Investigating the possibility of Notch signalling in the adult retina

by

Kara Ronellenfitch
BSc, University of Victoria, 2010

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Supervisory Committee

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Dr. Robert L. Chow, Department of Biology
Supervisor

Dr. Perry L. Howard, Department of Biology
Departmental Member

Dr. Robert D. Burke, Department of Biochemistry and Microbiology
Outside Member

Abstract

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The Notch signalling pathway is a highly conserved cell-to-cell signalling pathway involved in developmental cell fate determination in all metazons. When Notch is signalling, differentiation is inhibited and a progenitor-like state is favoured. This signalling pathway has been implicated in the developing retina, where the inhibition of Notch has been shown to skew the proportion of different retinal neuronal cell types. Although functional knockout studies have allowed us to characterize some of the roles of Notch in the retina, low protein levels have made it difficult to characterize the location of Notch receptors and ligands in neuronal tissue. Here we sought to characterize the localization of the Notch signalling pathway components in both the developing and the adult mouse retina. Using RT-PCR we were able to show the presence of mRNA for Notch receptors, ligands, and DNA binding cofactors for the Notch intracellular domain, CBF1, throughout postnatal development as well as in the adult retina. *In situ* hybridization confirmed the presence of Notch1, Notch2, and CBF1 mRNA in the embryonic (E14.5) and early postnatal (P1.5) retina similar to what has been reported in earlier studies, but in the adult retina (P40), levels were below detection. To further explore the role of Notch in the adult retina we used two transgenic mouse reporter models in which a Notch responsive element directs the expression of EGFP or Venus. In the adult retina of the NTR line (Tg(Cp-EGFP)25Gaia/J) reporter expression was detected in rod ON and cone type 2 OFF bipolar cells, as well as in a subset of both amacrine and ganglion cells. In the CBFRE:H2B-Venus line adult reporter expression was detected in photoreceptors, and a large proportion of both amacrine and ganglion cells. Together this data supports the conclusion that Notch is expressed and actively signalling in the retina throughout development and possibly in the adult retina, although

below levels of *in situ* hybridization detection. These results represent the possibility of a previously unknown role for Notch in the adult retina.

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List of Abbreviations

ANK	Ankyrin
Arc	Activity-regulated cytoskeleton-associated gene
BAC	Bacterial artificial chromosome
bHLH	Basic helix-loop-helix
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical l eukoencephalopathy
CBF1	C-promoter binding factor 1
CBP	Creb binding protein
CD	Coding domain
CoA	Co-activators
Compound E	{(S,S)-2-[2-(3,5- Difluorophenyl)-acetylamino]-N-(1-methyl-2-oxo-5- phenyl-2,3-dihydro-1H-benzo [e] [1,4] diazepin-3-yl)-propionamide}
CoR	Co-repressors
CSL	CBF1/Suppressor of Hairless/Lag-1 family\
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
Dx1-4	Deltex1-4
Dll	Delta-like ligand
DOS	Delta and OSM-11-like
DSL	Delta/Serrate/Lag2
E14.5	Embryonic day 14.5
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
GCL	Ganglion cell layer
GlcNAc	N-acetylglucosamine
H2B	Histone 2B

HES	Hairy/enhancer of split
HERP	Hes-related repressor protein
HDAC	Histone deacetylase
INL	Inner nuclear layer
INM	Interkinetic nuclear migration
IPL	Inner plexiform layer
LNR	Lin12-Notch repeats
LTD	Long term depression
LTP	Long term potentiation
MAM	Mastermind
Mash1/3	Mammalian achaete-scute homolog 1/3
Mib1/2	Mindbomb1/2
NBL	Neuroblastic layer
NECD	Notch extracellular domain
NEXT	Notch extracellular truncation
Neur1/2	Neuralized1/2
NICD	Notch intracellular domain
NLS	Nuclear localization signal
NRR	Negative regulatory region
NTR	Notch transgenic reporter
ONL	Outer nuclear layer
OPL	Outer plexiform layer
Otx2	Orthodenticle homeobox 2
P1.5	Postnatal day 1.5
PEST	Proline, glutamic acid, serine, threonine-rich motif
Pofut1	Protein *0*-fucosyltransferase 1
PTB	phosphotyrosine-binding domain
RAM	RBPJ/CBF1 association module
RBPJ	Recombination signal-binding protein 1
RPE	Retinal pigmented epithelium
RT+	Reverse transcriptase positive

RT-	Reverse transcriptase negative
RT-PCR	Reverse transcriptase polymerase chain reaction
S1/2/3	Site 1/2/3
SHH	Sonic hedgehog
Skip	Ski-interacting protien
SMRT	Nuclear receptor corepressor 2
SVZ	Subventricular Zone
TMD	Transmembrane domain
VZ	Ventricular Zone

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1. Introduction

The Notch signalling pathway is essential to metazoan development. It has been implicated in lateral inhibition for its role in inhibiting differentiation of certain cell types and in cell fate determination in developing systems. Recent studies have implicated a role for Notch in the mature nervous system. In this study we sought to explore a role for Notch signalling in the adult retina by examining mRNA expression and the cellular localization of active Notch signalling.

In this introduction I will provide background into the discovery and molecular basis of Notch signalling as well as its importance in human disease. I will then explore some of the regulatory mechanisms of this pathway. Subsequently, I will discuss the role of Notch signalling in the nervous system, both in development and in the adult. Finally I will summarize what is known about Notch's role in the retina.

1.1 Discovery and conservation of the Notch signalling pathway

The Notch mutation was first genetically described by Morgan (1917) when he identified the inherited alleles of notched wings in mutant *Drosophila*. Genetic analysis revealed that the "notching" was due to mutations in a gene with a role in determining the epidermal wing margin in *Drosophila*. Notch was later implicated in neurogenic development as fly mutants for Notch display a "neurogenic" phenotype, meaning that they have a larger population of neurons compared to wildtype flies (Lehmann et al., 1983; Poulson, 1945). This pathway is now known to be one of the key signalling pathways mediated by cell-to-cell communication and effects to cell-fate determination in a multitude of cellular niches. Notch plays a role in a variety of cellular contexts including nervous system (Yoon and Gaiano, 2005), heart (High and Epstein, 2008), and pancreatic (Apelqvist et al., 1999) development, intestine development and progenitor proliferation (Fre et al., 2005), somitogenesis (Barrantes et al., 1999), angiogenesis (Phng and Gerhardt, 2009), as well as a role in homatopoietic stem cell maintenance (Duncan et al., 2005)

The Notch signalling pathway is highly conserved and found in all metazoans (Gazave et al., 2009). Sequence analysis has shown homologous receptors for the signalling pathway in all metazoan species. *Drosophila* Notch, dNotch, was the first Notch receptor characterized, and sequence analysis has revealed two nematode paralogs, *Caenorhabditis elegans* LIN-12 and GLP-1 (Lambie and Kimble, 1991). Mammals have four Notch paralogs, Notch1-4. The canonical Delta/Serrate/Lag2 (DSL) ligands are responsible for the majority of Notch signalling. The two *D. melanogaster* ligands dSerrate and dDelta, have several mammalian orthalogs including the Serrate-like ligands Jagged1and Jagged2, and the Delta-like ligands Delta-like ligand1, Delta-like ligand3, and Delta-like ligand4. *C. elegans* ligand paralogs include the DSL only ligands lacking a delta and OSM-11-like (DOS) motif cLag-2, cARG-1, cAPX-1, and cDSL1-7, as well as DOS co-ligands DOS1-3, OSM7 and OSM11.

1.2 Mammalian Notch receptors and ligands

Mammalian Notch receptors are a type-one single transmembrane receptor containing an intracellular and extracellular domain (Figure 1A). The extracellular domain contains 29-36 epidermal growth factor (EGF) repeats, the number of which varies between the four paralogs. These regions are largely responsible for the interaction between the receptor and ligand (Rebay et al., 1991). Also in the extracellular domain is the negative regulatory region (NRR) which includes 3 Lin12-Notch repeats (LNR) and a heterodimerization domain. The NRR prevents proteolytic cleavage in the absence of ligand binding (Sanchez-Irizarry et al., 2004) by sterically inhibiting enzyme access to the cleavage site (Gordon et al., 2007).

An unstructured loop in the heterodimerization domain is the location of the site one (S1) cleavage where the single precursor protein is cleaved at the trans-Golgi apparatus into the Notch extracellular domain (NECD) and another subunit containing the transmembrane domain (TMD) and the Notch intracellular domain (NICD) (Logeat et al., 1998). These two subunits are non-covalently associated through a calcium mediated

interaction involving the LNR (Rand et al., 2000). The mature receptor is inserted into the membrane following the S1 cleavage and heterodimerization (Sanchez-Irizarry et al., 2004).

Within the intracellular domain, the RBPJ/CBF1 association module (RAM), along with seven ankyrin (ANK) repeats, are responsible for the interaction between the NICD and the transcriptional coactivator CBF1 in the nucleus (Tamura et al., 1995). The ANK repeats are flanked by two nuclear localization signals (NLS). The innermost region contains a proline, glutamic acid, serine, threonine-rich motif (PEST). This motif contains residues that are ubiquitination sites important for degradation and therefore controlling the half-life of the NICD (Fryer et al., 2004).

Like the receptors, canonical Notch ligands are also type-1 transmembrane proteins (Figure 1B). As previously noted, the mammalian Notch ligands can be broken down into two classes, Serrate-like (Jagged1 and Jagged2) and Delta-like1, 3, and 4. All mammalian Notch ligands contain a DSL motif at the N-terminus made up of EGF repeats necessary for binding to the Notch receptor (Shimizu et al., 1999). The extracellular domain also contains multiple EGF repeats independent of the DSL. The spacing and number of these EGF repeats differs between ligands and contributes to the interaction with the Notch receptors (Rebay et al., 1991). Interestingly, Jagged1 and Jagged2 contain roughly double the number of EGF repeats as the Delta-like like ligands (Weinmaster, 1997). Jagged ligands contain a cysteine-rich motif, which may help in forming cell aggregates between Notch and Jagged expressing cells (Rebay et al., 1991). Jagged1, Jagged2, and Delta-like ligand1 also contain a DOS motif, which may act along with the DSL domain to mediate activation of Notch signalling (Komatsu et al., 2008). The intracellular domain of Notch ligands have not been as well studied, but appear to be necessary for ligand signalling activity (Sun and Artavanis-Tsakonas, 1996; Heuss et al., 2008) as well as ligand trafficking (Heuss et al., 2008).

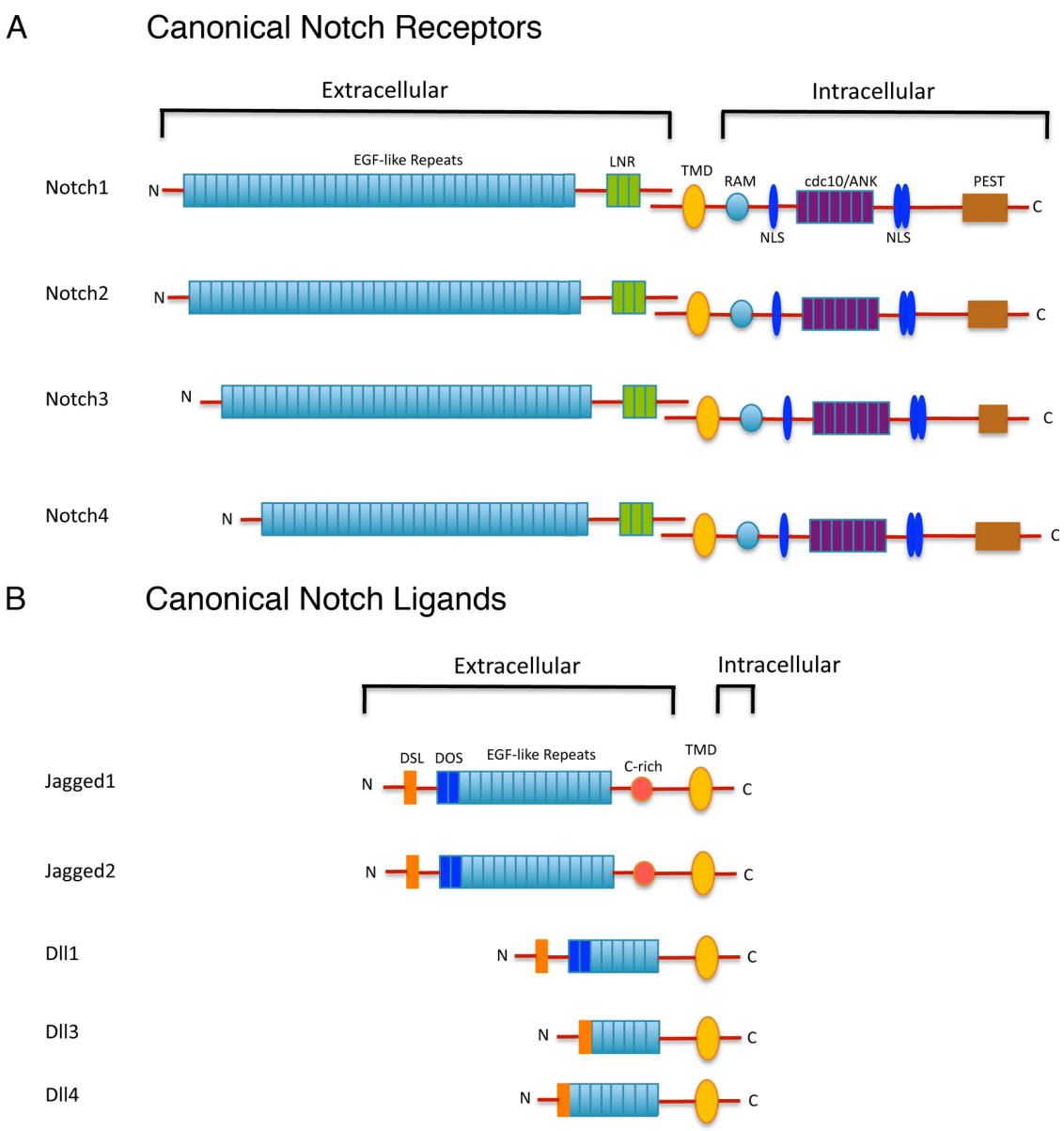

Figure 1. Protein structure of canonical mammalian Notch receptors and ligands. (A) The mammalian Notch receptors. Notch1-4 differ mainly in their extracellular domain where they vary in the number and composition of epidermal growth factor (EGF)-like repeats which is the interaction site with the Notch ligands. Also in the extracellular domain is the Lin12-Notch repeats (LNR), which is part of the negative regulatory region which acts to inhibit erroneous receptor activation. The extracellular and transmembrane/intracellular protein fragments are attached non-covalently though a calcium dependent interaction. The transmembrane domain (TMD) is followed by the RBPJ/CBF1-association module (RAM) which is the site of the intracellular domain's CBF1 interaction in the nucleus. A single nuclear localization sequence

