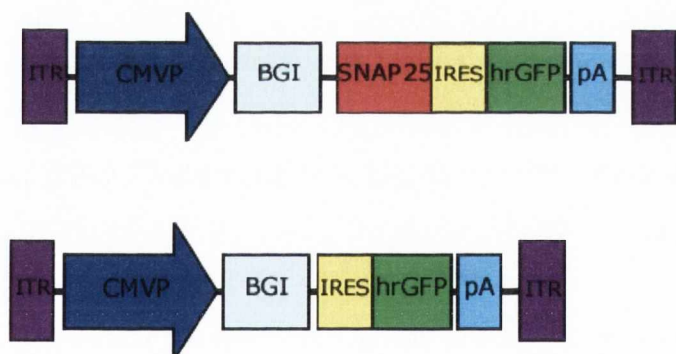


Figure 4.1: AAV constructs used in Chapter 4.

Top: AAV/SNAP-25; Bottom: AAV/hrGFP. Viral transcripts were expressed under the control of the CMV immediate early promoter. The β -globin intron (BGI) is located at the 5' end of the transcript and the human growth hormone polyadenylation signal (pA) is located at the 3' end of the transcript. A humanized recombinant green fluorescent protein reporter gene (hrGFP) linked by a internal ribosome entry site (IRES) was used, allowing both SNAP-25 and hrGFP proteins to be translated from the same transcript. In this way, hrGFP expression may be used to ascertain the infection efficiency for the desired target cell type and also serves as a useful expression marker for the SNAP-25 protein.

Adult (3-4 month old) C57/BJ6 or CD1 mice were injected subretinally with $\sim 3\mu\text{l}$ of each virus. Fluorescence microscopy of retinal sections at 4 weeks post injection shows high infectivity of AAV and hrGFP positive cells are clearly visible in the outer nuclear layer (ONL) (figure 4.2).

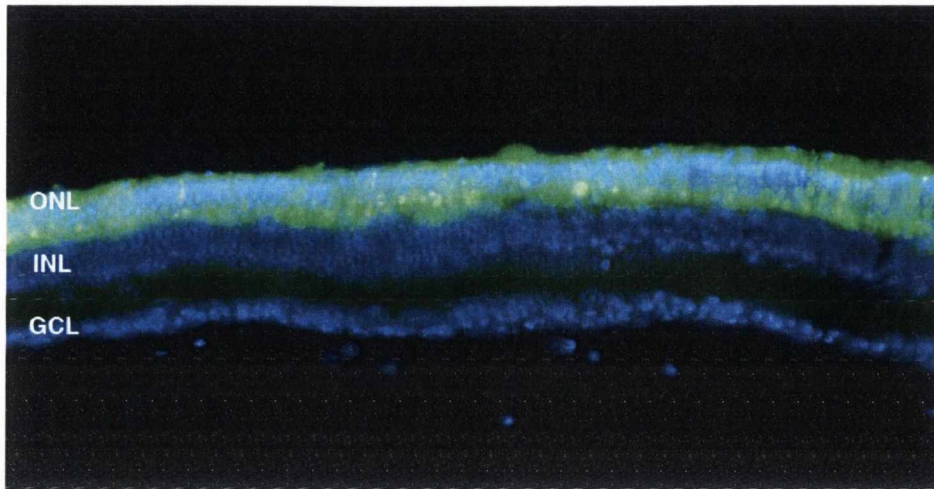


Figure 4.2: Fluorescent cross section of hrGFP expression in an AAV/SNAP-25 injected eye. Retinas were embedded in agar and cut into $\sim 50\mu\text{m}$ sections on a vibrotome. hrGFP expression is localized in the outer nuclear layer (ONL) and outer segments of the photoreceptors. INL = inner nuclear layer; GCL = ganglion cell layer.

In a separate cohort of injected animals ($n=6$) eyes were enucleated, the retinas dissected, and the cells dissociated by trypsinization for cell sorting using FACS. FACS analysis shows a clearly distinct population of fluorescent cells, allowing for efficient cell sorting (Figure 4.3).

RNA was extracted from GFP-positive cells for quantitative RT-PCR analysis. As SNAP-25 is endogenously expressed in the mouse retina, levels of expression in AAV/SNAP25 injected eyes were compared to control (AAV/hrGFP)-injected eyes (for each animal, AAV/SNAP25 was injected into the right eye, AAV/hrGFP was injected into the left, $n=6$). Using RDS/peripherin as a housekeeping gene, values were normalized to the relative amounts of RDS/peripherin present in each RNA sample and the increase in SNAP-25 expression was expressed as an average increase with endogenous levels expressed as a baseline (figure 4.4). A very significant (<30 fold,

p<0.00001) increase in SNAP-25 expression was observed.

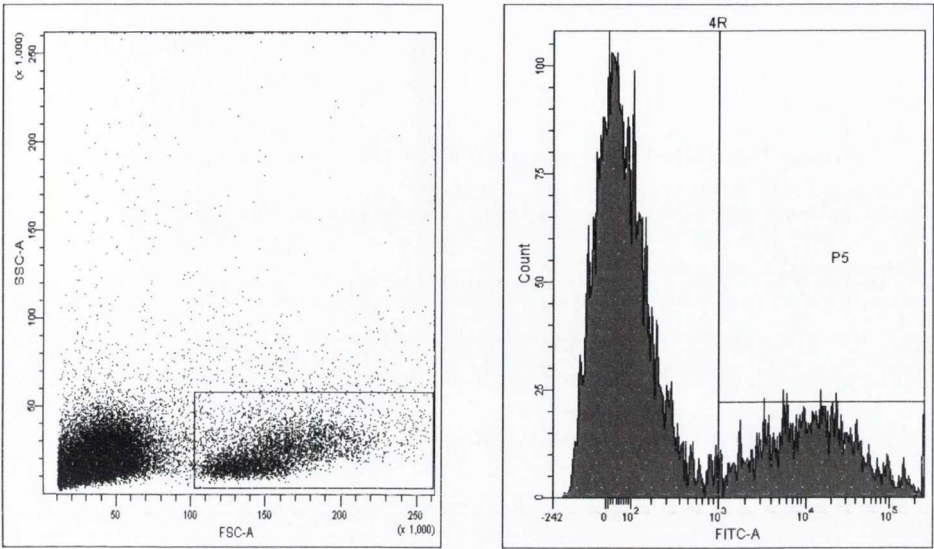


Figure 4.3: Fluorescent activated cell sorting (FACS) of hrGFP-labelled retinal cells.

Retinas were dissected 5 weeks post-injection, dissociated, and analysed by FACS. Scatterplot (left) and histogram (right) shows total hrGFP fluorescence in a representative rAAV-injected retina. A subpopulation of fluorescent green photoreceptor cells (P5) were sorted and RNA was extracted from these cells for quantitative RT-PCR analyses.

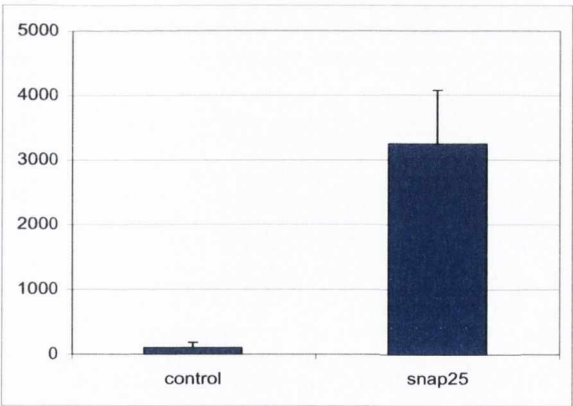


Figure 4.4: Quantitative real time RT-PCR analysis of SNAP-25 expression in transduced retinal cells.

RNA extracted from FACS sorted hrGFP expressing cells was analysed for SNAP-25 expression. As SNAP-25 is expressed endogenously in the mouse retina, levels of SNAP-25 expression in AAV/SNAP-25 transduced cells were compared to AAV/hrGFP controls, with SNAP-25 levels in control eyes set at 100%. There was an over 30-fold increase in SNAP-25 levels in GFP-expressing retinal cells compared to control (AAV/hrGFP) injected cells, quantified by RT-PCR. (Error bars represent SD values; P<0.00001; n=5-6 retina samples per group)

4.3.2 SNAP-25 overexpression results in impaired ERG responses

AAV-injected mice were subjected to ERG analysis 4 weeks post injection. Right eyes (injected with AAV/SNAP-25) showed significant attenuation of maximal intensity b-wave amplitudes, whereas left eyes (injected with AAV/hrGFP) appeared normal (figure 4.5), demonstrating that SNAP-25 overexpression appears to cause an impairment in rod photoreceptor function. Figure 4.5 illustrates the representative ERG responses recorded from two animals.

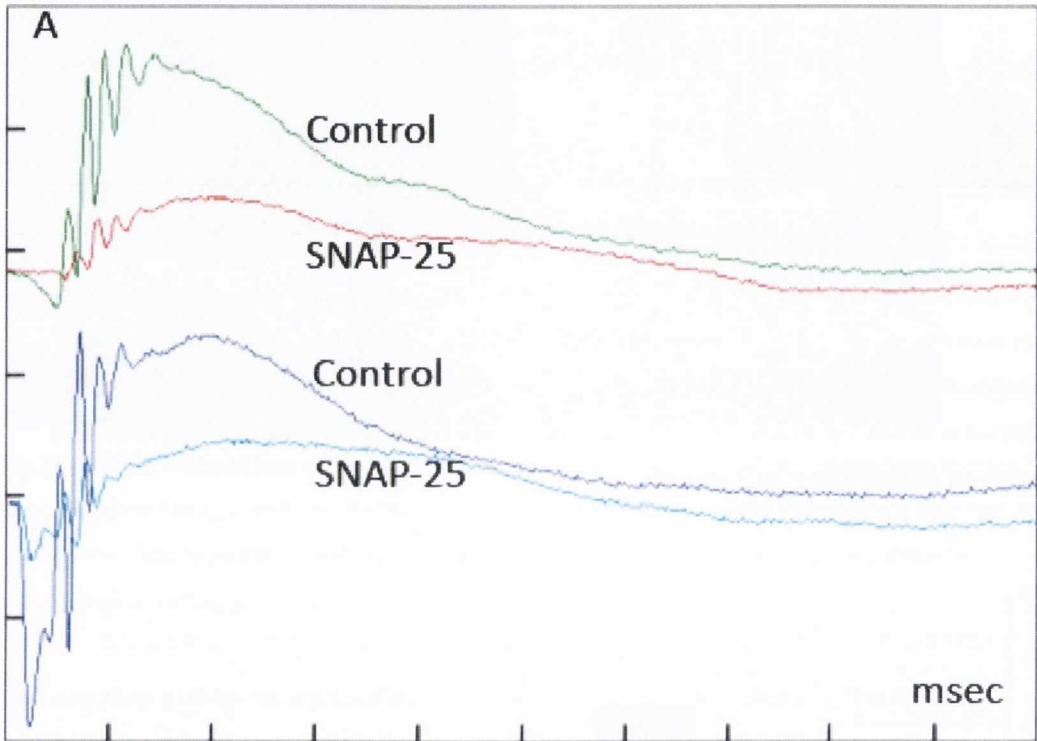


Figure 4.5: Representative rod-isolated ERG responses from 2 AAV-injected animals. Green and purple traces represent control AAV/hrGFP-injected left eye rod-isolated responses, whereas the reduced red and blue traces represent right eyes injected with AAV/SNAP-25. The range with use of our protocol is 349-720 μ V. Amplitude scale (Y-axis) is 200 microvolts per division, the time scale (X-axis) is 50 milliseconds per division.

4.3.3 SNAP-25 overexpression induces photoreceptor cell death

Eyes from AAV-injected animals were fixed and embedded in resin, then cut into $\sim 1\mu$ m thin sections and examined by light microscopy to better visualize retinal histology. Normal murine retinas typically have 10-12 rows of photoreceptor nuclei in the outer nuclear layer (ONL), and control AAV/hrGFP injected eyes appear to

have normal retinal histology (figure 4.6). This indicates that neither AAV infection or overexpression of hrGFP cause adverse side effects or kill cells. The subretinal injection procedure causes a retinal detachment but this usually has no adverse effects, apart from scarring at the site of injection.

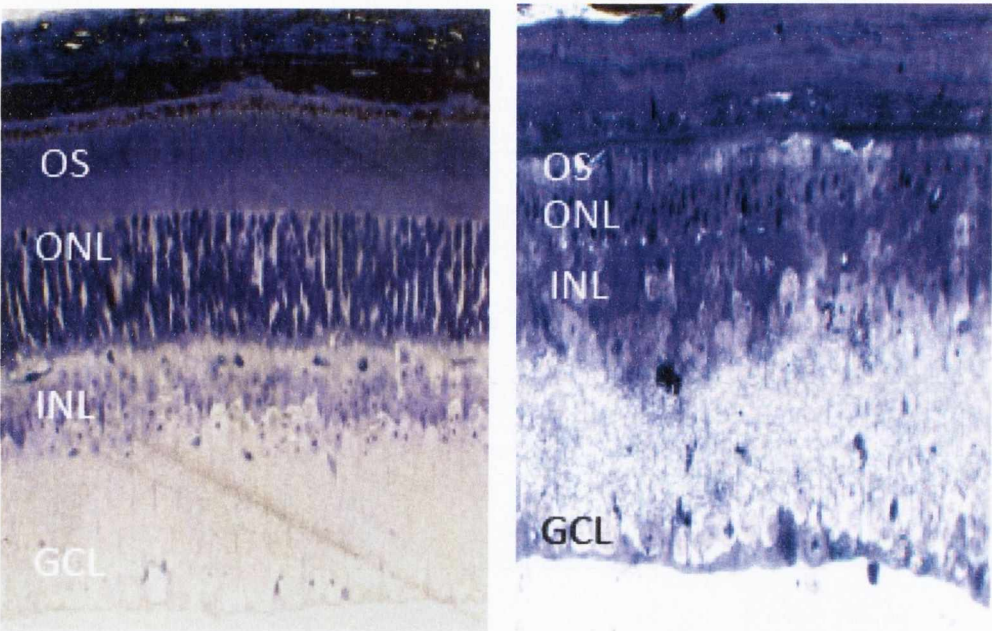


Figure 4.6: Retinal Histology. AAV-mediated SNAP-25 overexpression induces photoreceptor cell death. Representative 1 μ m-thick retinal sections from AAV-injected mouse eyes, 5 weeks post-injection. Left panel shows control AAV/GFP injected eye with normal retinal histology, whereas the right eye (injected with AAV/SNAP-25) shows significant photoreceptor degeneration with severely depleted outer segments (OS) only 1-2 rows of nuclei remaining in the outer nuclear layer (ONL).

Consistent with findings from ERG analysis, the retinas from AAV/SNAP-25 injected eyes showed a significant retinal degeneration, with only 1-2 rows of nuclei left in the ONL at 5 weeks post injection. It is also worth noting that the inner nuclear layer also appeared to be thinner in these animals, although the nuclei of the INL are more difficult to stain (figure 4.6).

To further investigate and quantify the extent of outer nuclear layer cell death, a separate cohort of C57 (wt) mice were injected with AAV/control and AAV/SNAP25 into the left and right eyes respectively (n=6), as before. Four weeks post-injection, animals were sacrificed and eyes enucleated and fixed in 4% paraformaldehyde

before being embedded in OCT for cryosectioning. To evaluate quantitatively the area per unit length (μm) of positively transduced ONL, retinal sections were viewed under a fluorescent microscope (Zeiss, Axioplan 2). GFP-positive ONL thickness from several representative sections from each eye could then be measured using and the average area per unit length for AAV/SNAP injected eyes could be compared with control injected eyes. In this way a quantitative measure of ONL degeneration as a result of viral-mediated SNAP-25 over-expression could be determined, as is shown in figure 4.7.

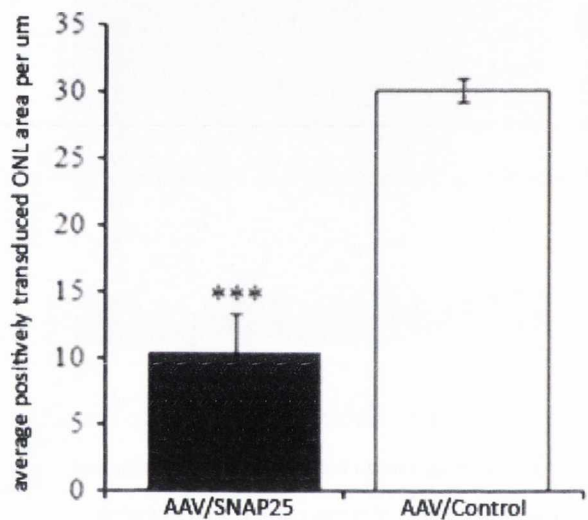


Figure 4.7: Quantifying ONL thickness in retinal sections.

Mice ($n=6$) were injected with $3\mu\text{l}$ of AAV/Control and AAV/SNAP-25 into left and right eye respectively. 4 weeks post injection eyes were enucleated and cryosectioned. GFP-positive ONL areas from 12 independent sections from each eye were measured using analytical software tools (Analysis B, Zeiss), the average area per unit length for each treated animal was grouped, and the average positively transduced ONL area per unit length was compared with control eyes. SNAP-25 ($10.13 \pm 3.4\mu\text{m}$) injected ONL is significantly thinner than control ($30.12 \pm 0.8\mu\text{m}$). Data represent average positively transduced ONL area per unit length (μm) $\pm$ SEM (** $P \leq 0.0001$, $n = 6$).

4.3.4 SNAP-25 overexpression does not alter retinal neurochemistry

SNAP-25 is a synaptic protein and plays an essential role in neurotransmitter release. In the brain, it is expressed primarily in glutamatergic neurons (Verderio *et al.*, 2004), and altered levels of SNAP-25 expression within these neurons has been shown to effect the cell's responsiveness to stimuli. In a similar experiment, we delivered

AAV/SNAP-25 to the rat hippocampus and using *in vivo* microdialysis, we collected dialysates and analyzed their neurochemistry using reverse-phase high performance liquid chromatography (HPLC). Interestingly, we found a significant increase in extracellular glutamate levels in the hippocampi of AAV/SNAP-25 injected rats (discussed further in chapter 5). Although microdialysis is not technically possible in the murine eye due to its small size, I attempted to prepare crude retinal lysates and analyze their neurochemical content using reverse-phase HPLC. To evaluate extracellular levels of glutamate and other retinal neurotransmitters, retinas were simply dissociated and sonicated, and the resulting supernatant was analyzed on the HPLC using a protocol similar to that used for hippocampal dialysates in chapter 5. Surprisingly, there were no significant differences observed in the concentrations of glutamate, GABA, or aspartate (figure 4.8 A,B & C respectively). Further work is needed to optimize the preparation of retinal lysates, and it would also be worthwhile investigating neurochemical concentrations at an earlier time point, perhaps two weeks post-injection.

