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Discovery and characterisation of the novel, pathological GNB3 mutation (D153del/ $G\beta_{3D}$),in the retinopathy globe enlarged (rge) chicken

Hemanth Tummala

In the attitude of silence the soul finds the path in a clearer light, and what is elusive and deceptive resolves itself into crystal clearness. Our life is a long and arduous quest after Truth.

Mahatma Gandhi
Indian political and spiritual leader (1869 - 1948)

UNIVERSITY
of
ABERTAY DUNDEE

School of Contemporary Sciences

Discovery and characterisation of the novel, pathological GNB3 mutation (D153del/ $G\beta_{3D}$), in the retinopathy globe enlarged (rge) chicken.

PhD Thesis

Hemanth Tummala (Student-ID: 0305418)

Supervisor: Dr. Douglas.H. Lester (UAD)

2nd Supervisor: Prof Chris. F. Inglehearn (University of Leeds)

Dundee, 11.09.08

University of Abertay Dundee School of Contemporary Sciences

CERTIFICATE

I certify that PhD student, HEMANTH TUMMALA has undertaken the work described herein and is based on the original work done at University of Abertay Dundee in collaboration with Roslin Institute and Univeristy of Leeds in partial fulfillment for the requirements for the award of the PhD in Medical Biotechnology at University of Abertay, Dundee. This has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or any similar title and represents independent work on the part of the candidate.

Signature: 

Date: 24/10/08

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University of Abertay Dundee **School of Contemporary Sciences**

Author: **Hemanth Tummala**

Title: **Discovery and characterisation of the novel, pathological GNB3 mutation (D153del/ $G\beta_{3D,}$), in the retinopathy globe enlarged (rge) chicken.**

Qualification: Dissertation to obtain a PhD in Medical Biotechnology

Year of Submission: 2008

I certify that the above mentioned report is my own original piece of work. and agree that a copy may be made of the whole or any part of the above mentioned project report without further reference to the undersig[redacted]

Signature: _____

Date: 24/10/08

Address: [redacted]

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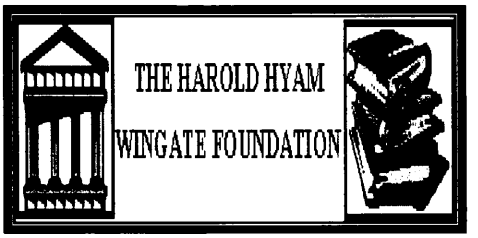
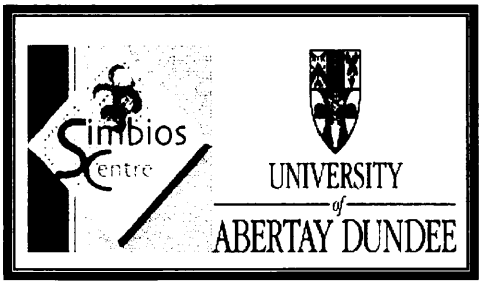




Something that has always puzzled me all my life is why, when I am in special need of help, the good deed is usually done by somebody on whom I have no claim.

William A. Feather (1889-1981)

Abstract

The common human GNB3 825C>T variant, which is present in ~50% of the world's chromosomes, has previously been shown to predispose individuals to hypertension, cardiac and neural disorders. This variant causes the production of a stable and gain of function protein $G\beta_{3S}$. This thesis describes the discovery of a novel D153del mutation that produces an unstable, loss of function, protein $G\beta_{3D}$ in the recessively inherited, retinopathy globe enlarged (rge) chickens. This thesis also demonstrates that the normal $G\beta_3$ downstream phosphorylation signalling pathways are significantly altered in a tissue specific manner in rge chicken organs and in a human GNB3 825TT lymphoblast cell line. In rge tissues expressing $G\beta_{3D}$ protein, the cAMP induced GRK2 phosphorylation activity is significantly altered. Moreover MAPK1 (ERK2) phosphorylation is significantly decreased compared to normal tissues. In contrast human 825TT cell lines expressing the $G\beta_{3S}$ protein, showed enhanced cAMP induced GRK2 and MAPK (ERK1 and ERK2) phosphorylation activity. These results confirm previous findings of 825C>T $G\beta_3$ studies, that $G\beta_{3S}$ is indeed a hyper-activating structural variant, in contrast to the D153del $G\beta_{3D}$ is a classical recessively inherited non-functional mutation.

Keywords: G-protein; Genetic polymorphism; GTPase; Pharmacogenetics; Splice variant; Signal transduction, neurological disorders, hypertension, and Cardiac disease

ABBREVIATIONS, SYMBOLS AND NOTATIONS

AC	-	Adenylyl cyclase
Ag	-	Agonist
APP	-	Amyloid precursor protein
AChE	-	Acetylchotinesterase
BARK/ βARK	-	β-Adrenergic receptor kinase
BSA	-	Bovine serum albumin
cAMP	-	Adenosine cyclic monophosphate
cGMP	-	Guanosine cyclic monophosphate
CNG	-	Cyclic nucleotide-gated
CREB	-	cAMP resposive element binding protein
DAG	-	Diacyglycerol
ENaC	-	Epithelial sodium channel
EPAC	-	Exchange protein activated by cAMP
ERK1/2	-	Extracellular-signal-regulated kinases 1/2
GC	-	Guanylyl cyclase
GDP	-	Guanosine diphosphate
GIRK	-	G protein regulated rectifier inward potassium channels
$G\beta_3$	-	Guanine nucleotide binding protein beta 3 subunit protein
$G\beta_{3D}$	-	Guanine nucleotide binding protein beta 3 subunit protein affected by D153del mutation
$G\beta_{3S}$	-	Guanine nucleotide binding protein beta 3 subunit splice variant protein
GPCR	-	G protein coupled receptor
GRK2	-	G protein coupled receptor kinase 2
GTP	-	Guanosine trisphosphate
©HT	-	Diagram modified by Hemanth Tummala
IOD	-	Intensity optical density

IP_3	-	Inositol(1,4,5)-triphosphate
LCA	-	Leber congenital amaurosis
MAPK	-	Mitogen-activated protein kinase
MW	-	Molecular weight
NeDD4-2	-	Neuronally Expressed Developmentally
Downregulated 4		
NRTK	-	Non-receptor tyrosine kinases
NO	-	Nitric oxide
PDE	-	Phosphodiesterase
PhLP	-	Phosducin-like proteins
PI3K	-	Phosphatidylinositol 3-kinase
PIP_2	-	Phosphatidyl-inositol(4,5)biphosphat
PKA	-	cAMP-dependant protein kinase
PKG	-	Protein kinase G
PLA_2	-	Phospholipase A_2
PLC	-	Phospholipase C
rge	-	Retinopathy globe enlarged
RGS	-	Regulators of G protein signaling
SGk	-	Serum and Glucocorticoid-inducible kinase
VSCC	-	Voltage sensitive calcium channels
V2R	-	Vasopressin type 2 receptor
WD	-	Tryptophan-Aspartic acid
wt	-	Wild type

Chapter I

The outcome of any serious research can only be to make two questions grow where only one grew before....

Thorstein Veblen (1857 - 1929)

Chapter 1

Discovery and characterisation of the novel, pathological GNB3 mutation (D153del/G$\beta_{3D,}$), in the retinopathy globe enlarged (rge) chicken

Significance of the topic

Inherited retinal dystrophies are a common cause of human blindness. To date, 161 unique human retinal diseases have been mapped to specific chromosomal loci and the defective genes for 111 of these have been identified (Hartong et al 2006). However, identification of the genes underlying the recessively-inherited diseases has tended to lag behind, due to a lack of large pedigrees for mapping studies (RetNet; http://www.sph.uth.tmc.edu/retnet/). Furthermore, the development of specific treatments for these conditions based on the genetic information generated has been limited in part by the lack of suitable animal models to test therapies.

In this project, I aimed to characterise the molecular defects in a chicken model of inherited retinal disease known as 'retinopathy globe enlarged' (rge) prior to identifying the analogous human phenotype for this condition, so that the value of these models for targeted therapy can be assessed.

The chicken has already proved a useful model animal for the study of eye development and disease (Judge SJ. 1990, Sander EJ & Wride MA. 1997, Mey J & Thanos S. 2000) and also has potential advantages as a clinical animal model (Semple-Roland et al. 1998). Chicken eyes are large, similar in size to human eyes, which facilitate pathological examination and should simplify the testing of

therapies for retinal diseases. Also, the chicken retina is cone-dominated, in contrast to the other widely used animal model the mouse, which has a primarily rod-dominated retina. Given that loss of cone-function is the most debilitating component of human retinal degeneration, the study of retinal degeneration in chickens could make a valuable contribution to the search for therapies for these diseases.

The rge chicken lines, which arose spontaneously in commercial chicken stocks, were originally reported around twenty years ago and the associated eye defects were shown to be inherited in a recessive manner (Randall & McLachlan 1979 Pollock, et al. 1982, Clayton, et al. 1983, Curtis, et al. 1988, Curtis, et al. 1987). Since then, this chicken lines has been maintained at the Roslin Institute, Edinburgh. The prospect of identifying the disease-causing genes in chickens, with large "family" sizes of 50 or more progeny, has recently been simplified with the publication of the chicken genome and the revelation that its physical size is only a third of the human genome (Hillier LW, et al. 2004).

Retinopathy globe enlarged (rge).

The rge chicken phenotype shares some features with human pathological myopia including the presence of retinal lesions known as "lacquer cracks" that extend from the optic nerve to the periphery (Curtis R, et al. 1988 Montiani-Ferreira F, et al. 2004). Affected birds have a variable degree of vision loss at hatch that deteriorates gradually until all the birds are functionally blind by 30 days. During these developmental stages there is minimal visible retinal atrophy though there is photoreceptor dysfunction, as demonstrated by abnormal electroretinogram (ERG)

responses, suggesting that the primary lesion may affect function rather than structure, (Curtis R, et al. 1988, Montiani-Ferreira F, et al. 2005) Montiani-Ferreira F, et al. 2003). As the disease progresses, there is a generalised thinning of the retinal layers as well as globe enlargement (Fig 1 & 1.1), loss of corneal curvature and cataracts in the older birds (Curtis PE, et al. 1987, Curtis R, et al. 1988, Montiani-Ferreira F, et al. 2003, Inglehearn CF et al. 2003, Montiani-Ferreira F, et al. 2005)

Figure 1: Pathology of rge

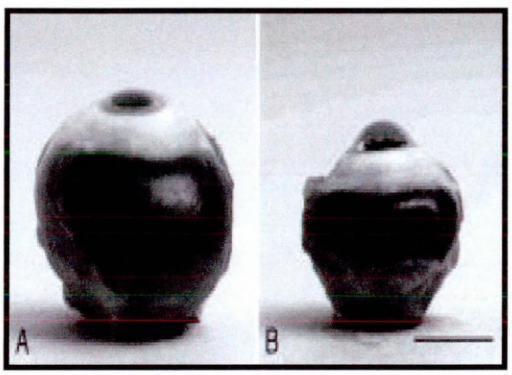


Figure 1.1: Histopathology of rge

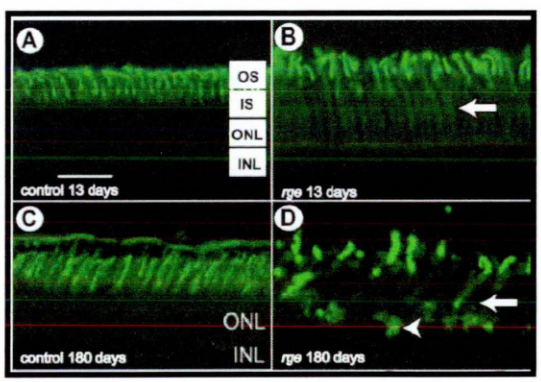


Fig 1: Phenotype retinopathy globe enlarged (rge) in chickens: [A] left. Globes of an rge affected (rge/rge) [LEFT] and [B] left Sighted heterozygote (similar to wild type) Carrier (right; rge/+) [RIGHT] chick at 180 days post-hatch. Scale bar represents 1cm and eyes are shown at the same level of magnification.(Inglehearn C.F etal 2003).

Fig 1.1: Gradual mislocalisation of opsin in rge+/+ retina was observed in the outer nuclear layer (ONL). Picture adapted form (Montiani-Ferrera etal 2005). A and B (top right) represent the 13 days old control normal and rge affected retina. C and D (bottom right) represent 180 days old the control normal retina and rge. Arrows indicate the mislocation in pattern of arrangement of retina in the rge when compared to normal.

Although the eye appears to be the only affected organ in rge homozygote birds, it was observed from the mating of a blind male homozygote with three sighted heterozygotes that of the 138 surviving offspring, 56 were blind and 82 were

sighted (Montiani-Ferreira F, et al. 2005). This significant (p=0.026) deviation from the expected 1:1 ratio for simple recessive inheritance suggested that the rge trait may be embryonic lethal in some birds, yet in birds that survive hatching there is no apparent difference in mortality from normal birds nor any other obvious abnormalities apart from the eyes. A genome-wide linkage screen identified linkage to a 13.7Mb region on chicken chromosome 1 between the polymorphic markers MCW0112 and LEI0101 (Fig 2 Inglehearn, et al. 2003).

Figure 2: Consensus mapping of rge locus

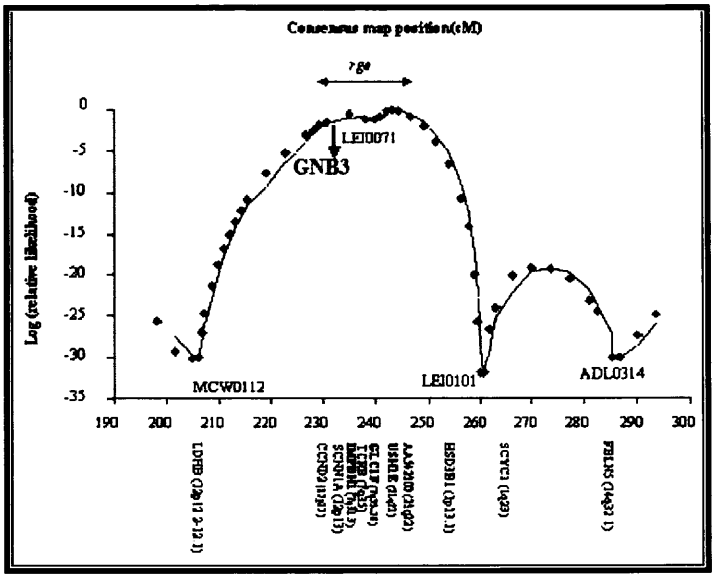
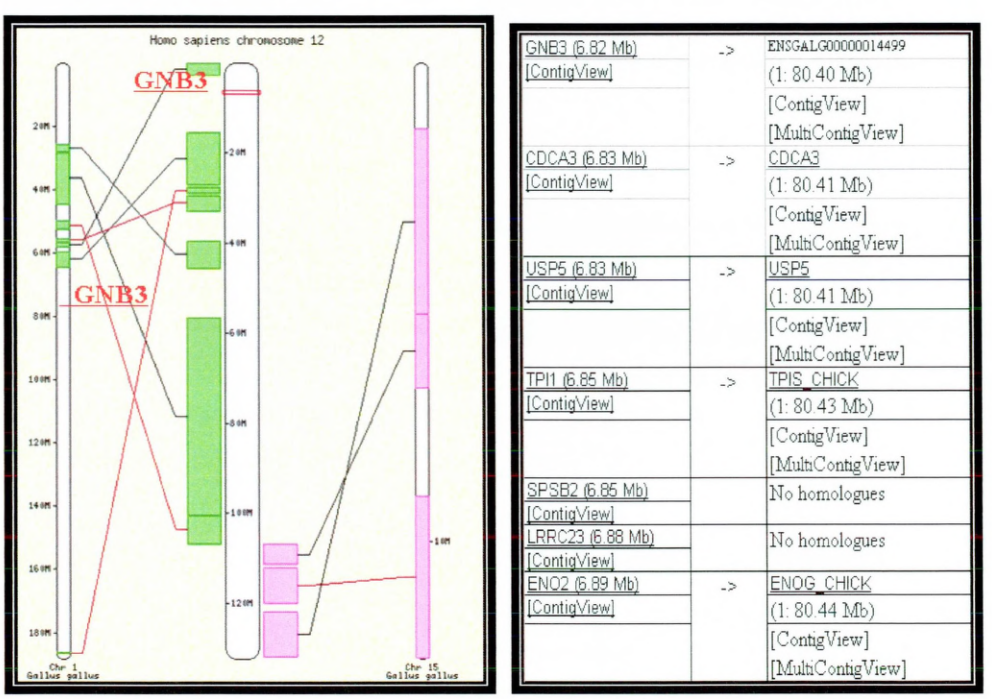


Fig 2: Consensus Map position of rge linkage studies on chicken chromosome 1 highlighted potential candiadate genes such as IMPDH1, GLC1F and USH1E along with GNB3 located in this region. Figure adapted and modified [HT] from Inglehearn et al 2003.

High-resolution genotyping was performed around the critical rge region using previously uncharacterised microsatellite markers to narrow the interval from 13.7Mb to a 3.8Mb region between 68.1Mb and 71.9Mb on chromosome 1. This region spanned the centromere and contained 73 annotated genes. A candidate

gene sequencing approach was pursued and literature search to highlight candidates that had been involved in vision research identified several molecules including a guanine nucleotide-binding protein (Gβ3), located at 71.3Mb that had previously been implicated in the cone (but not rod) visual transduction pathway (Peng YW, et al. 1992).

Figure 3: Synteny view



GNB3 (6.82 Mb) [ContigView]	->	ENSGALG00000014499 (1: 80.40 Mb) [ContigView] [MultiContigView]
CDCA3 (6.83 Mb) [ContigView]	->	CDCA3 (1: 80.41 Mb) [ContigView] [MultiContigView]
USP5 (6.83 Mb) [ContigView]	->	USP5 (1: 80.41 Mb) [ContigView] [MultiContigView]
TPI1 (6.85 Mb) [ContigView]	->	TPIS_CHICK (1: 80.43 Mb) [ContigView] [MultiContigView]
SPSB2 (6.85 Mb) [ContigView]		No homologues
LRRC23 (6.88 Mb) [ContigView]		No homologues
ENO2 (6.89 Mb) [ContigView]	->	ENOG_CHICK (1: 80.44 Mb) [ContigView] [MultiContigView]

Fig 3: The above Figure shows the latest release 50 of the high level of synteny between genes at around 80mb (80.29- 80.44Mb), from the telomere of the short arm of chicken chromosome 1 and the region of 6.65-6.90Mb from the telomere of the short arm of human chromosome 1 (http://www.ensembl.org/Homo_sapiens/syntenyview?otherspecies=Gallus_gallus;chr=12;loc=6893875.5;pre=1).

Following the linkage of the rge gene to this region ,of chicken chromosome 1 (Montiani-Ferreira, et al. 2003), and the subsequent sequencing of the chicken genome (Hillier, et al. 2004), An earlier release of this human/ chicken synteny

view was used by my supervisor, Dr Lester to help identify candidate genes for the rge trait (Fig 3). This was necessary as the chicken genes in this region had only been assigned ensembl numbers and not the actual gene name. For example the chicken GNB3 gene, was at that time only assigned the ambiguous code ENSGALG00000014499. By using this synteny view the names of the chicken genes in the linkage region were identified by looking at the syntenic human genes e.g. ENSGALG00000014499 = GNB3.