(NLS) and a bipartite NLS flank a series of ankyrin (ANK) repeats. Near the C-terminus is a proline/glutamic acid/serine/threonin-rich (PEST) motif which contains degradations signals that limit the stability of the activated receptor. **(B)** The mammalian Notch ligands. On their extracellular region, Jagged1 and Jagged2 contain a Delta/Serrate/Lag-2 (DSL) motif, followed by specialized EGF repeats called Delta and OSM-11-like (DOS) motif, and a series of EGF repeats, all of which have roles in receptor interactions. Both Jagged receptors also include a cysteine (c)-rich domain in the extracellular region. All delta-like ligands are lacking a c-rich domain and have a smaller number of EGF repeats than the Jagged ligands. Both Delta-like ligand3 and Delta-like ligand4 lack a DOS domain in addition to the c-rich domain.

1.3 The Notch signalling pathway

Notch signalling is a cell-to-cell signalling pathway (Figure 2). The signal cascade begins when the ligand and receptor on neighbouring cells interact, which is thought to be mediated through EGF-repeats on the receptor and EGF-repeats and the DSL domain of the ligands. This binding is thought to cause a conformational change in the receptor near the TMD which allows for the disintegrin metalloprotease ADAM to proteolyticly cleave the receptor at site 2 (S2). The entire extracellular domain except for a few amino acids stays associated with the ligand and is endocytosed into the ligand containing cell (Parks et al., 2000). The remaining Notch receptor is referred to as the Notch extracellular truncation (NEXT). NEXT is a substrate for γ-secretase at site 3 (S3) and then site 4 (S4) both within the TMD. The cleavages result in the free NICD which can localize to the nucleus, directed by the NLS. Here the NICD interacts with C-Promoter Binding Factor 1 (CBF1) also known as Recombination Signal-binding Protein 1 (RBPJ) in mammals, of the CSL family (CBF1 in veterbrates, Suppresser of Hairless in *Drosophila*, and Lag-1 in *C. elegans*) (Fortini and Artavanis-Tsakonas, 1994). In the absence of NICD, CBF1 acts as transcriptional co-repressor to inhibit the expression of Notch target genes. The transcriptional repressor complex includes Ski-interacting protein (Skip), nuclear receptor corepressor 2 (SMRT) and histone deacetylase (HDAC). The NICD associates with CBF1 through an interaction with the co-activator Mastermind (Mam). Together, these proteins recruit transcription factors and histone

acetyltransferases including p300/Creb binding protein (CBP) to promote the transcription of Notch target genes.

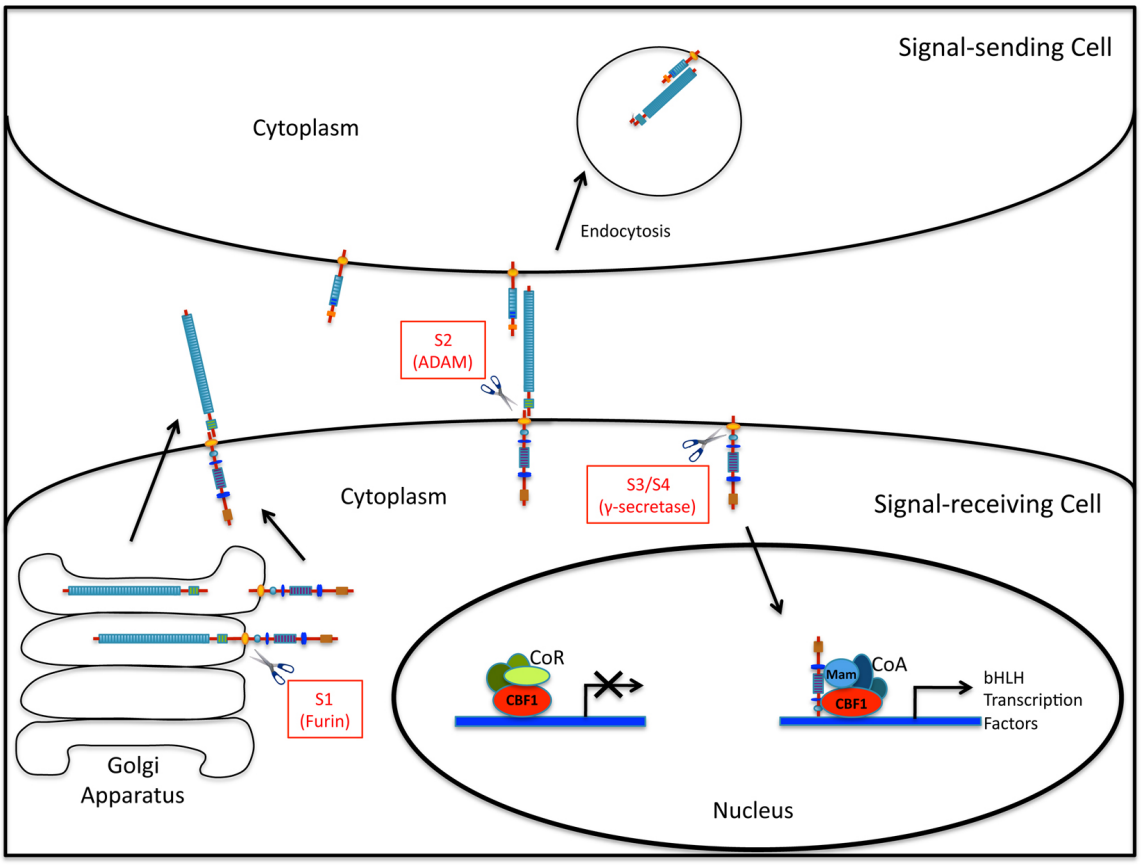

Figure 2. The canonical vertebrate Notch signalling pathway showing key components and proteolytic cleavages (as indicated by scissors). The Notch receptor first undergoes a site 1 (S1) proteolytic cleavage in the Golgi apparatus where the single polypeptide is cleaved by a furin-like protease and are then non-covalently linked to form the mature Notch heterodimer. The mature receptor is then translocated to the plasma membrane. When the Notch receptor and ligand interact through their extracellular epidermal growth factor-like repeats a conformational change occurs in the Notch receptor allowing the site 2 (S2) cleavage to occur by an ADAM metaloprotease, releasing the Notch extracellular domain from the receptor. The extracellular domain is endocytosed along with the ligand into the signaling cell. The S2 cleavage allows γ-secretase to access the site 3 (S3) and then site 4 (S4) cleavage sites. This final proteolytic cleavage results in the release of the Notch intracellular domain (NICD) which translocates to the nucleus of the signal receiving cell. Here, in the absence of NICD, C-promoter binding factor 1 (CBF1) along with transcriptional co-repressors (CoR) repress the expression of Notch target

genes. The NICD binds to CBF1 and Mastermind (Mam) and recruits a co-activator (CoA) complex which promotes the transcription of target genes including the basic helix-loop-helix (bHLH) family of transcription factors.

Notch target genes vary depending on the tissue and developmental contexts. In mammals this includes the basic helix-loop-helix (bHLH) family of transcription factors called Hairy/Enhancer of split (Hes) repressor (Hes1, Hes3, Hes5), and a closely related bHLH family HES-related repressor protein (HERP) (commonly referred to as Hey) which has recently been identified (Iso et al., 2003). These proteins act as transcriptional repressors to prevent the expression of pro-neuronal activator-type bHLH transcription factors such as Mash1, Math5, and Neurogenin, which act to promote neuronal differentiation. Notch may also regulate cell cycle progression by up-regulating the expression of cyclinD1 (Ronchini and Capobianco, 2001). Notch targets also include components of other signalling pathways including Sonic Hedgehog (Shh), Hippo, and Wnt (Li et al., 2012).

1.4 Notch Signalling and Human Disease

As Notch signalling is involved in so many biological contexts, and is highly conserved between species, it is not surprising that its function is critical to healthy development. Dysregulation of Notch signalling has been implicated in many human diseases. Due to its role in maintaining a progenitor-like population of cells, most Notch-related diseases manifest either as developmental defects when mutations in Notch pathway-related genes leads to a decrease in Notch signalling and the progenitor pool is depleted, or in cancer-like diseases, where Notch is constitutively activated and cells erroneously re-enter or fail to exit the cell cycle.

Alagille syndrome, a disorder affecting face, eye, skeleton, liver, and heart development is caused by various mutations to the human homolog of the ligand Jagged1 which prevent it from activating the Notch receptor (Li et al., 1997). Mutations in the EGF repeats of the human homolog of Delta-like ligand3, preventing Delta-like ligand3

interaction with Notch results in spondylocostal dysostosis, a disease resulting in skeletal defects due to improper somitogenesis (Bulman et al., 2000). Hajdu-Cheney syndrome, another disorder affecting skeletal development is caused by a truncation mutation in the Notch2 receptor (Isidor et al., 2011). Mutations in the EGF repeats of the Notch3 receptor making it non-functional have been linked to the adult-onset stroke disorder CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (Joutel et al., 1996). Notch mutations have also been linked to a large number of cancers, including a translocation mutation in the human form of Notch1 causing Notch to become constitutively active and act as an oncogene, leading to T-cell acute lymphoblastic leukemia (Ellisen et al., 1991). Notch has also been implicated in Alzheimer's disease where an upregulation of Notch1 has been seen the brain of sporadic cases (Berezovska et al*.,* 1998). However, in familial cases of Alzheimer's linked to mutations in presinilin-1, some of the disease phenotype can be rescued with activated Notch receptor, suggesting that in some cases, a downregulation of Notch signalling may be responsible for some of the disease characteristics (Veeraraghavalu et al., 2010).

1.5 Regulation of Notch signalling

Several components of the Notch signalling pathway are negatively regulated when Notch is actively signalling. Hes1 can bind directly to the N box sequences within its own promoter acting as a negative regulator of its own expression (Takebayashi et al., 1994). This leads to oscillations of Hes1 during active Notch signalling that in turn leads to oscillations in the proneuronal genes that Hes1 inhibits (Shimojo et al., 2008). Delta-like ligand1 is itself a pro-neuronal gene in addition to being a direct Notch ligand, and its expression is inhibited by Hes1 expression (Kageyama et al., 2008; Shimojo et al., 2008).

As CBF1 can interact with all four mammalian NICD proteins, much of the regulation of Notch occurs via direct modifications to the receptors and ligands. The EGF repeats can be modified by the addition of sugar moieties which can affect the ability of the Notch receptor and ligand to interact. A number of proteins can act as glycosyltransferases of the receptor and ligands. Protein *0*-fucosyltransferase-1 (Pofut1)

transfers *0*-fructose to specific EGF repeats following translation. This glycosylation step appears necessary for proper Notch signalling (Shi and Stanley, 2003; Stahl et al., 2008). There are three mammalian Fringe proteins: Lunatic, Manic, and Radical which add a N-acetylglucosamine (GlcNAc) to *O*-fructose on EGF repeats on the Notch receptor. This may affect the affinity of the Notch receptor for different ligands. For example, in *Drosophila* it has been demonstrated that this glycosylation decreases Notch binding with Serrate, while increasing the affinity for Delta (Xu et al., 2007).

RING-type E3 ubiquitin ligases such as Neuralized (Neur1 and Neur2 in mammals) and Mind Bomb (Mib1 and Mib2 in mammals), play an important role in Notch signalling through their interactions with Notch ligands. Both Neur and Mib interact with and directly ubiquitinate the intracellular domain of Notch ligands thereby enhancing their endocytosis and downregulating their membrane expression (Daskalaki et al., 2011). Since ligands expressed in the same cells as the receptor inhibit Notch signalling, endocytosis of the ligands enhances Notch signalling in these cells (Sakamoto et al., 2002). Although in *Drosophila* both Neur and Mib seem to be necessary for proper Notch signalling, in mammals it appears there may be redundant functions of the E3 ubiquitin ligases, and only Mib1 mutations display defects in Notch signalling (Koo et al., 2007). In addition to regulating ligand expression, Mib1 also acts to assist with the endocytosis of the Notch ligand along with the Notch extracellular domain of the receptor (Hansson et al., 2010) into the signal-sending cell, which must occur to relieve the LNR-mediated repression of the γ-secreatse cleavage in the signal-receiving cell (Parks et al., 2000).