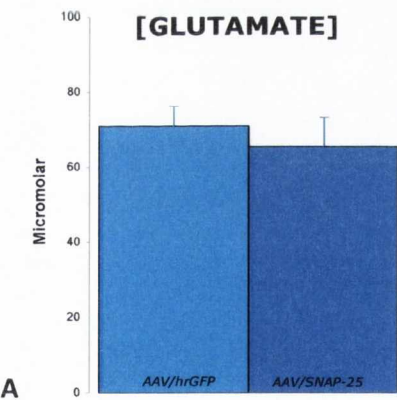


Figure 4.8A: Neurochemical analysis in the retina:

A: Reverse phase HPLC was used to assay for Glutamate concentration in crude retinal lysates. Histogram represents the mean micromolar concentration of glutamate $\pm$ SEM for each sample. No significant difference was observed using a paired student *t*-test. AAV/SNAP-25 = 65.527 μ M $\pm$ 7.699; AAV/hrGFP = 70.625 μ M $\pm$ 5.45, n=6. (continued on next page)

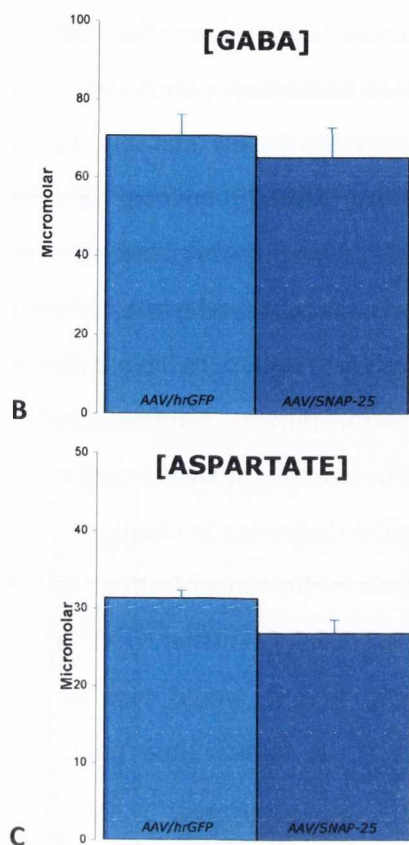


Figure 4.8 (continued):

Reverse phase HPLC assay for GABA (B) and Aspartate (C) concentrations in crude retinal lysates. Histogram represents the mean micromolar concentration of glutamate $\pm$ SEM for each sample. No significant differences were observed using a paired student *t*-test.

GABA: AAV/SNAP-25 = 26.899 μ M $\pm$ 1.654; AAV/hrGFP = 31.354 μ M $\pm$ 0.95, *n*=6.

ASPARTATE: AAV/SNAP-25 = 20.078 μ M $\pm$ 1.279; AAV/hrGFP = 24.676 μ M $\pm$ 2.54, *n*=6.

4.4 Discussion

The work presented in this chapter of my thesis describes the effects of SNAP-25 overexpression in the retina. Initially I utilized the subretinal injection of SNAP-25 simply to validate that the AAV/SNAP-25 vector efficiently transduced neuronal cells and that sufficient levels of SNAP-25 were expressed. However, upon examining retinal sections it became apparent that the ONL of the AAV/SNAP-25 injected retinas appeared significantly thinner (figure 4.2). Subretinal injection of AAV/SNAP-25 was then shown to efficiently induce over-expression of SNAP-25. Animals were allowed to recover and housed together until further analyses, 4-5 weeks post injection (also allows for good expression of the viral transgene). 4 weeks post injection, fluorescence microscopy of retinal sections shows high infectivity of AAV and hrGFP positive cells are clearly visible in the outer nuclear layer (ONL) (figure 4.2). Quantitative RT-PCR analysis of FACS sorted hrGFP positive cells (figure 4.3) show that SNAP-25 is expressed ~30 fold higher in retinas injected with AAV/SNAP25 than in control eyes injected with AAV/control (figure 4.4).

Electroretinogram (ERG) analysis confirms that the function of SNAP-25 infected retinas is severely disrupted, with significantly reduced a- and b- wave rod responses (figure 4.5).

Histological analyses of resin-embedded 1µm thick retinal sections show a marked degeneration of the photoreceptor outer-, and, to a lesser extent, inner nuclear layers (Figure 4.6). Using imaging analysis software the thinning of the ONL could accurately be measured and was found to be significantly reduced in the retinas of AAV/SNAP-25 eyes compared with controls (figure 4.7). However, as the viral transcript is only expressed in the ONL, we hypothesized that INL degeneration may be as a result of glutamate excitotoxicity. To investigate this further we prepared crude retinal lysates 4 weeks post-injection and examined the neurochemical concentrations using reverse-phase HPLC, but surprisingly, no differences in concentrations of glutamate (figure 4.8A), GABA (figure 4.8B), or aspartate (figure 4.8C) were observed. However, this was an experiment that would be worth repeating. The retinal lysates were prepared quite crudely, using an improvised and previously untested protocol. Further work should also examine the glutamate levels

at earlier time points because at 4-5 weeks only 1-2 layers of photoreceptor cells remain. Perhaps at 2 weeks there would be more photoreceptor cells and therefore higher glutamate concentrations in AAV/SNAP-25 transduced retinas.

It is interesting to note that glutamate excitotoxicity is a common feature of glaucoma. Glaucoma is a neurodegenerative disease typified by progressive loss of retinal ganglion cells (RGCs). Mild excitotoxicity has been implicated as one of the factors contributing to RGC death during the glaucomatous process. This type of excitotoxic cell death is due, at least in part, to somewhat excessive activation of N-methyl-D-aspartate (NMDA)-type glutamate receptors. A study by Wiggs *et al.*, (2004) identified 25 pedigrees with typical juvenile-onset OAG (JOAG), demonstrating autosomal dominant inheritance and, using multipoint linkage analysis of markers on chromosome 20 achieved a peak lod score of 4 on chromosome 20p12, which interestingly, is where the SNAP-25 gene is located.

Taken together these findings suggest that cell death can be induced in photoreceptors by over-expression of SNAP-25 and potentially implicate SNAP-25 related excitotoxicity as a general mechanism in a number of retinopathies. As altered gene expression profiles can result in disease pathology, future studies will determine if a possible epigenetic mechanism is involved in SNAP-25 over-expression.

4.5 Conclusions

SNAP-25 overexpression induces photoreceptor cell death. While it is clear that photoreceptors are degenerating, more work needs to be done to further elucidate the cause of cell death. Real time analysis of transduced cells shows a huge increase in SNAP-25 expression and this could perhaps be toxic. Glutamate excitotoxicity in the retina is a somewhat controversial subject, as the initial paper describing the effects was later shown to contain erroneous results. However, other groups have since shown that glutamate excitotoxicity does occur in the eye. Unfortunately, our analyses failed to detect increased levels of glutamate, which is surprising considering we found a ~3-fold increase in extracellular glutamate in a similar experiment in the rat hippocampus (see chapter 5). Interestingly, in the hippocampus, the total percentage of transduced cells was much smaller than the

percentage transduced in the retina, which makes the negative result in figure 4.8 so disappointing. More work is definitely needed in this regard and it would be most interesting to further investigate levels of glutamate at earlier timepoints, and perhaps using a more sophisticated method for collecting retinal fractions. Nevertheless, the finding that SNAP-25 induces a retinal degeneration is still in itself significant, and could have implications in helping to better understand the mechanisms of cell death in glaucoma and other retinopathies.

Chapter 5

AAV-mediated Overexpression of SNAP-25 in Rat Hippocampus

ALL INFORMATION CONTAINED HEREIN IS UNCLASSIFIED

DATE 02-01-2001 BY 60322

5.1 Introduction

The main aim of this chapter of my thesis was to inject the high-titre AAV/SNAP-25 virus generated as part of the work described in chapter 3 into the rat hippocampus and investigate its effects at a functional and molecular level.

This part of my research was concerned primarily with the role of SNAP-25 in memory and learning. SNAP-25 was initially identified as a candidate gene for further investigation by our collaborators in UCD and Wyeth as part of the Science Foundation Ireland (SFI)-funded Applied Neurotherapeutics Research Group (ANRG). The major goal of the ANRG is to focus on the molecular and cellular events relating to memory and learning with a specific objective of defining possible drug targets for psychiatric and neurological illness. To achieve this goal, the group first sought to identify changes in gene expression associated with the synaptic plasticity accompanying behavioural adaptation in the rat. Using comparative microarray analysis, expression changes in several genes were identified 12-24 hours post-training and these could potentially identify core events related to mechanisms of memory and learning, and in turn, psychiatric and mental illness. By over-expressing transcripts which are down-regulated post-training and observing behavioural changes, it should, in principle be possible to verify their potential as drug targets.

Using a minimum criterion of a two-fold change in gene expression, over 250 genes were found to be significantly altered in expression over the first 24 hours of hippocampal memory consolidation post-passive avoidance training (O'Sullivan *et al.*, 2007). Of these, SNAP-25 showed an almost 3-fold post-training decrease in expression. Given that SNAP-25 in the SNARE complex is known to be involved in neurotransmitter release at the synapse and because of the significant decrease in SNAP-25 expression post-training in rats, we therefore sought to over-express SNAP-25 in the dorsal hippocampus and to investigate its effects.

The ternary SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex, the minimal machinery required for vesicle exocytosis, consists of the synaptosomal protein of 25 kDa (SNAP-25), a presynaptic plasma membrane protein, together with synaptobrevin/VAMP and syntaxin-1A (Sollner *et al.*, 1993; Wang and Tang, 2006). The SNARE complex has been repeatedly implicated in

cognitive functions such as memory formation and sensory processing (Sollner *et al.*, 1993; Hou *et al.*, 2004; Verderio *et al.*, 2004; Gosso *et al.*, 2006; O'Sullivan *et al.*, 2007). By way of example, selective inhibition of SNAP-25 using antisense technology has been shown to prevent neurite outgrowth and elongation, a morphological correlate of memory consolidation in the hippocampus (Osen-Sand *et al.*, 1993; O'Malley *et al.*, 1998, 2000; Eyre *et al.*, 2003). Moreover, *in vitro* evidence in primary hippocampal culture and in brain slices suggests that SNAP-25 is expressed predominantly in the glutamatergic, and not GABAergic, synapses of hippocampal neurons (Verderio *et al.*, 2004). These studies used knockdown and knock-in methods to demonstrate that decreased and increased SNAP-25 expression augments and depresses neuronal calcium responsiveness, respectively. *In vivo*, up and down regulation of SNAP-25 mRNA expression has been noted to accompany hippocampal long-term potentiation (LTP) and passive avoidance learning, respectively, the former a widely accepted physiological model of memory-associated synaptic plasticity (Roberts *et al.*, 1998; Malenka & Bear, 2004; Cooke & Bliss, 2006; O'Sullivan *et al.*, 2007).

The contribution of developmental upset and regionally specific disruption to the motor and cognitive deficits associated with SNAP-25 activity remains unclear. In this chapter of my PhD thesis, I have deployed recombinant AAV-mediated gene delivery *in vivo* to evaluate the functional consequences of increased SNAP-25 expression restricted in space to the dorsal hippocampus and in time to the adult period of life. Specifically, we have evaluated the consequence of dorsal hippocampal SNAP-25 over-activity on short term plasticity and transmission tone as measured by paired pulse facilitation and glutamate levels, respectively. Finally, I establish the effects of SNAP-25 over-expression on a range of cognitive-associated behaviours selected to test functionality in different subregions of the hippocampus, prefrontal cortex and motor circuitry. Taken together, the results suggest that over-activity of SNAP-25 in the adult brain reduces the signal-to-noise ratio and potential for synaptic plasticity within circuitry controlling spatial memory consolidation resulting in cognitive deficits akin to those observed in schizophrenia, ADHD and other disorders of higher brain function.

In this chapter of my PhD thesis I show that AAV/SNAP-25 can be delivered to the target tissue, infect a significant number of cells in the dentate gyrus, and effectively over-express the viral transgene. Using *in vivo* microdialysis, I show that SNAP-25 overexpression is associated with an approximately 3-fold increase in extracellular glutamate levels, and I describe an associated electrophysiological change in post-pulse facilitation in the CA1 region of the hippocampus. I then show the functional consequences of SNAP-25 overexpression over a range of behavioural paradigms, in particular describing a selective impairment of spatial memory in the Morris water maze and in contextual fear conditioning paradigms.

5.2 Methods

5.2.1 Animals

The subjects of these experiments were male Wistar rats obtained from a colony in the Biomedical Facility in University College Dublin. All animals were postnatal day 49 on the day of surgery with subsequent testing, behavioural and microdialysis, beginning at postnatal days p80-84. Water and food were provided *ad libitum*. All procedures described were conducted in accordance with the regulations of the Animal Research Ethics Committee of University College Dublin and Trinity College Dublin and were carried out by individuals who held the appropriate licenses issued by the Department of Health.

5.2.2 Site directed injection to the dorsal hippocampus

Animals were anaesthetised by intraperitoneal administration of a solution (1ml/kg) containing 4 parts ketamine hydrochloride (Vetalar®) (100mg/ml) and 3 parts xylocaine (Dormitor®) (20mg/ml). The depth of anaesthesia was assessed by monitoring respiratory rate and palpebral and pedal withdrawal reflexes. Once the animal was fully anaesthetised they were placed in the stereotaxic frame and the skull was exposed. The skull was trepanned (1.4mm in diameter) bilaterally, at a position of 3.8mm posterior and 1.5mm lateral to Bregma. A cannula (28 gauge), loaded with either AAV/SNAP-25 or AAV/Control was slowly lowered in the burr holes to a depth of 3.5mm ventral to Bregma. The virus was then slowly infused over a 10 minute period. After the end of the 10 minute injection period the cannula was kept in place

for a further 2 minutes to minimise any drawback of the virus as the cannula was removed. After the bilateral viral injections, the incision was closed with interrupted silk sutures and the animal was placed in a heated cage (35°C) and monitored carefully. The anaesthetic was reversed by administration of atipamezole hydrochloride (Antisedan®) (2.5mg/kg) and carprofen (Rimadyl®) was administered (0.1ml/kg i.p) as a post-operative analgesic. The animals were then left for 5 weeks to allow time for good viral transfection. For the remainder of the experiment the animals were housed individually with ad libitum access to food and water.

5.2.3 Histological staining, immunohistochemistry, and *in situ* hybridizations

Coronal sections (12µm) of the dorsal hippocampus of both SNAP25-AAV and GFP-AAV were cut on a cryostat (HM 500 Microm, Walldorf, Germany) at -15°C and thaw-mounted onto SuperFrost Plus slides (VWR International, Leicestershire, UK). For histological staining, sections were stained with toluidine blue and imaged using an Aperio Slide Scanning system at the UCD Conway Institute, which was funded under a Health Research Board of Ireland Equipment Grant.

For immunohistochemistry, sections were fixed in 70% ethanol for 25mins, permeabilised using a solution of 0.02% Triton X in phosphate buffered saline (PBS) for 30mins, washed with PBS and incubated overnight (18 hours) with SNAP-25 primary antibody (S9684, Sigma, Dublin, Ireland). Following two 10min washes in PBS, sections were incubated for three hours with an anti-rabbit IgG (H+L) secondary antibody labeled with the fluorescent marker Cy3 (Jackson ImmunoResearch, PA, USA). The secondary antibody was then washed off with PBS, the sections were mounted with Citifluor® and the slides were stored in darkness at 4°C. Confocal images were taken using the 10x/0.8W water-dipping lens (Zeiss Achroplan).

A 275bp region of SNAP25 cDNA (563-822) was amplified using PCR, incorporating a SP6- and T7-RNA-polymerase promoter into the forward and reverse strand respectively. Sense and antisense digoxigenin-UTP labelled RNA probes were then generated by *in vitro* transcription using SP6- and T7-RNA polymerase respectively (DIG RNA labelling kit (SP6/T7), Roche, West Sussex, UK).

Coronal sections (30µm) of the dorsal hippocampus of both AAV/SNAP-25 and AAV/Control were cut on a cryostat (HM 500 Microm, Walldorf, Germany) at -15°C

and thaw-mounted onto SuperFrost Plus slides (VWR International, Leicestershire, UK), one each per slide. Sections were dried at room temperature and fixed in 4% paraformaldehyde at 4°C. Following washes and pre-hybridisation steps, hybridization was performed with 1µl probes in 100µl hybridisation buffer (Sigma, Dublin, Ireland) in a humidity chamber saturated with a 50% 2x SSC-50% formamide solution at 60°C for 16 h. The sections were washed twice in 2x SSC, 1x SSC, and 0.1x SSC all at 60°C. Slides then were soaked twice in buffer I (1M Tris HCl and 1.5M NaCl, pH 7.5) at room temperature. Buffer I, containing 1% goat serum and 0.1% Triton X-100, was then added to tissue sections, followed by an incubation with anti-digoxigenin-AP conjugate (1:1000, diluted with 1% normal goat serum and 0.1% Triton X-100 in buffer I) at room temperature for 2 h. Slides were rinsed in buffer I twice and once with buffer II (100 mM Tris HCl, 100mM NaCl and 50mM MgCl₂, pH 9.5). The colour reaction was developed in an NBT-BCIP solution (Roche) until a dark-blue reaction product was produced in the antisense but not sense slides. The slides then were rinsed in buffer III (10mM Tris HCl and 1mM EDTA, pH 8.1) to terminate the reaction, washed in distilled water, the sections were coverslipped and scanned using an Aperio Slide Scanning system.

5.2.4 Fluorescent-Activated Cell Sorting (FACS) and Quantitative RT-PCR

Animals were sacrificed 10 weeks post injection and the dorsal hippocampus was dissected and dissociated in trypsin (1mg/ml in HBSS) for 5 minutes at 37°C. Dissociated cells were resuspended in HBSS and GFP-expressing cells were identified and sorted by FACS (100,000-200,000 cells per hippocampus) using a Beckman-Coulter Altra fluorescence activated cell sorter (Beckman-Coulter Inc).

Gene-specific primers for RT-PCR were designed in exonic sequence and so that the product also spanned an intron, using the Primer3 algorithm (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). RNA was extracted using the RNeasy Mini Kit (Qiagen) and Quantitative PCR was performed on an ABI 7300 Real Time PCR System (Applied Biosystems) using the Quantitech PCR kit according to manufacturer's instructions (Qiagen). Serial dilutions of RNA were used to generate standard curves of crossing cycle number versus the logarithmus of concentration for each gene of interest. A linear regression line calculated from the

standard curves allowed relative transcript levels in each RNA sample to be determined. Values were normalized to the relative amounts of 18sRNA present in the same RNA preparations and a melting curve was also carried out after each amplification step, which provides confirmation that specific PCR products were formed in the reaction. Expression of SNAP-25 in injected hippocampus was expressed as an average increase with control values used as the baseline.