GNB3 was therefore an obvious candidate gene for the rge phenotype. Although in the retina GNB3 is cone specific (Peng, et al. 1992), it had previously been shown to be expressed in a wide variety of other tissues (Sun, et al. 2005). Any mutations in GNB3 may therefore alter signalling pathways in other non-retinal tissues, with likely pleitropic effects. In order to understand these possible alterations in signal transduction in both retinal and non-retinal tissues, one first has to understand the complex process of G protein signalling.

G proteins: History

The evolutionarily highly conserved group of molecules, known as heterotrimeric guanine nucleotide-binding proteins (G or GN proteins), are key determinants for the specificity and temporal control of many signalling processes in vertebrates (Neves, et al. 2002). Many hormones, neurotransmitters, chemokines, local mediators, and sensory stimuli exert their effects on cells by binding to one of the 865 different seven membrane receptors, which are coupled to such heterotrimeric G proteins (Lodish, et al. 2007). These highly specialised transducers can modulate

the activity of multiple signalling pathways leading to diverse biological responses. In vivo, specific combinations of many different Gα and Gβγ subunits are required for connecting individual receptors to signalling pathways (Wettschureck and Offermanns. 2005). 16 different vertebrate genes have been identified that encode for Gα subunits, five genes encode for Gβ (GNB1-5) and 12 genes encode for Gγ subunits (Gautam, et al. 1998).

G proteins: Structure and Function

Heterotrimeric G proteins consist of three different subunits which are associated in a basal state to a trimeric complex, containing α- (Gα), β- (Gβ) and γ-subunit (Gγ) (Fig 4). Furthermore, the Gα subunit is covalently bound to a guanosine diphosphate (GDP) molecule (Lodish, et al. 2007). This complex represents the G protein in its inactive state and can interact with a suitable G protein coupled receptor (GPCR) (Wettschureck and Offermanns, 2005). Under this temporary condition, G proteins are linked to the inner face of the plasma membrane by covalently bound lipids, interacting with the Gα and Gγ subunits (Lodish, et al. 2007). The basic structure of the Gβ subunit is composed of two distinct regions as follows: an amino-terminal a-helical segment, followed by 7 repeating units called WD repeats (Fig 4) (Gracia-Higuera, et al. 1998).Each WD configuration encloses 4 antiparallel β strands (Gautam, et al. 1998). The seven WD repeats are arranged in a ring form to a propeller structure with seven blades (Gracia-Higuera, et al. 1998).

Figure 4: Structure Heterotrimeric G protein complex

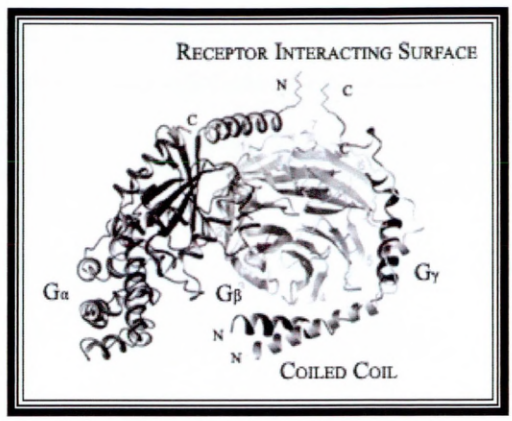


Fig 4: Three dimensional Structure of the αβγ-complex of heterotrimeric G proteins. (Gautam et al, 1998.)

Figure 5: Structure of Individual subunits of G protein heterotrimeric Complex

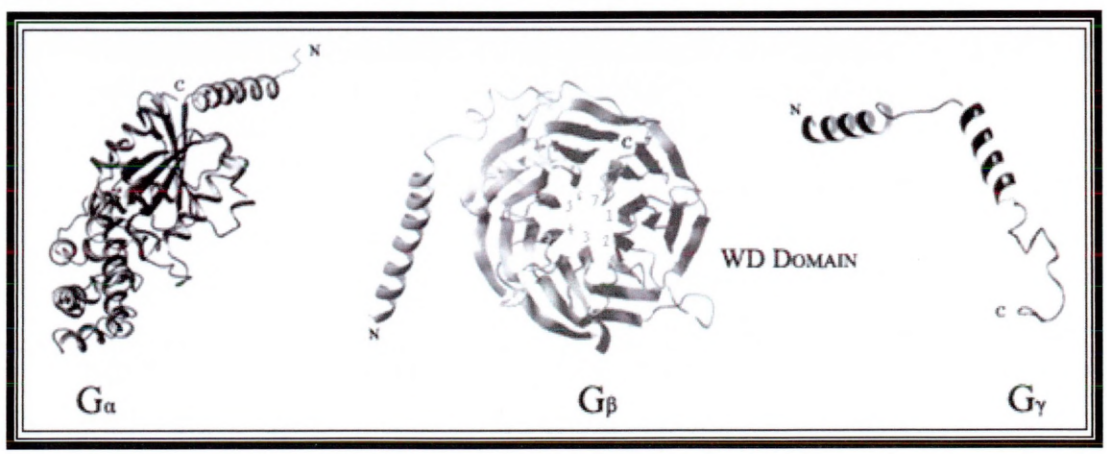


Fig 5: Three dimensional structures of heterotrimeric G protein subunits: [LEFT] Gα subunit, [MID] Gβ subunit, [RIGHT] Gγ subunit. Figure obtained from using by modification of 1B9X PDB id in deep view 3D modeller version 3.7

The Gγ subunit contains two helices and is bound to the G_{β}subunit by forming partial coiled structures between helices of each subunit at the N terminus (Fig. 5) (Gautam, et al, 1998). Today the exact coupling mechanism between G protein and its GPCR, i.e. the way in which ligand-induced conformational change in the

receptor molecule results in G protein activation, is still unclear (Wettschureck and Offermanns, 2005). However, already proven is the need of all three tightly coupled subunits as a requirement for receptor activation of a G protein (Gautam, et al, 1998). It has also been discovered that the Gβγ heterodimer serves to couple Gα to the receptor and also to inhibit its spontaneous release of GDP (i.e., acting as a guanine nucleotide dissociation inhibitor or "GDI" for Gα) and therefore remains the G protein in its inactive state (Siderovski and Willard, 2005).

The basic principle of G protein signalling is shown in Fig 6. In general the first step of G protein activation is the binding of a normal hormonal ligand (e.g. epinephrine) or an agonist (e.g. isoproterenol) to the GPCR (Lodish et al, 2007). As a consequence, a conformational change in the cytosol-facing loops of the GPCR occurs, that allows interactions between the receptor and the Gα subunit and terminates the binding of Gα (Lodish et al, 2007). The interaction promotes the release of GDP, which gets replaced by GTP. As a result of GTP binding, the Gα subunit changes its conformation. The additional binding of the GTP molecule acquired phosphate (termed terminal γ phosphate) interacts with backbone amide groups of a conserved threonine and glycine residue of two protein segments, called switch I and II (Lodish et al, 2007). Consequently, a conformational change of these segments occur, leading to dissociation of the Gα subunit form, the activated receptor as well as the βγ complex, both the Gα subunit and the βγ complex are now free to modulate the activity of a variety of effector target proteins, like ion channels or enzymes (Lodish et al, 2007 and Wettschureck and

Offermanns, 2005). To generalize, the triggered GPCR operates as a guanine-nucleotide-exchange factor (GEF) (Lodish et al, 2007).

Figure 6: Basic principle of G protein signaling

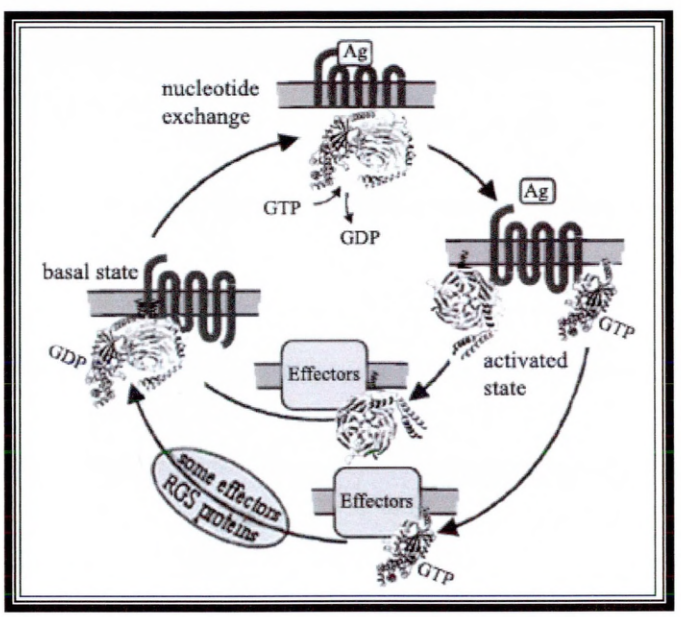


Fig 6: G proteins are bound to a 7-transmembrane domain receptor and in an inactive state. The binding of an agonist (Ag) to the receptor promotes the release of GDP from the α-subunit of the heterotrimeric G protein resulting in the formation of GTP-bound Gα. GTP-Gα and Gβγ dissociate and are able to modulate effector functions. Through an intrinsic GTPase activity, GTP is hydrolysed to GDP and inactivates the Gα subunit. The process can be accelerated by various effectors as well as by regulators of G protein signalling (RGS) proteins. GDP-bound Gα then reassociates with Gβγ. (Wettschurek and Offermanns, 2005 and Gautam et al., 1998, modified)

The progress of signal transduction via G proteins is terminated by an intrinsic GTPase activity that hydrolyses GTP to GDP. Hence, the γ-phosphorus atom of the γ-phosphate from GTP gets associated with oxygen of a water molecule (Farfel et al., 1999). This reaction can only be catalyzed, if the involved atoms are arranged precisely to neighbouring amino acids of Gα (Farfel et al., 1999). Most alterations

in these amino acids have been shown to cause a decreased ability to stabilize the arrangements of these critical atoms. This results in the slowing of GTP hydrolysis and termination of the signal (Farfel et al., 1999). As a result, these affected G protein show persistent and defective signalling that usually leads to a disease (Farfel et al., 1999).

After hydrolysis and conformational change, Gα reassociates with the βγ-complex and is able to act again with a ligand-activated GPCR. Thus, the kinetics of the hydrolysis determines the time of the activated state of the G protein. This is also influenced by effector proteins, regulators of G protein signalling (RGS) and the recently discovered so called GoLoco motif proteins, which help Gα dissociation and reassociation to Gβγ (Siderovski and Willard, 2005). The binding of the Gα subunit to its target proteins leads to activation of the intrinsic GTPase activity. Thus, the target protein acts as a GTPase-activating protein (GAP) (Lodish et al., 2007). The RGS protein superfamily modulate the duration of the activated state of G proteins by stimulating the GTPase activity of G protein Gα subunits (Fig 6) (Gold et al., 1997). All RGS possesses the characteristic RGS domain which binds to the Gα-subunits (Gold et al., 1997) and stabilize particular arginine and glumatine fingers in the active site of GTPase (Farfel et al., 1999). RGS are supposed to play an emphasized role in neuronal signal transduction and its regulation (Gold et al., 1997).

Gβγ subunit Regulation and function

The heterodimeric βγ-complex modulate an array of important effector proteins, including enzymes like phospholipase A_2 (PLA_2) which is involved in

inflammatory response, β-adrenergic receptor kinase (βARK, also called GRK2), diverse isoforms of (adenylate cyclase) AC and PLC, and ion membrane carrier like potassium, sodium and calcium channels (Ford et al., 1998, Gautam et al., 1998 and Morishita et al., 1994). The main downstream pathways are summarized in Fig 7. Furthermore, free βγ complexes stimulate central and significant signalling pathway of the cell which either stand in crosstalk with signalling pathways of the Gα subunit or appears to be overlapping with them.

Figure 7: cAMP and cGMP main messengers in G protein signalling pathway

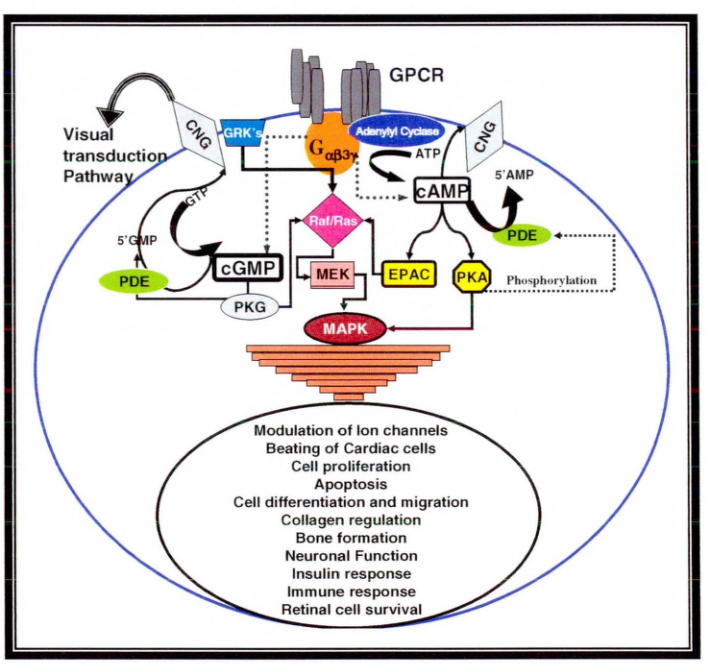


Fig. 7 – Main Downstream effector proteins and pathways of the Gαβ3γ-complex: pathways are represented independently of individual effectors regulating through cAMP and cGMP. Dotted lines represent secondary messengers activated to regulate downstream effectors.

Abbreviations: GRK's - G protein-coupled receptor kinases , cAMP - cyclic adenosine mono phosphate, cGMP–cyclic guanine mono phosphate, PKA protein kinase A, PKG protein kinase G, PDE phophodiesterases, CNG cyclic nucleotide gated ion channnel MAPK - mitogen-activated protein kinase, MEK - MAPK kinase, EPAC exchange protein activating cAMP.

Modulation of effector proteins can occur either in two ways, directly by covalent binding events or indirectly through mediator proteins. Functional selectivity of βγ complexes are characterised by a huge diversity and numerous combination variants of the diverse Gβ- and Gγ-Subunits, (Jones et al., 2004). But this diversity among various forms of βγ complexes seems to be the attribute of the 12 different Gγ rather than the five different Gβ -subunit (Morishita et al., 1994).

Interestingly, downstream targets of Gα subunit and βγ complex overlap in particular cases. For example the isoforms I, II and IV of AC are target proteins of diverse compositions of βγ complexes and of different Gα subunits. These interactions can trigger either negative (e.g. AC I) or positive regulation (e.g. II, IV) (Kroetze et al., 2003). Furthermore, isoforms 1 to 3 of PLCβ have also shown to be activated by particular βγ heterodimer compositions (Kroetze et al., 2003, Morris, 1997). It has previously been reported that activation PLC occurs by direct binding of the PLC catalytic domain to both the N terminus and propeller regions of Gβγ (Bonacci et al., 2005). As it has already proven that the βγ-complex shows equal importance to the Gα subunit in maintaining the equilibrium of cell physiology, it is not surprising that the significant MAPK-pathways are also stimulated by Gβγ (Bonacci et al., 2005).

Furthermore, it has been discovered that Gβγ directly acts with the Raf-1, a serine/ threonine protein kinase and Ras that also represents initiator of MAPK-cascades (Ito et al., 1995 and Pumiglia et al., 1995) and in case of over expression influence of the phosphorylated state of the MAPK itself (Ito et al., 1995). Additonal proteins

that directly act with Gβγ are calmodulin, dynamin, phosducin, phosducin-like proteins (PhLP) and the phosphatidylinositol 3-kinase (PI3K) which is important in lipid signalling by providing "docking stations" for target proteins at the cytoplasmic membrane (Gautam et al., 1998 and Kroetze et al., 2003). Binding of Gβγ to dynamin, which is suggested to play a role in synaptic-vesicle recycling, leads to its inactivation (Gautam et al., 1998). In general, it is suggested that diverse Gβγ complexes play significant roles in synaptic signal processing (Bertram et al., 2002).

A further target protein of G proteins with significant importance is beta adrenergic receptor kinase (βARK). βARK specifically phosphorylates agonist-occupied forms of the β-adrenergic receptor (βAR), as well as the light-bleached form of rhodopsin (Benovic et al., 1987) and is efficiently activated depending on the compounds of the heterodimer (Müller et al., 1993). Binding of Gβγ in addition PIP_2 targets βARK to its membrane bound receptor which phosphorylates a certain GPCR at particular amino acid residues (Gautam et al., 1998 and Pitcher et al., 1995). Phosphorylation of βAR results in desensitization and in particular leads to internalization and degradation through β-arrestin binding to this particular kind of GPCR and therefore to termination of G protein signalling (Vaughan, et al., 2006). Several lines of evidence have shown that the Gβγ complex directly covalent binds to βARK which leads to activation and subsequently desensitization of musacrinic cholingeric and β-adrenergic receptors (Koch et al., 1993 and Kozasa, 2004).

Mutations in Gβ subunits

Several inherited disorders in vertebrates have been identified in different Gα subunits e.g. McCune–Albright syndrome, pseudo-hypoparathyroidism type Ia/b, congenital cone dysfunction and night blindness (Reviewed by Weinstein et al. 2006). In contrast, before this thesis's findings (and Tummala et al. 2006) only three different vertebrate phenotypes have so far been reported for mutations in the GNB proteins, these are outlined in the following paragraphs.

Human 825C>T mutation

The first mutation found was the common GNB3 825C>T mutation, which was first reported by Siffert et al. in 1998. This synonymous mutation/variation changes a C nucleotide to a T nucleotide at position 825 in exon 10 of GNB3 (825C>T). The 825C>T mutation paradoxically causes GNB3 exon 9 RNA to misplice approximately half the time, deleting 123 nucleotides, causing 41 amino acids to be deleted in the $G\beta_3$ protein (Fig 8) and producing the structural variant protein $G\beta_{3S}$. (Fig 9). $G\beta_{3S}$ has a structure of circular propeller but with only 6 propeller blades, rather than 7 propeller blades. One propeller blade is absent because of the deletion of 41 amino acids (Siffert, 2000). $G\beta_{3S}$ is surprisingly structurally stable and has been postulated to enhance many GPCR signalling pathways (e.g.Rosskopf et al., 2003), that the $G\beta_3$ protein is involved in. Moreover the 825C>T mutation/variation is extremely common in Black Africans (e.g. 84% Nigerians studied in the HapMap project have the 825 T/T homozygous genotype while 66% of Europeans studied are either 825C/T heterozygous or 825T/T homozygous (http://www.hapmap.org/cgi-perl/snp_details?name=rs2301339) (Fig 8).