E3 ubiquitin ligases are also implicated in regulating Notch receptor endocytosis. Deltex (Dx1-4 in mammals). Dx1 can interact with the ANK repeats on Notch1 and Notch2 (Matsuno et al., 1998). However, the relationship of Dx and the Notch signalling pathway is complex and although some studies have suggested Dx may negatively regulate Notch (Mukherjee et al., 2005), it is likely also playing a positive regulatory role in the Notch signalling pathway. In addition to its role in receptor endocytosis, Dx1 can also act downstream of receptor activation as a transcriptional co-activator with the NICD in the nucleus (Matsuno et al., 1998; Yamamoto et al., 2001).

Notch ligands can interact with Notch receptors not only to activate Notch signalling in neighbouring cells, but also within the same plasma membrane where they act as negative regulators through cis-inhibition. Over-expression of very high levels of ligand results in reduced Notch signalling (Sakamoto et al., 2002). The molecular mechanism underlying cis-inhibition is largely unknown, but it is likely due to an orientation-specific physical interaction between the receptor and ligand which prevents Notch activation (Cordle et al., 2008). This downregulation of Notch receptors in cells expressing high levels of ligand explains how cells become predominately signal-sending (ligand rich) or signal-receiving (receptor rich) (Sprinzak et al., 2010). Interestingly, Delta-like ligand3 is likely unable to trans-activate Notch because it lacks the necessary DOS domain and lacks an intracellular lysine residue which can therefore not be ubiquitinated and activated for signalling, however, Delta-like ligand3 participates in cis-inhibition (Ladi et al., 2005).

One form of Notch signalling regulation is especially important for asymmetric cell division – a cell division where the two daughter cells adopt different cell fates. Numb and its mouse paralog Numblike are membrane bound proteins which contains a phosphotyrosine-binding (PTB) domain, which can directly interact with ubiquinated Notch thereby signalling the receptor for endocytosis and preventing Notch activation (Guo et al., 1996; McGill and McGlade, 2003). Numb and numblike are often inherited asymmetrically in dividing cells in the developing cortex and retina (Rhyu et al., 1994). When this occurs, the cells that inherit Numb/numblike undergo differentiation, whereas the cells that do not inherit Numb continue to undergo Notch signalling and become a self renewing daughter cell (Del Bene et al., 2008; Kechad et al., 2012). Mib, the E3 ubiquitin ligase discussed above, can be inherited in a similar asymmetric manner, and by promoting the endocytosis of ligands, can thereby promote Notch signalling in the cell containing Mib (Dong et al., 2012).

1.6 Notch in the nervous system

Notch was first implicated in neuronal development in *Drosophila* where Notch mutants display a phenotype in which too many neuroblasts develop at the expense of the ectoderm (Lehmann et al., 1983). This finding helped establish the dogma for Notch's role in lateral-inhibition – inhibiting one cell fate while allowing the cell to differentiate into another cell-type - that would lay the ground-work for future research for decades to come. The role of Notch in the nervous system has since been extensively studied. In this section the general role for Notch in neural development will be briefly explored along with a few implications in adult neurogenesis before focusing in on Notch's role in the retina.

1.6.1 The roles of Notch in the brain

The role of Notch in lateral inhibition has been well characterized in mammals for its function in inhibiting neuronal development while promoting progenitor proliferation (Grandbarbe et al., 2003; Kawaguchi et al., 2008; Yang et al., 2010). However, Notch has been implicated not only in promoting progenitor maintenance, but also in promoting glial cell fates (Furukawa et al., 2000; Gaiano et al., 2000). Similarly, differential Notch signalling can also promote binary neuronal cell-fate choices where two neuronal types will terminally differentiate (reviewed by Cau and Blader, 2009).

Within the developing cortex a complex relationship between interkinetic nuclear migration (INM) and Notch signalling also plays and important role in cell fate determination (Dong et al., 2012). INM is a process by which the cell nucleus migrates from the apical to basal surfaces in the developing neuroblastic layer (NBL). The cell cycle is closely linked to this process with S-phase occurring at basal locations and cell divisions occurring mostly in the apical region (Dong et al., 2012). In vertebrates, all neuronal development in the presumptive neural retina as well as radial glial differentiation in the developing central nervous system occurs while cells undergo INM (Dong et al., 2012). Notch is important for the migration of cells in the subventricular

zone and when NICD is prematurely degraded, developing cortical neurons cannot migrate properly (Hashimoto-Torii et al., 2008). As such, relative levels of Notch singalling affect whether a given progenitor cell will continue to undergo the cell-cycle or differentiate.

Although Notch was originally characterized for its role in neuronal development, Notch is now implicated in many adult, postmitotic cells, particularly in the central nervous system. A role for Notch in post-mitotic neurons was first identified by Sestan and colleagues with their study of the role of Notch in regulating outgrowth and dendritic arborization of neurites in the cerebral cortex (Sestan et al., 1999). High levels of Notch inhibits outgrowth in a dose-dependent manner, whereas low levels of Notch enhance arborization. Notch1 activation also decreases the number of synapses in the juvenile visual cortex (Dahlhaus et al., 2008).

Notch has also been seen to play a role in synaptic plasticity the adult hippocampus. Notch seems to enhance long term potentiation (LTP), while impairing long-term depression (LTD) (Wang et al., 2004). However, some studies have found that high levels of Notch activation, specifically in pyramidal neurons, decreases LTP in layer 4 of the cortex (Dahlhaus et al., 2008). These conflicting results suggest either that there is a dosage dependent effect of Notch signalling, or that there is a cell type specific effect of Notch on synaptic plasticity. Notch1 and the ligand Jagged1 have been shown to be present at the synapse, and Notch1 signalling occurs in response to synaptic activity, while disruption of Notch1 disrupts both LTP and LTD in the adult hippocampus (Alberi et al., 2011). Therefore, although the specific role is unclear, Notch clearly plays a role in LTP and LTD in the adult brain.

1.6.2 Cell Fate Determination in the Retina

Cell specific differentiation in the developing retina has been extensively studied. In the mouse, although eye development begins as early as gastrulation, neuronal cell birth in the retina doesn't begin until embryonic day 9.5 (E9.5) when the first retinal

progenitors appear. Differentiation is complete by postnatal day 14 (P14) (Young, 1985; Ohsawa and Kageyama, 2008).

In the retina, cell birth is defined as the cell's exit from the cell cycle after its terminal mitosis (Ohsawa and Kageyama, 2008). The common assumption is that once these cells exit the cell cycle they start on a developmental pathway towards a certain cell type. However, it is also known that once these cells complete their terminal mitosis their cell fate can still be altered when extrinsic factors are applied (Turner and Cepko, 1987; Brzezinski et al., 2010). This fate switch seems to be dependent on the developmental time-point of differentiation of the progenitor cells. As such, progenitors seem to pass through phases of competence in which they are responsive to extrinsic cues which can modify their cell fate to a limited extent (Belliveau et al., 2000).

The birth of retinal cell types is temporally ordered. Early born cell types (ganglion, cone photoreceptor, most horizontal and some amacrine cells) are born predominately during embryogenesis, whereas late born cell types (bipolar, Müller glia, and some amacrine cells), are born predominately postnatally. Rod photoreceptor birth spans almost the entire period of retinogenesis beginning around E13, peaking at P2, and finishing with the other late-born cell types around P14 (reviewed by Marquardt and Gruss, 2002; Young1985).

As in the rest of the nervous system, Notch plays a critical role in maintaining a pool of progenitor cells during retinal development (Artavanis-Tsakonas et al., 1999), but more recently Notch has been implicated in cell-fate specification (Furukawa et al., 2000; Jadhav et al., 2006a; Jadhav et al., 2006b; Yaron et al., 2006). As Notch is important in inhibiting differentiation and maintaining a progenitor state, it is not surprising that the role of Notch seems to be dependent on the developmental time point and the competency of the progenitor cells present at that time point. Due to the critical role of Notch signalling in development, global mutations in Notch1 or 2 lead to embryonic lethality (Swiatek et al., 1994; Hamada et al., 1999). Much of our knowledge of the role of Notch in retina development comes from studies of mice with retina-specific, loss-of-

function mutations for Notch receptors, ligands, or downstream signalling components such as CBF1.

As a key factor in the responsive element of the Notch signalling cascade, it is no surprise that retina-specific conditional CBF1 mutant mice show defects in retinal development. CBF1 appears to have a role in cell fate determination and inhibits ganglion cell and cone photoreceptor development (Riesenberg et al., 2009). However, CBF1 can interact the NICD from all three Notch receptors found in the mammalian retina, and so these phenotypic results could not be interpreted based on the homolog of receptor that was activated.

Notch1 has been shown to play an important role in the photoreceptor neuronal differentiation. Conditional inhibition of Notch1 in the retina leads to smaller eyes with rosettes in the ONL, a decreased INL thickness (Jadhav et al., 2006b; Yaron et al., 2006) and a decreased optic nerve diameter (Jadhav et al., 2006b). These phenotypic differences can be explained by a quantified increase in photoreceptors in these mutant retinas at the expense of bipolar and ganglion cell types (Jadhav et al., 2006b; Yaron et al., 2006). However, the developmental time point of Notch inhibition plays a role in the types of photoreceptors that are affected. Conditional inhibition of Notch signalling in the embryonic retina leads to an increase in cone photoreceptors, whereas inhibition in early postnatal development leads to an increase in rod photoreceptors at the expense of other neuronal cell types (Jadhav et al., 2006b). This increase in photoreceptor differentiation can be explained at least in part by the repression of Hes1 on Orthodenticle homeobox 2 (Otx2), a gene critical for photoreceptor differentiation. Hes1 can bind directly to the embryonic enhancer region of this gene and suppress expression (Muranishi et al., 2011).

Notch may also have a role, not only in determining neuronal cell fates, but also in the neuron-glial cell-fate decision. Constitutive Notch1 activity did not affect the gene profile of early progenitor cells in the developing retina, but did lead to a retina that developed an increased number of Müller glia cells. These Müller glia cells also contain many progenitor cell markers, meaning these cells were more retinal stem-cell like than

normally differentiated Müller glia (Jadhav et al., 2006a). Notch1 overexpression also leads to slightly smaller retinas but with an increased INL depth, and decreased photoreceptor production marked by a decreased ONL depth. (Jadhav et al., 2006a). There may also be a role for Notch signalling in the cell fate decision between bipolar cells and Müller glia. The proneuronal genes Mammalian achaete-scute homolog 1 and 3 (Mash1 and Mash3), which are downregulated in response to Notch signalling, have been shown to promote bipolar cell fate when they are co-expressed with Chx10. However, Mash1-null mutant mice show a decrease in the proportion of bipolar cells and an increase in Müller glia (Hatakeyama et al., 2001). Since Mash1 and/or Mash3 without Chx10 is not sufficient to promote bipolar cell differentiation, this suggests that Mash1 and 3 are necessary to promote neuronal differentiation, whereas Chx10 is necessary to specify cell fate.

The roles of Notch2 and Notch3 have been less well studied than Notch1, however, both are present in the retina during embryonic and early postnatal development (Lindsell et al., 1996; Bao and Cepko, 1997; Zhu et al., 2013). A Notch3 gene trap mutant allele revealed a role for Notch3 in suppressing ganglion cell fate, but unlike Notch1 did not play a role in photoreceptor differentiation (Riesenberg et al., 2009). Furthermore, in many patients with CADASIL, which results from a mutation in Notch3, retinal dysfunction and abnormal electroretinogram responses have been observed although defects are likely due to impairments in retinal vascularisation instead of retinal cell fate determination (Parisi et al., 2003). No conditional Notch2 studies have been reported, but this is probably due to the fact that Notch2 was only recently reported to be present in the postnatal retina during the time of cell fate determination in the retina (Roesch et al., 2008; Zhu et al., 2013).

As in the ventricular zone of the developing nervous system, progenitor cells in the retina also undergo INM (Baye and Link, 2007). These cells migrate in relation to their cell cycle, dividing at the apical region of the retina closest to the retina pigmented epithelium, and undergo DNA synthesis at the basal region of the NBL (reviewed by Baye and Link, 2008). In this region of the developing retina, Notch signalling is

predominately activated on the apical side and proper INM through this gradient is thought to be necessary for proliferation of progenitor cells to continue and for proper cell fate determination in the mature retina (Del Bene et al., 2008).

Numb inhibits Notch signalling and is asymmetrically inherited by one of the two daughter cells following division. This is necessary for asymmetric terminal differentiation in the retina where each daughter cell adopts a different cell fate (Kechad et al., 2012). This may be important in later retinal development and may affect the fate choice between photoreceptors and either bipolar or Müller glia differentiation (Kechad et al., 2012).

1.6.3 Expression patterns in the retina

Although knockout studies have allowed for the characterization of some of the roles of Notch signalling in the retina, determining the location of the Notch receptors and ligands has proven problematic for researchers. Antibodies for downstream bHLH transcription factors are often used as an indicator of Notch signalling (Hojo et al., 2000; Nelson et al., 2006; Del Bene et al., 2008; Riesenberg et al., 2009; Muranishi et al., 2011; Chiodini et al., 2013), however few antibodies for Notch receptors and ligands are available and many require enzymatic amplification in order to visualize any antibody localization. Another confounding factor is that although antibodies have been developed for the activated NICD, small amounts of NICD, below the level of detection by immunohistochemistry, are capable of activating the Notch target genes (Schroeter et al., 1998; Struhl and Adachi, 1998).