5.2.5 *In vivo* microdialysis

Prior to surgery on post-natal day 80 the rat was anaesthetised using a Univentor 400 anaesthesia unit (Univentor, Malta) under isoflurane inhalation (approximately 4% in air delivered at 6.7 ml/min, with an airflow of 500 ml/min) until the pedal withdrawal reflex is lost. Once anaesthetised, the animal is then placed in a Kopf stereotaxic frame (David Kopf Instruments, USA) and stabilised with blunt ear bars. Anaesthesia was maintained by continuous administration of isoflurane (2%, delivered at 3.4 ml/min, air flow 500 ml/min) via a modified mouthpiece, which allows the animal to breathe freely. The parietal and the frontal skull bones were exposed by a sagittal incision of the scalp at the midline, from the level of the eyes to the occipital protuberance. A burr hole of 0.8 mm diameter was drilled through the left side of the skull and the dura were carefully incised. A microdialysis probe of concentric design (CMA/12, Carnegie Medicin, Solna, Sweden) was employed with an outer diameter of 0.5 mm and a 1 mm length of dialysing membrane. The stereotaxic co-ordinates for the probe in the dorsal hippocampus (DH) were AP -3.8, ML -1.5, V -3.4 (mm from bone) with a 1 mm membrane length. The incisor bar was set at -3.3 mm for all probe implantations (Paxinos & Watson, 1998). During surgery the body temperature was continuously maintained at 37 °C by means of a thermostatically regulated heating pad (CMA 150, Carnegie Medicin). The flow rate (2 µl/min) of the perfusion medium (sterile Ringer solution, Baxter, UK, Formula per 1000 ml: sodium chloride 8.6 g; potassium chloride 300 mg; calcium chloride 300 mg; pH ~ 6) through the microdialysis probes was maintained constant by a microperfusion pump (CMA 100; Carnegie Medicin) during implantation and for the duration of the microdialysis experiments. The probe was fixed to the skull with stainless steel screws and metacrylic cement (Svedia, Enköping, Sweden). A new

microdialysis probe were used for each rat. At the end of the experiments the animals were sacrificed by pentobarbital (Euthatal, Rhone Merieux, Harlow, UK) overdose and the placement of the probe was verified by microscopic examination using a Leitz Cyrostat (Leitz, Germany). Animals with incorrect probe position were not included in this study.

The animals were given 24h to recover post-surgery before commencement of the microdialysis experiments. Following surgery the animals were housed individually in cages for the duration of the experiment. For the initial 24h after surgery, animals are given a high calorie (5% sucrose) solution to prevent dehydration and loss of body weight. On the day of the microdialysis experiment, the rat was placed in a large hemispherical bowl (diameter: 135cm) and the microdialysis probe was connected to a microinfusion pump (CMA 100, prior to sampling to allow for stabilisation of glutamate and GABA levels following a 24h recovery after probe implantation (Frantz *et al.*, 2002). Dialysate samples were then collected and split into two 20 µl aliquots and analysed separately for glutamate and GABA. The results reported are means of at least five experiments (n=5–8).

5.2.6 Neurotransmitter analysis

Glutamate was determined by precolumn derivatization of a 10 µl dialysate sample with o-phthalaldehyde/mercaptoethanol reagent and separation by reversed-phase HPLC on a Biophase ODS 5 µM particle column (Knauer, Berlin, Germany). The mobile phase contained 0.1 M sodium acetate, 6.25% methanol, 1.5% tetrahydrofurane, pH 6.95 and was perfused at a flow rate of 1 ml/min. A linear gradient system was used to clean the column after elution of glutamate. This involved switching to 100% methanol for 2 min before switching back to the original acetate buffer. The excitation wavelength in the fluorescence detector (Hitachi F1000, Tokyo, Japan) was set at 370 nm and the emission cut off filter was set at 450 nm. The limit of detection was 0.5 pmol/sample for glutamate (Morari *et al.*, 1994). The GABA assay employed was based on precolumn derivatization of a 10 µl sample with o-phthalaldehyde/tbutylthiol reagent and separation by reversed-phase HPLC on a Nucleosil 3 C18 column perfused under isocratic conditions at the flow rate of

0.8 ml/min. The mobile phase was 0.15 M sodium acetate, 1 mM EDTA, 50% acetonitrile, pH 5.4. The BAS LC4B electrochemical detector (Bioanalytical Systems, West Lafayette, IN) was set at + 0.75 V. The limit of detection was 50 fmol/sample (Morari *et al.*, 1994).

5.2.7 Paired Pulse Facilitation

Animals were blinded from the electrophysiological experimenters until statistical analysis was carried out. Animals were deeply sedated with a 1 ml intraperitoneal injection of pentobarbital (Euthatal, Abbott Laboratories Ireland Ltd.) and decapitated by guillotine. The brain was rapidly dissected and placed into ice-cold artificial cerebro-spinal fluid (aCSF) bubbled with 95% O₂ and 5% CO₂ (aCSF composition: 220 mM sucrose, 28 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 7 mM MgSO₄·7H₂O, 0.5 mM CaCl₂ and 7 mM D-glucose). Dorsal hippocampal slices (400 µm) corresponding to the injection sites were cut coronally from both hemispheres using a vibroslice (Campden Instruments). Slices were transferred to a submerged incubation chamber containing bubbled aCSF warmed to 30 ± 0.5°C (aCSF composition: 130 mM NaCl, 24 mM NaHCO₃, 1.25 mM NaH₂PO₄, 3.5 mM KCl, 2 mM MgSO₄·7H₂O, 1.5 mM CaCl₂ and 10 mM D-glucose) and allowed to recover for 60 min. Thereafter, slices were kept at room temperature (22 – 24°C).

Following the recovery period, slices were transferred to a recording chamber perfused with aCSF at a flow rate of 1 – 2 ml/min and heated to 30 ± 0.5°C (aCSF composition: 130 mM NaCl, 24 mM NaHCO₃, 1.25 mM NaH₂PO₄, 3.5 mM KCl, 1 mM MgSO₄·7H₂O, 1.5 mM CaCl₂ and 10 mM D-glucose). Extracellular field excitatory post-synaptic potentials (fEPSPs) were elicited by stimulation of the Schaffer collateral pathway of the CA1 using monopolar glass electrodes (2 – 4 MΩ resistance) at a frequency of 0.05 Hz. Paired pulse facilitation (PPF) at a stimulus interval of 50 msec was recorded. Baseline EPSPs were allowed to stabilise before an input/output curve was generated. Responses were recorded at stimulus strengths corresponding to 50% of the maximum output. PPF was recorded in the CA1 for 15 min and a digital average of 45 recordings was calculated for each hippocampal slice. PPF was determined by calculating the ratio between the

amplitudes of the second and first post-synaptic potential (second post-synaptic potential/ first post-synaptic potential).

5.2.8 Open field assessment

To ensure that all animals were of good health prior to anaesthesia, all animals were weighed and their general behaviour assessed in an open field apparatus (62cm x 62cm x 15cm) over a 5 minute period. Video tracking system, Ethovision 3.1 (Noldus) was used for automated recording and analyses of both distance moved and mean velocity. Other behaviours assessed included rearing, grooming, piloerection, defecation and posture. All observations were carried out in the quiet room under low-level red illumination between 08:00 and 12:00 to minimise circadian influence.

5.2.9 Water Maze paradigm

This spatial learning task has been described in detail previously (Murphy *et al.*, 1996). Briefly the water maze apparatus consisted of a large circular pool, one and a half meters wide and 80cm high. The pool was filled with warm water (26°C +/- 1°C) with a platform (11cm in diameter) submerged 1.5 cm below the level of the water. For all trials the platform remained in the same location, 30cm from the wall in the north east quadrant. To eliminate any intra-maze cues, both the pool floor and the platform were constructed of black poly-vinyl plastic. The experimental room however, contained several extra-maze visual cues. Spatial training consisted of four training session, spaced 24h apart, with each session consisting of five trials (intertrial interval, 5 mins). Each trial started with the rat being lowered into the pool facing the wall of the maze at one of three locations. The time taken by the animal to find the hidden platform within the criterion of 90 seconds was defined as the escape latency time. If an animal failed to find the platform within this time it was placed on the platform for 10sec. One day following the final training session a probe trial was conducted to examine the animals recall for the platform location. Here the platform was removed and the animals swim trace monitored by a computerised tracking system, Ethovision 3.1, over a 60sec period. The training data

was analysed by plotting the mean escape latency $\pm$ SEM for both the control and treated groups. The groups were then compared across the whole session by way of a Two-way ANOVA, with intra-trial differences calculated by Bonferroni *post-hoc* tests. For the recall data, the data was expressed as the mean percentage time spent in each of the four quadrants.

5.2.10 Passive avoidance conditioning

The one-trial, step-through, light-dark version of the passive avoidance paradigm was employed as we have described previously (Fox *et al.* 1995). The smaller illuminated compartment was separated from the larger dark compartment by a shutter that contained a small entrance. The floor of the training apparatus consisted of a grid of stainless steel bars that would deliver a scrambled shock (0.75 mA every 0.5 ms) for 5 seconds when the animal entered the dark chamber. Recall was tested 24 hours following training by placing the animal back in the lighted chamber and noting the time to enter the dark chamber. A criterion time of 600sec was employed. These data were analysed by Mann-Whitney U-test with $p < 0.05$ considered significant.

5.2.11 Prepulse inhibition of startle

The PPI protocol employed was based on a procedure previously described by Geyer and colleagues (1993). Each rat was restrained in an appropriately sized cylindrical holder that was placed on a movement-sensitive platform and maintained in a soundproof chamber. The rat was allowed to habituate to a white noise background of 70 dB for 5 min before receiving five 20 ms startle trials of 120 dB separated by randomized intervals of 10-20 sec. Immediately thereafter, each rat received five separate presentations with one of the prepulse stimuli of 72, 76, 80, or 84 dB followed 100 ms later by the 120 dB acoustic startle stimulus. Each trial was separated by a time interval of 10-20 sec. The four prepulse stimuli were delivered in a randomized manner and included periods in which there was no prepulse or startle stimulus. The session terminated with five further startle trials. Randomization of the sound signals delivered and quantification of the startle movements were recorded from the movement-sensitive platform. Signals were integrated by the

software supplied by the manufacturers of equipment hardware (MED-Associates Inc., St. Albans, Vermont, USA) with data analyzed by two-way ANOVA.

5.2.12 Contextual Fear Conditioning

Contextual fear conditioning was carried out in a rodent observation cage (30 x 37 x 25cm), the side walls of which were constructed of stainless steel, and the back walls and doors constructed of clear Plexiglass. The floor consisted of 20 steel rods through which a scrambled shock from a LETICA I.C shock generator (Model LI100-26, Spain) could be delivered. Each observation cage was cleaned with a 1% acetic acid solution before and after each session. The chambers were illuminated with a 20W bulb. On the training day, each rat was transported from the colony room to the laboratory (situated in an adjacent room) and placed into the observation cage. After 3 minutes, the rats received three 1 second shocks of 0.7mA intensity. The intershock interval was 60 seconds and the rats were removed from the conditioning chambers 30 seconds after the final shock and returned to their cages. Thus, a conditioning session lasted approximately 330 seconds. In experiments to determine the effects of SNAP-25 over-expression on memory consolidation, recall of contextual fear was tested 3 and 5 days after initial conditioning. At recall testing, rats were placed back into the same chamber as used in conditioning, in the absence of shock, for an 8 minute context test. The time spent by each rat freezing was measured. Freezing is defined as behavioural immobility except for movement needed for respiration. This data was used to calculate percent time freezing and the mean SEM calculated for each group. Difference between the groups was assessed by Student's t-test.

5.3 Results

5.3.1 Site-directed injection of AAV/SNAP-25 to the dorsal hippocampus results in enduring overexpression of SNAP-25

Seven week old (P49) male adult rats were injected bilaterally into the dorsal hippocampus with 1µl AAV/SNAP-25 (8×10^{12} vp/ml) or AAV/control (5×10^{12} vp/ml)

using stereotaxic injection coordinates according to the wistar rat brain atlas (figure 5.1).

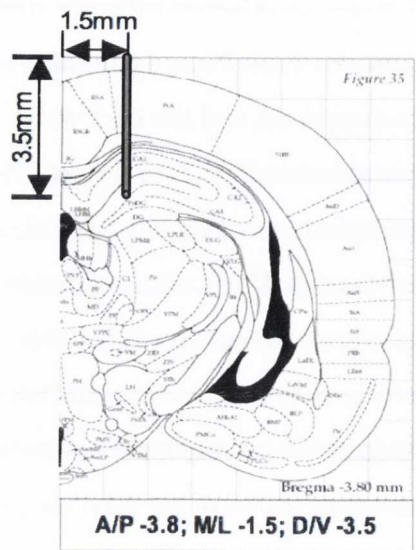


Figure 5.1: Brain Atlas diagram showing site of stereotaxic injection. The skull was trepanned (1.4mm in diameter) bilaterally, at a position of 3.8mm posterior and 1.5mm lateral to Bregma. A cannula (28 gauge), loaded with either rAAV/SNAP-25 or rAAV/Control was slowly lowered in the burr holes to a depth of 3.5mm ventral to Bregma. The virus was then slowly injected bilaterally into the brain at a rate of 0.1µl/min over a 10 minute period.

Animals were allowed to recover and housed singly until behavioural or neurochemical analyses (see below), 5 weeks post injection. Throughout the study neither viral vector adversely affected the health of the animals as indicated by normal weight gain (Fig 5.2).

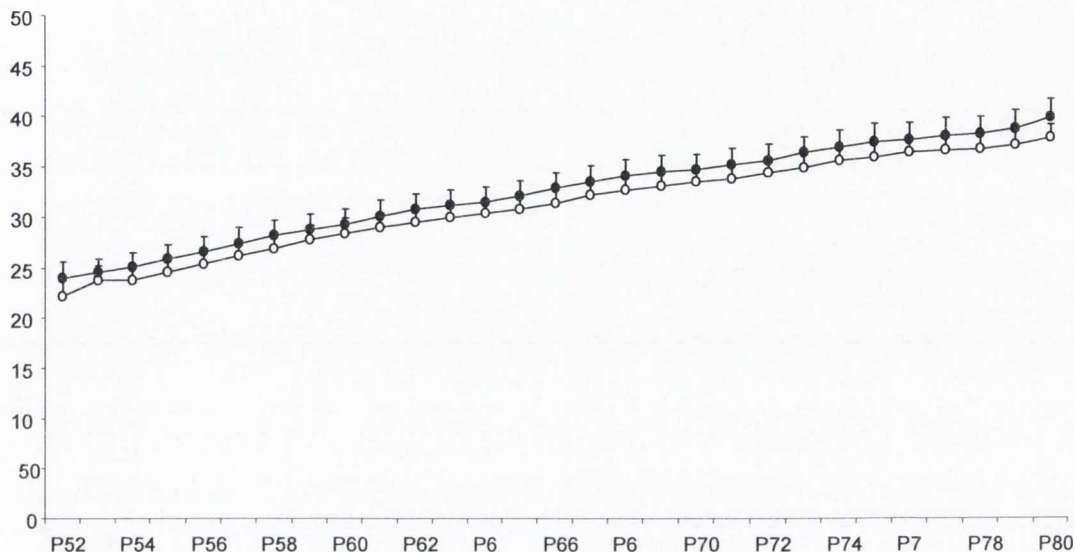


Figure 5.2: Change in body weight (grams) following a bilateral adeno-associated virus (AAV) SNAP 25 injection into the dorsal hippocampus on post partum day 52 (P52) (●) compared to that observed in sham-injected controls (○). Each data point represents the mean±SEM of eight animals per group. [X-axis = time (days); y axis = weight (grams)].Statistical analysis was performed by two-way ANOVA followed by Bonferoni post-hoc analysis on the mean value – there was no significant difference in body weight between the two groups of rats.

Following behavioural and neurochemical analyses, animals were sacrificed at 6-7 weeks post-injection and brains were assessed for effectiveness of viral infection and transgene delivery. In the first instance, the rAAV2/5 constructs proved to be effective delivery vehicles as judged by the significant number of cells in the hippocampus that expressed GFP (Figure 5.3). Specifically, approximately 5-10% of cells in the hippocampus were immunopositive for the GFP protein, with expression localized throughout the hippocampus, but mainly in the granule and hiller cells of the dentate gyrus, and the pyramidal cells of the CA1.

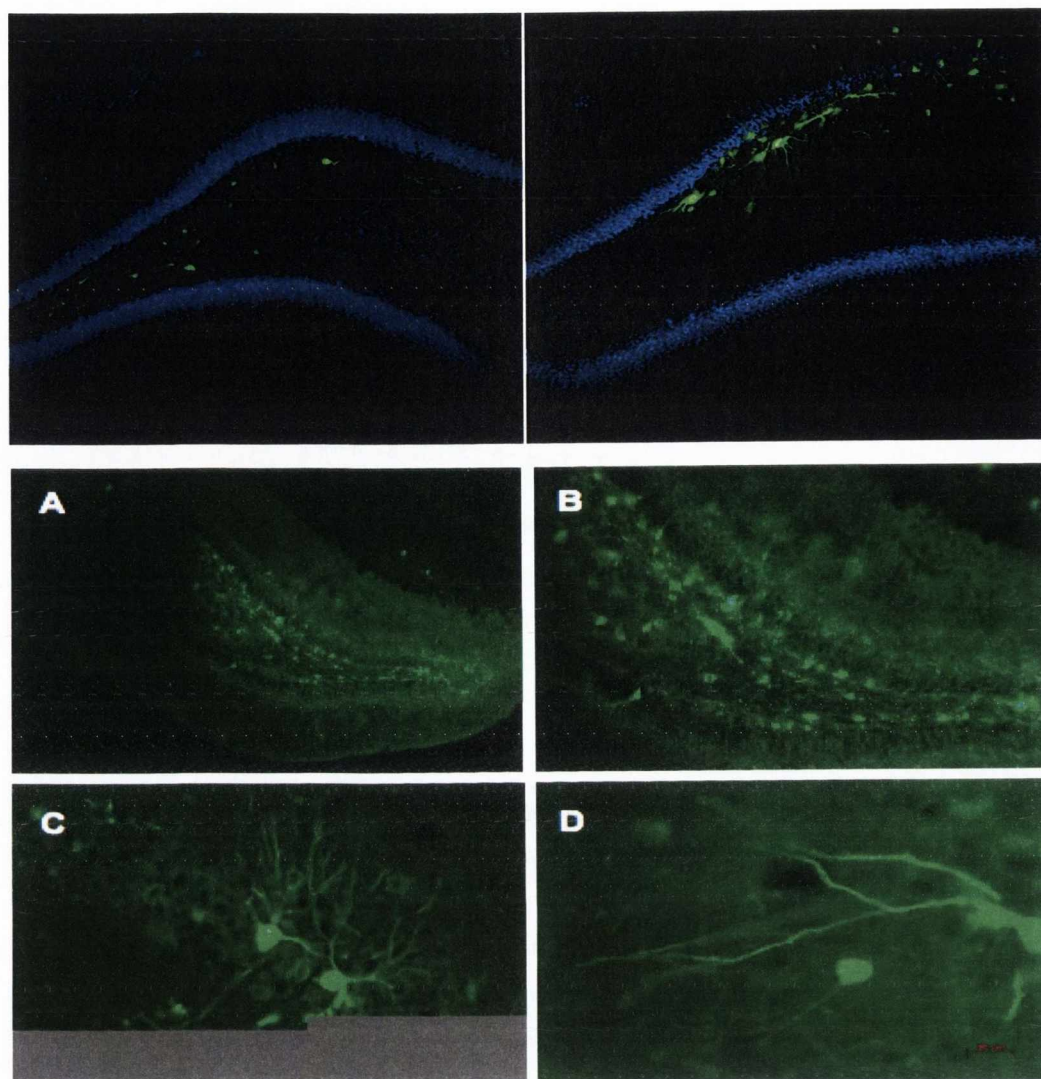


Figure 5.3: Representative fluorescent brain sections from rAAV injected rats. Top panel: AAV/SNAP-25 (left) and AAV/Control (right) show hrGFP and eGFP fluorescence, respectively. Nuclei are counterstained with DAPI Green AAV transduced cells are clearly visible in the dentate gyrus (DG) of the hippocampus. Higher magnification images (bottom panel) display transduced granule cells and hilar cells in the DG. A: 5x; B: 10x; C: 20x; D: 40x magnification. GFP fluorescence is distributed ubiquitously throughout the cell body and axonal projections.