Figure 8: 825C>T change misplices at Cryptic splice site in GNB3

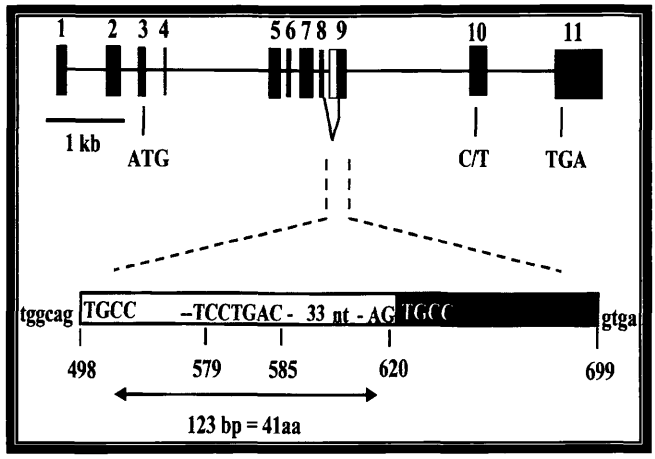


Fig 8: Gene coding for GNB3 consists of 11 exons with start codon ATG in exon 3 and stop codon TGA in exon 11. C to T change at 825th position in exon 10 misplices the gene at cryptic splice site at exon 9 there by deleting 123 nucleotides coding to 41 aminoacids (Siffert review 2000).

Figure 9: $G\beta_{3S}$ structure

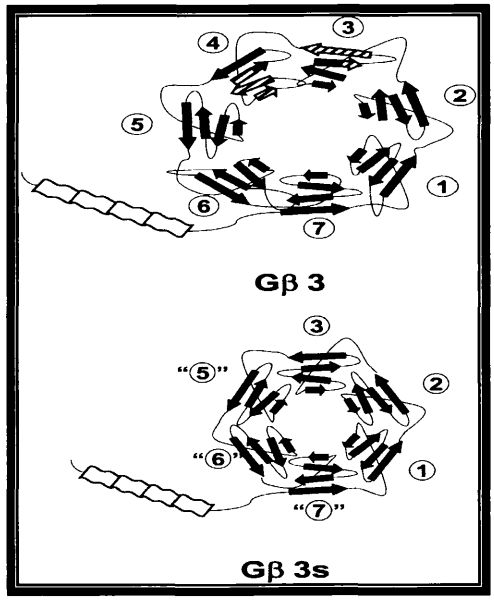


Fig 9: $G\beta_3$ secondary structure consists of 7 propeller structures each on designated as WD domain representing propeller. Due the deletion of 41 amino acids caused due to C to T change results in deletion of one of the 7 WD domain propellers generating $G\beta_{3S}$ structure (Siffert review 2000).

In both these population groups the 825T allele has been shown to be strongly associated with Hypertension (e.g. Bagos et al. 2007) and possibly diabetes (e.g. Fernandez-Real et al, 2003). The GNB3 825C>T variant is found in at least 30% of human chromosomes world wide. The 825T mutation accounts for a major portion of worldwide variation in blood pressure and is a major predisposing factor for hypertension, low birth weight, obesity, type II diabetes and atherosclerosis in the developing world. It has been hypothesised that the GNB3 825T allele probably has a selective advantage to people living in equatorial regions, but probably puts people at a selective disadvantage to people living in the cold higher latitudes, where both salt and calorific intake are generally higher (Young et al 2005).

Figure 10: World wide susceptibility in 825 C>T allele frequency

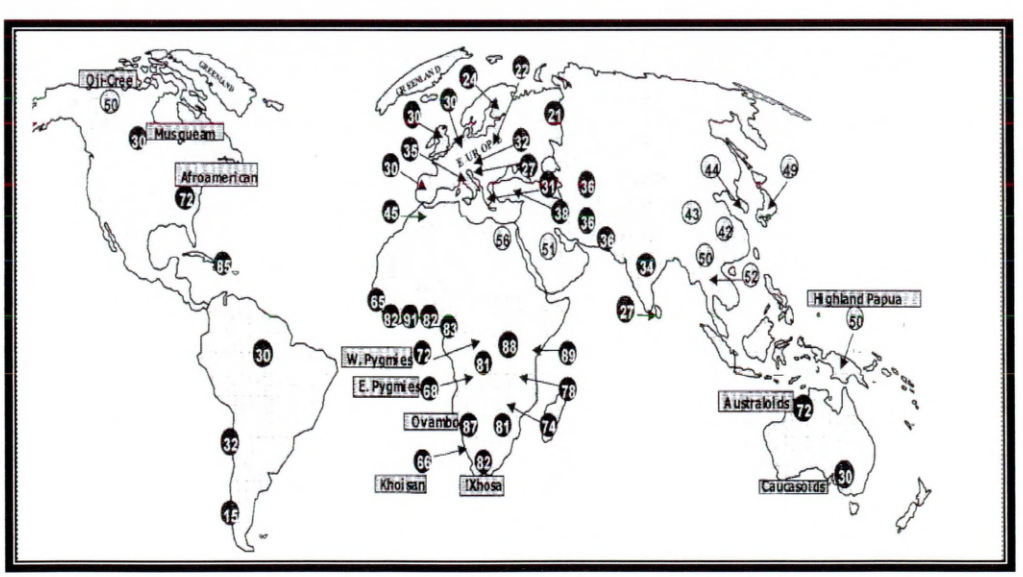


Fig 10: Figure showing the world wide 825C>T allele frequency (%) distribution and disease association such as diabetes hypertension and obesity. Figure obtained from Young etal 2005

It should be noted that to date no association has been made between diabetic (or any other retinopathy) and the $G\beta_3$ 825T mutation (Shcherbak and Schwartz 2001,

Gao Y et al 1998). This is probably due to the fact that only 40% of the $G\beta_3$ genes are mis-spliced in the cells of 825T/825T individuals and presumably the expression of 60% normal protein is sufficient for continuous, normal retinal function (Sun et al 2005).

$G\beta_5$ knockout mice

The second Gβ vertebrate mutation that has been reported with a phenotype is the $G\beta_5$ knockout mouse (Chen et al. 2003), which results in rod dysfunction and a "runty" low birth weight phenotype. Moreover 2/3 of $G\beta_5$ knockout mice die before 21 days of infancy, suggesting that these mice may be an animal model for some human predisposition to infant death and/or rod dysfunction (e.g. congenital stationary night blindness).

$G\beta_1$ Disruption in Mice

A large chromosomal inversion disrupting the $G\beta_1$ gene in mouse was recently identified as the third different phenotype. This mutation is homozygous lethal and only viable heterozygotes suffer from inherited retinal degeneration (Kitamura et al 2006).

Summary

Following linkage analysis carried out by the Roslin Institute GNB3 (which expresses the $G\beta_3$ protein), was identified by Dr Douglas H Lester as the prime candidate gene for the rge phenotype, using synteny analysis. The $G\beta_3$ protein is one of five transducin β (β) subunits, which interact with 18 alpha (α) and 12 gamma (γ) subunits that have so far been identified in humans (Downes & Gautam 1999). The diverse combinations of α, β and γ, subunits, form a diverse array of heterotrimers (and dimers) of guanine nucleotide-binding proteins (G-proteins). These diverse combinations of subunits couple the different signal transduction processes from the stimulated transmembrane receptors to the downstream effectors (Hamm 1998). Mutations and structural variants of the Gβ3 protein are therefore likely to have pleitropic effects.

For example a GNB3 polymorphism, 825C>T, that causes aberrant splicing in some transcripts and subsequent in-frame loss of 41 amino acids, is associated with essential hypertension, obesity, coronary heart disease, stroke and diabetic nephropathy (Siffert et al. 1998, Siffert 2005). Furthermore, a knockout mouse model of the related protein Gβ5 has retinal abnormalities and high pre-weaning infant mortality (Chen et al. 2003, Krispel et al. 2003), while RNAi knock-down of the $G\beta_1$ orthologue in C.elegans causes 50-80% embryonic lethality and an uncoordinated phenotype in the surviving worms (Simmer et al. 2003). These observations support the view that GNB protein mutations may have multiple deleterious effects.

It is noteworthy that human $G\beta_3$ had previously been screened in 164 patients with various retinal diseases including cone, cone-rod and macular dystrophies, and no disease-causing lesions were identified (Gao et al. 1998). One possible reason for this may be that the chicken phenotype is more consistent with a severe LCA type dystrophy. In addition, since the rge trait appears to be associated with embryonic mortality in chickens (Inglehearn et al. 2003), the human counterpart may also involve a predisposition to embryonic death leading to miscarriage, making this a rare disease or syndrome in surviving patients. On this point the GNB3 825T allele has previously been shown to be associated with a decrease in birth weight (Hocher et al. 2000).

Aims and Objectives

The general aim of my thesis was to identify and characterise the pathogenic mutation for the cause of the retinopathy globe enlarged (rge) phenotype disease in chickens, in the hope of making a discovery with a preferable clinical implication.

The first goal was to identify the underlying pathological mutation in the candidate GNB3 gene. Use of the traditional method of exon screening by classical sequencing of GNB3 gene was opted for. Any identified mutation would subsequently be confirmed due to its segregation with the rge phenotype in a large pedigree.

Secondly I set out to investigate the structural affects caused by any identified mutation in the $G\beta_3$ protein by using insilico secondary structure modelling tools such as deep view and What if secondary structure modelling programs.

Thirdly I chose to perform biochemical screening of the mutant $G\beta_3$ and its downstream effectors and there by attempt to demonstrate the effects of the rge mutation in the $G\beta_3$ protein.

Lastly I aimed to compare the signalling pathways affected by the rge $G\beta_3$ mutation, in different tissues, with the same pathways affected by the human $G\beta_{3S}$ variant. It was hoped that these experiments would help to prove whether the $G\beta_{3S}$ is indeed a gain of function variant and to shed light on why humans expressing this protein are predisposed to hypertension, Neurological and cardiac disorders.

Chapter II

The more original a discovery, the more obvious it seems afterwards.....

Arthur Koestler (1905 - 1983)

Chapter 2

DISCOVERY OF NOVEL D153del MUTATION IN GNB3 GENE OF rge DISEASE PHENOTYPE IN CHICKENS

2.1 Introduction

2.1.1 Discovery of D153del in $G\beta_3$ gene

Sequencing of GNB3 gene in wild-type and rge birds identified an in-frame 3bp deletion of codon. 153 which segregated with the rge phenotype in all birds tested (Fig 11). Deletion of this codon 153 which is an aspartic acid in chicken GNB3 causes the rge trait. According to the mutation nomenclature criteria by den Dunnen and Antonarakis (2001), the mutation is designated as D153del

Figure 11: Discovery of mutation D153del

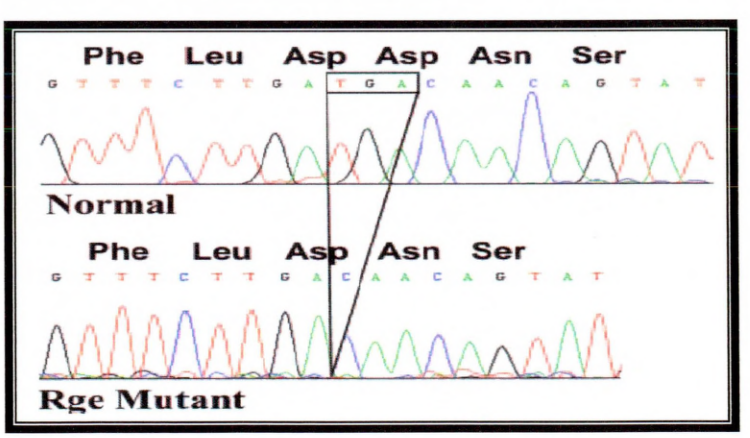


Fig 11: GNB3 mutation analysis in wild-type and rge chickens. The lower sequence chromatogram illustrates the homozygous D153del GNB3 mutation present in rge chickens. The upper image is the corresponding normal sequence

2.2 Results

2.2.1 D153del Mutation Analysis in rge chicken flocks by pcr-ARMS Amplification Refractory Mutation System test and heteroduplex analysis system.

Figure 12: PCR-ARMS analysis of the Gβ$_3$ mutation D153del in two rge flocks

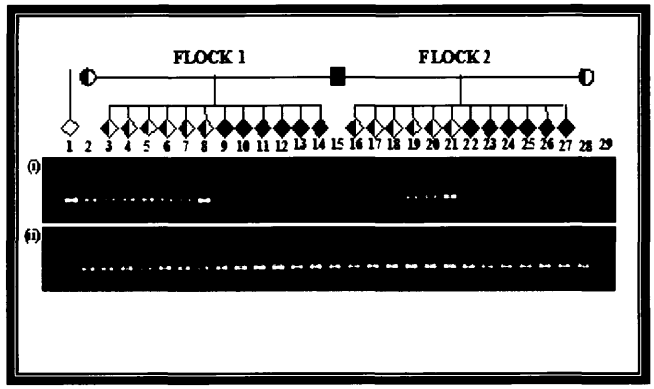


Fig 12: Lane 1 shows the banding pattern in a White Leghorn (wild-type) bird. Lanes 2 and 28 represent the asymptomatic heterozygous female parents that give rise to flocks 1 and 2. Lane 15 shows the banding pattern of the homozygous D153del male parent of the flocks. Lanes 3-8 and 16-21 represent the banding patterns for the asymptomatic offspring from flocks 1 and 2 respectively, and lanes 9-14 and 22-27 represent the pattern for the rge affected birds. Lane 29 is a no DNA control. This work was done by Dr. Manir Ali at Leeds Univeristy,

Pattern of inheritance for the D153del GNB3 mutation in flocks 1 and 2, appears that the D153del allele segregates with the rge phenotype in an autosomal recessive manner so that only D153del homozygotes have the disease phenotype (Fig 12 & 13).This mutation deletes one of two highly conserved aspartic acid residues in the Gβ$_3$ protein causing structural and functional instability.

Figure 13: Hetero Duplex Analysis for GNB3 Mutation D153del in rge affected flocks

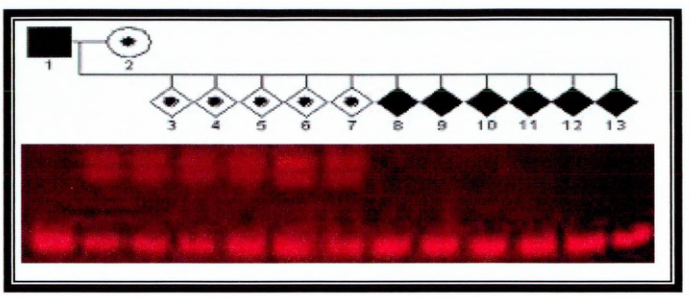


Fig 13: PCR heteroduplex genotyping of an rge family, using primers specific for GNB3 exon 6, which includes codon 153. Only unaffected heterozygote chicks show two extra slower moving bands characteristic of possessing both a normal and a GNB3 gene containing a 3bp deletion in exon 6. All homozygote affected chicks lack any heteroduplex bands, indicates that the D153del GNB3 mutation segregates with the RGE trait in this pedigree.

2.2.2 Deletion of Evolutionary Conserved Aspartic acid 'D' affects the stable $G\beta_3$ protein secondary structure

$G\beta_3$ belongs to a family called WD repeat proteins. WD repeats are highly conserved domains of 40-60 aminoacids that start with Glycine-Histidine (GH) dipeptide at 11-24 residues from N-terminus and end with a Tryptophan-Aspartic acid (WD) dipeptide at the C-terminus. Between GH and WD dipeptides, approximately 40 amino acids are conserved. The solved crystal structures of the β subunits of heterotrimeric G proteins, including bovine $G\beta_1$, have been shown to be made up of an amino-terminal α-helical segment followed by seven repeating units called WD (Trp-Asp) repeats (Smith et al. 1999).

Figure 14: D153del causes structural defects in $G\beta_3$

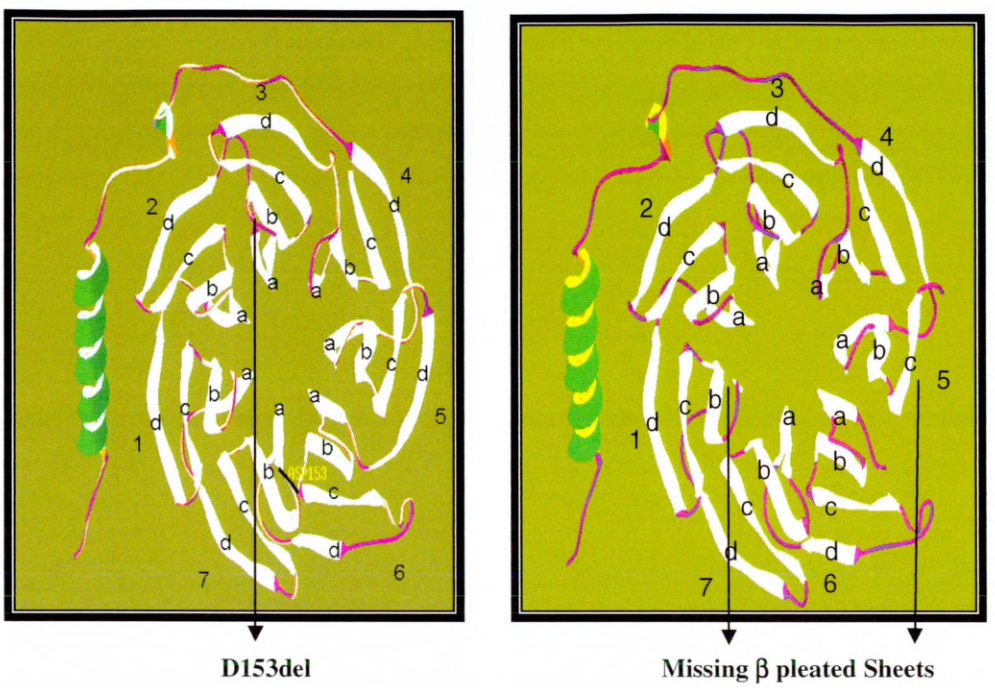


Fig 14: Normal structure of Bos taurus (bt) $G\beta_1$ (PDB ID: 1B9XA) showing the standard seven propeller structure (Sondek et al 1996), where each propeller domain contains a WD repeat motif and consists of 4 anti-parallel beta pleated sheets. A "What If" prediction of a Bos taurus D153del $G\beta_3$ mutation deletes two beta pleated sheets in propellers 1 and 5 of $G\beta_3$ proteins

As can be seen from (Fig 14) the seven WD repeats in these proteins form seven antiparallel β sheets which make up the blades of a toroidal propeller structure. The mutated aspartic acid residue D153del lies within the variable region II which is responsible for the turn between beta sheet **a** and **b** in the third propeller of GNB3. Although this region in WD proteins is in a so called variable region the sequence DDN (where the second D is D153) is very highly conserved in all Gβ proteins as evolutionarily conserved amino acid in almost all vertebrate species analysed in CLUSTALW alignment program (Fig 15).