Two parallel studies documenting mRNA expression in the developing retina were published in the late 1990s (Lindsell et al., 1996; Bao and Cepko, 1997). *In situ* hybridization studies performed on the rat revealed mRNA for *Notch1* and the ligands *Jagged* and *Delta-like ligand 1* in the ventricular zone of the embryonic and early postnatal neural retina up to postnatal day 5 (P5) (Bao and Cepko, 1997). Furthermore, the expression of the ligands and receptors were largely non-overlapping and

complementary as development progressed (Lindsell et al., 1996). *Notch2* mRNA was detected in non-neuronal tissues including the optic cup, ciliary body, retinal pigmented epithelium (RPE), and lens (Lindsell et al., 1996; Bao and Cepko, 1997). *Notch3* mRNA was also identified throughout the retina, lens and ciliary margin of the embryonic rat eye and this expression closely mirrored the expression of *Jagged* (Lindsell et al., 1996). By P5, *Notch1* mRNA was detected throughout the ventricular zone of the retina, whereas *Delta* was expressed in a smaller subset of cells within this zone (Bao and Cepko, 1997). *Notch2* and *Jagged* were restricted to the ciliary body (Bao and Cepko, 1997). By P27, no *Notch1* mRNA was detected (Bao and Cepko, 1997).

In recent years, the development of Notch antibodies has led to further localization studies of Notch. Unlike *in situ* hybridization data previously published, our lab was very recently able to detect Notch2 in the presumptive neural retina and lens at E9.5, after which levels decrease and become restricted to the RPE and peripheral retina (Zhu et al., 2013). Although this seems to contradict previously published data (Lindsell et al., 1996; Bao and Cepko, 1997), it has been suggested that the low level of Notch2 expression in the retina may be below the level of detection by *in situ* hybridization (Zhu et al., 2013). Notch2 was also detected using immunohistochemistry in a subset of Müller glia cells in the early postnatal and adult retina (Zhu et al., 2013).

The expression of Notch signalling components in the adult retina has been somewhat controversial. In 1995, Ahmed and colleagues reported *Notch1* mRNA using RT-PCR and *in situ* hybridization and also detected Notch1 using immunohistochemistry enhanced by immunoperoxidase in a subset of cells within the inner nuclear layer and the ganglion cell layer of the adult rat retina (Ahmad et al., 1995). More recently, Notch signalling components, including *Notch1* and *Notch2* were detected in Müller glia cells in the mature mouse retina using single cell RT-PCR and microarray analysis (Roesch et al., 2008). Notch2 has also been detected using immunohistochemistry in Müller cells in the adult retina (Zhu et al., 2013).

1.7 Notch reporter transgenic mouse lines

In an effort to characterize endogenous Notch signalling activity, several Notch signalling reporter transgenic mouse lines have recently been generated. The Notch Transgenic Reporter (NTR) mouse line (Duncan et al., 2005; Mizutani et al., 2007) and the CBFRE:H2B-Venus mouse line (Nowotschin et al., 2013) both contain transgenes designed to report canonical Notch signalling (Figure 3). Both transgenes encode a fluorescent reporter, which is driven by a four copies of a CBF1 responsive element (CBFRE) upstream of a basal SV40 promoter. When Notch signalling occurs, NICD (from any of the Notch1-4), associates with CBF1 bound to the responsive element and promote transcription of the reporter.

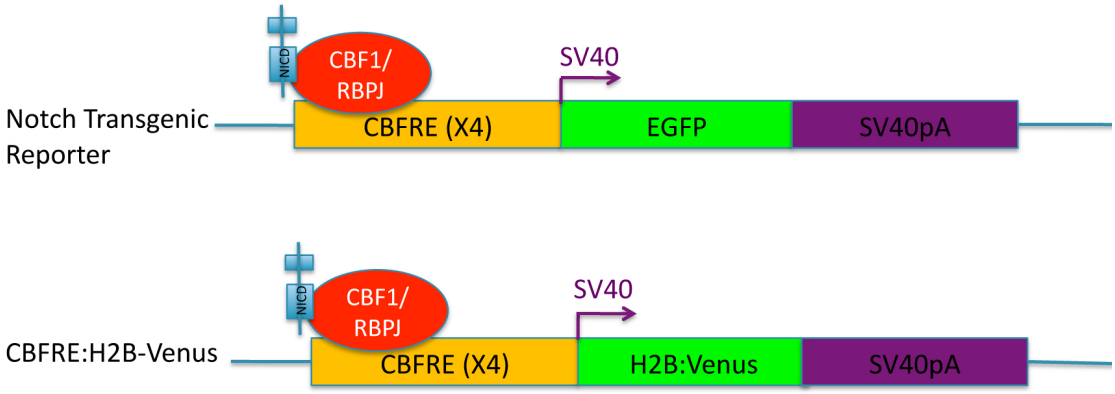

Figure 3. Transgene structure for the Notch signalling reporter mouse lines. Both transgenes contain 4 CBF1-responsive elements (CBFRE) upstream of a SV40 minimal promoter. When NICD is present, it can interact with the transgene via CBF1 to promote the expression of a fluorescent reporter. The reporter is flanked by an SV40 polyadenylation (pA) sequence. The Notch Transgenic Reporter transgene contains enhanced green fluorescent protein (EGFP) and CBFRE: H2B-Venus contains nuclear localized histone2B (H2B):Venus as its fluoresce reporter.

Enhanced green fluorescent protein (EGFP) levels produced by the NTR mouse line are diffuse and immunohistochemistry for EGFP must be used to detect low levels of fluorescent reporter in this mouse line. This Notch reporter has previously been reported in the E14.5 retina (Mizutani et al., 2007). Control experiments have also been performed on this mouse line including shRNA mediated knockdown of CBF1 in ventral telencephalic cells which led to a reduction in EGFP. Similarly, retroviral transfection of

NICD1-3 in mouse embryonic fibroblasts resulted in an upregulation of EGFP signal (Mizutani et al., 2007).

The CBFRE-H2B:Venus transgene contains nuclear localized venus due to its fusion with histone 2B (H2B) (Nowotschin et al., 2013). The venus reporter is bright enough to be detected without immunohistochemistry.

1.8 Objectives

The Notch signalling pathway is traditionally thought to play a role in maintaining a pool of undifferentiated progenitor cells in the developing nervous system and in cell-fate determination during neuronal differentiation. A new and distinct role for Notch has also been discovered in the fully developed nervous system, specifically in the brain where it plays a role in neurogenesis and synaptic plasticity. More recently, studies have presented evidence that Notch receptors may be expressed in Müller glia in the adult retina (Roesch et al., 2008; Zhu et al., 2013), whereas another study characterized Notch receptors in several cell-types of the adult neural retina (Ahmed et al., 1995).

I hypothesized that in addition to its previously characterized role in the developing retina, Notch functions throughout adult life in the mature retina. The primary goals of this research were to resolve the expression pattern of Notch receptors and ligands in the adult neural retina, examine whether these components were actively signalling, and if so, in what cell populations. My first aim was to identify whether mRNA was present for components of the Notch signalling pathway. This was examined by looking at mRNA expression through the use of RT-PCR and *in situ* hybridization. The expression of *Notch* receptors and ligands, as well as the transcription factor *CBF1*, which mediates the binding of the activated NICD, were examined throughout development and in the adult retina. My second aim was to determine whether the Notch pathway components were actively signalling in the adult retina, and in which retinal cell-types this was occurring. To examine this, I made use of two transgenic mouse lines, both of which have been reported to express fluorescent protein in response to the binding of both CBF1 and

activated NICD. I analyzed retinas at various time points throughout development and in the adult retina using cell-type specific markers to determine which retinal cell-types were actively undergoing Notch signalling.

2. Materials and Methods

2.1 Animal Breeding

All experiments were carried out in accordance with guidelines set out by the University of Victoria Animal Care Committee and the Canadian Council of Animal Care. Inbred wildtype 129SJ/SvImJ mice were used for all RT-PCR and *in situ* hybridization experiments. Notch Transgenic Reporter (NTR) mice STOCK Tg(Cp-EGFP)25Gaia/J were obtained from The Jackson Laboratory (Bar Harbor, Maine). Jackson Laboratory subsequently reported that continuous inbreeding of the line lead to transgene silencing. As such, the line was maintained by out-breeding hemizygous NTR animals to wildtype 129SJ/SvImJ mice at every generation. The second Notch reporter strain – CBFRE:H2B-Venus - were obtained from Dr. Anna-Katerina Hadjantonakis at The Sloan-Kettering Institute. These mice were out-bred to wildtype CD1 mice at every generation. For timed pregnancies, male and female mice were placed together in a breeding cage in the evening. The following morning females were checked for sperm plugs. If the female was plug-positive, the male was immediately separated out from the female and this time point was embryonic day 0.5 (E0.5). If no plug was found, the pair of mice remained together and the female was checked every morning until a plug was found.

2.2 Genotyping

Ear biopsies from mice older than 10 days or tail biopsies of mice younger than 10 days old were obtained. Tissue was denatured in 75 μl of 50 mM NaOH, incubated at 95°C for 10 minutes. 25 μl of 0.5 M Tris pH 8.0 was added to each sample which was then shaken vigorously. Samples were centrifuged for 3 minutes at 12,000 RCF. The PCR reaction for NTR mice was set up as described in Table 1. The PCR reaction for the CBFRE:H2B-Venus mice was set up as described in Table 2. PCR reactions were run on a T3 Thermocycler (Biometra) for 90 s at 94°C, then 35 × (30 s 94°C, 60 s 60°C (58 °C

for CBFRE:H2B-Venus), 60 s 72°C) then 120 s 72°C, and held at 4°C. PCR products were run on a 1% agarose gel with SYBR safe (Invitrogen, Cat. No. 533102) at 120V and 400 mAmps for 25 minutes then visualized using a UV Transilluminator (UVP).

Table 1. PCR reaction for Notch Transgenic Reporter eGFP

Reagent	Volume per reaction (μl)
Distilled water	3.39
10X PCR Buffer (Invitrogen Cat. No. 18038-042)	1.20
25 mM $MgCl_2$ (Invitrogen Cat. No. 18038-042)	0.96
2.5 μM dNTPs (Invitrogen Cat. No. 10297-018)	0.96
20 μM GFP Forward Primer 5' – AAGTTCATCTGCACCACCG – 3'	0.6
20 μM GFP Reverse Primer 5' – TCCTTGAAGAAGATGGTGCG – 3'	0.6
20 μM Positive Control Forward Primer 5' – CTAGGCCACAGAATTGAAAGATCT – 3'	0.3
20 μM Positive Control Reverse Primer 5' – GTAGGTGGAAATTCTAGCATCATCC – 3'	0.3
60% sucrose with cresol red	1.66
5 U/ul TAQ DNA polymerase (Invitrogen Cat. No. 18038-042)	0.03
DNA	2.0

Table 2. PCR reaction for CBFRE:H2B-Venus GFP

Reagent	Volume per reaction (μl)
Distilled water	13
10X PCR Buffer	2.5
50mM MgCl	1
4mM dNTPs	1
10μM CBFRE Forward Primer 5' – GCTGATTAATCGAGATCTGGTG TAAACAC– 3'	0.5
10uM SV40 Reverse Primer 5' – GCGAGCTAGCCAGCTTTTTGCA	0.5

AAAGCCTAG– 3’	
60% Sucrose with cresol red	3
5 U/ul TAQ DNA polymerase	0.3
DNA	2

2.3 Reverse Transciption PCR

Mice older than P14 were euthanized by 0.1 ml sub-peritoneal injection of euthanyl followed by cervical dislocation, and mice younger than P14 were euthanized by rapid decapitation. Eyes were enucleated and placed into chilled 1X PBS. The cornea, lens, vitreal body, and sclera were removed. Care was taken to ensure as much RPE as possible was removed from the retina. Retinas were collected from three mice aged P1.5, P8.5, and P14.5, and two P40 mice. As a positive control for RPE, the sclera and RPE from two P40 mice were also collected.

Tissue was placed into 1 ml TRIzol reagent (Invitrogen, Cat. No. 15596026) in Precellys 24 tissue homogenizing CKMix tubes (Precellys, Bertin Technologies, Cat. No. 03961-1-009) on ice. Tissue homogenization was performed using a Precellys-24 homogenizer by two 10 second pulses at 5000 RPM. Samples were incubated at room temperature for 5 minutes. 0.2 ml of chloroform was added and samples were shaken vigorously for 15 seconds, then incubated at room temperature for an additional 2-3 minutes. Samples were centrifuged at 12 000 X G for 15 minutes at 4 °C. The clear, upper aqueous phase was removed and placed into a clean tube. 0.5 ml of 100% isopropanol was added to each tube and samples were incubated at room temperature for 10 minutes. Tubes were centrifuged at 12 000 X G for 10 minutes at 4°C. The supernatant was removed and the pellet was washed with 75% ethanol, briefly vortexed, and centrifuged at 7500 X G for 5 minutes at 4°C. The supernatant was discarded and the RNA pellet was air dried for 5-10 minutes. RNA was resuspended in 50 μl of nuclease-free water (Ambion, Cat. No. 9937) and incubated at 60°C for 10 minutes. The concentration and purity of each sample measured on a UV spectrophotometer (Eppendorf BioPhotometer, Hamberg, Germany), and 500 ng of each sample was run on a 1.3% agarose gel at 120V for 30 minutes.

To make cDNA, 0.5 mg of RNA was placed in a tube and nuclease-free water was added to a final volume of 11.5 µl. 2 µl of 50 ng/ml cDNA poly A primer was added and tubes were heated at 65°C for 4 minutes then placed on ice. Duplicate tubes were prepared for each sample so that a reverse transcriptase (RT) negative control could be made for each tissue. A master mix of 1 µl 0.1M DTT (Invitrogen, Cat. No. Y00142), 1 µl 4mM dNTPs, 0.5 µl RNase inhibitor (Invitrogen, Cat. No. 15518-012) and 4 µl of 5x 1st strand cDNA synthesis buffer was prepared for each sample. 6.5 µl of the master mix was added to each RT negative (RT-) sample of RNA. 0.5 µl of SuperScript II RT (Invitrogen, Cat. No. 18064-014) per sample was added to the remaining master mix, and 7 µl of this mastermix was added to each RT positive (RT+) RNA sample. Tubes were gently mixed and incubated at 42°C for thirty minutes then placed on ice.