In addition to evaluation of GFP expression, these brains were used to assess SNAP-25 expression at both mRNA and protein levels by *in situ* hybridization and immunohistochemistry, respectively. Comparative *in situ* hybridization using a probe homologous to the SNAP-25 mRNA sequence showed increased levels of SNAP-25 mRNA in AAV/SNAP-25 injected rats (Figure 5.4). Transgene expression was widespread throughout the dorsal hippocampus with highest levels of expression in

the nuclei of the dentate granule neurons of the suprapyramidal blade and the CA3 pyramidal cells. Immunohistochemical analyses using a SNAP-25 antibody revealed an accompanying increase in SNAP-25 protein expression in the dendritic fields of the primary hippocampal cell populations, in particular, the synaptic field of the mossy fibre axons onto CA3 pyramidals (Figure 5.4F & I). Finally, histological analysis revealed normal cellular morphology of hippocampal neurons indicating that there were no adverse toxic effects caused by viral infection or overexpression of viral transgenes (Figure 5.4C & F).

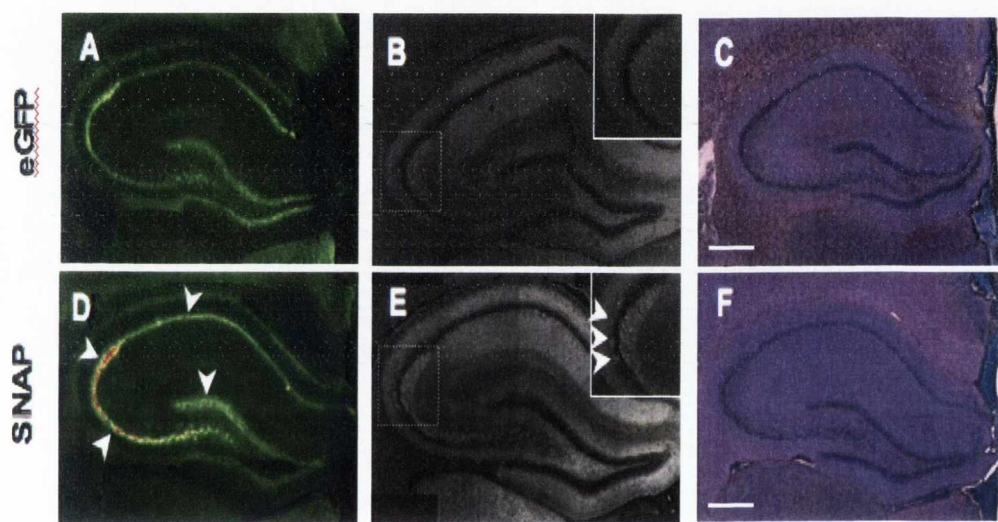


Figure 5.4: : Coronal sections through the rat dorsal hippocampus (approx. -3.3 mm w.r.t. bregma). Panels A and D show SNAP25 mRNA expression detected by *in situ* hybridization. The level of expression is indicated by pseudocolour, with areas of high expression coloured red and low expression dark blue. Areas of differential expression are indicated by arrowheads. Panels B and E: SNAP25 protein expression was detected by immunofluorescent labelling. SNAP25 was labelled using a Cy3-conjugated secondary antibody. Inset shows a close-up of the CA3-mossy fibre field and areas of differential expression are indicated by arrowheads. Panels C and F show toluidine blue stained sections that illustrate the overall morphology remained unaffected by either the control or SNAP-25 over-expressing vector. Scale bar = 500 µm.

The virally-mediated overexpression of SNAP-25 was specific to the dorsal extent of the hippocampal structure as no differences in SNAP-25 mRNA expression were evident by *in situ* analysis of the medial or ventral hippocampus at horizontal levels -

5.6mm and -7.6mm with respect to Bregma, respectively. By cutting serial 100 μ m thick sections using a vibrotome it was also possible to determine the spread of the virus from the injection site. By counting the number of serial sections containing GFP-positive cell bodies we were able to determine that the spread of the virus was between 1.2-2.0mm (ie: 12-20 sections).

In the remaining rAAV injected rats whole hippocampus was dissected, the cells dissociated and sorted using fluorescent activated cell sorting (FACS). When compared to an uninjected control hippocampus, both AAV/control (eGFP)- and AAV/SNAP-25 (hrGFP)-injected samples clearly had greater total fluorescence (Figure 5.5A). GFP-expressing cells comprised 8.1% ($\pm$ 1%, n=6) of the total cell population sorted by FACS. Total RNA was extracted from the GFP-positive cell-enriched fractions that resulted from the FACS analysis and quantitative RT-PCR analysis of this RNA using primers specific for SNAP-25 mRNA showed significantly increased levels of SNAP-25 expression in AAV/SNAP-25 injected rats compared to AAV/control (Figure 5.5B).

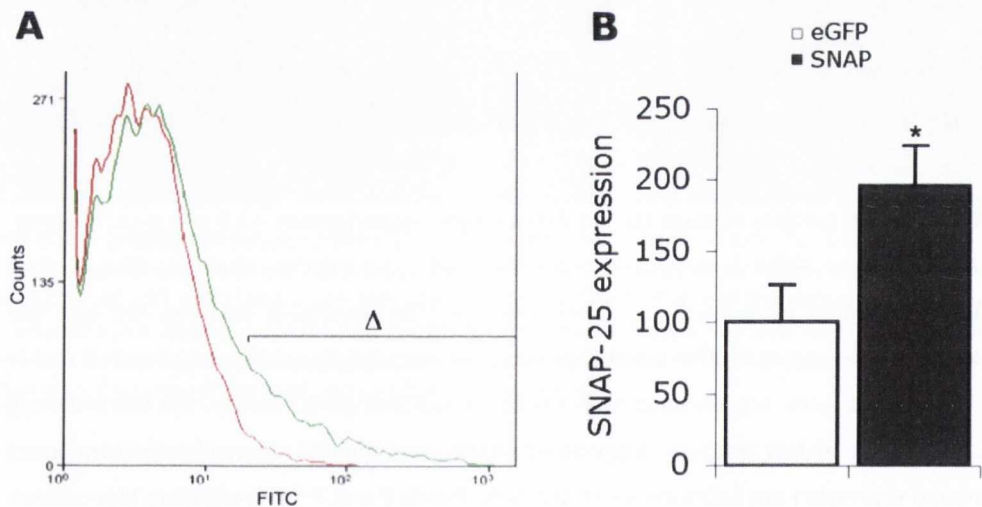


Figure 5.5: Fluorescent activated cell sorting (FACS). Rat dorsal hippocampi were dissected 10 weeks post-injection, dissociated, and analysed by FACS. Histogram (A) shows total GFP fluorescence in both an AAV/SNAP-25 injected sample (green) and an uninjected control animal. Fluorescent green cells (Δ) were sorted and RNA was extracted from these cells for quantitative RT-PCR analyses. B indicates an approximately 95% increase in SNAP-25 levels in GFP-expressing hippocampal cells quantified by RT-PCR. (Error bars represent SD values; $P < 0.0001$; n=6 animals per group).

These data indicated that a single 1 μ l injection of high-titre AAV/SNAP-25 into the dorsal hippocampus led to an approximate 100% increase in SNAP-25 expression within infected cells. Similarly, protein lysates were prepared from GFP-positive FACS sorted cells and western blot analyses confirmed that SNAP-25 was significantly up-regulated in AAV/SNAP-25 infected cells when compared with AAV/Control or non-infected cells (figure 5.5C)

5.3.2 AAV/SNAP-25-mediated SNAP-25 overexpression results in increased glutamate release.

SNAP-25 is a presynaptic membrane protein and is involved in neurotransmitter release. We therefore decided to investigate if increased expression of SNAP-25 caused an increase in the release of neurotransmitters within the injected area of the dorsal hippocampus. To do so, I injected another cohort of rats at the same time (injected at P49, analysed 5 weeks post-injection) as the group of rats used for the behaviour and electrophysiology experiments. As before, animals were injected with either AAV/SNAP-25 (n=6) or AAV/control (n=8). As before, a recovery time of 5 weeks was used to allow for sufficient viral expression to build up and for complete recovery post-surgery. A microdialysis probe (0.5mm in diameter, 1mm in length) was implanted unilaterally in the left dorsal hippocampus at precisely the same coordinates of the original viral injection (figure 5.1). The microdialysis technique used in these experiments allows for the determination of the precise chemical components of the fluids in the extracellular space of the hippocampus. A microdialysis probe (essentially a robust tube made of a semi-permeable membrane) is flushed with a dialysate and molecules within the extracellular fluid flow through the permeable membrane of the probe. Dialysate samples were collected and analyzed using reverse-phase HPLC to establish the levels of the excitatory neurotransmitter glutamate and the inhibitory neurotransmitter GABA. These studies revealed that levels of excitatory glutamate were significantly increased (Student's t-test, $p = 0.0388$) in the hippocampus of AAV/SNAP-25 injected rats (Figure 5.6) showing an approximate 3-fold increase in glutamate in rats over-expressing SNAP-25 ($1.868 \pm 0.965 \mu\text{M}$ vs $5.663 \pm 1.391 \mu\text{M}$ control vs SNAP-25,

respectively). In contrast, no difference was evident in the levels of GABA found in the dorsal hippocampus of the same animal cohort (9.872 ± 2.066 nM vs 9.742 ± 1.938 nM control vs SNAP-25, respectively). These findings suggest that chronic overexpression of SNAP-25 resulted in a functional increase in neurotransmitter release that was specific to the SNAP-25-dependent exocytosis of glutamate.

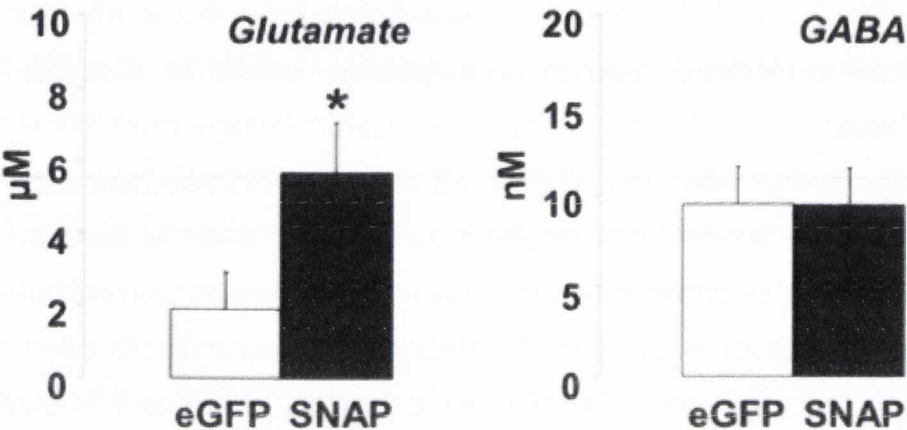


Figure 5.6: Neurochemical analysis of samples collected by microdialysis.

Overexpression of SNAP-25 causes an approx 3-fold increase in extracellular glutamate levels. Basal dialysate glutamate (left) and GABA (right) levels in the dorsal hippocampus. Each histogram represents the mean $\pm$ SEM (raw data) of four dialysate samples (4x20min) on post-partum day 81 commencing 160 mins after onset of perfusion. Statistical analysis was performed by Student's t-test. * $P < 0.05$.

5.3.3 AAV/SNAP-25-mediated SNAP-25 overexpression in the dorsal hippocampus selectively impairs spatial and contextual learning.

Rats were tested on a battery of behavioural paradigms 5 weeks post-bilateral injection to the dorsal hippocampus with either 1 μ l AAV/SNAP-25 (n=15) or control AAV (n=15) to assess the effect of chronic SNAP-25 over-activity on cognitive function. Both groups of rats were evaluated for general behaviour in the open-field arena, learning and memory abilities in the water maze, contextual fear conditioning, avoidance conditioning and sensorimotor gating as measured by pre-

pulse inhibition (PPI) of startle (Fox *et al.*, 1995; Murphy *et al.*, 1996; Geyer *et al.*, 2001). AAV/SNAP-25-injected animals exhibited a marked deficit in water maze acquisition with significantly slower times to platform across the second ($F[1,112]=7.060$, $p=0.0041$) and third ($F[1,112]=6.636$, $p=0.0156$) training session (Figure 5.7).

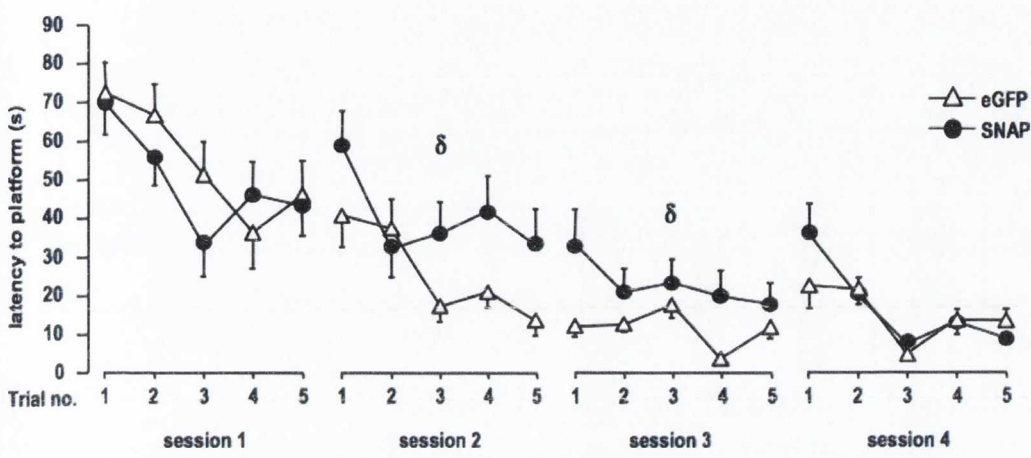


Figure 5.7: Spatial learning in the water maze over four sessions. When compared to controls (eGFP, clear triangles), animals over-expressing SNAP 25 (SNAP, black circles) displayed delayed acquisition of the water maze during session 2&3. Data represented as the mean ± SEM for each trial. δ signifies $p<0.05$ two-way ANOVA

By the fourth training session, however, the SNAP-25 animals performed as well as controls indicating that they were eventually able to acquire the task. This latter finding was supported by the equally robust recall exhibited by the two animal groups for the platform location when assessed 24h following the final training session (Figure 5.8).

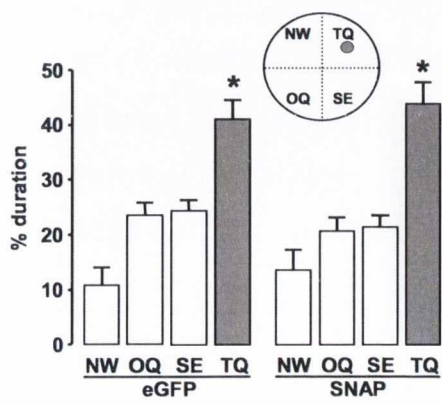


Figure 5.8: Water maze recall
 SNAP-25 over-expression did not lead to any deficits in recall (24h post-training) of this task. Data represented as % duration spent in each of the four quadrants (NW, OQ, SE and TQ, with TQ being the quadrant where the platform previously resided). * indicates $p<0.05$ One-way ANOVA.

The difference observed in time to platform during sessions two and three was not due to non-specific effects of SNAP-25 overexpression on general motor function as the animals exhibited equivalent swim speeds in these and other training sessions ($F[1,112]=0.379$, $p=0.539$; Figure 5.9).

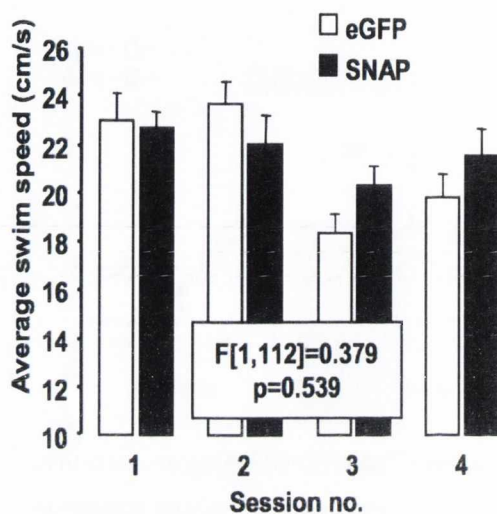


Figure 5.9: Swim speed across the four training sessions.

No difference in swim speed between groups. Data represented as mean swim speed per group (cm/s) $\pm$ SEM for each session.

In a separate cohort of animals ($n=8$), we evaluated the effect of SNAP-25 overexpression in the dorsal hippocampus on a second learning paradigm that is known to be dependent on this region, contextual fear conditioning (Fanselow, 2000). The foot shock stimulus employed in this study induced a robust freezing response in the rAAV/control-injected rats at both 3 and 5 days post training (figure 5.10). The high degree of freezing in response to the chamber indicated robust contextual memory. The percent time spent freezing upon re-exposure to the conditioning chamber was significantly reduced in the rAAV/SNAP-25-injected group at the 3 day recall but was no longer significantly different from control in the second recall session on day 5 post training (figure 5.10). Moreover, open field analysis revealed no significant differences in motor function with both locomotion and rearing appearing normal in these animals (Figure 5.8). The hyperactive phenotype of the *coloboma* mutant (Hess *et al.*, 1996) also led us to test these

animals for differences in locomotor activity in the open field apparatus but no significant difference was observed.

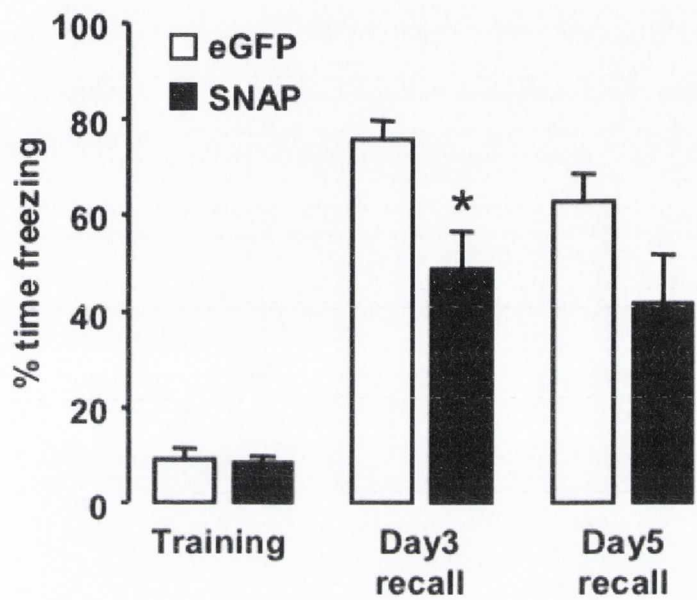


Figure 5.10: Effect of AAV-mediated SNAP-25 over-expression in the dorsal hippocampus on contextual fear conditioning.