Figure 15: Alignment of the third WD domain of Gβ proteins in various eukaryotic species using the programme ClustalW

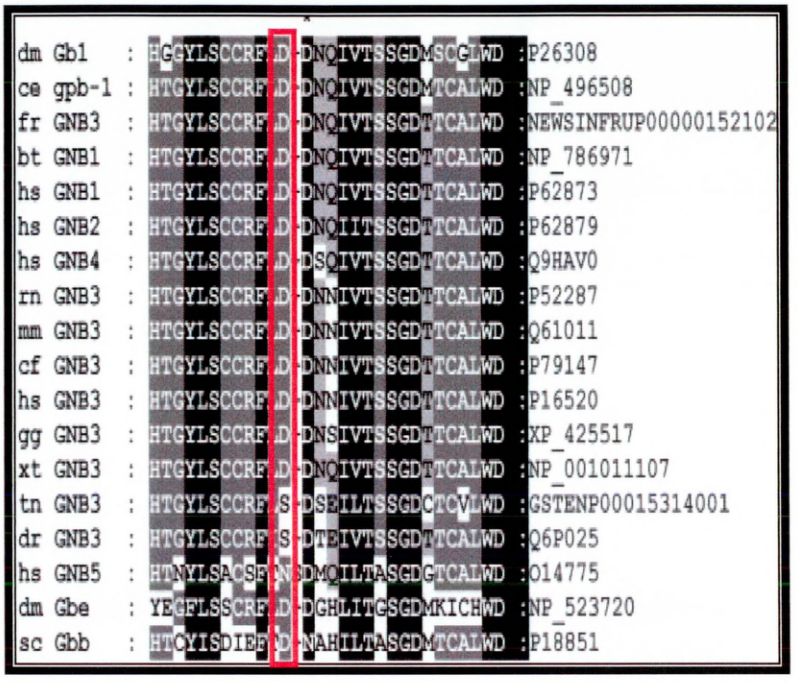


Fig 15: bt = Bos taurus, ce = Caenorhabditis elegans, cf = Canis familiaris, dm = Drosophila melanogaster, dr = Danio rerio, fr = Fugu rubripes, gg = Gallus gallus, hs = Homo sapiens, mm = Mus musculus, rn = Rattus norvegicus, sc = Saccharomyces cerevisiae, tn = Tetraodon nigroviridis, xt = Xenopus tropicalis. The asterix * indicates the position of the highly conserved aspartic acid residue at codon 153 in chicken (gg) $G\beta_3$. Numbers at the end of the sequence indicate the accession codes of the sequences used in the analysis.

The structure of Bos taurus GNB1, which is a close homolog of human $G\beta_3$, has been described by (Sondek et al 1996). Modeling the equivalent mutation in $G\beta_1$ using the "What If" (Vriend G. 1990) computer programme suggests that this deletion abolishes beta sheets in propellers 1 and 5 of the Gβ protein (appendix section 7.3 and Fig 14). Further analysis on secondary structure modeling of $G\beta_1$ molecule resulted in similar structure deleting β pleated sheets in propeller 1 and 5. (Fig14). On submitting this structure to the CASP5 committee (Critical Assessment

of methods for protein Structure Prediction (Tramontano A 2003), it was predicted that this Gβ protein would be unstable and liable to premature proteolysis. This was verified by comparing levels of $G\beta_3$ protein in retinal cell extracts from wild-type and rge birds.

2.2.3 D153del causes Protein haploinsufficiency due to structural instability in $G\beta_3$

RT-PCR of the $G\beta_3$ transcript done by our collaborators at Leeds University in a range of normal chicken tissues confirmed its ubiquitous expression and relatively high expression in the retina, but comparison of the transcript level in normal and rge tissues revealed no significant difference(Fig 16).

Figure 16: RT-PCR analysis of Gβ3 mRNA expression in multiple chicken tissues and in normal and rge retinas.

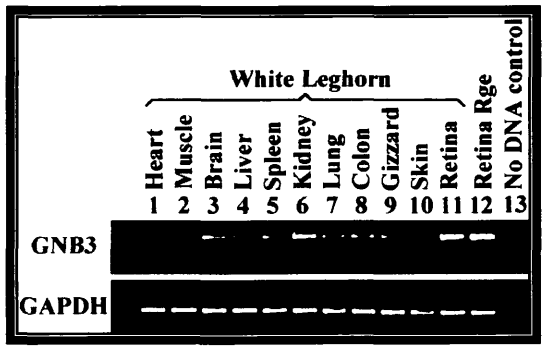


Fig 16: Lanes 1-10 show the $G\beta_3$ mRNA expression levels in tissues derived from a normal bird. Lanes 11 and 12 show the level of $G\beta_3$ mRNA expression in the retina of a White Leghorn and an rge affected bird respectively. Lane 13 represents the no DNA control.

In contrast a significant decrease in immunoreactive $G\beta_3$ protein levels is observed in rge tissues compared to normal tissues (Figure 17). This implies that the in silico modelling of an unstable D153del protein (Figure 14) is likely to be correct. The

$G\beta_{3D}$ protein is therefore likely to have a much shorter Ubiquitin proteasome, degradation half life than normal $G\beta_3$ protein (Obin et al 2002) .These results also imply that the D153del mutated transcript escapes nonsense mediated decay.

A significant reduction in $G\beta_3$ protein immuno reactivity was seen in affected retinas when compared to age and sex-matched normal retinas (Fig 17). This drop in $G\beta_3$ protein level and expression of $G\beta_{3D}$ protein in rge birds provides further proof that D153del is the rge mutation and strongly suggests haploinsufficiency due to protein instability as the primary disease mechanism.

Figure 17: D153del mutation causes degradation of Gβ3 molecule.

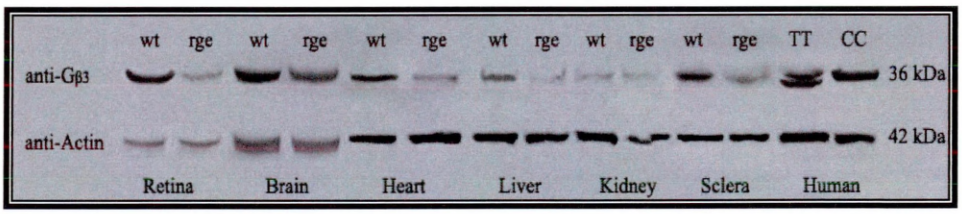


Fig 17: Western Blot analysis of D153del $G\beta_3$ affected (rge) and normal tissue (wt) protein extracts shows decreased immunoreactivity at 36kDa when blotted against the Leeds University Anti- $G\beta_3$. As a control human CC and TT alleles were analysed to see the truncated spliced gene $G\beta_{3S}$ of TT allele caused in $G\beta_3$ gene due to mutation 825C>T. Experiments represent a total of five other trails

In contrast to the unstable mutant chicken $G\beta_{3D}$ protein, the common smaller $G\beta_{3S}$ variant protein expressed in the 825TT lymphoblast cell line appears to be equally stable to the larger $G\beta_3$ normal protein, as 2 bands of equal intensity were found on Western blotting (Fig 17).

Figure 18: Densitometry analysis of GNB3 immunoreactivity

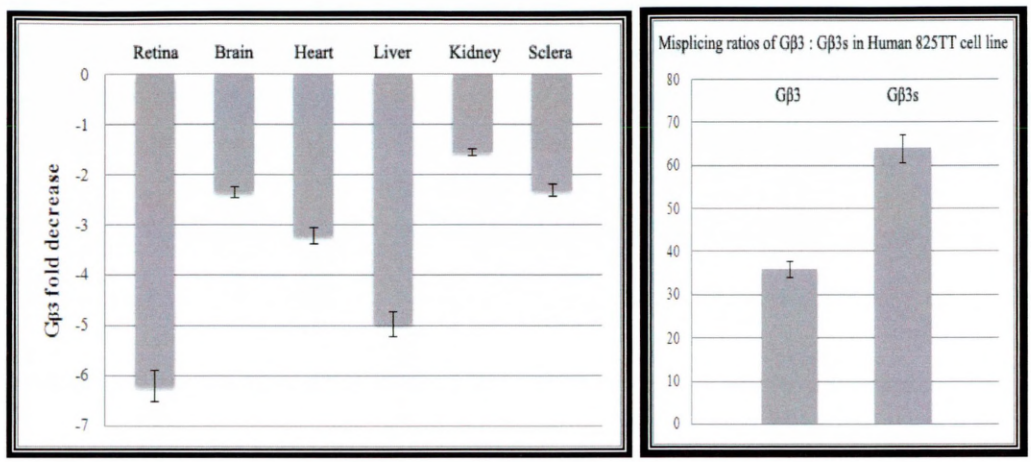


Fig 18: Quantitative densitometry analyses proved the lack of $G\beta_3$ immuno reactivity in the D153del affected tissues. Significant fold decrease is observed in D153del affected tissues when compared to normal. Misplicing ratios 35:65% was observed between $G\beta_3$ allele and $G\beta_{3S}$ variant in Human 825TT cell line. Data presented in all panels are the mean with standard error (bars) of four or five independent experiments.

These results confirm the findings of Sun et al. 2005, who showed equal intensities of RT-PCR bands for both the normal $G\beta_3$ and $G\beta_{3S}$ RNA transcripts in RNA extracted from 825TT tissues.

2.3 DISCUSSION

The in-frame deletion in $G\beta_3$, designated D153del, deletes one of two highly conserved aspartic acid residues in the third of seven WD repeat motifs in the $G\beta_3$ protein (Fig 15). The eye phenotype observed shifts interest in $G\beta_3$ back towards a potential human cone disease phenotype and illustrates the strength of the chicken as a model organism for studying inherited human retinal disease, as previously suggested (Semple-Rowland and Lee 2000). This finding is the first use of Mendelian genetics in chickens to highlight a gene of potential medical significance (the mutation responsible for the rd chicken was found using a candidate gene approach (Semple-Rowland and Lee 2000). The absence of $G\beta_3$ mutations in cone-rod and macular dystrophy patients in a previous study (Gao et al 1998), together with the relatively severe phenotype in the rge chickens, could imply a stationary achromatopsia or early onset Lebers amaurosis phenotype. A new mutational screen of UK achromatopsia and cone-rod patients is currently being carried out by our collaborators, Dr Manir Ali and Prof Chris Inglehearn at the University of Leeds.

However the reduced embryonic viability seen in rge chickens, alongside similar observations in other Gβ mutant animal models (Chen, C.K. et al 2003, Simmer, F. et al 2003,), suggests that Gβ mutations in humans may also predispose to infant mortality and/or pregnancy loss.

A similar outcome has been reported for two drosophila missense mutations in the retina specific G-beta protein Gβ, both of which cause marked reductions (95% and 99.5%) in the amount of protein produced, resulting in a dramatic loss in light

sensitivity (Dolph PJ 1994). In D153del mutant eye it's been studied that corneal flattening in rge chickens may be related to biomechanical changes caused by disarrangement of collagen at the corneal periphery (Boote C 2008). Lack of $G\beta_3$ protein expression level in the retina and sclera explains the regulation of collagen content in the eye and the altered molecular modelling of sclera. Both the human and chicken $G\beta_3$ Western blot results are also consistent with the human $G\beta_{3S}$ protein being a stable structure, whereas the chicken $G\beta_{3D}$ mutant protein as previously predicted (Tummala et al 2006) is probably unstable, with a shorter half life and probably non or less functional than normal $G\beta_3$ protein.

Summary of Chapter 2

This chapter has outlined the successful identification and characterisation at the DNA, RNA and protein level, of the D153del $G\beta_3$ mutation responsible for the retinopathy globe enlarged (rge) phenotype. The following chapters (3 and 4), show further biochemical and physiological investigations of the downstream pathways affected by both the $G\beta_{3D}$ and $G\beta_{3S}$ proteins.

Chapter III

Every day you may make progress. Every step may be fruitful. Yet there will stretch out before you an ever-lengthening, ever-ascending, ever-improving path. You know you will never get to the end of the journey. But this, so far from discouraging, only adds to the joy and glory of the climb....

Sir Winston Churchill (1874 - 1965)

Chapter 3

Estimation of cAMP and cGMP levels in rge affected tissues to identify the molecular defects caused due to D153del mutation

3.1 Introduction

21 human isoforms of PDE that are categorized into 12 families differently hydrolyse after activation the cellular concentration of both secondary messengers, cAMP and cGMP (Ionita and Pittler, 2007 and Wang et al., 2007). PDE families 4, 7 and 8 possess higher affinity to cAMP than to cGMP and PDE families 5, 6 and 9 prefer to hydrolyse cGMP (Wang et al., 2007). PDE families 1, 2, 3 and 10 process both cAMP and cGMP but also show minor varieties in their efficiency (Wang et al., 2007). Alterations in the appropriate ratio of cyclic to hydrolysed form, directly affects essential signalling transduction pathways like visual, neuronal or transcriptional. Both nucleotide derivates, cAMP and cGMP are important mediators of cell signalling and responsible for signal amplification. Thus, the following section will provide a brief insight into the central importance of cGMP and cAMP with regards to the phototransduction and neuronal transmission process.

cGMP, which is synthesized by guanyl cyclase (GC) from guanosine trisphosphate (GTP) in response to diverse signals, such as nitric oxide (NO), peptide ligands and fluxes in intracellular Ca^{2+}, regulates protein kinases (PKG – protein kinase G and PKA – protein kinase A with 50 times lower efficiency), directly cyclic nucleotide-gated (CNG) cation channels and determines its own cellular concentration by

autoregulation due to activation of PDEs (Andreopoulus and Papapetropolous, 2000 and Lucas et al., 2000).

Figure 19: Visual transduction pathway

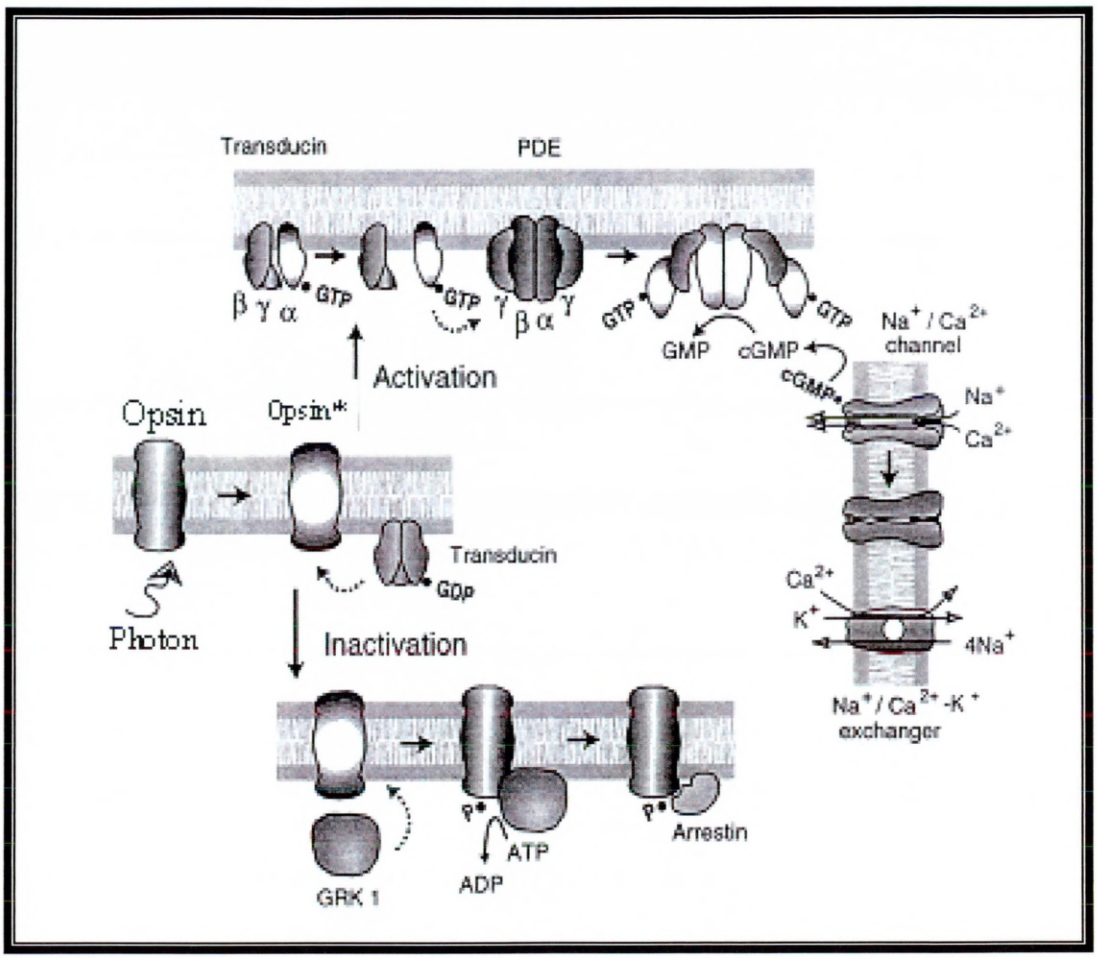


Fig 19: Schematic diagram illustrating mechanisms of activation and inactivation in vertebrate photoreceptors. Diagram modified ©HT from Fain.G.L etal 2001 showing the cone cell photoreceptor getting photoactivated and there activating the transducin α subunit of G protein complex. This results in the activation of PDE6 cleaving cGMP to 5'GMP on the cyclic nucleotide gated ion channel (Na^+ Ca^{2+} -K^+).

But all of the three target proteins differ in their affinity for cGMP binding, dependant to their function in cell physiology. It has been reported that CNG cation

channels require 10-100 times higher cGMP levels than those needed to activate PKG (Fiscus, 2002). cGMP has central importance in diverse physiological processes like smooth muscle relaxation, synaptic transmission and retinal signal transduction (Andreopoulus and Papapetropolous, 2000).

The main impact of cGMP in human cell physiology is found in the phototransduction process. In the outer segments of the rod and cone cells, cGMP is responsible for Na^+ and Ca^{2+} flux by modulating CNG cation channels and therefore regulates the recovery phase of visual excitations and adaption to background light. During dark conditions retinal cells having extremely high basal cGMP levels of around 10–40 μM, which maintain the continuous opening of the CNG cation channels (Fiscus, 2002). Light induction leads to rapid hydrolysis of cGMP by PDE 6 causing closure of CNG cation channels resulting in hyperpolarisation of the rod and cone plasma membrane (Ionita and Pittler, 2007). (Ionita and Pittler, 2007). Alteration of cGMP levels in retinal cells have shown to result in retinal dystrophies, caused by drastically elevated cytotoxic Ca^{2+} levels (Ionita and Pittler, 2007 and Lucas et al., 2000).

In neuronal cells and many other, cGMP acts through activating PKG. It has been discovered that in neuronal signal transduction, stimulated PKG leads to phosphorylation of a 23,000-dalton protein (G-substrata) (Fiscus, 2002) which has been suggested to effect diverse downstream effector proteins that are involved in neuronal transmission.

As already mentioned, glutamatergic signal transduction in neurons is coupled to cGMP signaling, where it is mentioned that glutamatergic hypoactivity exists in the

Alzheimer's disease brain which may contribute to accumulation of APP and impaired neuronal function (Mills and Reiner, 1999). Unfortunately, until today no evidence has shown a direct linkage between altered cGMP levels and therefore decreased release of glutamate that contributes to Alzheimer's disease. On the contrary, it is suggested that low basal levels of cGMP induces reduced activity of PKG, which is partial responsible for neuroprotection and thereby preventing stimulation of proapoptotic pathways (Fiscus, 2002).

Like cGMP, cAMP represents as universal messenger and gets regulated in its concentration by Adenylyl cyclase (AC) and PDE-isoforms. As mentioned above, cAMP is synthesized in cell from ATP by cell membrane bound enzyme Adenylyl Cyclase. It is also continuously hydrolysed to Adenosine 5'-monophosphate (5'-AMP) by cyclic AMP phosphodiesterases. G proteins are mainly involved in the regulation of levels of cAMP in a cell. Even though, α subunit activates adenylyl cyclase mostly, $\beta\gamma$ dimer is also involved equally in the reaction. Upon activation of G protein, activated Gα-GTP binds to Adenylyl cyclase enzyme and regulates the synthesis of cAMP. AC after activation through G proteins, which serves as an activator for CNG cation channels, the GTP exchange factor EPAC (exchange protein activated by cAMP) and mainly PKA. Activated PKA stimulate a huge diversity of effector proteins (Patterson, et al., 2000) down stream cAMP dependent PKA pathway.