Reverse transcriptase PCR was performed on each sample for the Notch receptors Notch1, Notch2, Notch3, Notch4, and the Notch ligands Jagged 1, Jagged 2, Delta-like 1, Delta-like 3, and Delta-like 4. Table 3 shows a complete list of the primer sequences used for each gene. All primers were designed so that the amplified PCR product would span at least one intron. A primer mix containing 20 µM of forward and reverse primer for each gene was created. For each gene, a master mix containing 19.5 µl nuclease-free water, 2.5 µl 10X PCR buffer, 0.75 µl 50 mM MgCl, 0.5 µl 10 mM dNTPs, 1 µl primer mix, 0.2 µL 5 U/ul TAQ DNA polymerase and 2 µl DNA per sample was made. PCR reactions were run on a T3 Thermocycler for 2 minutes at 94°C, then 29 × (30 s 94°C, 30 s 60°C (62°C for Notch3), 60 s 72°C) then 10 minutes 72°C, and held at 4°C. 25 cycles instead of 29 were used for CBF1 and RPE65 primers. Each sample was mixed with 2 µl 30% sucrose/cresol red and loaded onto a 1% agarose gel with SYBR safe at 120V and 400 mAmps for 35 minutes then visualized using a UV transilluminator.

Table 3. Reverse transcriptase PCR primers

Gene	Sequence of Forward (F) and Reverse (R) Primers (5'→3')	Design Credit
Notch 1	F5488N1 – GGACGACAATCAGAACGAGTGG	Dr. M Zhu
	R5728N1 - CACTGTTGCCTGTCTCAAGGCC	
Notch 2	F5700N2 – GGAGGCAGCTCAGACCTGAGTG	Dr. M Zhu
	R5954N2 - CAGATCGGTTACACGGTTGCGG	
Notch 3	Notch3F1 - TAATACGACTCACTATAGGGAGAACAAGGACATAGCTGCTGGC	K Ronellenfitch
	Notch3R2 - ATTTAGGTGACACTATAGAAGAGAGGATCAGTGCAGTAGAGC	
Notch 4	F4676N4 – GAGGACAACATTGGTCTTAAGG	Dr. M Zhu
	R4984N4 - CCCTCTATCCAGCAGTGGTTCC	
Jagged 1	Jag1F – GTTTCGCCTGGCCGAGGTCC	Dr. E Star
	Jag1R - GCCCTGCCAACCGTACTGGC	
Jagged 2	Jag2RT-F – ATCAACCCCGAGGACCGCTGG	Dr. E Star
	Jag2RT-R - GCTGCACCTGCACTCCCCAG	
Delta-like ligand1	Dll1F – GACCCCGCCTTCAGCAACCC	Dr. E Star
	Dll1R - GTCCCCGCAGGTGAAGTGGC	
Delta-like ligand3	Dll3F – ATGCTTGGCCGGGCACCTTC	Dr. E Star
	Dll3R - TCCCATGTGCCTGTGCGCTG	
Delta-like ligand 4	Del4RT-F – TGGCACACACCGGGAGACGAC	Dr. E Star
	Del4RT-R - GCGACCCTGCCAACCTGGAC	
MitF	m_mitf_f – GGAACAGCAACGAGCTAAGG	Dr. B Chow
	m_mitf_r2 - ATCTTCCTGGGGATGCTGTAGG	
HPRT	HPRT-F – CACAGGACTAGAACACCTGC	Dr. M Zhu
	HPRT-R - TTGCTGGTGAAAAGGACCTC	
CBF1 (Probe B)	RBPJF4 – TAATACGACTCACTATAGGGAGAATAATCCAATTTCAGGCCACTCC	K Ronellenfitch
	RBPJR2 – ATTTAGGTGACACTATAGAAGAGTTCAAGTCAGTTTCAGTCCCAGC	

2.4 Histological Preparation

Mice older than P14 were euthanized using a 0.1 ml sub-peritoneal injection of euthanyl, followed by cervical dislocation. Mice younger than P14 were euthanized by rapid decapitation. Eyes were quickly enucleated and placed into chilled 1X phosphate buffered saline (PBS) pH 7. The cornea, lens, and vitreous body were removed. The remaining eye cup and retina was fixed in 4% PFA pH 7 (Electron Microscopy Sciences, Cat. No. 157-8) at room temperature for 25 minutes, or overnight at 4°C. Following fixation, samples were rinsed 3 times in 1X PBS, then submerged in 30% sucrose in 1X PBS overnight at 4°C. Samples were rinsed 3 times in Tissue Tek O.C.T. Compound (Sakura Finetek, Cat. No. 4583) and embedded and rapidly frozen using dry ice in Tissue-Tek O.C.T. Compound. Sections were cut on a cryostat (Leica CM1860UV) to a thickness of 14 μM and mounted on saline adhesive coated slides (Newcomer Supply, Cat. No. 5070).

For embryo collection, pregnant dams were euthanized as described above by euthanyl followed by cervical dislocation. An incision was made through the abdominal wall, the uterus was removed using forceps and embryos were isolated. The embryo heads were decapitated and fixed overnight, submerged in sucrose, and rinsed as described above. When embedding in O.C.T. Compound, heads were oriented nose down so that all eyes would be in the same plane when sectioning and tissue was frozen and sliced as described above. Slides were stored at -20°C.

2.5 *In Situ* Hybridization

2.5.1 Probe Preparation

Using Conserved Domain (CD) coding sequences for the Notch receptors, Notch ligands, and CBF1, forward and reverse primers were designed using Amplify 3X (W.

Engels, University of Wisconsin-Madison). mRNA target sequences were chosen to be approximately 400-700 bps in size and to span at least one intron in order to easily discern desired products from DNA amplification. Consensus sequences for T7 and SP6 polymerases were added to the 5' end of all forward and reverse primers respectively (Table 4). Primer sequences were examined on BLAST (National Centre of Biotechnology Information) to ensure specificity for the gene of interest.

Table 4. In situ probe sequences

Gene	Primer Sequence
CBF1 (probe A)	RBPJF1 – TAATACGACTCACTATAGGGAGATCACTGAAGAATGCTGACTTGTGC
	RBPJR1 – ATTTAGGTGACACTATAGAAGAGTAAGTTCAAGCATTGCTACGTCC
CBF1 (probe B)	RBPJF4 – TAATACGACTCACTATAGGGAGAATAATCCAATTTCAGGCCACTCC
	RBPJR2 – ATTTAGGTGACACTATAGAAGAGTTCAAGTCAGTTTCAGTCCCAGC
Notch1	Notch1F4 – TAATACGACTCACTATAGGGAGAACTGCCATGGCCAGAATTGC
	Notch1R3 – ATTTAGGTGACACTATAGAAGAGTCTGGCGAGTCCACAATGC
Notch2 (probe A)	Notch2F7 – TAATACGACTCACTATAGGGAGATGGACGTGAATGTCCGAGGC
	Notch2R7 – ATTTAGGTGACACTATAGAAGAGTGGCCGCTTCATAACTTCC
Notch2 (probe B)	Notch2F2 – TAATACGACTCACTATAGGGAGATCACACGTATGTCTCCCATGC
	Notch2R3 – ATTTAGGTGACACTATAGAAGAGGATAAGCTGGAAAGTCACGAT
Notch3	Notch3F2 – TAATACGACTCACTATAGGGAGAACGTGGAGGCTACCTTGGC
	Notch3R3 – ATTTAGGTGACACTATAGAAGAGACCGGTGACAGTGTGACAGAGC
Jagged1	Jagged1F2 – TAATACGACTCACTATAGGGAGACGGTCCTGGATGACCAGTGT
	Jagged1R3 – ATTTAGGTGACACTATAGAAGAGTGTCACCAAGCAACAGACC

Jagged2	Jagged2F1 – TAATACGACTCACTATAGGGAGAAGCTGCAGATCCGAGTACG
	Jagged2R1 – ATTTAGGTGACACTATAGAAGAGAGATGCACTCGAAGCCGTCC
Delta-like ligand1	Delta1F3 – TAATACGACTCACTATAGGGAGATATGGCAAGGTCTGTGAGC
	Delta1R3 – ATTTAGGTGACACTATAGAAGAGAGCAGCAGCAGGAGGACAAGC
Delta-like ligand4	Delta4F1 – TAATACGACTCACTATAGGGAGATACTCTTACCGGGTCATCTGC
	Delta4R1 - ATTTAGGTGACACTATAGAAGAGTGGCCCACTGTTGGAACACG

RNA was extracted and cDNA was synthesized from adult whole-eye samples as described in section 2.3 above. For each probe, a master mix containing 39 µl nuclease-free water, 5 µl 10X PCR buffer, 1.5 µl 50 mM MgCl, 1 µl 10 mM dNTPs, 1 µl primer mix (20 µM each forward and reverse primer), 0.4 µL 5 U/ul TAQ DNA polymerase and 3 µl whole eye cDNA per sample was made. Samples were run on a T3 Thermocycler for 2 minutes at 94°C, then 35 × (30 s 94°C, 30 s 60°C (62°C for Notch3), 60 s 72°C) then 10 minutes 72°C, and held at 4°C. PCR products were run on a 1.5% agarose gel for 35 minutes and then excised using a UV Transilluminator. Products were purified using a QIAquick Gel Extraction Kit (Qiagen, Cat. No. 29704). Briefly, gel products were dissolved in QG buffer at 50 °C, then precipitated using isopropanol. Samples were bound to and washed in a QIAquick column using a benchtop centrifuge, then precipitated using nuclease free water. Purity and concentration was determined using a UV spectrophotometer. To increase purity and concentration, a second PCR amplification was performed as stated above, using 3 µl of the product from the first round of each amplification as cDNA template. Samples were again gel purified, run on a UV spectrophotometer, and were run again on a 1.5% gel for 35 minutes to ensure the correct product size. A sample from each purified product was sequenced using both SP6 and T7 as primers by Eurofins Mwg/Operon. Sequence results were examined using BLAST to verify that the PCR products were correctly amplifying the genes region of interest.

Labelling reactions were set up for each probe with 2 µl 10X Dig RNA labelling mix (Roche, Cat No. 11 277 073 910), 2 µl 10X transcription buffer (NEB, Cat No. B9012S),

1 µl 0.75 M DTT, 1 µl RNase inhibitor, 0.2 µg DNA, and the mix was topped up to 29 µl with nuclease free water. 1 µl T7 (NEB, Cat No. M0251s) or SP6 (NEB, Cat No. M0207S) RNA polymerase was added to each sense and antisense probe mix respectively. Samples were incubated at 37°C for 1 hour, another 1 µL RNA polymerase was added, and samples were incubated for an additional hour at 37°C. Probes were then eluted with LiCl and ethanol, and re-suspended in 100µl nuclease-free water. Finally, probes were run on a 1.5% gel for 35 minutes to evaluate probe synthesis.

2.5.2 *In Situ* Hybridization

Slides were thawed for 30 minutes then heated to 50°C for 20 minutes. Slides were then post-fixed using 4% PFA in 1X PBS for 20 minutes, then washed twice in 1X PBS for 5 minutes. Tissue was permeablized for 2-3 minutes in TE/Proteinase K (50 mM Tris-HCL pH8, 5 mM EDTA pH8, 0.5 mg/ml Proteinase K (Ambion, Cat No. 25530-015)). Permeabilization time was optimized for each tissue type to ensure maximum permeability without affecting the integrity of the section throughout the procedure. Slides were rinsed for 5 minutes in 1X PBS, fixed again with 4% PFA in PBS for 5 minutes, then rinsed in distilled water for 1 minute. The slides were acetylated with TEA/AA (135 mM Triethanolamine, 22.4 mM NaOH, 0.3% acetic anhydride) for 10 minutes, and finally slides were rinsed 3 times in 1X PBS for 5 minutes.

Tissue was pre-hybridized with hybridization solution (50% formamide, 5X SSC, 5X Denhardts (Invitrogen, Cat No. 750018), 0.25 mg/ml yeast tRNA (Invitrogen, Cat NO. 15401-029), and 0.5 mg/ml salmon sperm single stranded DNA (Invitrogen 15632-011)), overnight at 55°C.

For hybridization, 15 µl of probe was added to each slide mailer. Slides were added, enough hybridization solution was added to cover the tissue, and slides were incubated overnight at 55°C.

Slides were washed in 5X SSC for 15 minutes at 55°C, then in 0.2X SSC for 60 minutes at 62°C. Slides were rinsed in 1X RNA (0.1 M NaCl, 5 mM M Tris, 0.25 mM EDTA) for 10 minutes at 37°C, then 1X RNA with 20 μg/mL RNaseA for 30 minutes at 37°C, then once again in 1X RNA at 37°C for 5 minutes. Slides were rinsed in 2X SSC at 37°C for 10 minutes, and finally in 0.2X SSC at 37°C for 10 minutes.

For the antibody reaction, slides were first incubated at room temperature for 5 minutes with NT (0.1 M Tris pH7.5, 0.15 M NaCl), then blocked with 1% Blocking Reagent (Roche, Cat No. 11 096 176 001) in NT for 60 minutes at 37°C. Anti-Digoxigenin-AP Fab fragments (Roche, Cat No. 11 093 274 910) was diluted 1:250 in 1% Blocking Reagent in NT and incubated with slides at room temperature for 60 minutes. Slides were then rinsed with NT wash three times for 22 minutes at room temperature. Slides were rinsed in NTMT (0.1 M NaCl, 0.1 M Tris pH9.5, 50 mM MgCl, 0.1% Tween20) for 15 minutes at room temperature. A solution of 1 mg/ml NBT and 1 mg/ml BCIP (Invitrogen, Cat No. 00-2209) in NTMT was incubated at room temperature for 30 minutes and this mixture was then added to the slides and they were incubated overnight at room temperature.