AAV/SNAP-25 injected animals (filled columns) have a significantly reduced % time freezing on day 3, when compared to control injected animals (open columns), although there is no significant difference by day 5. Data represented as the mean $\pm$ SEM for each day, statistical analysis was performed by Student's t-test, * indicates $P < 0.05$

The animals were then assessed in the passive avoidance paradigm and again no difference was apparent in recall for association between the darkened chamber and the foot shock (median latency 600s with interquartile range 0,0 seconds vs median latency 600s, with interquartile range 89,0 seconds, control vs SNAP-25, respectively) (figure 5.11). Finally, PPI performance was evaluated as it is a predominantly prefrontal dependent sensory processing task and is regarded as an important endophenotype for schizophrenia and because several studies have linked SNAP-25 to schizophrenia (Lewis *et al.*, 2003). Although there was a tendency toward increased PPI in the SNAP-25 animals at the lower prepulse intensities, there was no significant effect on sensory processing ($F[1,96]=1.441$, $p=0.2329$) (figure 5.13).

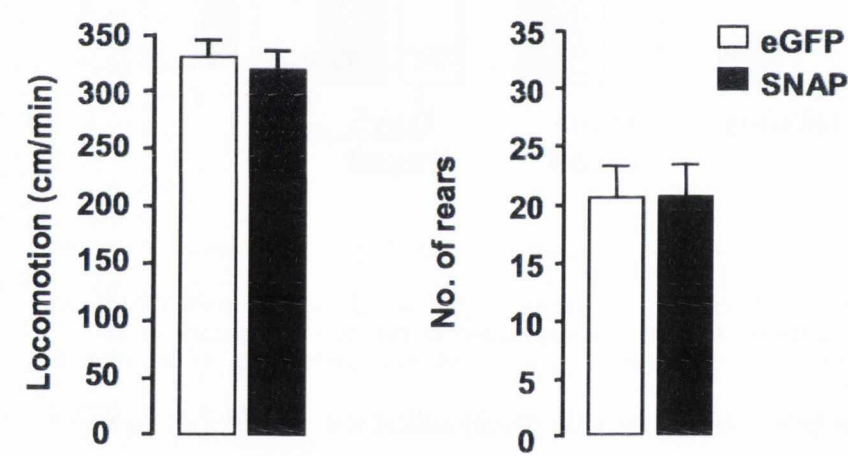


Figure 5.11: Exploratory behaviour in the open field.

To assess general behaviour, animals were placed in a box and their locomotion measured (cm/min). Healthy, alert animals should move around the box normally. The number of times an animal “rears” on its hind legs was also counted, as another rough measure of stress. No differences were observed between the two groups.

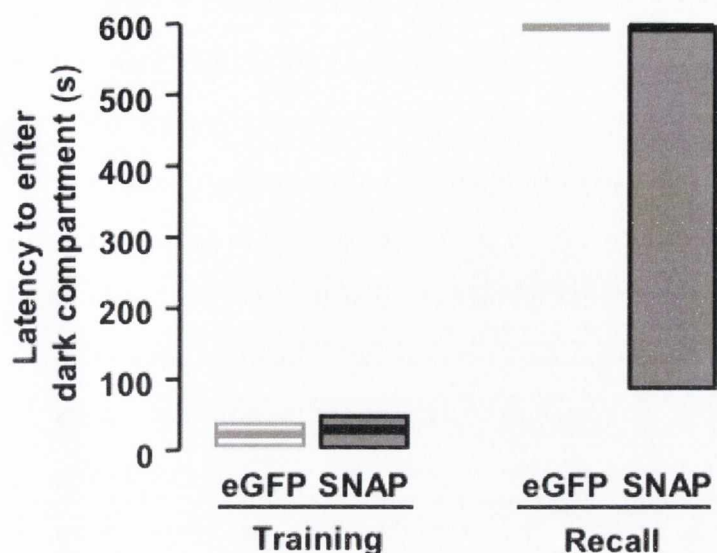


Figure 5.12: Passive avoidance training and 24h recall latencies.

Data represented as the median (thick line) latency to enter the dark compartment and the interquartile range illustrated by the box plot. Although there is no significant difference between the two groups of rats, AAV/Control-injected (eGFP) rats showed total recall ie: none entered the dark compartment on the second day.

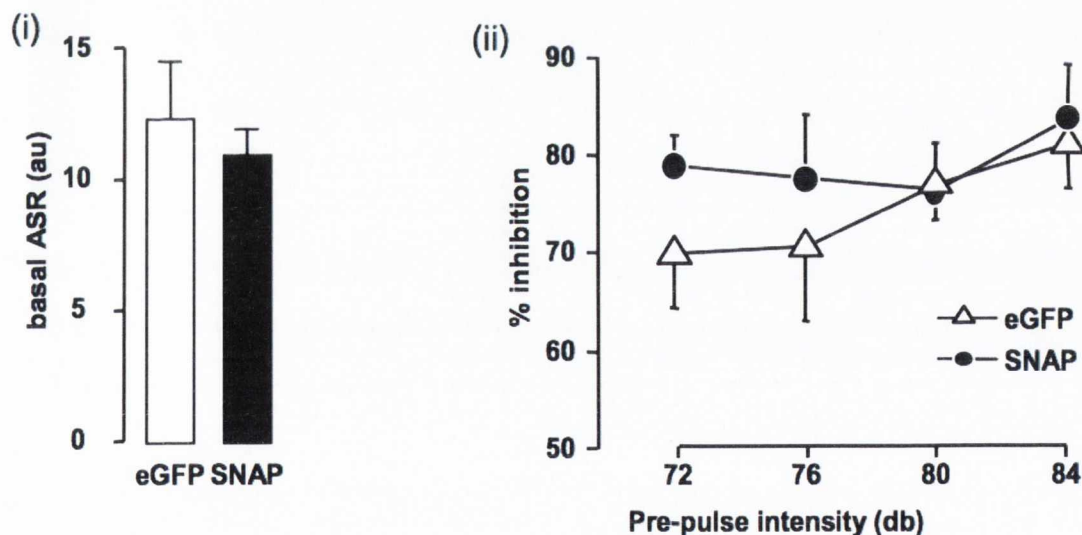


Figure 5.13: Prepulse Inhibition (PPI) of startle

(i) basal acoustic startle response (ASR) to pulse tone (120db) and

(ii) (ii) % pre-pulse inhibition. Data represented as the mean $\pm$ SEM.

No significant differences were observed.

5.3.4 Ex Vivo Electrophysiological Analyses of AAV-injected Hippocampi

Next, we assessed the effect SNAP-25 over-expression on parameters pertinent to synapse function and plasticity in the dorsal hippocampus. Paired-pulse facilitation (PPF) and depression (PPD) are well known short-term forms of synaptic plasticity. They are expressed as changes in the amplitude of a test evoked excitatory postsynaptic current (EPSC), evoked by a second presynaptic spike that follows the first (conditioning) one in the paired-pulse paradigm. The second post synaptic potential (PSP) can be up to five times the size of the first. Facilitation has been shown to be a phenomenon that occurs solely at the presynaptic side of a synapse, where synaptic vesicles containing neurotransmitter are released. As such, facilitation is thought to be the result of an increase in probability of vesicle release from this pool, and this in particular makes it a useful assay to study the effects of SNAP-25 overexpression on neurotransmitter release. Using acute slice electrophysiology, we observed a significant decrease in the magnitude of paired pulse facilitation in the Schaffer collateral synaptic input field to the CA1 pyramidal neurons. Figure 5.14 A & B present representative traces from rAAV/eGFP control and rAAV/SNAP25 injected animals, respectively.

While the paired facilitation obtained in the control animals was 2.1 fold that seen in the rAAV/SNAP-25 injected was only 1.75 fold (Figure 5.14C). Post-electrophysiology analysis of the acute slices indicated robust infection rates for both viral constructs as assessed by the number of GFP-positive cells that could be observed (Figure 5.14D & E). As before, GFP protein is clearly produced in and distributed throughout the infected cells, and throughout the hippocampus itself.

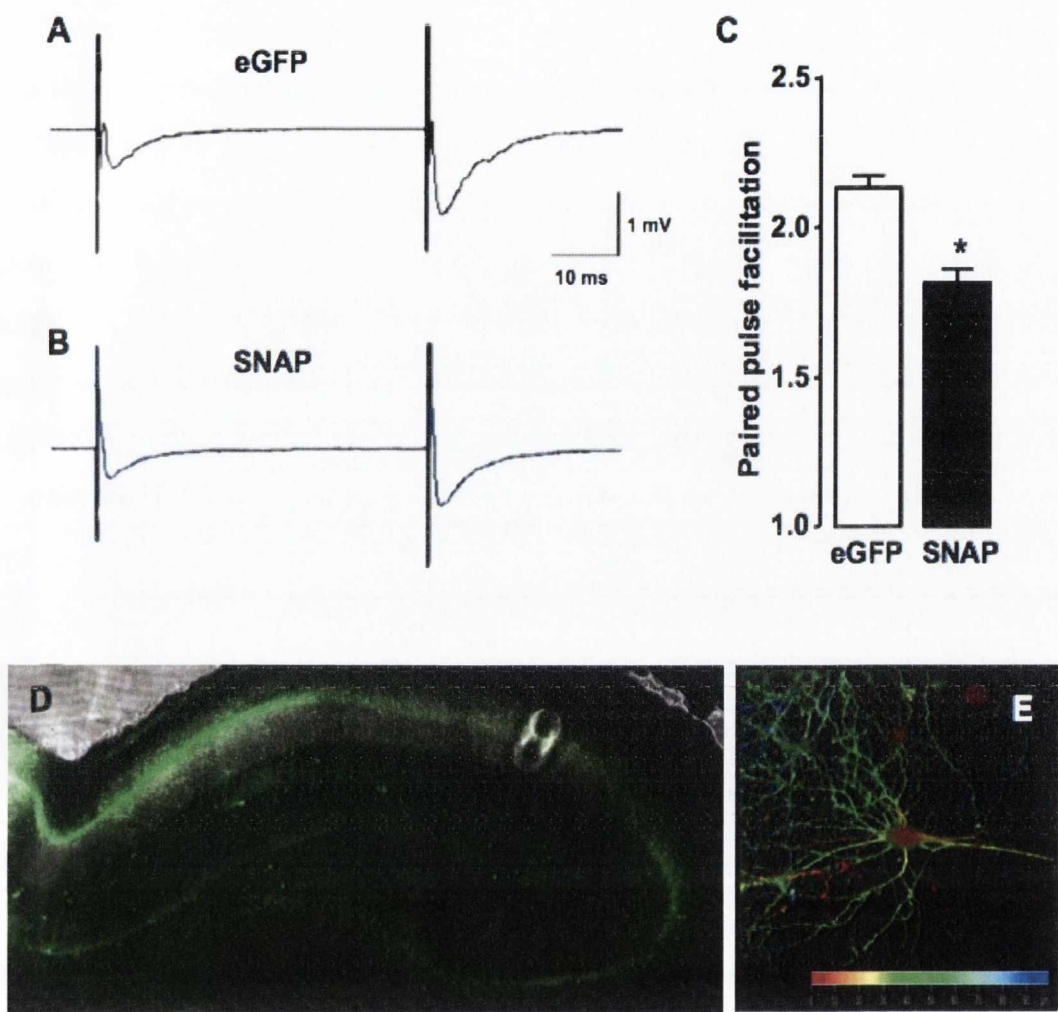


Figure 5.14: Paired Pulse Facilitation.

Panels A and B: representative traces from AAV/Control (eGFP) and AAV/SNAP25 (SNAP-25) injected animals, respectively. C: quantification of the paired pulse facilitation obtained in the rAAV/Control and rAAV/SNAP-25 injected animals. Panel D is a composite image overlaying a phase contrast image and a fluorescence image showing eGFP expression in an acute slice from a rAAV/Control injected animal. GFP protein was clearly produced in and distributed throughout the infected cells (E).

5.4 Discussion

Long-term memory is formed by alterations in glutamate-dependent excitatory synaptic transmission, which is in turn regulated by SNAP-25, a key component of the SNARE complex essential for exocytosis of neurotransmitter-filled synaptic vesicles (Lamprecht and LeDoux, 2004). Moreover, SNAP-25 has been shown to play a significant role in memory-associated plasticity (Hou *et al.*, 2004; Verderio *et al.*, 2004; Gosso *et al.*, 2006; O'Sullivan *et al.*, 2007). Both reduced and excessive SNAP-25 activity has been implicated in various disease states that involve cognitive dysfunctions such as ADHD, schizophrenia and Alzheimer's disease (Greber *et al.*, 1999; Barr *et al.*, 2000; Thompson *et al.*, 2003; Lewis *et al.*, 2003; Mill *et al.*, 2004).

In the current studies we wished to assess the functional implications on cognitive behaviour of SNAP-25 over-activity in the adult hippocampus in the absence of such dysregulation during the developmental period. In the first instance this required the construction and validation of viral-mediated gene delivery tools to allow the local overexpression of SNAP-25 *in vivo*. In particular, high titre recombinant AAV design and production was necessary as large bolus injection volumes cannot be used in the hippocampus without risk of tissue damage and subsequent impact on behavioural parameters. Here, rAAV viral titres of $\sim 5 \times 10^{12}$ or higher were routinely achieved. The use of these high titre preparations resulted in efficient infection rates *in vivo* and the robust overexpression of SNAP-25 at both a message and protein level in the absence of tissue damage to the dorsal hippocampus.

The specificity of the role of SNAP-25 in neurotransmitter vesicular release is still a topic of some debate (Verderio *et al.*, 2004; Wang and Tang, 2006). In particular, in the cortical regions such as hippocampus it remains unclear as to whether SNAP-25 functions exclusively in glutamatergic synapses or if it is also a component of the GABA release machinery. In the current study, the functional impact of SNAP-25 overexpression was indeed restricted to glutamatergic transmission as our microdialysis

studies revealed a selective increase in extracellular glutamate levels in the dorsal hippocampus with no effect on the inhibitory neurotransmitter GABA. The possibility exists, of course, that this differential effect on the cardinal neurotransmitter systems of the brain resulted from an AAV infection preference for glutamatergic granule and pyramidal cells of the hippocampus over GABAergic interneurons. No such preference has been reported previously in the literature and from our imaging studies there were equivalent numbers of these cell populations expressing GFP following infection (figure 5.2).

It is also important to note that the increase in glutamate levels achieved by SNAP-25 overexpression ($\sim 6\mu\text{M}$) is not in the range (mM) required to mediate excitotoxicity (Choi *et al.*, 1985), as indicated by the toluidine blue-stained sections illustrating no difference in overall morphology between the control or SNAP-25 over-expressing vector thus ensuring that effects noted on behaviour were not due to neuronal cell death.

The GFP visualization and FACS studies suggested that approximately 5-10% of cells in the hippocampus were infected by AAV/SNAP-25 virus and these expressed 100% additional SNAP-25 compared to normal. Unfortunately, because some of the rats used for FACS analyses were almost a year old, there was high levels of autofluorescence in the FACS dissociated brain cells, making it difficult to sort a specific population of GFP-positive cells. This task proved much more straightforward in the mouse retina, where lower levels of autofluorescence were observed, and a higher proportion of cells were GFP-positive (See Chapter 4, Figure 4.3). To overcome this problem and to show that a green population of cells could be sorted, neurons were dissociated from an age-matched uninjected rat and used as a control. Total fluorescence from a cell population of 30,000 cells was plotted on a histogram (Figure 5.5) and when compared to an AAV-injected sample, a “shift” in fluorescence can clearly be seen when compared to an uninjected control.

The overexpression of SNAP-25 in the dorsal hippocampus mediated a very specific detrimental effect on spatial memory consolidation, and contextual fear conditioning, both memory forms long established to be dependent on this region of the hippocampus (Moser *et al.*, 1995; Bannerman *et al.*, 2004). There were clear decrements in time to platform across sessions 2 and 3 in the water maze paradigm (figure 5.7). Moreover, this effect was particularly pronounced on the first trial of each session. By the fourth session and in the recall probe test AAV/SNAP-25 injected animals performed as well as their control counterparts (figure 5.8). Similarly, we observed a comparable deficit in contextual fear conditioning, with a significant deficit in recall shortly after the initial trial, but with no difference between SNAP-25 and control injected animals over longer periods of time (figure 5.10). Taken together, these findings implicate a dysfunction of memory consolidation that is normally ongoing between the training sessions and is reflected in gradual improvement in animal performance as the knowledge of platform location is acquired and refined.

In contrast, no influence on avoidance conditioning memory (figure 5.12) or PPI behaviour (figure 5.13) was observed. While the passive avoidance conditioning paradigm shares hippocampal dependence with the spatial water maze, lesion studies have clearly implicated the ventral reaches of this brain structure in learning of this aversive association (Bertoglio *et al.* 2006). In the present study no change was seen in SNAP-25 expression in the ventral hippocampus by *in situ* hybridization analysis and so it is not surprising that this region should have normal function. Similarly, the PPI behaviour is a sensorimotor gating test often employed both in animals and humans as a test of proper prefrontal cortical function (Geyer *et al.*, 2001). In particular, abnormality in PPI has been seen in individuals with psychotic disorders like schizophrenia. Again, no significant alteration in this behavioural readout was observed in the AAV/SNAP-25 animals (figure 5.13). Finally, given the association between SNAP-25 function and general physical activity, in relation to ADHD and hyperlocomotion, it was possible that the deficits in water maze were due to non-specific effects on motor

activity (Hess *et al.*, 1996; Barr *et al.*, 2000; Mill *et al.*, 2004;). No differences were seen in general activity parameters (figure 5.11). Taken together these findings suggest high SNAP-25 levels in the dorsal hippocampus mediate a very specific dysregulation of the spatial memory consolidation processes. Moreover, the cognitive dysfunction mediated by SNAP-25 over-expression was restricted to the dorsal hippocampal region targeted by the viral intervention as no deficits were observed in avoidance conditioning or prepulse inhibition of startle, functions dependent on the ventral hippocampus and prefrontal cortex, respectively

There are several possible explanations for the nature of the SNAP-25 overexpression-mediated deficit on spatial memory consolidation. In the first instance, as approximately 5-10% of cells were infected and a significant number of these are granule or pyramidal neurons, it is clear that a proportion of glutamatergic neurons in the dorsal hippocampus now release excessive quantities of glutamate and would thus falsely appear highly active within the nerve circuitry. In most computational models, it is concluded that any given memory trace will involve activation of approximately 5% of the cells in the dentate granule or CA fields (Treves and Rolls, 1994; Rolls and Kesner, 2006). Overexpression of SNAP-25 likely creates distractor cell groups that, during the memory trace lay down, will greatly reduce the signal-to-noise ratio and therefore the fidelity of the memory trace itself.

A second possibility relates to the need for SNAP-25 levels to decrease transiently following a memory trace (O'Sullivan *et al.*, 2007). Using microarray analysis, quantitative RT-PCR and western blotting techniques, SNAP-25 expression was shown to be markedly reduced at 6-12h following passive avoidance training, coinciding with the time window for memory consolidation. These changes were shown to be learning-specific as they were not observed in time-matched control animals (O'Sullivan *et al.*, 2007). Given the role of SNARE in glutamate release and the influence of SNAP-25 on neuronal calcium responsiveness, a coordinated decrease in expression of SNAP-25 may

function to modulate neuronal excitability during the 12h post-training period (Verderio *et al.*, 2004; O'Sullivan *et al.*, 2007). Thus, the chronic high expression of SNAP-25 in a significant proportion of the hippocampal glutamatergic neurons would impede such a down-regulation and again mediate a high transmission signal during a phase of memory consolidation when quiescence may be required to facilitate the synapse selection process initiated at this time (Murphy and Regan, 1998; Regan, 2004).