In most of the cells, cAMP shows its effects by activating cAMP dependent Protein/phospho Kinase A (PKA), but sometimes it directly activates ion channels in plasma membrane.

PKA activates different substrates in different cells. So, the effects of cAMP vary differently based on the type of cell. In inactivated state, PKA has a complex structure of two catalytic subunits and two regulatory subunits. Cyclic AMP binds to regulatory subunits of PKA and releases the catalytic subunits. The activated catalytic subunits phosphorylate specific protein substrate molecules in cell.

Figure 20: cAMP dependent PKA mediated pathway

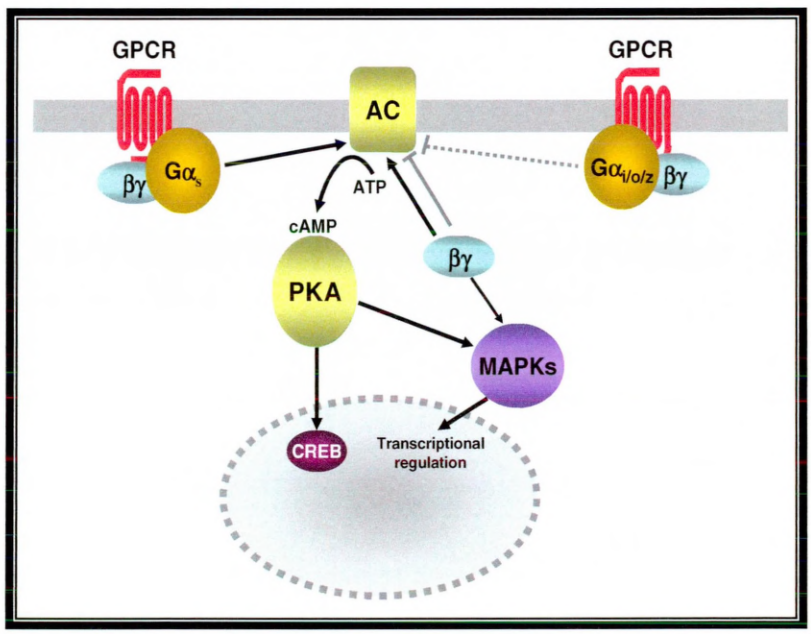


Fig 20: Activation of G_s-coupled receptors promotes increased AC activity and cAMP accumulation. Subsequent PKA activation leads to the activation of the transcription factor CREB and effector MAPKs through RAS/RAF pathway. Figure obtained from Wong 2007 review modified ©HT

The regulatory subunits are also useful in localizing the kinase inside the cell. cAMP response elements (CRE) are short DNA sequences that are found in regulatory regions of various genes. The cAMP is required to activate these CRE. PKA activates CRE-binding (CREB) protein, upon which it binds to CRE along

with CREB-binding proteins (CBP) and transcribes the genes (Fig 20). Direct involvement of $G\beta_3$ in controlling cAMP levels have previously shown by Bullido et al., (2004). These authors proved that there is a significant increase in cAMP levels in a human embryonic kidney cell simultaneously transfected with plasmids expressing $G\beta_{3S}$ protein and a variant of the β-andrenergic receptor protein, In addition other studies have also shown, in several tissues, that the $G\beta_{3S}$ protein appears to hyperactivate signalling pathways (Siffert W1995, S. Virchow 1999, Siffert W & Rosskopf D 1995, Pietruck F 1996, Rosskopf D 1993).

From these previous findings and results I therefore hypothesised that both cAMP and cGMP levels would be likely to be significantly altered in both tissues extracted from homozygote D153del $G\beta_{3D}$ chickens and in 825TT $G\beta_{3S}$ human cell lines.

3.2 Results

3.2.1 Lack of $G\beta_3$ protein causes altered adenylyl cyclase activity (cAMP)

An increase in cAMP levels such as 24.04 ± 1.2 folds in D153del affected retina 2.17 ± 0.17 folds increases in D153del affected brain was observed (Fig 21). Around ~2 fold decrease in cAMP levels was observed in liver kidney and heart tissue of rge chickens suggesting the nonfunctional $G\beta\gamma$ dimer in regulating $G\alpha$ subunit and failing subsequent down stream effector activation of (AC's) which synthesize cAMP.

Figure 21: Elevated cAMP levels observed in $G\beta_{3D}$ chicken retina and Human TT allele.

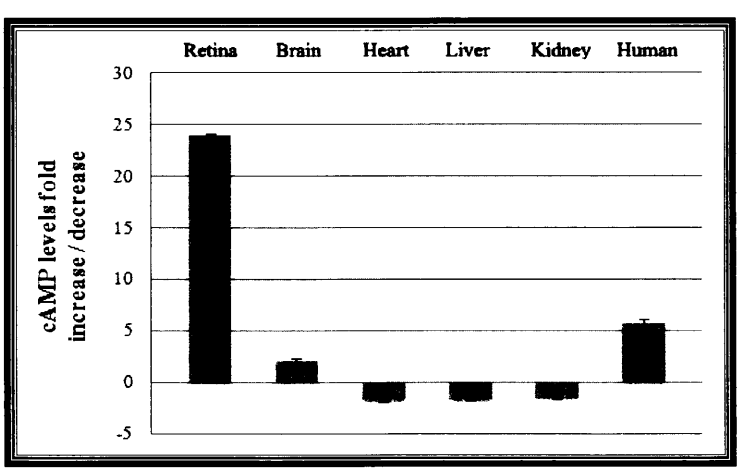


Fig 21: cAMP levels are determined and significant high fold increase around 24.04 ± 1.2 folds is found in D153del affected retina and 2.1± folds in brain samples when compared to normal. Around 2.17 ± 0.17 folds decrease in cAMP was observed in D153del affected heart sample when compared to normal. Human 825TT samples showed significant >5 fold increase of cAMP over 825CC allele confirming bullidos work (2004). Data are the mean with standard error (bars) of three independent experiments performed in triplicate.

3.2.2 cGMP analysis key mediator of intracellular signalling in visual transduction process

Since cGMP showed a pivotal role as key mediator of intracellular signalling in visual transduction process, in addition it also participates highly in synaptic signalling and neuronal cell physiology (Fiscus, 2002), cGMP levels were determined in retina, brain and heart tissue protein extracts. Both, rge-affected brain and retinal cells showed abnormal levels of cGMP. Interestingly, in comparison of D153del-affected brain to retinal protein extract a 1.44 ± 0,23 fold decrease in cGMP concentration was observed in comparison to normal brain . Retinal protein extract of rge-affected chickens showed a significant high fold (2.7 ± 1.27) increase in comparison to normal (Fig 22).

Figure 22: Elevated cGMP levels observed in $G\beta_{3D}$ Chicken Retina and Human 825TT allele

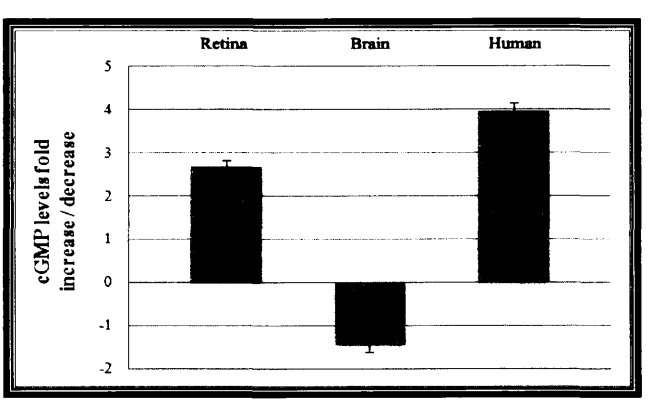


Fig 22: cGMP levels are determined and significant high fold increase around 2.7 ± 1.27 folds is found in D153del affected retina and 1.44 ± 0.23 folds decrease in brain samples when compared to normal. Interestingly Human 825TT samples showed a significant 4 fold increase of cGMP when compared to 825CC samples. Data are the mean with standard error (bars) of three independent experiments performed in triplicate.

3.3 Discussion

3.3.1 Elevation in cAMP level could be an impaired function of the βγ-complex in neural signalling

cAMP is involved in many signal transmission processes and especially in regulation of the hormone-stimulated generation of glucose from glycogen (Lodish et al., 2007). Neuronal signalling pathways of cAMP are also significant because they are ubiquitous, extremely efficient, versatile, multi modal and have an essential role in learning and memory (Mansuy, 2004). Inhibition of a certain G_i protein function that normally regulates negatively AC activity has shown to result in an increased cAMP level and impairing certain forms of memory, also with long-term effects (Mansuy, 2004). In addition to that, it has reported that Alzheimer's is associated with a general elevation in cAMP level (Bullido et al., 2004) and found to be increased in the cerebrospinal fluid of patients suffering from this neurodegenerative disorder (Martínez et al., 1999).

Taking the assumption that due to structural alteration caused by D153del in $G\beta_3$ (Tummala et al 2006), in this particular case, AC as direct downstream effector of G_α would be prolonged active and therefore increased synthesis of cAMP occurs. A secondary explanation for the elevation in cAMP level could be an impaired function of the βγ-complex.

3.3.2 High toxic levels of cAMP cause of retinal cell disruption in rge phenotype

In the cone cells of the homozygous D153del $G\beta_{3D}$ chicken, probably less $G_t\alpha_2$ subunits will bind to the unstable $G\beta_{3D}$ subunit. As photo-activated $G_t\alpha_{2\ is}$ known to

be an inhibitor of Adenylate cyclase (Wilkie et al., 2007), the production of less $G_t\alpha_2$ will cause the production of more cAMP (Fig 23). High local level cAMP can be toxic to photoreceptor cells and become unresponsive to survival factors resulting due to altered signalling, and may contribute to retinal cell disruption (Traverso, 2002) thereby causing retinal enlargement and leading to progressive blindness in these chickens.

3.3.3 Reduced cAMP causes glycogen accumulation in D153del affected tissues

In contrast decreased cAMP levels were observed in D153del affected heart liver and kidney tissues when compared to normal (Fig 21). Both the G_α-subunit, the $\beta\gamma$-conjugate exert effects to particular AC isoforms, which can be either negative (AC I) or positive regulated (II, IV) (Kroetze et al., 2003). This present data should be analyzed further in order to investigate the counteract feedback mechanisms between G_α-subunit, and the $\beta\gamma$-conjugate in regards to the isoforms of Adenylyl cyclases (AC I, II, & IV). As reduced levels of cAMP in liver protein samples of rge chicks observed suggesting the effect on Glycogenolysis pathway which is regulated by cAMP in liver cells (Jiang G and Zhang B. 2003). Also, Borba-Murad et al (2004) postulated that the inhibition of glycogen catabolism is associated with the reduced levels of cAMP in rat liver cells i.e. with the decrease in levels of cAMP in cell; there is a proportionate increase in the levels of glycogen in cell. Hence, it can be assumed that there could be an increase in the levels of glycogen in liver cells of rge phenotype $G\beta_{3D}$ chicken. There may be a chance of decrease in levels of glucose and also equal chances of increase in glucose levels, may be due to other factors, in rge phenotype $G\beta_{3D}$ chickens. So, there may be some direct or

indirect correlation of diabetes disorder with the increased levels of glycogen of $G\beta_{3D}$ chicken.

Also, Montiani-Ferreira, et al (2003) reported a pathological feature of abnormal localization and accumulation of glycogen in the peri-nuclear region of photoreceptor in rge chicken retina. As the levels of cAMP are low in D153del affected $G\beta_{3D}$ chickens kidney , the levels of glycogen might increase in these cells. This might be the probable explanation of glycogen accumulation in photoreceptors, and can be considered as one of the reasons for accumulation of glycogen in photoreceptors of rge phenotype $G\beta_{3D}$ chickens, which are to be confirmed by further experiments.

cAMP activates cAMP response element (CRE), a short DNA sequence that is found in regulatory regions of many genes. PKA activates CRE-binding (CREB) protein, a specific gene regulatory protein that binds and regulates CRE to synthesize renin (Tamura et al, 1994). As the levels of cAMP in kidney $G\beta_{3D}$ chickens are low, the synthesis of renin may be affected due to lack of transcriptional regulation mediated through inadequate cAMP and the concentration of renin may decrease in kidney. Increased levels of cAMP will increase the transcription of specific genes (Alberts, et al. 2002) and mediate several responses like glycogen breakdown (glucagon hormone in liver) salt(aldosterone), water reabsorption (vasopressin hormone in kidney), and increase in heart rate and force of contraction (adrenaline hormone in heart) (Alberts, et al. 2002). In kidney binding of vasopressin on V2R receptor activate $G_{s\alpha}$ adenylyly cyclises to release cAMP (Boone M and Deen PM 2008). Increase

of cAMP will stimulate the activity of PKA to phosphorylate Nedd 4-2 (Neuronally Expressed Developmentally Downregulated 4), a down stream mediator down regulates epithelial Na^+ transport through inhibition of the epithelial sodium channel (ENaC) (Snyder et al 2004). This mechanism of increased cAMP and PKA activity increases ENaC activity due to synergistic effects of SGK (Bonny O and Hummler E, 2000). Another contributing factor for hypertension will be the increased rate of cAMP synthesis activates EPAC pathway which, stimulates PI3K effector to cause increased ENaC activity (Helms et al 2006). Hypertensive patients carrying TT allele have an increased PKA mediated Na/H exchanger (NHE) activity with increased Na^+ reabsorption (Siffert 2000). Hence, the reduced levels of cAMP in kidney of $G\beta_{3D}$ chickens may reduce hypertension and blood pressure which, should be confirmed by testing the ENaC activity in kidney of the $G\beta_{3D}$ chickens

3.3.4 cGMP defines the pathogenesis of rge phenotype

Earlier discovery of D153del was found to be responsible for the autosomal recessive ocular disease phenotype retinopathy globe enlarged (rge) in chickens that causes progressive retinal degeneration and total vision loss (Inglehearn et al., 2003, Montiani-Ferreira et al., 2003 and Tummala et al., 2006). cGMP plays a crucial role as a key mediator of intracellular signalling in visual transduction process and has previously shown to be significantly increased in other animal models possessing recessively inherited visual transduction mutations (e.g. Semple-Rowland 1998). As cGMP, also participates in synaptic signalling and neuronal cell physiology, any alterations in levels may also change brain cognition

(Fiscus, 2002). From figure 22 it can be seen that there is a significant increase in cGMP in retinal and and significant decrease in brain tissue. As the unstable $G\beta_{3D}$ protein is unlikely to form stable heterotrimers with $G_t\alpha 2$ and $G\gamma$ in retinal cones, much less or no $G_t\alpha 2$ will be photo activated by cone opsin. This $G_t\alpha 2$ is responsible for activating PDE6; therefore any decrease in its activation will result in an increase in cGMP due to less cleavage of this molecule by PDE6. This resultant increase of cGMP through impaired hydrolysis may cause a continuous opening of CNG channels and leads to drastically elevated Na^+ and Ca^{2+} flux (Fig 23). The extremely elevated ion levels contribute primarily to disturbance in vision and secondarily, result in retinal dystrophies caused by drastically elevated cytotoxic Ca^{2+} levels (Ionita and Pittler, 2007; Lucas et al., 2000).

To conclude, the increase in retinal cGMP concentration is probably one of the most likely causes of the retinopathy/ blindness phenotype in rge chickens. By prominent examination in whole brain tissue a general decrease in cGMP level was observed. It has previously been shown that the cGMP protein kinase G pathway to be important in the regulation of synaptic transmission and plasticity, brain development and neuroprotection.

cGMP mediated pathways have also been shown to be functional in glial cells and may indicate that they control important glial cell physiology associated with neuronal function. (Baltrons et al.2008). In addition it has been suggested that low basal levels of cGMP is partially responsible for neuron protection (Fiscus, 2002).

Figure 23: Downstream effects of the D153del $G\beta_{3D}$ mutation

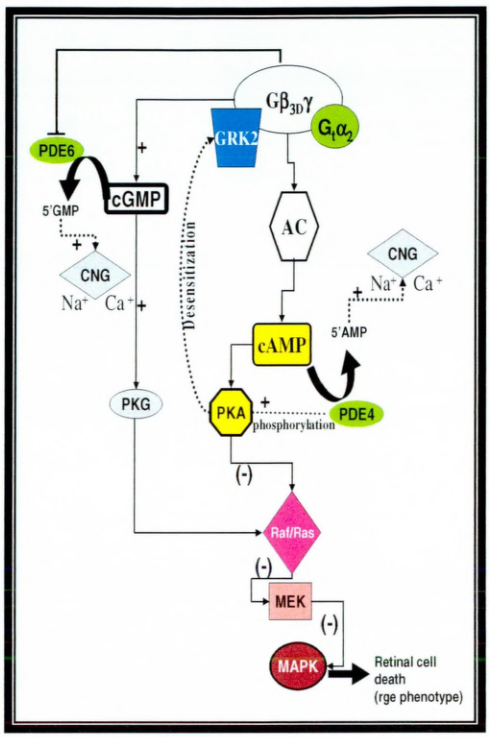


Fig 23: Pathway shows the probable downstream effects of the $G\beta_{3D}$ mutation in cone retinal tissue e.g. brain, where $G\beta_{3D}$ - $G_t\alpha2$ is exclusive. D153del mutation inhibits the function of $G_t\alpha2$ resulting in increase of cAMP and cGMP in retina. Key: (+) indicate more than basal fold Due to increased expression/activity, (-) less than basal fold due to decreased Expression /activity, Dotted arrow represents Phosphorylation and desensitization, AC, adenylyl cyclase; Gβ, G protein Beta subunit, GPCRs, G protein–coupled receptors; PKA, protein kinase A, CNG-channel, cyclic nucleotide-gated channel $G_t\alpha2$= Gαtransducin subunit.

Interestingly the lymphoblast 825TT cell line had significantly increased (~4 fold) cGMP level compared to the 825CC cell line (Fig 22). If this differential of cGMP levels also occurs in the brain cells in 825TT individuals, it may help explain their predisposition to brain diseases, e.g. Alzheimer's (Bullido 2004), Major depression disorder López-León (2008) and appear to have a differential response to drug treatments for psychiatric disorders (Anttila S et al. 2006, Prestes AP et al. 2007, Lee HJ et al. 2004, Müller DJ et al 2005, Schürks M et al. 2007). Moreover as our

unstable $G\beta_{3D}$ mutant chicken brains shows a decrease in cGMP levels compared to normal $G\beta_3$ brains, it is unikely that human brains expressing $G\beta_{3S}$ stable protein will have the same effect. Further investigations are necessary to determine a distinct cerebral pathways affected by the decrease in cGMP level.

Summary of results

From our results in the previous chapters (2 and 3), I have provided further evidence that the structural changes in the $G\beta_{3S}$ protein cause a gain of function in comparison to the normal $G\beta_3$ protein, as I have clearly demonstrated that the $G\beta_{3S}$ protein is highly stable and cells expressing this protein have increased adenylate cyclase activity. In contrast the $G\beta_{3D}$ protein is almost certainly unstable causing a loss of function. This is demonstrated by both its decrease in immunoreactivity and the significant increase in cGMP levels in homozygote D153del $G\beta_3$ retinal tissue. The latter result demonstrates that the PDE6 enzyme is not being sufficiently activated by cone alpha transducin (Fig 19) due to the structural alterations in the $G\beta_{3D}$ protein.