To mount, slides were rinsed three times at room temperature with 1X PBS and fixed for 2 hours at room temperature with formaldehyde/1X MEM (3.7% formaldehyde, 0.1 M MOPS, 0.2 mM EGTA, 1 mM $MgSO_4$). Tissue was dehydrated in subsequent 30%, 70%, 90%, and 100% ethanol solutions, for 1 minute each. To reduce background, slides were incubated for 1 minute at room temperature with 1:1 xylene/100% ethanol, then twice in 100% xylene at room temperature for 2 minutes each. Slides were mounted with 1:1 xylene/Permount (Fisher, Cat No. SP15-500). Slides were imaged using DIC brightfield illumination on a Nikon inverted microscope (Nikon Eclipse TE2000-U, Japan), using either a 4X/NA0.13, or 10X/NA0.30 Nikon objective. ACT-2U imaging software was used to acquire images and they were optimized for brightness and contrast in Adobe Photoshop.

2.6 Immunohistochemistry

Slides were stored at -20°C. Prior to staining, slides were rehydrated in 3 washes of 1X PBS pH7.4, then permeabilized in 1% Triton X-100 in 1X PBS, pH 7.4 for 30 minutes. Slides were washed again 3 times in 1X PBS, then blocked for 30 minutes using 10% horse serum (Sigma, Cat. No. H0146). The primary antibodies were diluted (Table 5) in 1X PBS, pH 7.4 (Gibco, Cat. No. 10010-031) and incubated on the slides at 4°C overnight. Slides were then washed 3 times in 1X PBS pH 7.4 and secondary antibodies were diluted 1:1000 (Molecular Probes, 488, 555, or 647) and nuclear stain was diluted 1:2500 (DRAQ5, Enzo Life Sciences, Cat. No. BOS-889-001-R050), were applied and incubated overnight at 4°C. Slides were then washed 3 times in 1X PBS, mounted with Immumount (Thermo Scientific, Cat. No. 9990402), and coverslipped (Fisherbrand 24 X 50 X 1.5, Cat. No. 12-544-E).

Table 5. Primary antibodies used for immunohistochemistry

Antibody	Target	Species/Dilution	Company/Catalogue Number
Brn3b	Ganglion	Goat 1:100	Santa Cruz Sc-31989
Cabp5	Cone Type 3 OFF Bipolar Cone Type 5 ON Bipolar Rod ON Bipolar	Rabbit 1:500	Gift in kind from F. Haeseler
Calbindin	Horizontal Subset of amacrine	Rabbit 1:500	Sigma C2724
Chx10	All bipolar cells, subset of Müller glia	Sheep 1:500	Exalpha Biologicals X1180P
GFP	GFP	Goat 1:500	Rockland 600-102-215
PKCα	Rod ON Bipolar	Rabbit 1:5000	Sigma P4334
PKCα	Rod ON Bipolar	Mouse 1:500	Sigma P5704

Recoverin	Photoreceptors Cone Type 2 OFF Bipolar	Rabbit 1:2500	Chemicon AB5585
Syntaxin	Amacrine	Mouse 1:500	Sigma S0664

Slides were imaged using a confocal microscope (Nikon Eclipse TE2000-U, Japan), equipped with a 488 nm Ar lazer, a HeNe 543 nm and HeNe 633 nm laser. All fluorescent images were collected using a 20X/NA0.75 or 60X/NA1.49 Nikon objective. EZ-C1 3.60 imaging software was used to quire the image and Adobe Photoshop was used to adjust brightness and contrast.

2.7 Notch Knockdown

P0.5 and P1.5 CBFRE:H2B-Venus mice were euthanized by rapid decapitation. Heads were sprayed with 70% ethanol and transferred into a sterile laminar flow hood (Labcano, Kansas City). Eyes were enucleated and placed into warm 1X Hanks Balanced Salt Solution, calcium, magnesium, no phenol red (HBSS) (Gibco, Cat. No. 14025-092). Each retina was removed using a dissecting microscope (Nikon, SMZ645) and transferred single nucleopore 25MM, 0.2 μm pore, polycarbonate membranes (WHATMAN, Cat. No. 110606) in a 6-well culture dish, each well containing explant media [Neurobasal (Gibco, Cat. No. 21103-049) with 1X Glutamax supplement (Gibco, Cat. No. 35050061) and 1X B27 supplement (Gibco, Cat. No. 17504-044)]. Experimental wells also contained 10 μM N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Sigma, Cat. No. D5942) dissolved in DMSO (Sigma, Cat. No. D2650), while control wells contained the same volume of DMSO. DAPT has previously been shown to inhibit Notch signalling by inhibiting γ-secretase cleavage and thereby preventing NICD cleavage in neural tissue including retinas (Nelson et al., 2006). From each mouse, one retina was cultured in DAPT and one in DMSO control conditions for two or four days in a CO_2 incubator (Thermo Electron Corp, Marietta, Ohio) at 37°C. A 1 ml media change was performed every 24 hours using explant media freshly mixed with DAPT or DMSO.

1 ml was removed from the well, 1 ml of fresh media was added and 100 µl of freshly changed media from the well was added to the top of each explanted retina.

Following culturing, retinal explants were floated off of their filters, and fixed in chilled 4% PFA for 25 minutes at room temperature. Explants were then rinsed three times in 1X PBS pH7, and submerged overnight in 30% sucrose in 1X PBS. Explants were rinsed three times in Tissue Tek OCT and then rapidly frozen on dry ice. The blocks were sectioned at 14 µm on a cryostat and mounted on saline adhesive coated slides. Slides were rinsed three times in 1X PBS pH 7.4, permeablized in 1% Triton-100X for 30 minutes, rehydrated three times in 1X PBS then stained overnight with DRAQ5 (1:2500 dilution). Slides were rinsed three times in 1X PBS, then mounted with Immumount, and coverslipped. 3-4 representative images from one section of each retina were taken using the Nikon confocal microscope described above, using a 60X/NA1.49 Nikon objective, ensuring the same gain levels and pinhole sizes were used for all images. ImageJ (Schneider et al., 2012) was used to analyze the average venus fluorescence intensity within the retina for each image. Briefly, IDS files for the GFP channel in grayscale for each image were opened up in ImageJ using and ICD/IDS input/output plug-in. Colour/brightness was adjusted so that the image was in 12-bit format, to match the format the picture was taken in. A square was drawn around the total retina area in the image and the Measure tool was used to obtain the mean intensity value of the venus fluorescence for the selected area. Average mean values were pooled and averaged for DAPT and DMSO control retinas and the standard error was calculated. R version 3.0.0 (www.r-project.org) was used to determine if there was a statistical difference between mean average fluorescence intensities of venus reporter in DAPT and DMSO groups.

Chapter 3: Results

3.1 mRNA for Notch receptors, ligands, and signalling components are present throughout development.

As very little investigation has been done into the characterization and roles, if any, of Notch signalling in the adult retina, we sought to determine whether mRNA for the Notch signalling pathway components are present in the adult and throughout postnatal development. Through the use of reverse-transcriptase (RT)-PCR, and *in situ* hybridization we examined the developing late embryonic, early postnatal, and the adult retina for the expression of the receptors *Notch1* and *Notch2*, the ligands *Jagged1*, *Jagged2, Delta-like ligand 1, Delta-like ligand 4*, and the transcriptional coactivator *CBF1*.

3.1.1 Reverse Transcriptase-PCR detection of Notch and Notch signalling components throughout development.

RT-PCR was used to look for expression of the Notch signalling pathway throughout development. mRNA was isolated from retina samples at P1.5, P8.5, P14.5, and P40. mRNA was also isolated from a combined sample of sclera and retina pigmented epithelium (RPE) from adult eyes. Samples were amplified using PCR primers for *Notch* receptors, ligands, and *CBF1* the Notch transcriptional co-activator (Figure 4). Low PCR cycle repeats (25 cycles for *RPE65* and *CBF1*, and 29 cycles for all other primers) were used to obtain semi-quantitative sub-saturation level PCR products. *Notch1* mRNA was detected in the retina at all four time points although levels decreased slightly as development progresses. *Notch1* mRNA was also detected at low levels in the P40 RPE/sclera. *Notch2* mRNA was detected at all developmental time points and was enriched in the adult RPE/sclera. *Notch3* mRNA was detected in the retina at P1.5, P8.5, and P14.5, was downregulated at P40, and was detected in P40 RPE/sclera. *Jagged1* was detected in the retina at all time points, in the P40 RPE/sclera and was most strongly expressed at P14.5. *Jagged2* mRNA expression was detected in the retina at all time points and was also present in the RPE/sclera. *Delta-like ligand 1* mRNA expression in the retina was detected most strongly at P1.5 and expression decreased as development

progressed. *Delta-like ligand 1* was also present weakly in the P40 RPE/sclera. Similarly, *Delta-like ligand 4* mRNA expression was detected most strongly in the retina at P1.5 and decreased as the retina developed. The expression of *Delta-like ligand 4* mRNA in the P40 RPE/sclera was higher than levels seen in the adult retina. *CBF1* mRNA was detected most strongly at P1.5 and levels decreased as development progressed, with the weakest expression in the adult retina and P40 RPE/sclera. RPE65 is a marker specific to the pigmented epithelium and was used to assay for possible RPE contamination in the retinal sections. In contrast to it's strong expression in the RPE/sclera, *RPE65* mRNA was not detected at P1.5 and detected very weakly at P8.5, P14.5, and P40. The housekeeping gene *HPRT* was used as a mRNA loading control. *HPRT* mRNA expression was detected in all RT-positive samples at comparable levels. These results indicate that mRNA is present for the Notch receptors, ligands, and transcription co-activators throughout development and in the adult retina.

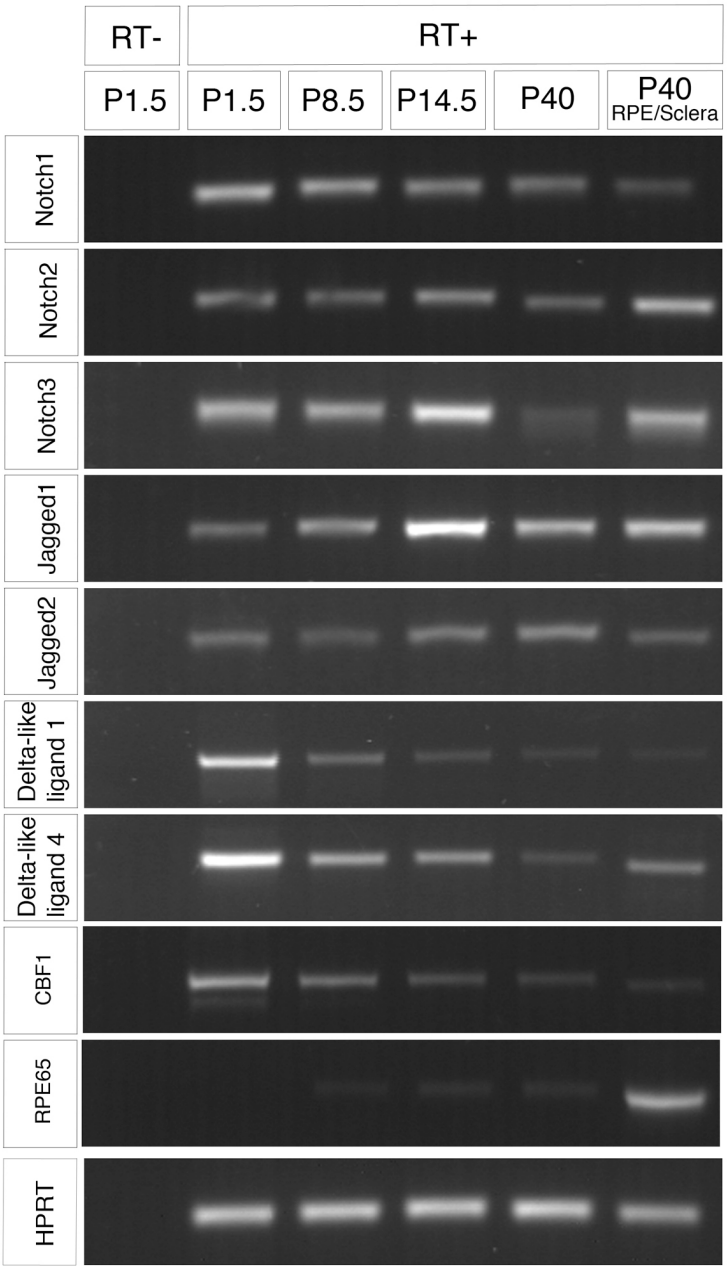

Figure 4. mRNA for Notch signalling components are present throughout postnatal retina development. RT-PCR performed on RNA isolated from retina samples from mice aged P1.5, P8.5, P14.5, P40, and retina pigmented epithelium (RPE)/sclera from a P40 mouse. Primers for the *Notch receptors 1-3*, the ligands *Jagged1* and *2*, and *Delta-like ligands 1*and *4*, as well as the canonical Notch transcriptional co-activator *CBF1* were amplified. All samples were through 29 PCR cycles except *CBF1* and *RPE65* which were run through 25 cycles. The RPE specific gene *RPE65* and the housekeeping gene *HPRT* were used as contamination and RNA loading controls, respectively. Reverse transcriptase negative samples (RT-) were used as a negative control.