Finally, this study shows that intervention using high titre AAV vectors delivered locally to brain regions like the hippocampus can be effectively used to explore the role of overexpression (or under-expression/knockdown) of candidate genes in cognitive processes such as learning and memory.

One of the main limitations of our AAV delivery protocol was the low volume in which the virus was to be infused and the variability between injections. Because of the low injection volume and the size of the target site (the dentate gyrus) in some cases, a slightly bent injection needle or even a small blockage in the infusion tubing would result in lower transfection rates. In the case of subretinal injections (described in chapters 3 & 4), "bad" injections (off target, excessive bleeding, retinal damage etc) can immediately be noted and if necessary the animal can be excluded from the experiment. However, with stereotaxic injection to the hippocampus, the relative success of each individual injection can only be realized at least 5 weeks after the procedure. If behavioural testing is carried out, it may be as late as 10 weeks before histology or FACS analysis is carried out. To overcome this variability and to ensure greater numbers of cells are transfected, work is currently underway to infuse larger volumes of virus, over a longer time period, using osmotic pumps. These small (~1cm) pumps can be primed with up to 100µl of AAV. Subsequent to subdermal implantation the pump will slowly infuse the viral load over the course of a week before being surgically removed, which should result in significantly greater numbers of AAV infected cells.

The research described in this chapter of my PhD thesis represents a first attempt to utilize information obtained from gene expression profiling studies to direct *in vivo* exploration of specific candidate genes implicated in memory and learning. This work highlights the value of vectors such as AAV that enable overexpression of candidate genes such as SNAP-25. Future work will also enable knockdown of other candidate genes using RNAi-based AAV vectors.

5.5 Conclusions

In this chapter of my thesis I have described results from a study investigating the effects of overexpression of SNAP-25 in the hippocampus. It is important to note that although this has given rise to some interesting set of results regarding the role of SNAP-25 in neurotransmitter release, and it's subsequent effects on memory and learning, the proof-of-principle described herein and the AAV technology platform developed in our lab provide an excellent set of molecular tools for further studies. We have shown that high doses of AAV can be delivered efficiently to a small region of the brain (the hippocampus) to alter gene expression. By including a fluorescent tag we can visualize the precise infection pattern within the target tissue, and also use FACS sorting to isolate these cells and efficiently quantify the changes in gene expression. The gene chosen for study in this experiment was initially identified as being differentially expressed in the dentate gyrus during memory consolidation. It was just one of a number of candidate genes identified, and we have since made several other rAAV vectors in this lab to overexpress (or knock down, using RNAi) other candidate genes. By altering expression of a given candidate gene in the hippocampus and investigating its effects on behaviour using a variety of memory and learning paradigms, it is possible to elucidate many of the molecular mechanisms involved in the memory process.

The first of these is the fact that the...
...the second is the fact that the...
...the third is the fact that the...
...the fourth is the fact that the...
...the fifth is the fact that the...
...the sixth is the fact that the...
...the seventh is the fact that the...
...the eighth is the fact that the...
...the ninth is the fact that the...
...the tenth is the fact that the...
...the eleventh is the fact that the...
...the twelfth is the fact that the...
...the thirteenth is the fact that the...
...the fourteenth is the fact that the...
...the fifteenth is the fact that the...
...the sixteenth is the fact that the...
...the seventeenth is the fact that the...
...the eighteenth is the fact that the...
...the nineteenth is the fact that the...
...the twentieth is the fact that the...
...the twenty-first is the fact that the...
...the twenty-second is the fact that the...
...the twenty-third is the fact that the...
...the twenty-fourth is the fact that the...
...the twenty-fifth is the fact that the...
...the twenty-sixth is the fact that the...
...the twenty-seventh is the fact that the...
...the twenty-eighth is the fact that the...
...the twenty-ninth is the fact that the...
...the thirtieth is the fact that the...
...the thirty-first is the fact that the...
...the thirty-second is the fact that the...
...the thirty-third is the fact that the...
...the thirty-fourth is the fact that the...
...the thirty-fifth is the fact that the...
...the thirty-sixth is the fact that the...
...the thirty-seventh is the fact that the...
...the thirty-eighth is the fact that the...
...the thirty-ninth is the fact that the...
...the fortieth is the fact that the...
...the forty-first is the fact that the...
...the forty-second is the fact that the...
...the forty-third is the fact that the...
...the forty-fourth is the fact that the...
...the forty-fifth is the fact that the...
...the forty-sixth is the fact that the...
...the forty-seventh is the fact that the...
...the forty-eighth is the fact that the...
...the forty-ninth is the fact that the...
...the fiftieth is the fact that the...
...the fifty-first is the fact that the...
...the fifty-second is the fact that the...
...the fifty-third is the fact that the...
...the fifty-fourth is the fact that the...
...the fifty-fifth is the fact that the...
...the fifty-sixth is the fact that the...
...the fifty-seventh is the fact that the...
...the fifty-eighth is the fact that the...
...the fifty-ninth is the fact that the...
...the sixtieth is the fact that the...
...the sixty-first is the fact that the...
...the sixty-second is the fact that the...
...the sixty-third is the fact that the...
...the sixty-fourth is the fact that the...
...the sixty-fifth is the fact that the...
...the sixty-sixth is the fact that the...
...the sixty-seventh is the fact that the...
...the sixty-eighth is the fact that the...
...the sixty-ninth is the fact that the...
...the seventieth is the fact that the...
...the seventy-first is the fact that the...
...the seventy-second is the fact that the...
...the seventy-third is the fact that the...
...the seventy-fourth is the fact that the...
...the seventy-fifth is the fact that the...
...the seventy-sixth is the fact that the...
...the seventy-seventh is the fact that the...
...the seventy-eighth is the fact that the...
...the seventy-ninth is the fact that the...
...the eightieth is the fact that the...
...the eighty-first is the fact that the...
...the eighty-second is the fact that the...
...the eighty-third is the fact that the...
...the eighty-fourth is the fact that the...
...the eighty-fifth is the fact that the...
...the eighty-sixth is the fact that the...
...the eighty-seventh is the fact that the...
...the eighty-eighth is the fact that the...
...the eighty-ninth is the fact that the...
...the ninetieth is the fact that the...
...the ninety-first is the fact that the...
...the ninety-second is the fact that the...
...the ninety-third is the fact that the...
...the ninety-fourth is the fact that the...
...the ninety-fifth is the fact that the...
...the ninety-sixth is the fact that the...
...the ninety-seventh is the fact that the...
...the ninety-eighth is the fact that the...
...the ninety-ninth is the fact that the...
...the hundredth is the fact that the...

Chapter 6

Conclusions and Future Studies

There were several projects within my PhD thesis but despite working with two different tissues, the overall “theme” of each project was similar. In brief, SNAP-25 was identified as a downregulated gene from microarray study investigating genes differentially expressed during memory and learning and I sought to investigate the significance of the observed decrease in expression. I attempted to overexpress SNAP-25 to better understand its function in both the hippocampus and the retina. One of the key methods utilized in this thesis was the generation of pure high titre rAAV preps. These undoubtedly provide the most useful means of targeted gene delivery to neuronal tissues, and the proof-of-principle described in chapters 3, 4 and 5 provides an excellent basis for future studies delivering genes to both the retina and the brain. Future work will involve using this AAV technology platform to investigate the functions of other candidate genes for memory and learning in the hippocampus, and other candidate genes for retinal protection in the eye. Our rAAV production protocol is constantly being modified and improved to generate higher titres and ultra-pure preps and future work in this lab will continue to develop this method. The rAAV viruses used in this study are all AAV-2/5 based, utilizing the AAV-5 capsid protein. While AAV-2/5 is certainly the most efficient of the different AAV pseudotypes at transducing photoreceptor cells in vivo, some recent evidence suggests that AAV-2/8 and AAV2/9 infect significantly more cells in the hippocampus. Therefore, future studies using rAAV vectors to deliver genes to the brain will involve redesigning and generating newer viruses to improve our infection efficiency.

I have shown that rAAV vectors can be delivered to both the retina and the hippocampus, that transduced cells can be sorted using a GFP-label, allowing for precise quantification of the changes in gene expression induced by the rAAV transgene. One of the limitations of my AAV/SNAP-25 construct is that it contained an internal ribosome entry site (IRES). IRES elements are useful as both genes are translated from the same transcript allowing for detection of hrGFP expression to ascertain the infection

efficiency in the target cell and as a useful expression marker for the inserted gene of interest. However, IRES elements also significantly reduce levels of expression of the gene downstream of the IRES, and hrGFP expression is often 10-50 fold lower than if it were simply driven from a CMV promoter without the IRES. Because of this, hrGFP fluorescence was often difficult to detect, especially in the brains of aged rats where levels of autofluorescence are high. Therefore, if I was to redesign my rAAV construct, I would certainly use a bicistronic approach, utilizing an alternative promoter driven eGFP marker, rather than using IRES-hrGFP.

Another improvement in our rAAV protocol is to dialyse viral preps with a polymer known as F68, which effectively prevents virus from binding to the walls of the tubing used for our stereotaxic injection surgery (Alloca *et al.*, 2008). Increased transduction can also be achieved by adding mannitol to viral preps (Mastakov *et al.*, 2002) and both of these techniques will be included in our protocols for future studies.

I have shown that altering gene expression can cause quantifiable phenotypic changes, providing evidence of SNAP-25 induced behavioural changes as measured by memory & learning paradigms. Not only that, but this altered behaviour was then explained at the synaptic and molecular level by describing changes neurochemistry and synaptic transmission. The behavioural changes observed in the morris water maze are interesting because spatial memory is considered to be primarily processed in the dorsal hippocampus. There was no significant change in fear conditioning memory as assessed using passive avoidance and this is unsurprising as this is a type of memory usually associated with the ventral hippocampus. Therefore it would be interesting to investigate if SNAP-25 overexpression in the ventral hippocampus would cause a change in the passive avoidance paradigm.

In the retina, SNAP-25 overexpression led to the serendipitous observation of photoreceptor cell death, and this degeneration of the retina was further characterized by ERG analysis and histological evaluation. Unfortunately we were unable to detect a significant change in extracellular glutamate levels, despite our hypothesis that the mode of cell death might be linked to glutamate excitotoxicity. However, this was a

preliminary experiment, using a improvised method to generate retinal extracts for analysis on reverse-phase HPLC, future studies will improve this technique. HPLC analysis was carried out at 5 weeks post-injection and further studies at earlier time points are needed before ruling out the possibility that the retinas of AAV/SNAP-25 injected mice contain excess glutamate.

Ultimately, the aim of completing a PhD thesis is to obtain an in depth understanding at the edge of current scientific knowledge, and in turn, to make a valid contribution to that knowledge. I hope that the findings of these thesis are a worthwhile contribution and may help to better understand the molecular mechanisms of neuronal function and perhaps aid in the development of therapies for neurodegenerative and neuropsychiatric disorders.

the first of these is the fact that the...
the second is the fact that the...
the third is the fact that the...
the fourth is the fact that the...
the fifth is the fact that the...
the sixth is the fact that the...
the seventh is the fact that the...
the eighth is the fact that the...
the ninth is the fact that the...
the tenth is the fact that the...
the eleventh is the fact that the...
the twelfth is the fact that the...
the thirteenth is the fact that the...
the fourteenth is the fact that the...
the fifteenth is the fact that the...
the sixteenth is the fact that the...
the seventeenth is the fact that the...
the eighteenth is the fact that the...
the nineteenth is the fact that the...
the twentieth is the fact that the...
the twenty-first is the fact that the...
the twenty-second is the fact that the...
the twenty-third is the fact that the...
the twenty-fourth is the fact that the...
the twenty-fifth is the fact that the...
the twenty-sixth is the fact that the...
the twenty-seventh is the fact that the...
the twenty-eighth is the fact that the...
the twenty-ninth is the fact that the...
the thirtieth is the fact that the...
the thirty-first is the fact that the...
the thirty-second is the fact that the...
the thirty-third is the fact that the...
the thirty-fourth is the fact that the...
the thirty-fifth is the fact that the...
the thirty-sixth is the fact that the...
the thirty-seventh is the fact that the...
the thirty-eighth is the fact that the...
the thirty-ninth is the fact that the...
the fortieth is the fact that the...
the forty-first is the fact that the...
the forty-second is the fact that the...
the forty-third is the fact that the...
the forty-fourth is the fact that the...
the forty-fifth is the fact that the...
the forty-sixth is the fact that the...
the forty-seventh is the fact that the...
the forty-eighth is the fact that the...
the forty-ninth is the fact that the...
the fiftieth is the fact that the...
the fifty-first is the fact that the...
the fifty-second is the fact that the...
the fifty-third is the fact that the...
the fifty-fourth is the fact that the...
the fifty-fifth is the fact that the...
the fifty-sixth is the fact that the...
the fifty-seventh is the fact that the...
the fifty-eighth is the fact that the...
the fifty-ninth is the fact that the...
the sixtieth is the fact that the...
the sixty-first is the fact that the...
the sixty-second is the fact that the...
the sixty-third is the fact that the...
the sixty-fourth is the fact that the...
the sixty-fifth is the fact that the...
the sixty-sixth is the fact that the...
the sixty-seventh is the fact that the...
the sixty-eighth is the fact that the...
the sixty-ninth is the fact that the...
the seventieth is the fact that the...
the seventy-first is the fact that the...
the seventy-second is the fact that the...
the seventy-third is the fact that the...
the seventy-fourth is the fact that the...
the seventy-fifth is the fact that the...
the seventy-sixth is the fact that the...
the seventy-seventh is the fact that the...
the seventy-eighth is the fact that the...
the seventy-ninth is the fact that the...
the eightieth is the fact that the...
the eighty-first is the fact that the...
the eighty-second is the fact that the...
the eighty-third is the fact that the...
the eighty-fourth is the fact that the...
the eighty-fifth is the fact that the...
the eighty-sixth is the fact that the...
the eighty-seventh is the fact that the...
the eighty-eighth is the fact that the...
the eighty-ninth is the fact that the...
the ninetieth is the fact that the...
the ninety-first is the fact that the...
the ninety-second is the fact that the...
the ninety-third is the fact that the...
the ninety-fourth is the fact that the...
the ninety-fifth is the fact that the...
the ninety-sixth is the fact that the...
the ninety-seventh is the fact that the...
the ninety-eighth is the fact that the...
the ninety-ninth is the fact that the...
the hundredth is the fact that the...

References:

Ali RR, Reichel MB, Thrasher AJ, Levinsky RJ, Kinnon C, Kanuga N, Hunt DM, Bhattacharya SS. (1996). Gene transfer into the mouse retina mediated by an adeno-associated viral vector. *Hum Mol Genet.* 5:591-4.

Ali RR, Reichel MB, De Alwis M, Kanuga N, Kinnon C, Levinsky RJ, Hunt DM, Bhattacharya SS, Thrasher AJ. (1998). Adeno-associated virus gene transfer to mouse retina. *Hum Gene Ther.* 9:81-6.

Allocca M, Doria M, Petrillo M, Colella P, Garcia-Hoyos M, Gibbs D, Kim SR, Maguire A, Rex TS, Di Vicino U, Cutillo L, Sparrow JR, Williams DS, Bennett J, Auricchio A. (2008). Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J Clin Invest*, 118, 1955-64.

Andang M, Hinkula J, Hotchkiss G, Larsson S, Britton S, Wong-Staal F, Wahren B, Ahrlund-Richter L. (1999). Dose-response resistance to HIV-1/MuLV pseudotype virus ex vivo in a hairpin ribozyme transgenic mouse model. *Proc Natl Acad Sci U S A*, 96, 12749-53.

Babcock AM, Standing D, Bullshields K, Schwartz E, Paden CM, Poulsen DJ. (2005). In vivo inhibition of hippocampal Ca²⁺/calmodulin-dependent protein kinase II by RNA interference.

Mol Ther, 11, 899-905.

Bainbridge JW, Smith AJ, Barker SS, Robbie S, Henderson R, Balaggan K, Viswanathan A, Holder GE, Stockman A, Tyler N, Petersen-Jones S, Bhattacharya SS, Thrasher AJ, Fitzke FW, Carter BJ, Rubin GS, Moore AT, Ali RR. (2008). Effect of gene therapy on visual

function in Leber's congenital amaurosis. *N Engl J Med*, 358, 2231-9.

Balakirev MY, Tcherniuk SO, Jaquinod M, Chroboczek J. (2003).

Otubains: a new family of cysteine proteases in the ubiquitin pathway.

EMBO Rep, 4, 517-22.

Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, Zhang WN, Pothuizen HH, Feldon J. (2004). Regional dissociations within the hippocampus-memory and anxiety. *Neurosci Biobehav Rev*. 28, 273-283.

Bantel-Schaal U, zur Hausen H. (1984). Characterization of the DNA of a defective human parvovirus isolated from a genital site. *Virology*. 134, 52-63.

Bantel-Schaal U, Delius H, Schmidt R, zur Hausen H. (1999). Human adeno-associated virus type 5 is only distantly related to other known primate helper-dependent parvoviruses. *J Virol*, 73, 939-47.

Barr CL, Feng Y, Wigg K, Bloom S, Roberts W, Malone M, Schachar R, Tannock R, Kennedy JL. (2000). Identification of DNA variants in the SNAP-25 gene and linkage study of these polymorphisms and attention-deficit hyperactivity disorder. *Mol Psychiatry*, 5, 405-409.

Bartsch U, Oriyakhel W, Kenna PF, Linke S, Richard G, Petrowitz B, Humphries P, Farrar GJ, Ader M. (2008). Retinal cells integrate into the outer nuclear layer and differentiate into mature photoreceptors after subretinal transplantation into adult mice. *Exp Eye Res*, 86, 691-700.

Benes FM. (2007). Searching for unique endophenotypes for schizophrenia and bipolar disorder within neural circuits and their molecular regulatory mechanisms. *Schizophr Bull.* 33, 932-936.

Bennett J, Maguire AM, Cideciyan AV, Schnell M, Glover E, Anand V, Aleman TS, Chirmule N, Gupta AR, Huang Y, Gao GP, Nyberg WC, Tazelaar J, Hughes J, Wilson JM, Jacobson SG. (1999). Stable transgene expression in rod photoreceptors after recombinant adeno-associated virus-mediated gene transfer to monkey retina. *Proc Natl Acad Sci U S A.* 96:9920-5.

Bennett J. (2000) Gene therapy for retinitis pigmentosa. *Curr Opin Mol Ther.* 2:420-5. Review.

Berson EL. (1996). Retinitis pigmentosa: unfolding its mystery. *Proc Natl Acad Sci U S A,* 93, 4526-8.

Berns K.I (1996) Parvoviridae: the viruses and their replication. In: B.N. Fields, Editor. *Fundamental Virology.* Lippencot-Raven. Philadelphia, New York: 1017-1042

Bertoglio LJ, Joca SR, Guimaraes FS. (2006). Further evidence that anxiety and memory are regionally dissociated within the hippocampus. *Behav Brain Res.* 175, 183-188

Bessant DA, Ali RR, Bhattacharya SS. (2001). Molecular genetics and prospects for therapy of the inherited retinal dystrophies. *Curr Opin Genet Dev,* 11, 307-16. Review.

Björklund A, Kirik D, Rosenblad C, Georgievska B, Lundberg C, Mandel RJ. (2000). Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. *Brain Res,* 886, 82-98. Review.