Chapter IV

We advance on our journey only when we face our goal, when we are confident and believe we are going to win out....

Orison Swett Marden (1850 - 1924)

Chapter 4

D153del mutation causes tissue specific pleiotropic effects affecting the phosphorylation target effectors in $G\beta_3$ controlled signalling pathways in comparison to 825C>T mutation

4.1 Introduction

Despite the evidence for a substantial genetic contribution and population based studies to the risk of developing brain disorders, hypertension and coronary heart disease in humans, the discovery of the $G\beta_{3S}$ protein and its involvement in these disorders has not produced a definitive pathway from genetic mutation to the resultant pathology (reviewed by Weinstein 2006). Most papers support a G protein enhanced signalling hypothesis for $G\beta_{3S}$ (reviewed by Dobrev et al. 2000).

As $G\beta_3$ has previously been shown to bind both $G\alpha_i$ and $G\alpha_s$ subunits (Rosskopf et al. 2003), the activating $G\beta_{3S}$ subunit is likely to show different tissue specific effects on signalling pathways, depending on the cellular ratios of these α subunits. To back this hypothesis up, Krumins and Gilman (2006), found a reduced accumulation of both $G\alpha_i$ and $G\alpha_s$ subunits following the RNAi inhibition of $G\beta$ subunits. Disappointingly very few studies (Siffert 2001) have been carried out, at the cellular level, using human organ tissues, such as heart and brain, which

are likely to have altered signalling pathways in humans expressing the $G\beta_{3S}$ protein.

Understanding the effect of $G\beta_3$ mutant proteins on signalling pathways in such tissues, may help to explain the pathological basis of the many complex disorders that 825T $G\beta_3$ individuals are predisposed to. Due to the limited access to affected human tissues, D153del $G\beta_3$ mutant chickens may therefore prove to be a useful animal model for clinical studies for $G\beta_3$ affected pathways. Any alterations in tissue specific signalling pathways caused by the $G\beta_{3D}$ protein are however, likely to have the opposite effect to cells expressing the $G\beta_{3S}$ protein, but may still reveal the likely method of pathogenesis. In this chapter I therefore aimed to study the pleiotropic effects of alterations in $G\beta_3$ controlled pathways, such as cAMP induced GRK2 pathway involving downstream effector MAPK, in tissues previously implicated in $G\beta_3$ disease association.

4.2 Results

Immuno Blot Analysis

Our western blot and slot blot studies on various tissue protein extracts (Brain, Retina, Heart Liver and Kidney) from D153del affected and normal chicken tissues revealed significant signalling affects.

4.2.1 D153del affects the G protein phosphorylation activity of MAPK1 (ERK2)

This study for the first time demonstrated the multi-step alterations of signalling pathways connecting D153del mutant $G\beta_3$ subunit to MAPkinases and entire cellular phosphorylation activity in chickens, a genetic model for defective G protein disorders. Furthermore, the study has revealed significant alterations in ERK 2, βARK (GRK2) activity, which is activated promoting Gs-mediated stimulation of adenylyl cyclase (Bullido etal 2004) and entire cellular protein activity, when compared Human 825C>T. cAMP and cGMP levels are observed to relate the level of activity affected by these messengers in signal regulation to function of the organ.

I compared the altered signalling effect due to mutations in Human cell line 825CC, 825TT, chicken normal $G\beta_3$ and mutant $G\beta_{3D}$ tissue protein extract samples from heart, brain and retina were blotted against the anti-MAPK phosphorylated and unphosphorylated (abcam biosciences) antibodies in 2μg/ml

concentration with blocking reagent for expression and activity analysis (Fig 24 & Fig 25). Anti-MAPK phosphorylated is specific for the active, dually-phosphorylated form of MAP kinase and does not recognise the non-phosphorylated or the monophosphorylated forms of the MAP kinase molecule. Decreased MAPK phospho activity is observed in D153del affected chicken tissues and increased phospho activity observed in Human 825TT allele compares to CC (Fig 4b) suggesting the evidence of $G\beta_{3D}$ association in increasing the intracellular signal transduction via G protein-coupled receptors (Winfried Siffert 2003). Thus Quantitative densitometric analysis showed significant fold decrease of EKR2 in D153del affected chicken heart (-1.5 ± 0.2 fold), Brain (-2.1 ± 0.4 fold) and retinal (-1.7 ± 0.25 fold) tissues (Fig 26).

Figure 24: D153del exert no effect on Unphosphorylated MAPK molecules

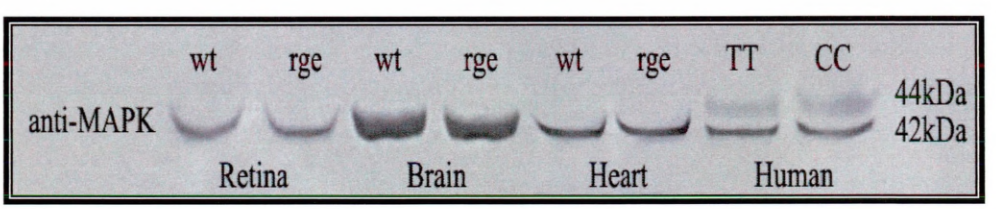


Fig 24: Western blot analysis of total MAPK (only ERK2 in chickens) for normalisation in D153del affected retina brain and heart chicken protein extracts showed no significant changes when compared to normal. Human samples CC and TT alleles showed no difference (Both ERK1 & ERK2). Experiments represent a total of five other trails

Figure 25: Phospho ERK2 decreased in D153del affected tissues

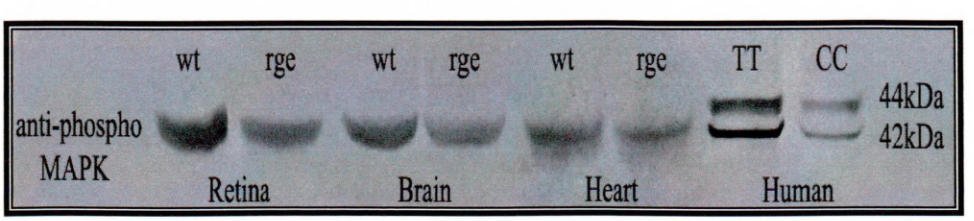


Fig 25: Western blot analysis of Phopho ERK2 in D153del affected retina brain and heart chicken protein extracts showed significant changes when compared to normal. Human samples TT showed increased MAPK activity (ERK1 + ERK2) when compared CC allele. Experiments represent a total of five other trails

Figure 26: Determination of Biochemical activity of Phospho MAPK activity

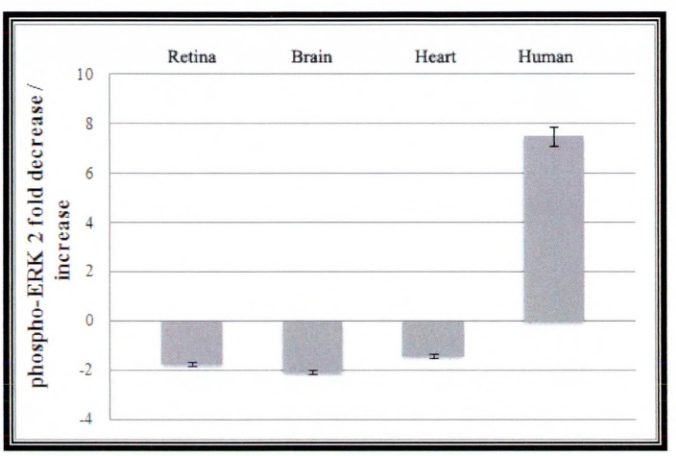
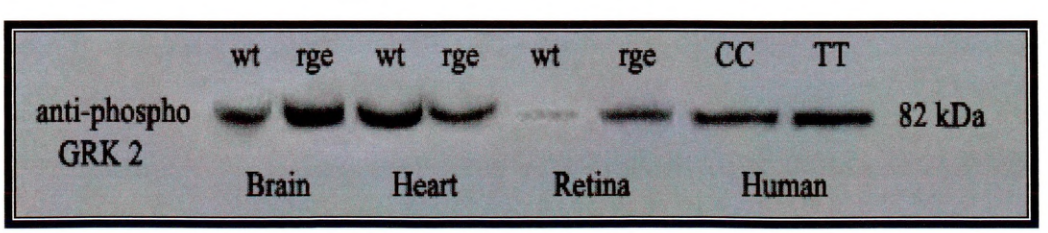


Fig 26: Quantitative densitometry analyses proved the lack of Phospho ERK2 immuno reactivity in the D153del affected tissues. Significant fold decrease is observed in D153del affected tissues when compared to normal. Data presented in all panels are the mean with standard error (bars) of four/five independent experiments.

4.2.2 cAMP Induced GRK2 phosphorylation observed in D153del affected tissues

Since 825C>T was found to be involved in alteration of β-adrenergic signalling in neural (Bullido et al., 2004 and Willeit et al., 2003) and cardiovascular system (Jaber et al., 1996) the study examined the βARK (GRK 2) activity.

Figure 27: Tissue specific phosphorylation of GRK2 molecules



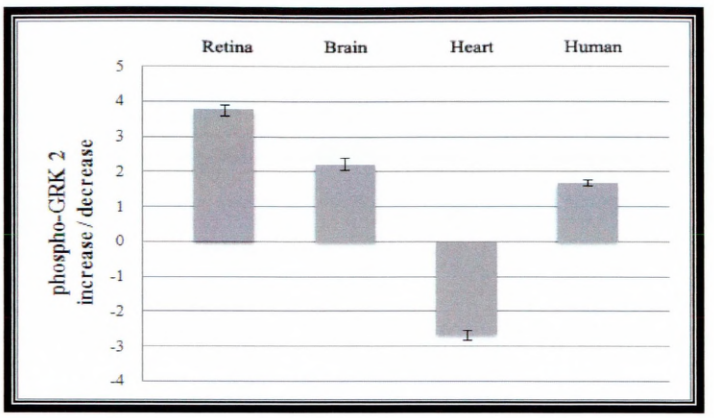


Fig 27: Immunoblot analysis of D153del affected brain heart and retina protein extracts along with human cell extracts. Quantitative densitometry analyses showed significant fold increase in the immunoreactivity against the phospho specific GRK2 residue in brain retina and human TT allele whereas D153del affected heart tissue showed significant ~2.8 fold decrease when compared to normal. Experiments represent a total of three as above trails.

Tissue extracts of D153del mutants showed a significant increase in quantity of activated (phosphorylated) βARK molecules. D153del affected Brain showed 1.64 ± 0.17 folds increase, and in D153del affected retinal protein extract, a 1.76 ± 0.16 fold increase of immunoreactivity respectively (Fig 27).

4.2.3 D153del affects whole cellular protein phosphorylation

An insight into the entire cellular status of phosphorylated protein residues, such as phosphoserine, -threonine and -tyrosine residues (PSTT) were assessed by slot blot analysis. This analysis was preffered due to the low sensitivity of the antibody in recognition to the specific PSTT residues. This study revealed an association of D153del with alterations in cellular balance of entire PSTT residues. In D153del brain around 1.3 ± 0.07 folds and in D153del-mutants retina a significant high (3.9 ± 0,97) folds increase in immuno reactivity of PSTT residues were observed (Fig

28). In heart around 2.5 ± 1.2 folds decrease in immuno reactivity was observed when compared to normal (Fig 28).

Figure 28: Insight into the entire cellular status of phosphorylated protein residues

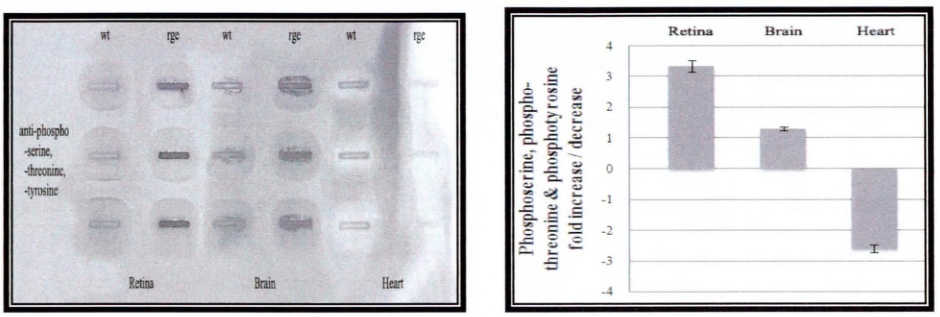


Fig 28: Slot blot analysis of D153del affected retina and brain protein extracts Quantitative densitometry analyses proved significant fold increase in the immunoreactivity against the phospho specific anti-serine, threonine and tyrosine residues where as D153del affected heart tissue showed significant fold decrease when compared to normal. Experiments represent a total of three other trails.

4.3 Discussion

MAPK has defined central importance in cellular growth, differentiation, survival and the pathological effects on various downstream pathways, which lead to stimulation or repression (Bando et al., 2006 and Werry et al., 2005). Our study proved again that chickens lack expressing mammalian homologue of MAPK ERK1 but express ERK2 (Sanghera et al., 1992). Previous studies elucidated the G-protein mediated -activated kinase (MAP kinase)coupled signalling profile in a mice genetic model showed decrease ERK2 phospho activity causing congestive heart failure (CHF) mimicking a similar disease in humans (Kacimi R 2003). In contrast there is a significant increase of phospho activity in the human 825TT lymphoblast cell line compared with the 825CC cell line. This confirms previous evidence of an association of $G\beta_{3S}$ and an increase in the intracellular signal transduction via G protein-coupled receptors (Siffert 2003, Bullido 2004).

Since 825C>T was found to be involved in alteration of β-adrenergic signalling in neural (Bullido et al., 2004 and Willeit et al., 2003) and cardiovascular system (Jaber et al., 1996) I examined βARK (GRK 2) activity. GRK2 plays a pivotal role in desensitization of its associated GPCR. Recent studies showed that a close interaction of $G\beta_{3}\gamma$ and PKA is required for efficient receptor desensitization (Cong et al., 2001). Moreover, GRK 2 activity was potentiated by decreased ERK activity (Pitcher et al., 1999). Thus, the amplified activity of GRK 2 that was found in brain and retinal protein extract of $G\beta_{3D}$ mutants is expected to alter the kinetics of (photo-) receptor desensitization and therefore to redundancy of activation of PDE6α. In the $G\beta_{3D}$ heart tissue GRK2 activity was found to be

relatively decreased when compared to normal (Fig 27). As GRK2 is a cytosolic protein that is recruited to the cell membrane in part by the dissociated $G\beta_{3D}\gamma$ subunits upon receptor stimulation, lack of $G\beta_3$ expression in $G\beta_{3D}$ heart tissue stimulates minute responses in the GRK2 receptor..

The Phosphorylated serine threonine and tyrosine (PSTT) activity which were determined by slotblot analysis (Fig 28) correlate with the differential mechanism of GRK2 activity in cardiovascular system (Fig 27 βARK activity). The obtained data do not aid examining alterations in particular signalling pathways, but instead demonstrate the wide range of molecular effects, caused by a single amino acid deletion in $G\beta_3$ subunit. The changes in protein activity could include phosphatases as well as kinases. Thus, $G\beta_{3D}$ could not be distinctly characterized as a protein that causes exclusively decreasing or increasing effects in the alterations of signalling pathways.

Summary and future prospects

This study demonstrates the downstream multi-step alterations caused by the multifaceted mutant protein subunit $G\beta_{3D.}$ The demonstrated alteration of several signalling pathways, which connect to MAP kinases and entire cellular phosphorylation in the rge chicken, makes this animal a very useful genetic model for defective G protein disorders. These molecular findings suggest that a complex interplay exists between the protein targets (MAPK and GRK2) and cellular messengers (cAMP and cGMP), due to altered signalling by D153del.

$G\beta_{3D}$ mutant chicken may be a conclusive model for helping understand pathogenic disorders caused by 825C>T $G\beta_3$ mutation in humans.

Table 1: Tissue Specific Effects caused by D153del

signaling member	*D153del-affected tissue*					*C825T-affected cell line*
	Retina	**Brain**	**Heart**	**Liver**	**Kidney**	**Yoruba population**
wild type Gβ3	---	---	---	---	-	---
Gβ3s	not expressed in del153D-affected chickens					+++
phospho-GRK 2	+	+	-	n. d.	n.d.	+
phospho-MAPK 3	not expressed in chickens					+++
phospho-MAPK 1	-	-	-	n.d.	n.d.	+++
phospho-STT	+++	+	--	n.d.	n.d.	n.d.
cGMP	++	-	=	=	=	+
cAMP	+++	++	-	-	-	++

Table 1 – Alterations in activity or concentration of particular cell signalling members are observed in homozygous 825C>T-affected cell lines and different $G\beta_{3D}$ affected tissues, respectively. $G\beta_{3D}$ Mutation significantly decreases immunoreactive $G\beta_3$ protein in all homozygous rge/rge tissues examined. We can also clearly see that the Yoruba $G\beta_3$ 825TT cell line has significant decreased immunoreactivity of $G\beta_3$ when compared to 825CC cell line
Symbols: +/- 1-2 fold increase/decrease, ++/-- 2-3 fold increase/decrease, +++/--- >3 fold increase/decrease, = - no significant changes. Abbreviation: n.d. – no data

The observed thesis results, which are summarized in Table 1, relate to the level of activity affected by the defective $G\beta_3$ protein in signal regulation to the function of the organ.

Furthermore, the study has revealed significant alterations in ERK 2-, βARK (GRK2) activity, which is activated and promotes Gs-mediated stimulation of adenylyl cyclase (Bullido et al., 2004) and entire cellular protein activity, when compared human 825C>T. The pathway shown in Figure 23 will be involved to a greater or lesser extent in disease onset, development and therapeutic control. As

the $G\beta_{3D}$ mutant shows tissue specific signalling effects when compared to 825C>T for example the rge affected chickens maintains a lower altered $G\beta_3$ signalling with decreased intracellular cAMP levels. The D153del mutation may therefore interfere with a negative feedback loop in the $G\beta_3$ signalling and preserve the integrity of the diverse cells in the specific tissues studied in rge mutant chickens.

Finally, the identification of naturally occurring D153del mutation of $G\beta_3$ protein has already had major implications for understanding the structure and function of $G\beta_3$ signaling. But unfortunately, the implications of identifying G protein mutations for diagnosis and treatment for G protein disorders are, as yet, rather limited. This thesis has however, shown the value of the D153del chicken in helping to elucidate the complex tissue specific pathways that are probably involved in the predisposition of GNB3 825TT individuals to hypertension, cardiac and neural disorders. A critical goal in future studies is to provide molecular explanation for these different diseases, which will require comprehensive understanding the role of individual G protein subunit regulation on their downstream target effectors. This can probably be achieved by identifying the ratios of $G\alpha$, $G\beta$ and $G\gamma$ individual subunits in each specific tissue. This should help reveal the molecular basis of these diseases, as there is no clear evidence of single interaction that is responsible for all of these diseases.

This report however, does not rule out indirect effects of the $G\beta_{3S}$ on controlling ion channel function, many of which are controlled by either cAMP or cGMP. Future transfection and electrophysiological experiments using constructs that express the $G\beta_{3S}$ protein in normal mammalian cells should help prove or disprove

the importance of this variant protein to ion channel function. In vivo studies should also be explored to know the mechanism of such interactions. For example D153del and 825C>T knock-in genetically engineered mice would be extremely useful animal models to try to further elucidate the pathways involved in the early stages of disease associated biochemical systems in 825C>T affected individuals.