3.1.2 Preparation of *in situ* hybridization probes for Notch1, Notch2 and CBF1.

Although *in situ* probes had been published by other research groups (Williams et al., 1995; Bao and Cepko, 1997), we were unable to obtain these probes. As such, we designed our probes based on the design of those previously published, and verified the specificity of our probes to the early time points that had been previously analyzed. Primers for *Notch1, Notch2*, and *CBF1* were designed to amplify mRNA products between 400-700 bps in length and whenever possible span at least one intron in order to prevent amplification of genomic DNA. Each forward primer had the T7 consensus sequence added to the 5' end and each reverse primer had the SP6 consensus sequence added to the 5' end. *Notch1* and *Notch2* primers were designed so the probes would hybridize to the regions between the ANK repeats and the PEST domain, because it had previously be reported that this region is the least conserved sequence region between the mammalian *Notch* genes (Bao and Cepko, 1997). The *CBF1* probes were designed to hybridize to all four transcript variants. The *Notch1* probe corresponded to nucleotides 8294-8781, Notch2-A to nucleotides 5620-6223, and Notch2-B to nucleotides 6635-7078 of the *Mus musculus* mRNA sequences. The CBF1-A probe corresponded to 599-1190 of transcript variant 1 and CBF1-B corresponded to 922-1593 of transcript variant 1 for the *Mus musculus* mRNA nucleotide sequences for *CBF1*. All probes hybridization sites are shown in relation to where these mRNA nucleotide regions correspond to the protein structure in Figure 5.

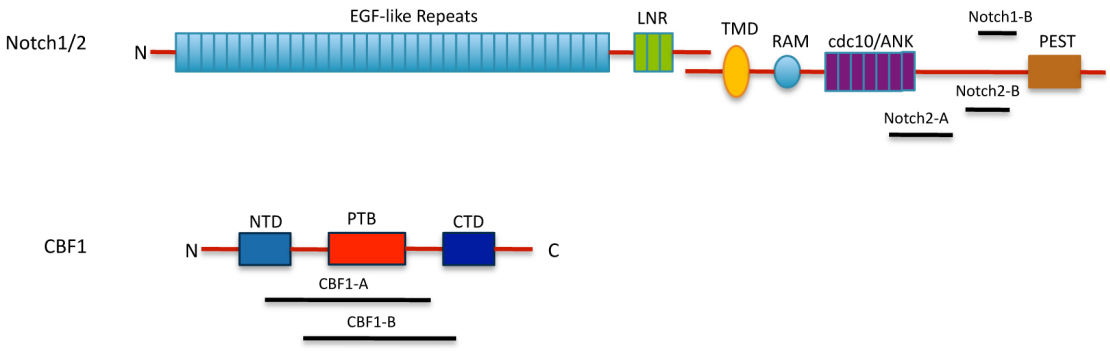

Figure 5. *In situ* hybridization probe binding sites in relation to protein motifs of Notch and CBF1. The Notch1-B probe binds in a region in between the ankyrin repeat (ANK) domain and the proline, glutamic acid, serine, threonine (PEST) domain of Notch1, Notch2-B bind in a

similar region of *Notch2*, and Notch2-A binds a region within the ANK domain and spans into the region between the ANK domain and the PEST domain. *Notch* probes do not span the highly conserved epidermal growth factor (EGF)-like repeats, the Lin12-Notch repeats (LNR), transmembrane domain (TMD), or RBPJ association module (RAM) regions. CBF1-A spans part of the N-terminus domain (NTD), the beta-trefoil DNA binding (PTB) domain, and part of the region between the PTB and the C-terminus domain (CTD). CBF1-B spans the PTB and part of the CTD. Note that the relative size of Notch1/2 to CBF1 and their probes is not to scale.

mRNA was isolated from adult whole eye samples and a PCR was run with each primer set using the cDNA constructed from this mRNA as template. Two primer pairs were designed for *Notch1, Notch2*, and *CBF1*, however, the second *Notch1* probe primer pair produced a large primer dimmer and amplified RT-negative samples (data not shown) so it was excluded from subsequent analysis. A second round of PCR was run with the purified product from the first round of PCR as a template to increase specificity of the PCR product. The final product was run on a gel (Figure 6) and the nucleotide sequence was confirmed via sequencing.

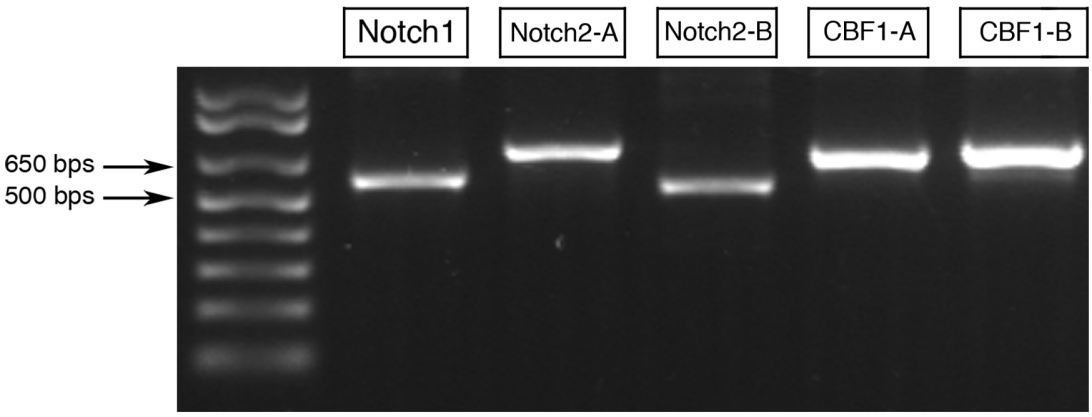

Figure 6. PCR products for Notch pathway probes. Purified PCR samples were run on a 1.2% agarose gel for 30 minutes at 120V following two rounds of amplification and purification. Notch1 was approximately 530 bps, Notch2-A was approximately 650 bps, Notch2-B was approximately 480 bps, CBF1-A was approximately 640 bps, and CBF1-B was approximately 650bps.

Each PCR product was synthesized into an mRNA probe using digoxigenin-labelled nucleotides and SP6 DNA polymerase to create the antisense probe, and T7 RNA polymerase to create the sense probe. The synthesized probes were run on a gel (Figure 7).

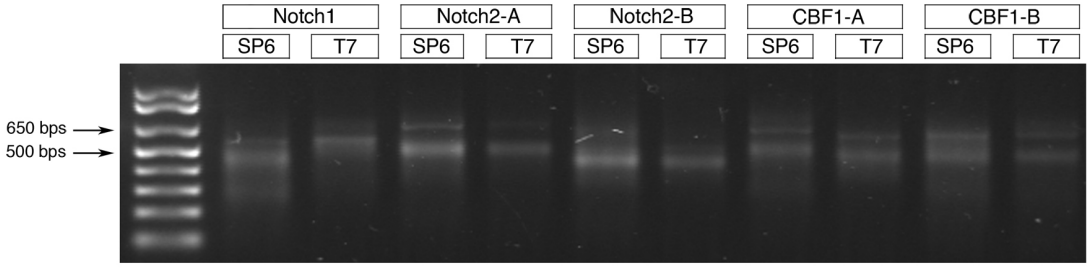

Figure 7. Fully synthesized in situ probes for Notch signalling components. Probes were run on a 1.2% agarose gel for 30 minutes at 120V. An antisense and sense probe for each nucleotide sequence was created using RNA polymerase SP6 and T7 respectively.

3.1.3 *In situ* hybridization of Notch1, Notch2 and CBF1 at E14.5, P1.5, and P40.

In situ hybridization was performed on embryonic tissue from E14.5 (Figure 8). CBF1-A and CBF1-B both showed similar hybridization patterns. Both probes detected *CBF1* mRNA throughout the neural retina, and weakly in the lens epithelium (8B,E). In the ventricle region of the brain, both probes detected *CBF1* throughout the ventricular/subventricular zone (VZ/SVZ) (8C,F). *Notch1* mRNA was detected in the apical region of the neural retina closest to the retina pigmented epithelium (RPE) but was absent from the lens (8H). *Notch1* mRNA was also detected in the VZ/SVZ (8I). The Notch2-A probe detected the strongest *Notch2* expression in the VZ/SVZ (8L), but was also able to detect signal in the neural retina (8K). Notch2-B detected lower levels of *Notch2* mRNA compared to Notch2-A. *Notch2* mRNA was not detected in the neural retina with Notch2-B (8N) and low mRNA levels were detected in the VZ/SVZ (8O).

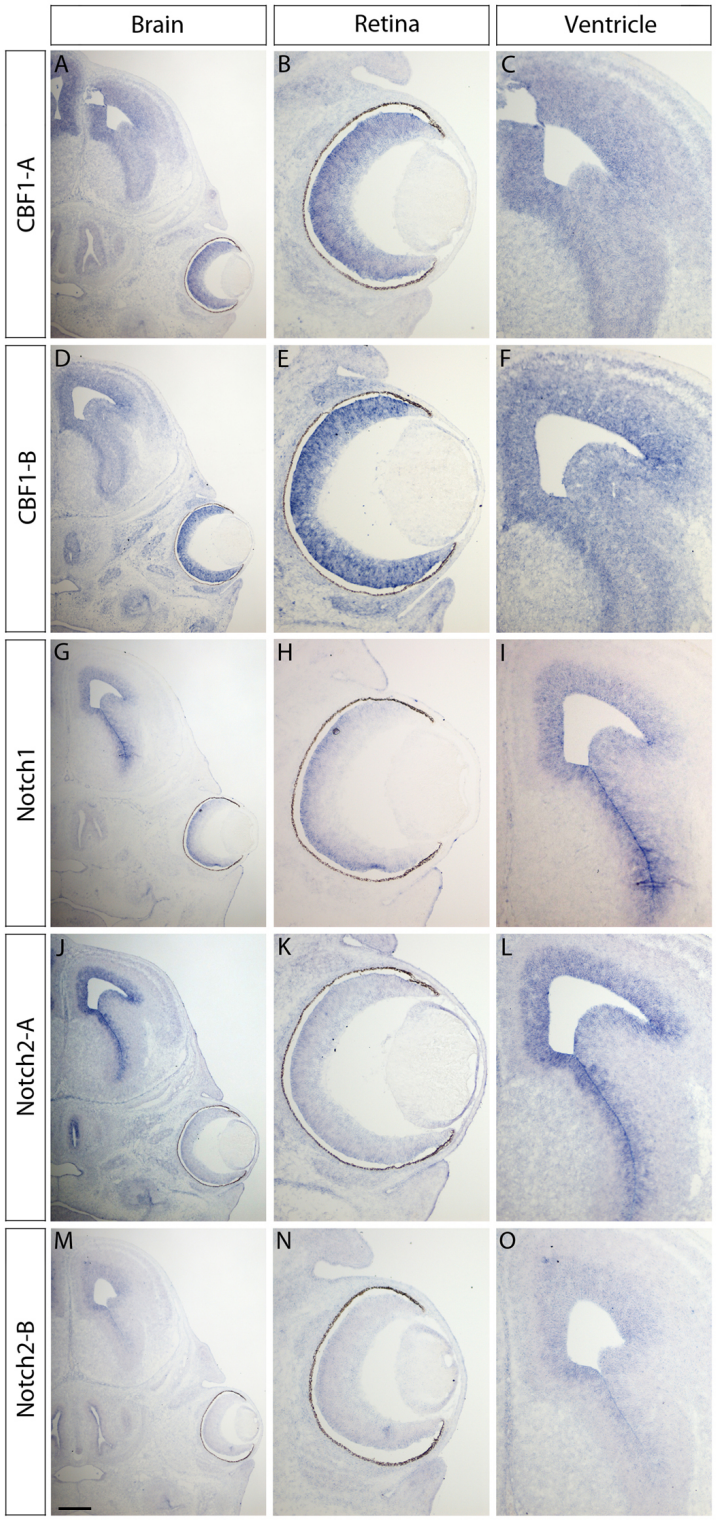

Figure 8. *CBF1, Notch1,* and *Notch2* mRNA expression in E14.5 neural tissue. *In situ* hybridizations of coronal sections of E14.5 embryos. Two *CBF1* probes, CBF1-A **(A-C)**, and CBF1-B **(D-F)** are detected in the retina **(B,E)**, ventricles **(C,F)**, and brain tissue immediately

surrounding the ventricles **(A,D)**. *Notch1* is detected in the apical region of the retina **(H)**, and in the ventricular zone **(I)**. *Notch2* is detected at varying levels in the ventricular zone **(L,O)**, and at low levels in the retina with Notch2-A **(K)** but not Notch2-B **(N)**. Scale bar = 40 μm in Brain sections, 15 μm in retina and ventricle sections.

At P1.5 *Notch1, Notch2*, and *CBF1* were all detected in the retina (Figure 9). The CBF1-A probe was able to detect *CBF1* mRNA throughout the neuroblastic layer (NBL) and more weakly within the ganglion cell layer (GCL) (9A). *Notch1* mRNA was detected within the mid-NBL but was only very weakly detected in the GCL (9B). Both *Notch2* probes detected mRNA within the NBL and in the GCL, although the expression detected by Notch2-B was weaker in the apical regions of the NBL and stronger basally (9C,D).