Blackshaw S, Snyder SH. (1997). Developmental expression pattern of phototransduction components in mammalian pineal implies a light-sensing function. *J Neurosci*, 17, 8074-82.

Blechacz B, Russell SJ. (2004). Parvovirus vectors: use and optimisation in cancer gene therapy. *Expert Rev Mol Med*, 23, 1-24. Review.

Brinster RL, Chen HY, Warren R, Sarthy A, Palmiter RD. (1982). Regulation of metallothionein--thymidine kinase fusion plasmids injected into mouse eggs. *Nature*, 296, 39-42.

Brinster RL, Chen HY, Trumbauer M, Senear AW, Warren R, Palmiter RD. (1981). Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell*, 1 (pt 2), 223-31.

Brophy K, Hawi Z, Kirley A, Fitzgerald M, Gill M. (2002). Synaptosomal-associated protein 25 (SNAP-25) and attention deficit hyperactivity disorder (ADHD): evidence of linkage and association in the Irish population. *Mol Psychiatry*, 7, 913-917.

Buch PK, Bainbridge JW, Ali RR. (2008). AAV-mediated gene therapy for retinal disorders: from mouse to man. *Gene Ther*, 15, 849-57. Review.

Büning H, Perabo L, Coutelle O, Quadts-Humme S, Hallek M. (2008). Recent developments in adeno-associated virus vector technology. *J Gene Med*, 10, 717-33.

Burger, C, Gorbatyuk, OS, Velardo, MJ, Peden, CS, Williams, P, Zolotukhin, S *et al.* (2004). Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and

5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Mol Ther*, 10, 302–317

Burger C, Nash K, Mandel RJ. (2005). Recombinant adeno-associated viral vectors in the nervous system. *Hum Gene Ther*, 16, 781-91. Review.

Carter BJ. (2005). Adeno-associated virus vectors in clinical trials. *Hum Gene Ther*, 16, 541-50. Review.

Cao L, Jiao X, Zuzga DS, Liu Y, Fong DM, Young D, During MJ. (2004). VEGF links hippocampal activity with neurogenesis, learning and memory. *Nat Genet*, 36, 827-35.

Cearley, CN and Wolfe, JH (2006). Transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9, and Rh10 in the mouse brain. *Mol Ther*, 13, 528–537.

Chakarova CF, Hims MM, Bolz H, Abu-Safieh L, Patel RJ, Papaioannou MG, Inglehearn CF, Keen TJ, Willis C, Moore AT, Rosenberg T, Webster AR, Bird AC, Gal A, Hunt D, Vithana EN, Bhattacharya SS. (2002). Mutations in HPRP3, a third member of pre-mRNA splicing factor genes, implicated in autosomal dominant retinitis pigmentosa. *Hum Mol Genet*, 11, 87-92.

Chang B, Hawes NL, Hurd RE, Davisson MT, Nusinowitz S, Heckenlively JR. (2002). Retinal degeneration mutants in the mouse. *Vision Res*, 42, 517-25. Review.

Chang GQ, Hao Y, Wong F. (1993). Apoptosis: final common pathway of photoreceptor death in rd, rds, and rhodopsin mutant mice. *Neuron*, 11, 595-605.

Chiorini JA, Yang L, Liu Y, Safer B, Kotin RM. (1997). Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. *J Virol.* 71, 6823-33.

Chiorini JA, Kim F, Yang L, Kotin RM. (1999). Cloning and characterization of adeno-associated virus type 5. *J Virol.* 73, 1309-19.

Cho YH, Friedman E, Silva AJ. (1999). Ibotenate lesions of the hippocampus impair spatial learning but not contextual fear conditioning in mice. *Behav Brain Res*, 98, 77-87.

Choi DW. (1985). Glutamate neurotoxicity in cortical cell culture is calcium dependent. *Neurosci Lett.*, 58, 293-7.

Conley S, Nour M, Fliesler SJ, Naash MI. (2007). Late-onset cone photoreceptor degeneration induced by R172W mutation in Rds and partial rescue by gene supplementation. *Invest Ophthalmol Vis Sci*, 48, 5397-407.

Conrad CK, Allen SS, Afione SA, Reynolds TC, Beck SE, Fee-Maki M, Barrazza-Ortiz X, Adams R, Askin FB, Carter BJ, Guggino WB, Flotte TR. (1996). Safety of single-dose administration of an adeno-associated virus (AAV)-CFTR vector in the primate lung. *Gene Ther*, 3, 658-68.

Cooke SF, Bliss TV. (2006). Plasticity in the human central nervous system. *Brain* 129, 1659-1673.

Curcio CA, Allen KA. (1990). Topography of ganglion cells in human retina. *J Comp Neurol*, 300, 5-25.

Daniels DM, Shen WY, Constable IJ, Rakoczy PE. (2003). Quantitative model demonstrating that recombinant adeno-associated virus and green fluorescent protein are non-toxic to the rat retina. *Clin Experiment Ophthalmol*, 31, 439-44.

Davidson, BL, Stein, CS, Heth, JA, Martins, I, Kotin, RM, Derksen, TA *et al.* (2000). Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proc Natl Acad Sci USA*, 97, 3428–3432

Di Pasquale G, Davidson BL, Stein CS, Martins I, Scudiero D, Monks A, Chiorini JA. (2003). Identification of PDGFR as a receptor for AAV-5 transduction. *Nat Med*. 9, 1306-12.

Djamgoz MB, Cunningham JR, Davenport SL, Neal MJ. (1995). Nitric oxide inhibits depolarization-induced release of endogenous dopamine in the rabbit retina. *Neurosci Lett*, 198, 33-6.

Dryja TP, Li T. (2005). Molecular genetics of retinitis pigmentosa. *Hum Mol Genet*. 4, Spec No:1739-43. Review.

Duan D, Yue Y, Engelhardt JF. (2001). Expanding AAV packaging capacity with trans-splicing or overlapping vectors: a quantitative comparison. *Mol Ther*. 4: 383-91.

Dudus L, Anand V, Acland GM, Chen SJ, Wilson JM, Fisher KJ, Maguire AM, Bennett J. (1999). Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV. *Vision Res*. 39, 2545-53.

Eslamboli A, Cummings RM, Ridley RM, Baker HF, Muzyczka N, Burger C, Mandel RJ, Kirik D, Annett LE. (2003). Recombinant adeno-associated viral vector (rAAV) delivery of

GDNF provides protection against 6-OHDA lesion in the common marmoset monkey (*Callithrix jacchus*). *Exp Neurol*, 184, 536-48.

Eslamboli A, Georgievska B, Ridley RM, Baker HF, Muzyczka N, Burger C, Mandel RJ, Annett L, Kirik D. (2005). Continuous low-level glial cell line-derived neurotrophic factor delivery using recombinant adeno-associated viral vectors provides neuroprotection and induces behavioral recovery in a primate model of Parkinson's disease. *J Neurosci*, 25, 769-77.

Favre D, Provost N, Blouin V, Blancho G, Chérel Y, Salvetti A, Moullier P. (2001). Immediate and long-term safety of recombinant adeno-associated virus injection into the nonhuman primate muscle. *Mol Ther*, 4, 559-66.

Fiandaca M, Forsayeth J, Bankiewicz K. (2008). Current status of gene therapy trials for Parkinson's disease. *Exp Neurol.*, 209, 51-7.

Fox GB, O'Connell AW, Murphy KJ, Regan CM. (1995). Memory consolidation induces a transient and time-dependent increase in the frequency of neural cell adhesion molecule polysialylated cells in the adult rat hippocampus. *J Neurochem*. 65, 2796-2799.

Frantz K, Harte M, Ungerstedt U, O'Connor WT. (2002). A dual probe characterization of dialysate amino acid levels in the medial prefrontal cortex and ventral tegmental area of the awake freely moving rat. *J. Neurosci. Methods* 119:109– 119.

Furukawa T, Morrow EM, Li T, Davis FC, Cepko CL. (1999). Retinopathy and attenuated circadian entrainment in *Crx*-deficient mice. *Nat Genet*. 23, 466-70.

Gallagher M, Holland PC. (1992). Preserved configural learning and spatial learning impairment in rats with hippocampal damage. *Hippocampus*, 2, 81-8.

Galli-Resta L, Ensini M. (1996). An intrinsic time limit between genesis and death of individual neurons in the developing retinal ganglion cell layer. *J Neurosci*, 16, 2318-24.

Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. (2002a). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci U S A*. 99, 11854-9.

Gao GP, Lu F, Sanmiguel JC, Tran PT, Abbas Z, Lynd KS, Marsh J, Spinner NB, Wilson JM. (2002b). Rep/Cap gene amplification and high-yield production of AAV in an A549 cell line expressing Rep/Cap. *Mol Ther*. 5, 644-9.

Gao G, Vandenberghe LH, Wilson JM. (2005). New recombinant serotypes of AAV vectors. *Curr Gene Ther*. 5, 285-97. Review.

Garey LJ, Ong WY, Patel TS, Kanani M, Davis A, Mortimer AM, Barnes TRE, Hirsch SR (1998) Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. *J. Neurol. Neurosurg. Psychiatr*. 65, 446-453.

Geyer MA, Krebs-Thomson K, Braff DL, Swerdlow NR (1993) Pharmacological studies of prepulse inhibition models of sensorimotor gating deficits in schizophrenia: a decade in review. *Biol Psychiatry* 34, 361-372.

Geyer MA, Krebs-Thomson K, Braff DL, Swerdlow NR. (2001) Pharmacological studies of prepulse inhibition models of sensorimotor gating deficits in schizophrenia: a decade in review. *Psychopharmacology (Berl)*. 156:117-54.

Glantz LA, Lewis DA (2000) Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Arch. Gen. Psychiat.* 57, 65-73.

Gosso MF, de Geus EJ, van Belzen MJ, Polderman TJ, Heutink P, Boomsma DI, Posthuma D. (2006) The SNAP-25 gene is associated with cognitive ability: evidence from a family-based study in two independent Dutch cohorts. *Mol Psychiatry.* 11, 878-886.

Greber S, Lubec G, Cairns N, Fountoulakis M. (1999). Decreased levels of synaptosomal associated protein 25 in the brain of patients with Down syndrome and Alzheimer's disease. *Electrophoresis* 20, 928-934.

Guy J, Qi X, Muzyczka N, Hauswirth WW. (1999). Reporter expression persists 1 year after adeno-associated virus-mediated gene transfer to the optic nerve. *Arch Ophthalmol.* 117, 929-37.

Ham DI, Kim SJ, Chen J, Vistica BP, Fariss RN, Lee RS, Wawrousek EF, Takase H, Yu CR, Ekwuagu CE, Chan CC, Gery I. (2004). Central immunotolerance in transgenic mice expressing a foreign antigen under control of the rhodopsin promoter. *Invest Ophthalmol Vis Sci,* 45, 857-62.

Hartong DT, Berson EL, Dryja TP. (2006). Retinitis pigmentosa. *Lancet,* 368, 1795-809. R

Hermonat PL, Muzyczka N. (1984). Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells. *Proc Natl Acad Sci U S A,* 81, 6466-70.

Hermonat PL, Quirk JG, Bishop BM, and Han L. (1997). The packaging capacity of adeno-associated virus (AAV) and the potential for wild-type-plus AAV gene therapy vectors. *FEBS Lett*, 407, 78–84.

Hess EJ, Collins KA, Wilson MC. (1996). Mouse model of hyperkinesis implicates SNAP-25 in behavioral regulation. *J Neurosci*, 16, 3104-11.

Hildinger M, Auricchio A, Gao G, Wang L, Chirmule N, Wilson JM (2001) Hybrid vectors based on adeno-associated virus serotypes 2 and 5 for muscle-directed gene transfer. *J Virol* 75:6199–6203

Hou Q, Gao X, Zhang X, Kong L, Wang X, Bian W, Tu Y, Jin M, Zhao G, Li B, Jing N, Yu L. (2004). SNAP-25 in hippocampal CA1 region is involved in memory consolidation. *Eur J Neurosci*. 20, 1593-1603.

Humphries MM, Rancourt D, Farrar GJ, Kenna P, Hazel M, Bush RA, Sieving PA, Sheils DM, McNally N, Creighton P, Erven A, Boros A, Gulya K, Capecchi MR, Humphries P. (1997). Retinopathy induced in mice by targeted disruption of the rhodopsin gene. *Nat Genet*, 15, 216-9.

Humphries P, Kenna P, Farrar GJ. (1992). On the molecular genetics of retinitis pigmentosa. *Science*, 256, 804-8. Review.

Ichsan AM, Kato I, Yoshida T, Takasawa K, Hayasaka S, Hiraga K. (2005). Rhodopsin promoter-EGFP fusion transgene expression in photoreceptor neurons of retina and pineal complex in mice. *Neurosci Lett*. 375, 138-43.

Ikawa M, Tanaka N, Kao WW, Verma IM. (2003). Generation of transgenic mice using lentiviral vectors: a novel preclinical assessment of lentiviral vectors for gene therapy. *Mol Ther.*, 8, 666-73.

Jaenisch R. (1988). Transgenic animals. *Science*, 240, 1468-74.

Jeans AF, Oliver PL, Johnson R, Capogna M, Vikman J, Molnar Z, Babbs A, Partridge CJ, Salehi A, Bengtsson M, Eliasson L, Rorsman P, Davies KE. (2007). A dominant mutation in Snap25 causes impaired vesicle trafficking, sensorimotor gating, and ataxia in the blind-drunk mouse. *Proc Natl Acad Sci U S A*, 104, 2431-6.

Jung M, Zhang Y, Lee S, Dritschilo A. (1995). Correction of radiation sensitivity in ataxia telangiectasia cells by a truncated I kappa B-alpha. *Science*, 268, 1619-21.

Kalloniatis M, Fletcher EL. (2004). Retinitis pigmentosa: understanding the clinical presentation, mechanisms and treatment options. *Clin Exp Optom*, 87, 65-80.

Kennan A, Aherne A, Palfi A, Humphries M, McKee A, Stitt A, Simpson DA, Dentreder K, Orntoft T, Ayuso C, Kenna PF, Farrar GJ, Humphries P. (2002). Identification of an IMPDH1 mutation in autosomal dominant retinitis pigmentosa (RP10) revealed following comparative microarray analysis of transcripts derived from retinas of wild-type and Rho(-/-) mice. *Hum Mol Genet*, 11, 547-57.

Kirik D, Rosenblad C, Björklund A. (1998). Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. *Exp Neurol*, 152, 259-77.

Klugmann M, Symes CW, Leichtlein CB, Klaussner BK, Dunning J, Fong D, Young D, During MJ. (2005). AAV-mediated hippocampal expression of short and long Homer 1 proteins differentially affect cognition and seizure activity in adult rats. *Mol Cell Neurosci*. 28, 347-60.

Kotin RM, Linden RM, Berns KI. (1992). Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. *EMBO J.* 11, 5071-8.

Kolluri N, Sun Z, Sampson AR, Lewis DA (2005) Lamina-specific reductions in dendritic spine density in the prefrontal cortex of subjects with schizophrenia. *Am. J. Psychiat.* 162, 1200-1202.

Kustanovich V, Merriman B, McGough J, McCracken JT, Smalley SL, Nelson SF. (2003) Biased paternal transmission of SNAP-25 risk alleles in attention-deficit hyperactivity disorder. *Mol Psychiatry*, 8, 309-15.

Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, Williams NM, Schwab SG *et al.*, (2003). Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. *Am J Hum Genet*, 73, 34-48.

Liang FQ, Aleman TS, Dejneka NS, Dudus L, Fisher KJ, Maguire AM, Jacobson SG, Bennett J. (2001). Long-term protection of retinal structure but not function using RAAV.CNTF in animal models of retinitis pigmentosa. *Mol Ther.* 4, 461-72.

Linden RM, Ward P, Giraud C, Winocour E, Berns KI. (1996). Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci USA.* 93, 11288-94. Review.

Linton SD. Caspase inhibitors: a pharmaceutical industry perspective. (2005). *Curr Top Med Chem*, 5, 1697-717.

Livesey FJ, Furukawa T, Steffen MA, Church GM, Cepko CL. (2000). Microarray analysis of the transcriptional network controlled by the photoreceptor homeobox gene *Crx*. *Curr Biol*, 10, 301-10.

Lochrie MA, Tatsuno GP, Arbetman AE, Jones K, Pater C, Smith PH, McDonnell JW, Zhou SZ, Kachi S, Kachi M, Campochiaro PA, Pierce GF, Colosi P. (2006). Adeno-associated virus (AAV) capsid genes isolated from rat and mouse liver genomic DNA define two new AAV species distantly related to AAV-5. *Virology*, 353, 68-82.

Lotery AJ, Yang GS, Mullins RF, Russell SR, Schmidt M, Stone EM, Lindbloom JD, Chiorini JA, Kotin RM, Davidson BL. (2003) Adeno-associated virus type 5: transduction efficiency and cell-type specificity in the primate retina. *Hum Gene Ther*. 14:1663-71.

Maguire AM, Simonelli F, Pierce EA, Pugh EN Jr, Mingozzi F, Bennicelli J, Banfi S, Marshall KA, Testa F, Surace EM, Rossi S, Lyubarsky A, Arruda VR, Konkle B, Stone E, Sun J, Jacobs J, Dell'Osso L, Hertle R, Ma JX, Redmond TM, Zhu X, Hauck B, Zeleniaia O, Shindler KS, Maguire MG, Wright JF, Volpe NJ, McDonnell JW, Auricchio A, High KA, Bennett J. (2008). Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med*. 358:2240-8.

Maguire AM, High KA, Auricchio A, Wright JF, Pierce EA, Testa F, Mingozzi F, Bennicelli JL, Ying GS, Rossi S, Fulton A, Marshall KA, Banfi S, Chung DC, Morgan JJ, Hauck B, Zeleniaia O, Zhu X, Raffini L, Coppieters F, De Baere E, Shindler KS, Volpe NJ, Surace EM, Acerra C, Lyubarsky A, Redmond TM, Stone E, Sun J, McDonnell JW, Leroy BP, Simonelli F, Bennett J. (2009). Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. *Lancet*. 374(9701):1597-605

MacLaren RE, Pearson RA, MacNeil A, Douglas RH, Salt TE, Akimoto M, Swaroop A, Sowden JC, Ali RR. Retinal repair by transplantation of photoreceptor precursors. *Nature*, 444, 203-7.

Mastakov MY, Baer K, Symes CW, Leichtlein CB, Kotin RM, During MJ. (2002). Immunological aspects of recombinant adeno-associated virus delivery to the mammalian brain. *J Virol*, 76, 8446-54

Maita H, Kitaura H, Keen TJ, Inglehearn CF, Ariga H, Iguchi-Ariga SM. (2004). PAP-1, the mutated gene underlying the RP9 form of dominant retinitis pigmentosa, is a splicing factor. *Exp Cell Res*, 300, 283-96.

Makarova KS, Aravind L, Koonin EV. (2000). A novel superfamily of predicted cysteine proteases from eukaryotes, viruses and *Chlamydia pneumoniae*. *Trends Biochem Sci*, 25, 50-2

Malenka, R.C. and Bear, M.F. 2004. LTP and LTD: an embarrassment of riches. *Neuron* 44, 5–21.

Martin KR, Klein RL, Quigley HA. (2002). Gene delivery to the eye using adeno-associated viral vectors. *Methods*. 28, 267-75. Review.