Chapter V

It is common sense to take a method and try it. If it fails, admit it frankly and try another. But above all, try something....

Franklin D. Roosevelt (1882 - 1945)

Chapter 5

MATERIALS AND METHODS

5.1 Genotyping and identification of candidate gene

High-resolution genotyping was performed using previously uncharacterized microsatellite markers that have been downloaded as simple repeat sequences from the chicken genome database at UCSC (http://www.genome.ucsc.edu/). Primer sequences for the PCR were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/). The primers were labelled with one of the dyes, FAM, HEX or TET (Invitrogen). For the PCR, initial optimisation was followed by analysing the markers in the parent DNA to check for informativity. Only the polymorphic markers were amplified using the progeny DNA as template. For genotyping, the PCR products were migrated through a polyacrylamide gel on the ABI377 gene sequencer (Applied Biosystems) and fragment length analysis was carried out using the Genescan V. and Genotyper V. softwares. Refinement of the rge critical interval was achieved by following the segregation pattern of the polymorphic markers with the disease phenotype.

5.2 Sequencing

Genomic DNA was extracted from normal and rge affected bloods using DNAzol solution (Invitrogen). 50ng of this Genomic DNA was then added to 12.5ul of 2 X master mix solution (Promega) for each 25µl PCR reaction. The intronic flanking

primers (1μM of each), used for PCR amplification of all exons, of the chicken GNB3 gene were

GNB3EX1-seqF CTTTCTACCTCTCATCAG
GNB3EX1F TTGAGCAGTCCTATTGTTGGC
GNB3EX1R TTTAGAGGGCTGTACGGTGCT

GNB3EX2-seqF ACAAACTAAGCAAAACTC
GNB3EX2F ATGCCTTGTGCTGGGTACAAT
GNB3EX2R ACAATCTGAGGAAAGAGCAGG

GNB3EX3-4-seqF CAGGTTGGAAGCAGTTAG
GNB3EX3-4F ATCACTTCCAAGGGGACTCTC
GNB3EX3-4R AAAGGGATGGCATGAACCTG

GNB3EX5-6-seqF CCCGCTCTCCTATCTACT
GNB3EX5-6F ATGCAGGATTGGAACCCTTCA
GNB3EX5-6R TGAGATCACACAGCACCCTGA

GNB3EX7-seqF TACCCAGGCCCATGACTT
GNB3EX7F ATGTGGTACCCAGGCCCAT
GNB3EX7R TTCGTTCCTCCCGGACTCAA

GNB3EX8-seqF CACCCAGGAAGCACGTAT
GNB3EX8F TTGATTTTTGCTCTGGGGGT
GNB3EX8R GGAAGAATTGCTGAGTTTTGG

GNB3EX9-seqF CAGAATGATGCACAAGAG
GNB3EX9F TTGCTAATCACATTTTGCAGG
GNB3EX9R TGTGTGTACTCCCCCTCCTT

PCR was performed for 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The reaction products were visualised following agarose gel electrophoresis under ultraviolet illumination. QIAquick PCR Purification Kit (Qiagen) was used to clean up PCR fragments generated and remove unincorporated primers. 10ng of cleaned PCR product, together with 3.2 micromoles (per reaction) of the forward or revere GNB3 primers or an internal sequencing primer, were sent to The Sequencing Service, School of Life Sciences,University of Dundee (http://www.dnaseq.co.uk/) for commercial sequencing.

5.3 Heteroduplex Analysis

Heteroduplex analyses method is popular electrophoretic method for identifiaction of mutated sequence. Technical simplicity and relative high sensitivity has enabled us to carry this experiment for the detection mutated GNB3 sequence. The PCR amplified DNA form normal and rge samples were subjected to heteroduplex analysis., The PCR product was heated for 10minutes at 95°C for 10 minutes and immediately cooled on ice before mixing with 6X DNA loading buffer. The samples were then loaded on a 10% polyacrylamide gel which is made from 40% stock solution containing 40% acrylamide and 0.4% N,N'-methylenebisacrylamide. Gels were run at room temperature 0.6x TBE with 7.5 µl aliquot of PCR products obtained after clean up process mixed with equal amount of loading buffer until fragments migrated at least 25cm. Gels were stained in gel red (sigma) and photographed under UV light

5.4 PCR-ARMS Analysis

The PCR was carried out using the PCRx Enhancer Kit (Invitrogen) with 1 x enhancer solution, 1 x amplification buffer and 1.5mM $MgSO_4$ as well as 0.2mM dNTPs and 2.5U Taq DNA polymerase for each 10µl reaction. The oligonucleotide primers used in the reaction were dGCAGGGAACTCTCAGCTCATA and either (i) dAGCTAGTCACAATACTGTTGTGATC (wild-type) or (ii) dAGCTAGTCACAATACTGTTGTGAAG (mutant) to give reaction products of 175 and 172 bp respectively. 20ng DNA was used as a template for each reaction which was performed for 30 cycles at 94°C for 30 seconds, 61°C for 30 seconds

and 72°C for 30 seconds. The reaction products were visualised following agarose gel electrophoresis under ultraviolet illumination.

5.5 Bioinformatics

Multiple sequence alignments were carried out using ClustalW, available through http://www.ebi.ac.uk/clustalw/index.html. Clustal W alignments were viewed using Jalview and saved in MSF format. The generated MSF file was then opened in GENDOC, available through http://www.psc.edu/biomed/genedoc/. Selected blocks of the generated alignment were then saved in rtf format and edited in Microsoft Word.

5.6 RT-PCR Analysis

Reverse-transcription of chicken RNA that had been isolated from various tissues was performed using oligo $(dT)_{15}$ primer and Superscript II reverse-transcriptase (Invitrogen). Amplification of 3720p of GNB3 cDNA was carried out using the primers dCTGGTCAGTGCCTCACAAGA and dGGGAAACTGCCAAGCTCATA. As normalisation control, amplification of 237bp of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was performed using the primers dGGAAAGTCATCCCTGAGCTG and dCATCAAAGGTGGAGGAATGG.

5.7 Structural Prediction

The D153del GNB3 mutation was modelled in the homologous Bos Taurus GNB-1 (1B9X) protein, using the both the Deepview (http://spdbv.vital-it.ch/) and the

"What If" programme (http://btcpxx.che.unibayreuth.de/COMPUTER/Software/WHATIF/html/).

5.8 Protein Extraction

The protein extraction was performed in regard to Biosource tissue extraction protocol (http://www.biosource.com, catalog number: FNN0071).

1. Tissue samples of normal and rge-affected birds (chicken lines: wt – 1011, rge – 1005, Roslin Institute) were weighted and placed in tissue extraction buffer (Pierce Biotechnology) containing a protease and phosphatase inhibitor cocktail (Pierce Biotechnology) at a concentration of 10 ml extraction buffer for each g of tissue. Subsequently, tissue samples were homogenized by grinding. Cell extract was centrifuged at 10,000 RPM for 5 minutes to pellet the tissue debris. Supernatant were collected, aliquoted and stored at -80°C.

2. Human transformed B-lymphocyte cell lines, with known GNB3 825 genotypes (http://www.ncbi.nlm.nih.gov/SNP/snp_ss.cgi?ss=ss69099869); 825CC (GM19116) and 825TT (GM18500) were obtained from Coriell Cell Repositories, USA. These cell lines were cultured in 10% FCS DMEM media (Sigma) in culture flasks and were harvested after 48hrs. The media containing cells were centrifuged at 2500g for 5 min and the pellet was washed twice with ice cold PBS and centrifuged again at 2500g for 5 min. 1 ml of RIPA buffer was added for 40 mg of wet cell pellet, mixed well and were then sonicated for two cycles 5 seconds at 50% pulse. The final mixture was shaken gently on ice for 15 min and the Protein supernatant was obtained by centrifuging the cells at 14000g for 15 min. Subsequently all protein extract samples were quantified with the use of a spectrophotometer, with BSA as a standard.

5.9 Protein Quantification

The protein quantification of the extracts were carried-out in regard to Bradford standard protocol with Coomassie (Bradford) Protein Assay Kit (Pierce Biotechnology). Protein extract were photometrical analyzed with the use of a spectrophotometer at a wavelength of 595 nm, with BSA (Pierce Biotechnology) as a standard.

5.10 Immunoblotting

5.10.1 Western Blotting

For Western blotting, Western Breeze Immunodetecion Kit (Invitrogen) were employed, following standard methods.

1. Protein extract of normal and rge-affected chickens was electrophoretic separated by a 10% Bis-Tris gel (Invitrogen).
2. Gel was placed into transfer buffer (Invitrogen) and transferred to a nitrocellulose membrane (Amersham pharmacia).
3. Proteins were blotted by 45V for 45minutes.
4. Membrane was further processed by incubation with blocking solution (contents view chapter 7.1.1) for 30 minutes at room temperature on a rotary shaker
5. Subsequently, membrane was washed 2 times for 5 minutes with ultra pure water and incubated for 2 hours by room temperature on a rotary shaker by 100rpm with primary antibody of interest diluted in blocking solution
6. After incubation, membrane was 4 times washed for each 5 minutes with antibody wash solution (Invitrogen) and incubated for 2 hours at 37°C in secondary antibody solution (Invitrogen) on rotary shaker.

7. Afterwards, membrane was washed 4 times for each 5 minutes with antibody wash solution and rinsed 2 times each 2 minutes with ultra pure water.
8. Bands were visualized by treating membrane for 0-60 minutes with 5ml chromogen solution (Invitrogen).
9. Resultant bands were digitalized and quantified by densitometry analysis (Gelpro software; Image Processing Solutions, North Reading, MA).

5.10.2 Slot Blotting

For slot blotting, this procedure was mainly opted to detect the immuno reactivity for the antibodies which have low sensitivity to detect specific bands.15 µL equal amounts of normal and Del153D -affected protein extracts were blotted, in triplicate, on a nitrocellulose membrane (Hybond ECL; GE Healthcare Life Sciences, Little Chalfont, UK) with a slot blot apparatus (PR600; Hoefer Scientific Instruments, San Francisco, CA). Blots were then incubated with blocking solution supplied in the kit (WesternBreeze™ Chromogenic Detection Kit, Invitrogen. catalogue no: WB7105, WB7103) before the addition 2µg/ml Phosphoserine/threonine/tyrosine antibody (Abcam) for 2 hours at room temperature or overnight at 4°C, followed by incubation with appropriate secondary antibody (anti-mouse/anti-rabbit). Finally, bands were visualized with the enhanced chromogenic substrate analysis system supplied by the kit. For internal control, the levels of β-actin were examined by repeating the same procedure using anti-β-actin (Sigma) and anti-mouse secondary antibody supplied in the kit.

5.11 Antibodies

5.11.2 GNB3 Expression Analysis

For GNB3expression analysis, four different antibodies were employed. For detection of overall expression, including $\beta_3\gamma$ complexes, GNB3 polyclonal antibody (A01) (Abnova) in a dilution of 1:1000 was used, raised against the full length of the $G_{\beta 3}$ protein with GST tag. In addition a GNB3 monoclonal chicken specific antibody (peptide - MGEMEQMKQEA) raised and affinity purified at Leeds University has been used to detect the GNB3 expression levels in Human CC, TT cell protein extracts, normal and rge tissue protein extracts. Some commercial antibodies which are available from Santacruz biotech and Calbiochem proved no response to specific GNB3 antigen.

5.11.3 MAPK Activity and Expression analysis

MAPK (ERK 1/2) activation was detected by using polyclonal ERK 1/2 (phospho T183+Y185) antibody solution (0.54µg/ml) (Abcam). ERK 1/2 (phospho T183+Y185) antibody interacts only with phosphorylated MAPK isoforms ERK 1 and 2. For determination of ERK 1/2 expression, ERK 1/2 polyclonal antibodies (Abcam) have been used.

5.11.4 β-ARK (GRK2) Activity

β-ARK (GRK2) activation has been determined by using polyclonal GRK2 (phospho S670) antibody solution (1.5µg/ml) (Abcam). GRK2 (phospho S670) interacts only with phosphorylated species of β-ARK.

5.11.5 Protein Phosphorylation Activity

The entire cellular protein phosphorylation status, was measured by using 0.75µg/ml monoclonal phosphoserine/threonine/tyrosine antibody solution (Abcam) that specific binds to phosphorylated serine, threonine and tyrosine residues on rge affected and normal tissues protein extracts as well as human CC and TT cell lines.

5.12 Enzyme Immuno Assays for cAMP & cGMP Level Determination

Enzyme immuno assays were performed with a commercial Cyclic AMP/GMP EIA Kits (Cayman Chemicals) in accordance to the instruction protocol (http:// www.caymanchem.com).

1. For the assay, 50 µl of each tissue sample and as well as a standard, together with 50 µl of cyclic AMP/GMP tracer (cGMP/cAMP coupled to acetylcholinesterase) and 50 µl of a rabbit anti-cyclic GMP antiserum was added to microplate wells precoated with mouse monoclonal anti-rabbit immunoglobulin G (IgG) antibodies.
2. The plate was incubated at room temperature for 18 h.
3. Wells were emptied and washed five times. Subsequently, 200 µl of Ellman's reagent (acetylthiocholine+5, 5′-dithio-bis-2-nitrobenzoic acid) was added to the wells.
4. The plate was developed for 120 minutes at a room temperature in the dark using a rotary shaker.
5. The concentration of the product (5-thio-2-nitrobenzoic acid) was assayed by measuring the absorbance at 412 nm with the use of a spectrophotometer.

Each tissue sample, as well as a standard was assessed in triplicates for quantification of cAMP and cGMP levels. Assays were repeated twice.

5.13 Imaging Analysis & Data presentation

Calculation of quantified data and statistical analysis on resultant blots were performed by capturing the images in high resolution TIFF format files using a charge-coupled-device camera (AxioCam MRc, Carl Zeiss). Densitometry analysis of the images is done with the computer program Gelpro (Gelpro Software, San Diego, USA). cAMP/cGMP ELISA based experiments shown are representative for a total of at least three experiments in triplicates of samples. The microplate wells were read on an Anthos HTIII Microplate Reader (Denley Instruments, Billingshurst) utilising its integral software to calculate results. All the results are statistically proved by calculating the appropriate standard deviation/error represented in the graphs

Chapter VI

Our test of truth is a reference to either a present or imagined future majority in favour of our view. . . .

Oliver Wendell Holmes Jr. (1841 - 1935)

Chapter 6

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- **Chicken Genome**: http://www.genome.ucsc.edu/
- **Primer designing:** http://frodo.wi.mit.edu/cgi-bin/primer3/
- **DNA sequencing:** http://www.dnaseq.co.uk/
- **CLUSTAL view:** http://www.ebi.ac.uk/clustalw/index.html
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- **DEEP VIEW:** http://spdbv.vital-it.ch/
- **WHAT IF:** http://btcpxx.che.unibayreuth.de/COMPUTER/Software/WHATIF/html/

Chapter VII

Someone's boring me. I think it's me....

Dylan Thomas (1914 - 1953),

CHAPTER 7

APPENDIX

7.1 BUFFERS AND CHEMICAL LIST

The following list represents the commercial chemicals that were mainly employed. Manufactured buffers are listed under chapter 7.1.2.

7.1.1 Consumables

- Antibody wash solution (Invitrogen # 46-7005)
- Chromogen solution (Invitrogen # SO-W001)
- Cyclic AMP EIA Kit (Caymanchem.#581001.1)
- Cyclic GMP EIA Kit (Caymanchem.#581022.1)
- Coomassie (Bradford) Protein Assay Kit (Pierce Biotech # 23200)
- Blocker/Diluent A (Invitrogen # 46-7003)
- Blocker/Diluent B (Invitrogen # 46-7004)
- Bovine serum albumin (BSA) standard (Pierce Biotech # 23209)
- ERK 1/ERK 2 antibody (Abcam # ab17942)
- ERK 1/ERK 2 (phospho T183+Y185) antibody (Abcam # ab50011)
- GNB3 polyclonal antibody (A01) (Abnova#H00002784A01)
- GRK2 (phospho S670) antibody (Abcam # ab4473)
- Halt® Phospatase inhibitor cocktail (Pierce Biotech. # 78420)
- Halt® Protease inhibitor cocktail (Pierce Biotech. # 78415)
- Hybind™ ECL™ Nitrocellulose membrane (AmershamP# RPND/98/11)
- NuPage® 10% Bis-Tris Gel (Invitrogen # NP0301)
- NuPage® MES SDS page running buffer 20x (Invitrogen # NP0002)
- Novex® Sharp Pre- Stained protein Standard (Invitrogen # LC5625)
- NuPage® Transfer buffer 20x (Invitrogen # NP0005-1)
- Phosphoserine/threonine/tyrosine antibody (Abcam # ab15556)
- RIPA Buffer (Pierce Biotech # 89901)

- Secondary antibody solution (mouse) (Invitrogen # 46-7006)
- Secondary antibody wash solution (rabbit) (Invitrogen # 46-7007)
- Western Breeze Immunodetecion Kit (Invitrogen # WB7103)

7.1.2 Manufactured buffers

Blocking solution: ultra filtered water, Blocker A (Invitrogen) and Blocker B (Invitrogen), components blended in a ratio 7 : 2 : 1

7.1.3 A table of various parameters used for perfecting SDS-PAGE with the Invitrogen family of electrophoretic products.

Assay	% polyacrylamide	Well loading volume	Voltage	Time
1	10%	20µl	80v	90min
2	10%	20µl	120v	90min
3	20%	15µl	30v	120min
4	10%	30µl	80v	60min
5	10%	20µl	80v	60min
6	10%	10µl	80v	60min

A table to summarise the discrete adjustments that were made to the immunoassay procedure when performing the western strip blots derived from two PVDF membranes.

7.1.4 PVDF membrane blotting procedure

Immunoassay Stage	Strip 1 heterozygous	Strip 2 rge	Strip 3 rge	Strip 4 rge
Blocking Stage	**Blocked at 4°C overnight**	**Blocked at 4°C overnight**	**Blocked at 4°C overnight**	**Blocked at 4°C overnight**
Initial rinsing stage	As per protocol	As per protocol	As per protocol	As per protocol
1°AB Incubation stage	**1:500 dilution at 37°C for 120 minutes**	**1:2000 dilution at 37°C for 120 minutes**	**1:3000 dilution at 37°C for 120 minutes**	**1:5000 dilution at 37°C for 120 minutes**
Post-1°AB Washing stage	As per protocol	As per protocol	As per protocol	As per protocol
2°AB Incubation stage	**37°C for 60 minutes**	**37°C for 60 minutes**	**37°C for 60 minutes**	**37°C for 60 minutes**
Post-2°AB Washing stage	As per protocol	As per protocol	As per protocol	As per protocol
Final rinsing Stage	As per protocol	As per protocol	As per protocol	As per protocol

7.2 Cyclic AMP/ Cyclic GMP assay

Cyclic AMP/ Cyclic GMP assay is the assay carried out to find variations in the concentration of cAMP/cGMP in the samples when compared to standard curve. This assay was performed with Cayman's "Cyclic AMP/Cyclic GMP EIA Kit" and according to its protocol (Appendix Four). The cAMP/cGMP in sample competes with cAMP/cGMP tracer for the secondary antibody which combines with pre-coated mouse monoclonal primary antibody. The kit has Cyclic AMP/ Cyclic GMP EIA Antiserum (1

vial / 100 dtn), Cyclic AMP/Cyclic GMP AChE Tracer (1 vial / 100 dtn), Cyclic AMP/ Cyclic GMP EIA Standard (1 vial), EIA Buffer Concentrate (10X) (2 vials / 10ml), Wash Buffer Concentrate (400X) (1 vial / 5ml), Tween 20 (1 vial / 3ml), one Mouse Anti-rabbit IgG Coated plate, one plate cover, Ellman's Reagent (3 vials / 100 dtn), Acetic Anhydride (1 vial / 2.5ml), KOH (1 vial), EIA Tracer Dye (1 vial), EIA Antiserum Dye (1 vial).

7.2.1 Principle of cayman cAMP/ cGMP assay

This assay is an ACE^{TM} Competitive Immunoassay, where there is a competition between free cAMP/cGMP (cAMP/cGMP in sample) and a cAMP/cGMP - acetylchotinesterase (AChE) conjugate (cAMP/cGMP tracer) for a constant number of cAMP/cGMP -specific rabbit antibody binding sites. The kit contains a 96-well plate where each well was pre-coated with mouse monoclonal antibody which serves as a primary antibody in the assay. The wells were also coated with a proprietary solution of proteins. The EIA antiserum will act as a cAMP/cGMP -specific rabbit secondary antibody that binds to primary antibody coated on the walls of well. The cAMP/cGMP tracer and the cAMP/cGMP of sample compete for the secondary antibody.

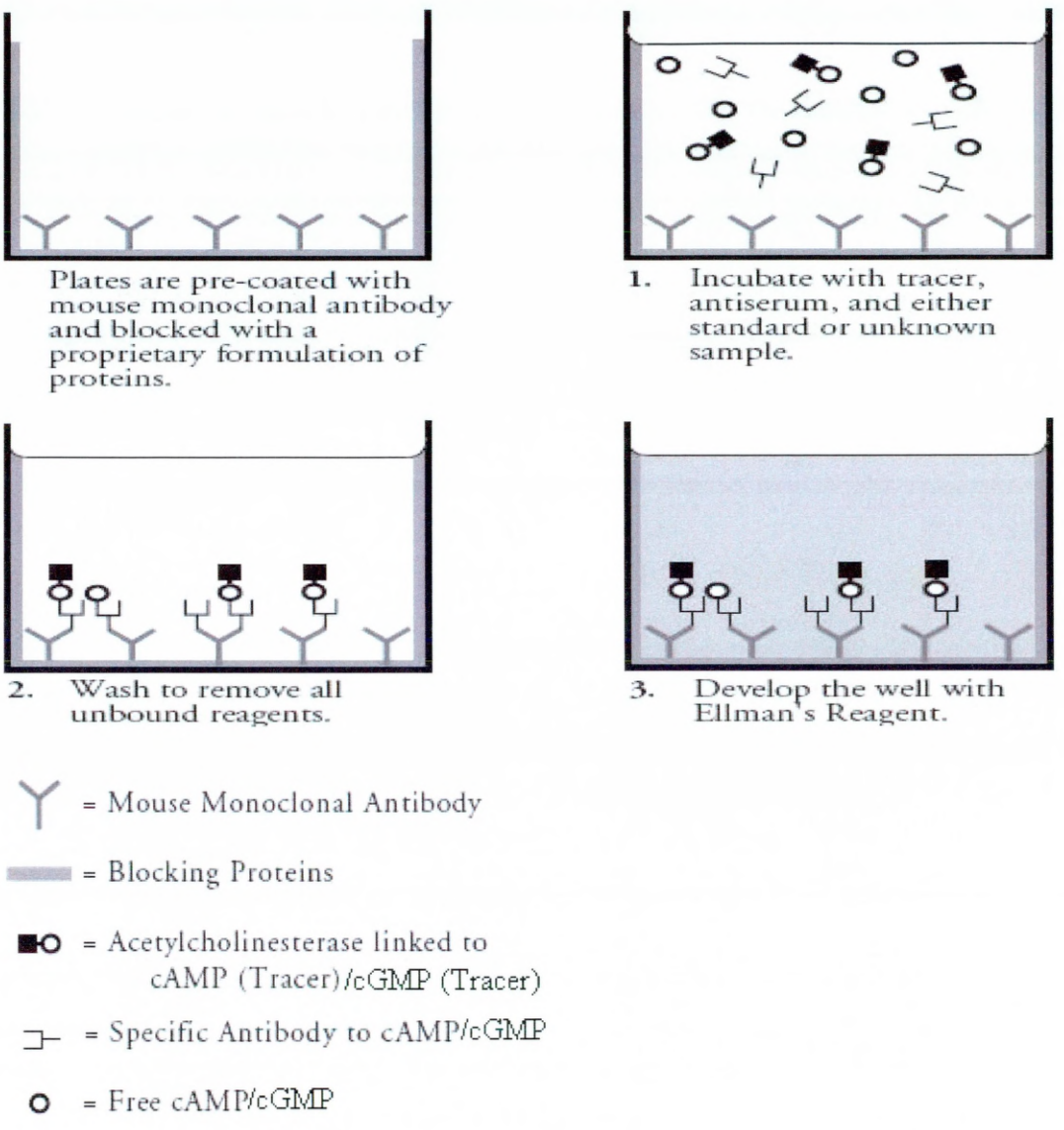


Fig. Schematic diagram of the ACE™ Enzyme Immunoassay

Picture adapted from: Cyclic AMP/Cyclic GMP EIA kit manual [Online]. Cayman Chemicals. Available at http://www.caymanchem.com/app/template/Product.vm/catalog/581001/a/z.[Accessed on 10/09/2008]

The cAMP tracer reacts with the ellman's reagent and produces a distinct colour that absorbs strongly at 412nm. The principle of cAMP was represented pictorially in Fig: (2.3). So, the absorbance reading denotes directly the quantity of cAMP tracer bound to

mouse antibody and the reading is indirectly proportional to the quantity of cAMP in sample in the well. This can be interpreted as the absorbance reading increases, the quantity of cAMP tracer increases and the quantity of cAMP in sample decreases. This can be represented as an equation as below:

$$\text{Absorbance} \propto [\text{Bound cAMP Tracer}] \propto 1/[\text{cAMP/cGMP}]$$

The cAMP/cGMP standard curve was calculated by plotting a graph between $\%B/B_O$ and cAMP/cGMP concentrations of the standard. The cAMP/cGMP levels in samples were estimated by comparing the obtained readings against the standard curve.

7.2.2 Biochemistry of ACE™ EIA assay

Electric eel, Electrophorus electricus, contains electric organ which produces AChE that has a capacity of high turnover for the hydrolysis of acetylcholine. This AChE is covalently attached to a molecule of analyte and act as a Tracer in immunoassasy. Ellman's Reagent, which has acetylcholine and 5,5'-dithio-bis-(2-nitrobenzoic acid), can measure the activity of AChE and in turn the quantity of tracer that is binding to antibodies in the well. AChE hydrlyses acetylcholine to thiocholine, which on non-enzymatic reaction with 5,5'-dithio-bis-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid. It has a distinct yellow colour and strong absorption at 412nm. The biochemistry of ACE™ EIA Assays was represented in Figure below.

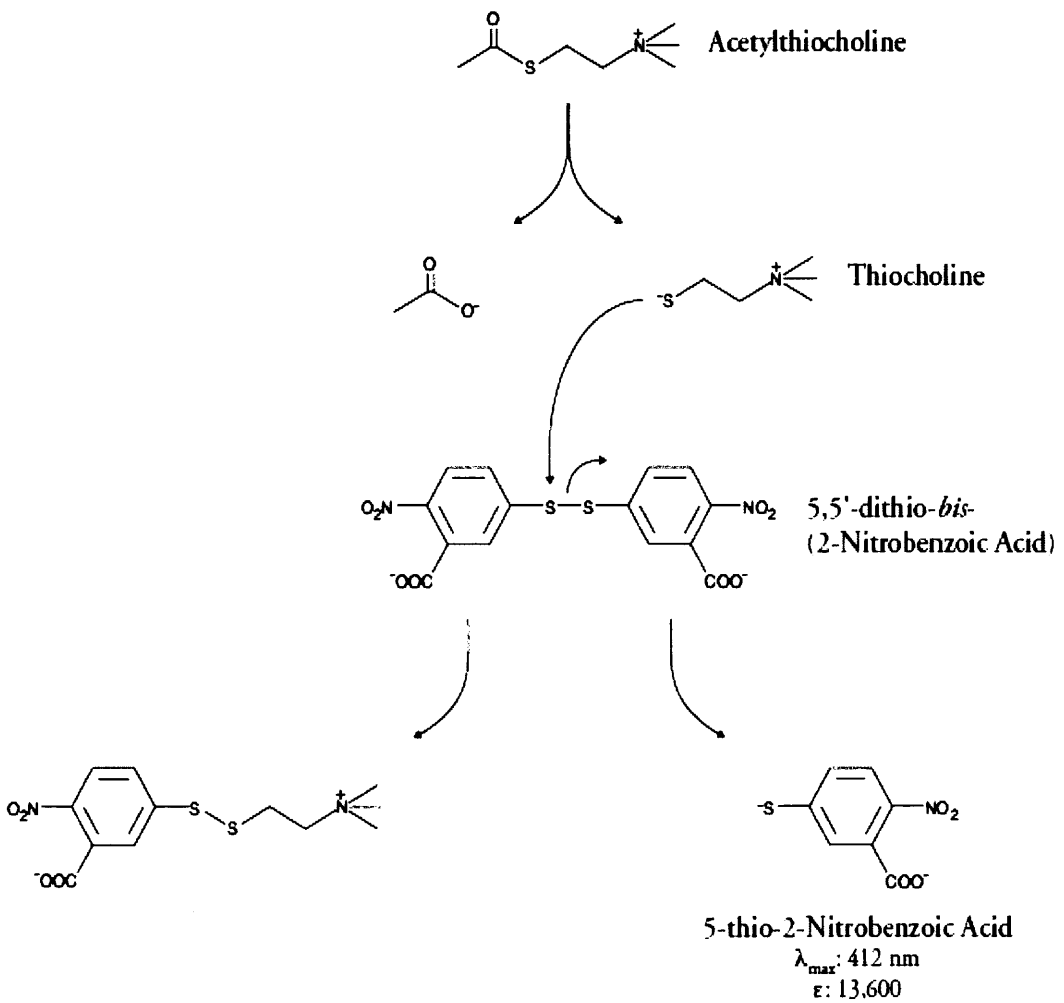


Fig. Schematic diagram of reaction catalyzed by Acetylycholinesterase
Picture adapted from: Cyclic AMP/Cyclic GMP EIA kit manual [Online]. Cayman Chemical. Available at http://www.caymanchem.com/app/template/Product.vm/catalog/581001/a/z. [Accessed on 10/09/2008].

As the amount of cAMP/cGMP tracer is constant and the amount of cAMP/cGMP in sample (free cAMP) varies, the amount of cAMP/cGMP that binds to rabbit antibody will be inversely proportional to the quantity of cAMP/cGMP of sample in the well. The cAMP/cGMP assay was carried without any acetylation.

7.2.2.1 cAMP/cGMP standard curve

The cAMP/cGMP standard curve is the curve obtained by running assay with cAMP/cGMP standard. This standard curve is useful in estimating the quantities of cAMP/cGMP in the sample.

7.2.2.2 Preparation of reagents

The reagents EIA Buffer, Wash Buffer, cAMP/cGMP tracer, and cAMP/cGMP antiserum were prepared as per the requirements of the experiment.

7.2.2.3 EIA buffer preparation

The vial (10ml) of EIA buffer concentrate was diluted with 90ml of ultra pure water. The vial was rinsed well to remove any salts that may have precipitated.

7.2.2.4 Wash buffer preparation

The vial (5ml) of Wash Buffer concentrate was diluted at 1:400 dilutions with ultra pure water. Later Tween 20 was added around 0.5ml/L of wash buffer. The quantity of wash buffer prepared was based on usage in the experiment every time.

7.2.2.5 cAMP/cGMP AChE tracer

cAMP/cGMP tracer (100dtn) was diluted with 6ml of EIA buffer. Tracer dye was added to the reconstituted tracer at a final dilution of 1:100 (60µl of dye to 6ml tracer) and stored at 4^0C in refrigerator.

7.2.2.6 cAMP/cGMP antiserum

cAMP/cGMP Antiserum (100dtn) was diluted with 6ml of EIA buffer. Antiserum dye was added to the reconstituted antiserum at a final dilution of 1:100 (60µl of dye to 6ml tracer) and stored at 4^0C in refrigerator.

7.2.2.7 cAMP/cGMP standard

cAMP/cGMP standard was diluted with 1ml of EIA buffer to make the concentration to 3000pmol/ml. The standard was stored at 4^0C.

7.2.3 Preparation of standard samples

Neatly labelled 8 eppendorf tubes were taken with 900µl of EIA Buffer in tube 1 and 500µl of EIA Buffer in tubes 2-8. In tube 1, 100µl of reconstituted standard (3000pmol/ml) was added and mixed thoroughly to make the concentration to 300pmol/ml.

Tubes	1	2	3	4	5	6	7	8
Concentration (pmol/ml)	300	150	75	37.5	18.8	9.4	4.7	2.3

Table showing the concentration in each tube due to serial dilution.

500µl of aliquot was taken fro tube 1 and serially diluted into tube 2 and mixed well. Again 500µl of the aliquot was taken form tube 2 and transferred into tube 3 and mixed

thoroughly. This serial dilution was continued until tube 8, and 500µl of aliquot was removed and discarded from tube 8. The quantity of the aliquot in each tube was 500 µl. These diluted standards were with different concentrations (table 2.5) and were used immediately for the experiment.

7.3 ADDITIONAL DATA

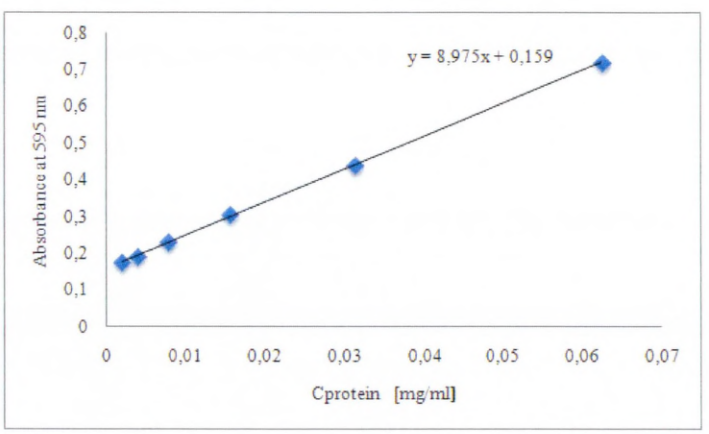
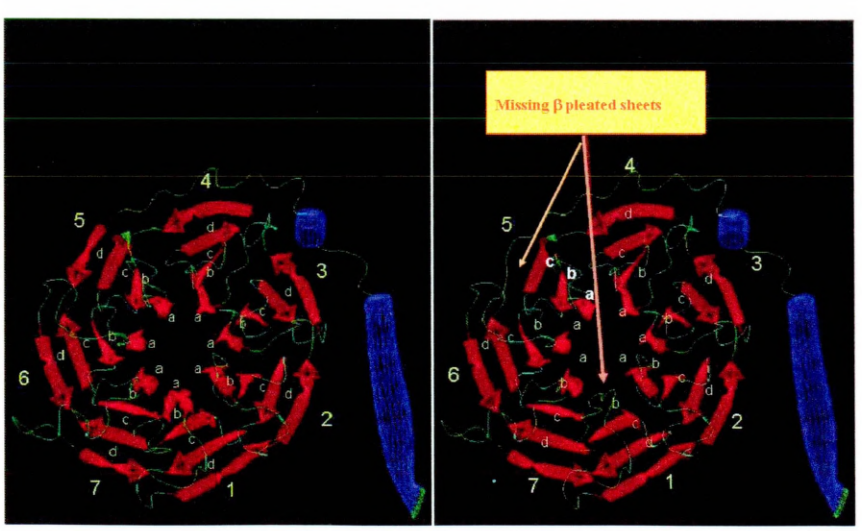


Fig. – BSA standard curve for protein quantification



Molecular modeling of GNB3 molecule 1B9X PDB ID in J vriend WHATIF program

List of Publication and Presentations

Publications

- **Tummala, H.,** Wehner, D., Zhelev, N., Hocking, P., Ali, M., Inglehearn, C.F. and Lester, D.H. Characterization of the D153del GNB3 affected pathways reveals novel tissue specific changes that may determine GNB3s associated diseases. **(Submitted).**

- **Tummala, H.,** Getty, P., M. Ali, Hocking, P. M., Burt, D. W., Ingelhearn, C. F. & Lester, D. H. (2006) Mutation in the guanine nucleotide-binding protein beta-3 causes retinal degeneration and embryonic mortality in chickens. Investigative Opthalmology and Visual Science Vol. 47, pp 4714-4718.

- **H. Tummala,** C.F. Inglehearn, M. Ali, P. Getty, P.M. Hocking, D.W. Burt, and D.H. Lester (2007) Discovery of a Novel GNB3 mutation that causes the retinopathy globe enlarged phenotype and possibly hypertension in chickens. Genetical Research 2007

Poster Presentations

- C.F. Inglehearn, **H. Tummala,** M. Ali, P. Getty, P.M. Hocking, D.W. Burt, and D.H. Lester. A Mutation in the Guanine Nucleotide–Binding Protein Beta–3 (Gnb3) Causes Retinal Degeneration and Embryonic Mortality in Chickens. Invest. Ophthalmol. Vis. Sci. 2006 47: May 2006 ARVO conference Florida USA

- **Tummala H,** Adusumalli V, Getty P, Hocking P, Burt DW , Ali M, Inglehearn C, Lester DH. A novel GNB3 mutation that causes retinal degeneration and predisposition to embryonic mortality in chickens. Horizons in Molecular Biology 2006 - International PhD Student Symposium, 14. - 16. Sept. 2006, Max Planck Institute for biophysical chemistry, Göttingen, Germany.

- **H Tummala,** C.F. Inglehearn, M. Ali, P. Getty, P.M. Hocking, D.W. Burt, and D.H. LesterA mutation in a ubiquitously expressed, trimeric G protein, beta subunit (GNB3), causes both retinal degeneration and predisposition to embryonic mortality in chickens. British Society of Developmental Biology (BSDB). Induction and the origin of developmental genetics in embryonic development. 13–15 September 2006 Apex City Quay and Spa Hotel, Dundee.

Oral Presentation

- **H. Tummala,** C.F. Inglehearn, M. Ali, P. Getty, P.M. Hocking, D.W. Burt, and D.H. LesterDiscovery of a Novel GNB3 mutation that causes the retinopathy globe enlarged phenotype and possibly hypertension in chickens. 17th Mammalian genetics and Development. A meeting of the Genetics Society. University College London 2006 Institute of Child Health, 30 Guilford Street, London WC1N 1^{E} 9 and 10 Nov 2006.

I always wanted a happy ending... Now I've learned, the hard way, that some poems don't rhyme, and some stories don't have a clear beginning, middle and end. Life is about not knowing, having to change, taking the moment and making the best of it without knowing what's going to happen next. Delicious ambiguity....

Gilda Radner (1946 - 1989)