Martin SJ, Grimwood PD, Morris RG. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu Rev Neurosci*.23, 649-711.

Mandel RJ, Spratt SK, Snyder RO, Leff SE. (1997). Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. *Proc Natl Acad Sci U S A*, 94, 14083-8.

Mandel RJ, Rendahl KG, Spratt SK, Snyder RO, Cohen LK, Leff SE. (1998). Characterization of intrastriatal recombinant adeno-associated virus-mediated gene transfer of human tyrosine hydroxylase and human GTP-cyclohydrolase I in a rat model of Parkinson's disease. *J Neurosci*, *18*, 4271-84.

Mandel RJ, Rendahl KG, Snyder RO, Leff SE. (1999). Progress in direct striatal delivery of L-dopa via gene therapy for treatment of Parkinson's disease using recombinant adeno-associated viral vectors. *Exp Neurol*, *159*, 47-64. Review.

Matsuda T, Cepko CL. (2004). Electroporation and RNA interference in the rodent retina *in vivo* and *in vitro*. *Proc Natl Acad Sci U S A*, *101*, 16-22.

Matsuda T, Cepko CL. (2007). Controlled expression of transgenes introduced by *in vivo* electroporation. *Proc Natl Acad Sci U S A*, *104*, 1027-32

Matsuda T, Cepko CL. (2008). Analysis of gene function in the retina. *Methods Mol Biol*, *423*, 259-78.

McKie AB, McHale JC, Keen TJ, Tarttelin EE, Goliath R, van Lith-Verhoeven JJ, Greenberg J, Ramesar RS, Hoyng CB, Cremers FP, Mackey DA, Bhattacharya SS, Bird AC, Markham AF, Inglehearn CF. (2001). Mutations in the pre-mRNA splicing factor gene *PRPC8* in autosomal dominant retinitis pigmentosa (RP13). *Hum Mol Genet*, *10*, 1555-62.

McWilliam P, Farrar GJ, Kenna P, Bradley DG, Humphries MM, Sharp EM, McConnell DJ, Lawler M, Sheils D, Ryan C, *et al.* (1989). Autosomal dominant retinitis pigmentosa (ADRP): localization of an ADRP gene to the long arm of chromosome 3. *Genomics*, *5*, 619-22.

Metsaranta M, Garofalo S, Decker G, Rintala M, de Crombrughe B, Vuorio E. (1992). Chondrodysplasia in transgenic mice harboring a 15-amino acid deletion in the triple helical domain of pro alpha 1(II) collagen chain. *J Cell Biol*, 118, 203-12.

Mill J, Curran S, Kent L, Gould A, Hockett L, Richards S, Taylor E, Asherson P. (2002). Association study of a SNAP-25 microsatellite and attention deficit hyperactivity disorder. *Am J Med Genet*, 114, 269-71.

Mill J, Richards S, Knight J, Curran S, Taylor E, Asherson P. (2004). Haplotype analysis of SNAP-25 suggests a role in the aetiology of ADHD. *Mol Psychiatry*, 9, 801-10.

Millington-Ward S, O'Neill B, Tuohy G, Al-Jandal N, Kiang AS, Kenna PF, Palfi A, Hayden P, Mansergh F, Kennan A, Humphries P, Farrar GJ. (1997). Strategems in vitro for gene therapies directed to dominant mutations. *Hum Mol Genet*, 6, 1415-26.

Molday RS. (1998). Photoreceptor membrane proteins, phototransduction, and retinal degenerative diseases. The Friedenwald Lecture. *Invest Ophthalmol Vis Sci*. 39, 2491-513. Review.

Moser E, Moser MB, Andersen P. (1993). Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions. *J Neurosci*, 13, 3916-25.

Moser MB, Moser EI, Forrest E, Andersen P, Morris RG. (1995). Spatial learning with a minilab in the dorsal hippocampus. *Proc Natl Acad Sci U S A*, 92, 9697-701.

Morari M, O'Connor WT, Ungerstedt U, Fuxe K. (1994). Dopamine D1 and D2 receptor antagonism differentially modulates stimulation of striatal neurotransmitter levels by N-methyl-D-aspartic acid. *Eur J Pharmacol*. 256:23-30.

Mori S, Wang L, Takeuchi T, Kanda T. (2004). Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. *Virology*, 330, 375-83.

Morris RG, Garrud P, Rawlins JN, O'Keefe J. (1982). Place navigation impaired in rats with hippocampal lesions. *Nature*. 297:681-3.

Moser M-B, Moser EI, Forrest E, Andersen P, Morris RGM. (1995). Spatial learning with a minislab in the dorsal hippocampus. *Proc Natl Acad Sci USA* 92: 9697–9701.

Mukaetova-Ladinska EB, Hurt J, Honer WG, Harrington CR, Wischik CM. (2002). Loss of synaptic but not cytoskeletal proteins in the cerebellum of chronic schizophrenics. *Neurosci Lett*, 317, 161-5.

Muramatsu S, Mizukami H, Young NS, Brown KE. (1996). Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. *Virology*. 221, 208-17.

Murphy KJ, O'Connell AW, Regan CM (1996) Repetitive and transient increases in hippocampal neural cell adhesion molecule polysialylation state following multi-trial spatial training. *J Neurochem* 67, 1268-1274.

Murphy KJ, Regan CM (1998) Contributions of cell adhesion molecules to altered synaptic weightings during memory consolidation. *Neurobiol Learn Mem* 70, 73-81.

Nagy A, Gertsenstein M, Vintersten K, Behringer R. (2003). Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor, New York, New York: Cold Spring Harbor Laboratory Press.

Ogden TE. (1989). Topography of the retina. In: *Retina Volume I. Basic science and inherited retinal disease*. Ryan SJ, Ed. The CV Mosby Co., St Louis, pp 32-37.

Olsson JE, Gordon JW, Pawlyk BS, Roof D, Hayes A, Molday RS, Mukai S, Cowley GS, Berson EL, Dryja TP. (1992). Transgenic mice with a rhodopsin mutation (Pro23His): a mouse model of autosomal dominant retinitis pigmentosa. *Neuron*, 9, 815-30.

O'Neill B, Millington-Ward S, O'Reilly M, Tuohy G, Kiang AS, Kenna PF, Humphries P, Farrar GJ. (2000). Ribozyme-based therapeutic approaches for autosomal dominant retinitis pigmentosa. *Invest Ophthalmol Vis Sci*. 41, 2863-9.

O'Reilly M, Palfi A, Chadderton N, Millington-Ward S, Ader M, Cronin T, Tuohy T, Auricchio A, Hildinger M, Tivnan A, McNally N, Humphries MM, Kiang AS, Humphries P, Kenna PF, Farrar GJ. (2007) RNA interference-mediated suppression and replacement of human rhodopsin in vivo. *Am J Hum Genet*. 81, 127-35.

Osen-Sand A, Catsicas M, Staple JK, Jones KA, Ayala G, Knowles J, Grenningloh G, Catsicas S. (1993). Inhibition of axonal growth by SNAP-25 antisense oligonucleotides in vitro and in vivo. *Nature*, 364, 445-8.

O'Sullivan NC, McGettigan PA, Sheridan GK, Pickering M, Conboy L, O'Connor JJ, Moynagh PN, Higgins DG, Regan CM, Murphy KJ. (2007) Temporal change in gene expression in the rat dentate gyrus following passive avoidance learning. *J Neurochem*, 101, 1085-98.

Palfi A, Chadderton N, McKee AG, Blanco-Fernandez A, Humphries P, Kenna PF, Farrar GJ. (2012). *Hum Gene Ther*. Apr 30. [Epub ahead of print]

Passini MA, Lee EB, Heuer GG, Wolfe JH. (2002). Distribution of a lysosomal enzyme in the adult brain by axonal transport and by cells of the rostral migratory stream. *J Neurosci.* 22, 6437-46.

Passini, MA, Watson, DJ, Vite, CH, Landsburg, DJ, Feigenbaum, AL and Wolfe, JH (2003). Intraventricular brain injection of adeno-associated virus type 1 (AAV1) in neonatal mice results in complementary patterns of neuronal transduction to AAV2 and total long-term correction of storage lesions in the brains of beta-glucuronidase-deficient mice. *J Virol*, 77, 7034–7040.

Paterna JC, Feldon J, Büeler H. (2004). Transduction profiles of recombinant adeno-associated virus vectors derived from serotypes 2 and 5 in the nigrostriatal system of rats. *J Virol.* 78, 6808-17.

Paxinos G, Watson C. (1998). *The Rat Brain in Stereotaxic Co-Ordinates*, Academic Press, New York.

Peden CS, Burger C, Muzyczka N, Mandel RJ. (2004). Circulating anti-wild-type adeno-associated virus type 2 (AAV2) antibodies inhibit recombinant AAV2 (rAAV2)-mediated, but not rAAV5-mediated, gene transfer in the brain. *J Virol*, 78, 6344-59.

Perry VH, Henderson Z, Linden R. (1983). Postnatal changes in retinal ganglion cell and optic axon populations in the pigmented rat. *J Comp Neurol.* 219, 356-68.

Peters K, Werner S, Liao X, Wert S, Whitsett J, Williams L. (1994). Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. *EMBO J*, 13, 3296-301.

Poulsen DJ, Standing D, Bullshields K, Spencer K, Micevych PE, Babcock AM. (2007). Overexpression of hippocampal Ca²⁺/calmodulin-dependent protein kinase II improves spatial memory. *J Neurosci Res*, 85, 735-9.

Qing K, Mah C, Hansen J, Zhou S, Dwarki V, Srivastava A. (1999) Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nat Med*. 5:71-7.

Rabinowitz JE, Rolling F, Li C, Conrath H, Xiao W, Xiao X, Samulski RJ. (2002). Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol*. 76, 791-801.

Rabinowitz JE, Samulski RJ. (2002). The adeno-associated virus crystal: impact inversely proportional to size. *Mol Ther*. 6, 443-5

Raychaudhuri S, Remmers EF, Lee AT, Hackett R, Guiducci C, Burt NP, Gianniny L, Korman BD, Padyukov L, Kurreeman FA, Chang M, Catanese JJ, Ding B, Wong S, van der Helm-van Mil AH, Neale BM, Coblyn J, Cui J, Tak PP, Wolbink GJ, Crusius JB, Horst-Bruinsma IE, Criswell LA, Amos CI, Seldin MF, Kastner DL, Ardlie KG, Alfredsson L, Costenbader KH, Altshuler D, Huizinga TW, Shadick NA, Weinblatt ME, de Vries N, Worthington J, Seielstad M, Toes RE, Karlson EW, Begovich AB, Klareskog L, Gregersen PK, Daly MJ, Plenge RM. (2008). Common variants at CD40 and other loci confer risk of rheumatoid arthritis. *Nat Genet*. 2008 Sep 14. [Epub ahead of print]

Redmond TM, Yu S, Lee E, Bok D, Hamasaki D, Chen N, Goletz P, Ma JX, Crouch RK, Pfeifer K. (1998). Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nat Genet*, 20, 344-51.

Regan C. M. (2004) Cell adhesion molecules. In Riedel, G., Blatt, B., (eds), *Memories are made of these: from messengers to molecules*. Kluwer Academic/ Plenum Publishers.

Richichi C, Lin EJ, Stefanin D, Colella D, Ravizza T, Grignaschi G, Veglianesi P, Sperk G, During MJ, Vezzani A. (2004). Anticonvulsant and antiepileptogenic effects mediated by adeno-associated virus vector neuropeptide Y expression in the rat hippocampus. *J Neurosci.* 24, 3051-9.

Roberts LA, Morris BJ, O'Shaughnessy CT. (1998). Involvement of two isoforms of SNAP-25 in the expression of long-term potentiation in the rat hippocampus. *Neuroreport*, 9, 33-6.

Rohr UP, Wulf MA, Stahn S, Steidl U, Haas R, Kronenwett R. (2002). Fast and reliable titration of recombinant adeno-associated virus type-2 using quantitative real-time PCR. *J Virol Methods.* 106, 81-8.

Rolling F. (2004). Recombinant AAV-mediated gene transfer to the retina: gene therapy perspectives. *Gene Ther*, 11 Suppl 1, S26-32.

Romano C, Chen Q, Olney JW. (1998). The intact isolated (ex vivo) retina as a model system for the study of excitotoxicity. *Prog Retin Eye Res.* 17,465-83

Ruitenbergh MJ, Blits B, Dijkhuizen PA, te Beek ET, Bakker A, van Heerikhuizen JJ, Pool CW, Hermens WT, Boer GJ, Verhaagen J. (2004). Adeno-associated viral vector-mediated gene transfer of brain-derived neurotrophic factor reverses atrophy of rubrospinal neurons following both acute and chronic spinal cord injury. *Neurobiol Dis*, 15, 394-406.

Rutledge EA, Halbert CL, Russell DW. (1998). Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. *J Virol.* 72, 309-19.

Sanftner LM, Suzuki BM, Doroudchi MM, Feng L, McClelland A, Forsayeth JR, Cunningham J. (2004). Striatal delivery of rAAV-hAADC to rats with preexisting immunity to AAV. *Mol Ther*, 9, 403-9.

Schmidt M, Grot E, Cervenka P, Wainer S, Buck C, Chiorini JA. (2006). Identification and characterization of novel adeno-associated virus isolates in ATCC virus stocks. *J Virol*, 80, 5082-5.

Skapek SX, Lin SC, Jablonski MM, McKeller RN, Tan M, Hu N, Lee EY. (2001). Persistent expression of cyclin D1 disrupts normal photoreceptor differentiation and retina development. *Oncogene*, 20, 6742-51.

Skinner DM, Martin GM, Harley C, Kolb B, Pridgar A, Bechara A, van der Kooy D. (1994). Acquisition of conditional discriminations in hippocampal lesioned and decorticated rats: evidence for learning that is separate from both simple classical conditioning and configural learning. *Behav Neurosci*, 108, 911-26.

Söllner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE. (1993). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell*, 75, 409-18.

Summerford C, Samulski RJ. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol*. 72, 1438-45.

Summerford C, Bartlett JS, Samulski RJ. (1999). AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat Med*. 5, 78-82.

Surace EM, Auricchio A. (2008). Versatility of AAV vectors for retinal gene transfer. *Vision Res*, 48, 353-9.

Taymans, JM, Vandenberghe, LH, Haute, CV, Thiry, I, Deroose, CM, Mortelmans, L *et al.* (2007). Comparative analysis of adeno-associated viral vector serotypes 1, 2, 5, 7, and 8 in mouse brain. *Hum Gene Ther*, 18, 195–206.

Thompson PM, Egbufoama S, Vawter MP. (2003). SNAP-25 reduction in the hippocampus of patients with schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry*, 27, 411-7.

Tratschin JD, Miller IL, Carter BJ. (1984). Genetic analysis of adeno-associated virus: properties of deletion mutants constructed in vitro and evidence for an adeno-associated virus replication function. *J Virol*, 51, 611-9.

Verma IM, Weitzman MD. (2005). Gene therapy: twenty-first century medicine. *Annu Rev Biochem*, 74, 711-38.

Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U, Coco S, Proux-Gillardeaux V, Galli T, Rossetto O, Frassoni C, Matteoli M. (2004). SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. *Neuron*, 41, 599-610.

Vithana EN, Abu-Safieh L, Allen MJ, Carey A, Papaioannou M, Chakarova C, Al-Maghtheh M, Ebenezer ND, Willis C, Moore AT, Bird AC, Hunt DM, Bhattacharya SS. (2001). A human homolog of yeast pre-mRNA splicing gene, PRP31, underlies autosomal dominant retinitis pigmentosa on chromosome 19q13.4 (RP11). *Mol Cell*, 8, 375-81.

Walsh DM, Selkoe DJ. (2004). Deciphering the molecular basis of memory failure in Alzheimer's disease. *Neuron* 44, 181-193.

Walters RW, Yi SM, Keshavjee S, Brown KE, Welsh MJ, Chiorini JA, Zabner J. (2001). Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. *J Biol Chem*. 276:20610-6.

Wang Y, Tang BL. (2006). SNAREs in neurons-beyond synaptic vesicle exocytosis (Review). *Mol Membr Biol*. 23, 377-384.

Washbourne P, Thompson PM, Carta M, Costa ET, Mathews JR, Lopez-Bendito G, Molnar Z, Becher MW, Valenzuela CF, Partridge LD, Wilson MC. (2002). Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. *Nat Neurosci*, 5, 19-26.

Wang XS, Ponnazhagan S, Srivastava A. (1995). Rescue and replication signals of the adeno-associated virus 2 genome. *J Mol Biol*. 250, 573-80.

Warrington KH Jr, Herzog RW. (2006). Treatment of human disease by adeno-associated viral gene transfer. *Hum Genet*, 119, 571-603.

Weber M, Rabinowitz J, Provost N, Conrath H, Folliot S, Briot D, Chérel Y, Chenuaud P, Samulski J, Moullier P, Rolling F. (2003). Recombinant adeno-associated virus serotype 4 mediates unique and exclusive long-term transduction of retinal pigmented epithelium in rat, dog, and nonhuman primate after subretinal delivery. *Mol Ther*. 7, 774-81.

Wechsler D (ed). (1997). WAIS-III Wechsler Adult Intelligence Scale. Psychological Corporation: San Antonio, TX.

Weindler FW, Heilbronn R. (1991). A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. *J Virol.* 65, 2476-83.

Weitzman MD, Kyöstiö SR, Kotin RM, Owens RA. (1994). Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. *Proc Natl Acad Sci U S A.* 91, 5808-12.

Weleber RG, Gregory-Evans K, (2001). Retinitis pigmentosa and allied disorders. In: Ryan SJ Ed. *Retina*. St Louis, MO: Mosby 2001: 362-470

Wing SS. (2003). Deubiquitinating enzymes--the importance of driving in reverse along

Wright JF. (2008). Manufacturing and characterizing AAV-based vectors for use in clinical studies. *Gene Ther*, 15, 840-8.

Yang GS, Schmidt M, Yan Z, Lindbloom JD, Harding TC, Donahue BA, Engelhardt JF, Kotin R, Davidson BL. (2002). Virus-mediated transduction of murine retina with adeno-associated virus: effects of viral capsid and genome size. *J Virol.* 76, 7651-60.

Yang JN, Chen JM, Luo L, Lin SC, Li D, Hu SX. (2005). Tetramethylpyrazine protected photoreceptor cells of rats by modulating nuclear translocation of NF-kappaB. *Acta Pharmacol Sin.*, 26, 887-92.

Xie Q, Bu W, Bhatia S, Hare J, Somasundaram T, Azzi A, Chapman MS. (2002). The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proc Natl Acad Sci U S A*. 99, 10405-10.

Zack DJ, Bennett J, Wang Y, Davenport C, Klaunberg B, Gearhart J, Nathans J. (1991). Unusual topography of bovine rhodopsin promoter-lacZ fusion gene expression in transgenic mouse retinas. *Neuron*, 6, 187-99.

Zhou X, Muzyczka N. (1998). In vitro packaging of adeno-associated virus DNA. *J Virol*. 72, 3241-7.

Znoiko SL, Rohrer B, Lu K, Lohr HR, Crouch RK, Ma JX. (2005). Downregulation of cone-specific gene expression and degeneration of cone photoreceptors in the Rpe65^{-/-} mouse at early ages. *Invest Ophthalmol Vis Sci*, 46, 1473-9.