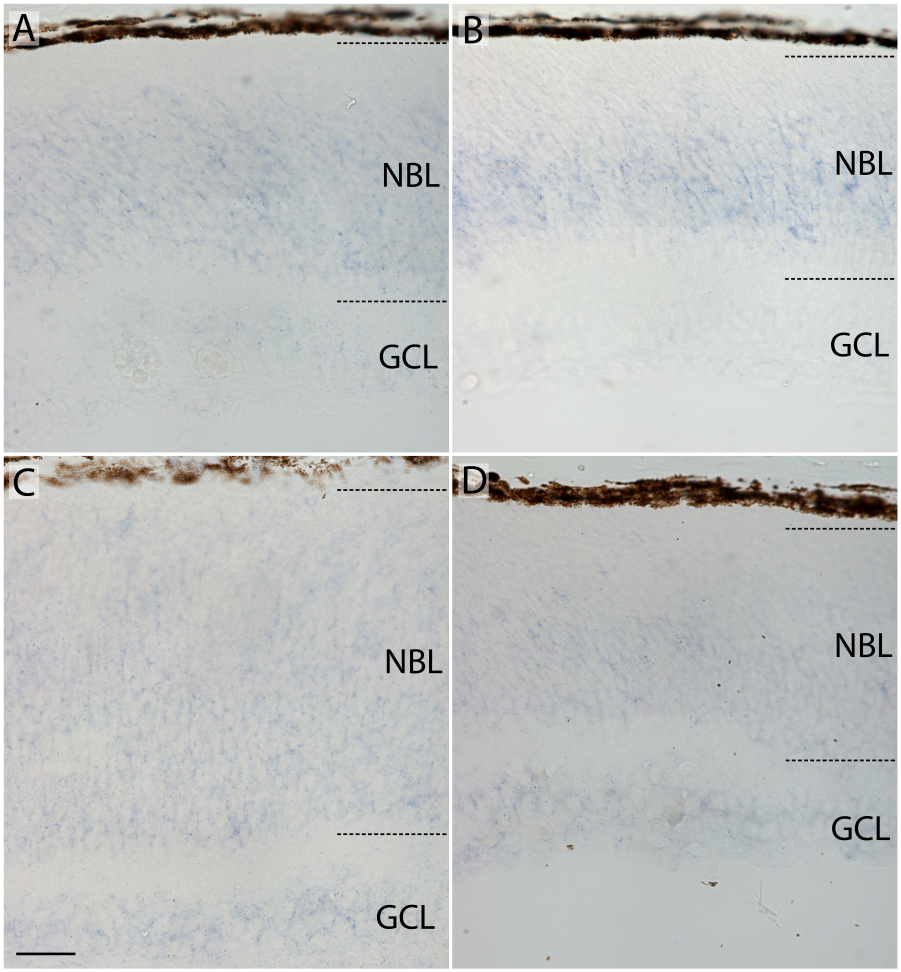

Figure 9. Expression of *CBF1, Notch1*, and *Notch2* mRNA in P1.5 retina. *CBF1* is detected throughout the neuroblastic layer (NBL) and weakly in the ganglion cell layer (GCL) **(A)**. *Notch1* is detected in the mid-region of the NBL but only weakly in the GCL **(B)**. Both *Notch2* probes, Notch2-A **(C)**, and Notch2-B **(D)**, detect *Notch2* throughout the NBL and in the GCL. Scale bar = 30 µm.

In situ hybridization in the adult retina (Figure 10), revealed much lower detection levels of detection with all probes than seen in previous developmental time points (Figure 8 and Figure 9). The two *CBF1* probes were only very weakly able to detect mRNA. The CBF1-A probe, weakly labelled the outer nuclear layer (ONL) (10A). The CBF1-B probe labelled at a slightly higher level, and was detected in the ONL, inner nuclear layer (INL), and very weakly in the GCL, however the background staining was a bit higher in this section and blue precipitate can be observed in the outer segments of the

photoreceptors (apical to the ONL) (10B). *Notch1* mRNA was not detected in any nuclear layer (10C). Both *Notch2* probes showed extremely weak signal in both the INL and ONL (10D,E).

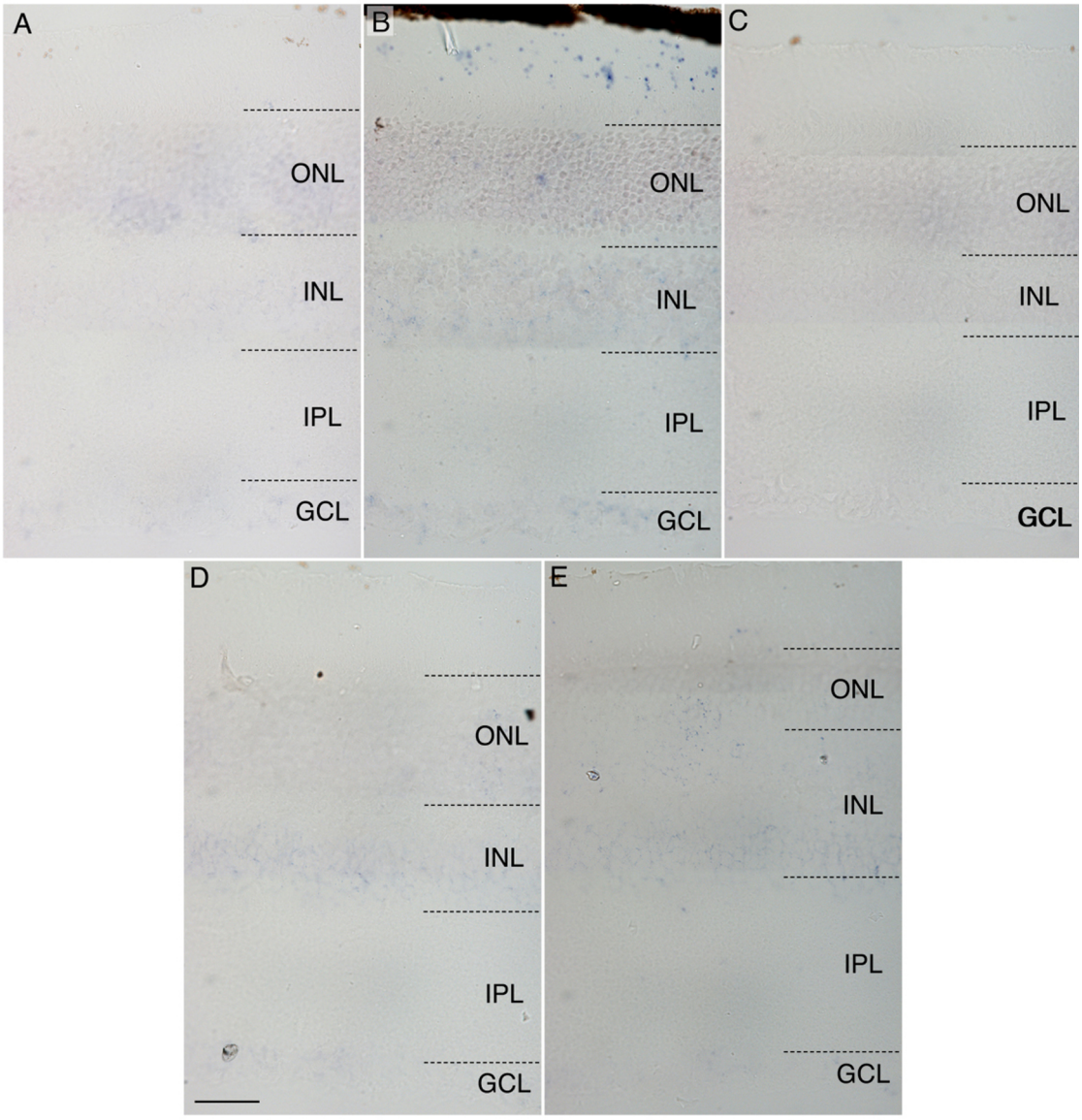

Figure 10. *In situ* hybridization of adult (P40) retinas for *CBF1, Notch1*, and *Notch2*. *In situ* hybridizations with two probes for *CBF1,* CBF1-A **(A)**, and CBF1-B **(B)**, Notch1 **(C)**, and two probes for *Notch2,* Notch2-A **(D)** and Notch2-B **(E)**. Scale bar = 35 µm.

To check the specificity of the probes, several control *in situ* hybridization experiments were performed (Figure 11). Two T7 sense probes were used. Both the CBF1-A sense and Notch2-A sense probes showed strong, possibly non-specific hybridization at both P1.5 and P40. The CBF1-A sense probe showed strong labelling in the NBL at P1.5 (11A), and in the INL and GCL at P40 (11B). The Notch2-A sense probe showed strong labelling in the NBL and in the GCL at P1.5 (11C), and in all three nuclear layers at P40 (11D). A final control experiment was performed to test for background signal caused by the alkaline-phosphatase/anti-digoxigenin antibody colour reaction. Here, the entire *in situ* hybridization protocol was completed but no probe was added to the hybridization buffer. At both P1.5 (11E) and P40 (11F), there was no signal detected.

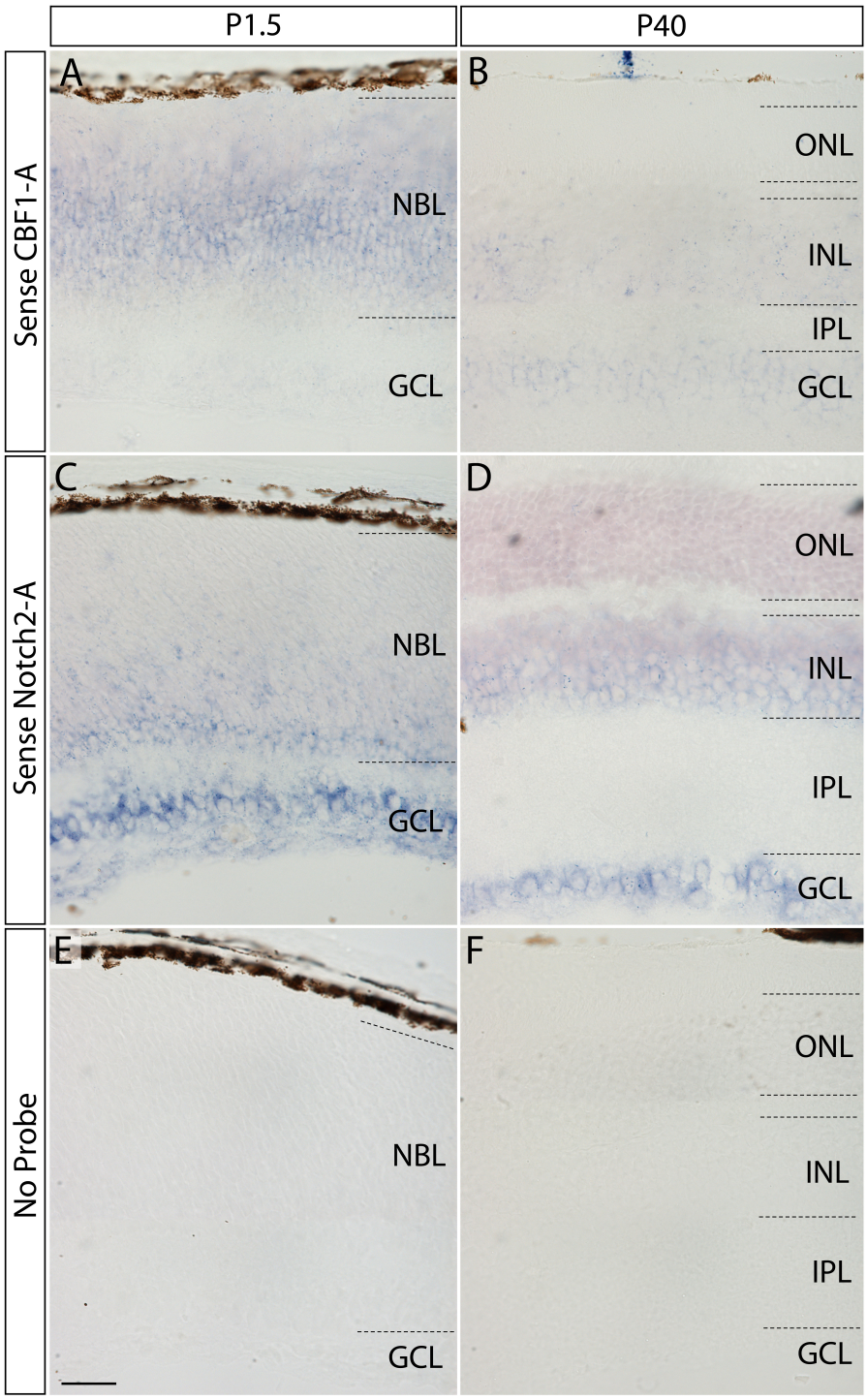

Figure 11. Control *in situ* hybridizations in P1.5 and P40 retinas. *In situ* hybridization with CBF1-A sense probe shows signal in the neuroblastic layer (NBL) at P1.5 **(A)** and in the inner nuclear layer (INL) and ganglion cell layer (GCL) at P40 **(B)**. Notch2-A sense probe shows signal throughout the NBL and GCL at P1.5 **(C)** and in the outer nuclear layer (ONL), INL, and GCL at P40 **(D)**. Slide prepared without probe **(E,F)** show no background signal. Scale bar = 30 μm.

Cumulatively, these results indicate that there is mRNA signal detected for *Notch1* in the embryonic retina and VZ/SVZ, in the NBL of the early postnatal retina. *Notch2* mRNA is detected in the VZ/SVZ of the embryonic brain, in the NBL and ganglion cell layer of the early postnatal retina. *CBF1* mRNA is detected in the VZ/SVZ and throughout the embryonic neural as well as throughout the NBL of the early embryonic retina. In the adult retina, mRNA signal may be weakly detected for *Notch2* and *CBF1,* but not for *Notch1* mRNA. Although the sense probes detected a strong signal, the slides prepared without primary antibody did not show background signal due to the alkaline-phosphatase reaction with the digoxigenin antibody.

3.2 Transgenic reporters for Notch signalling activity show reporter expression throughout development.

Although the adult *in situ* hybridization results were not able to show conclusive evidence for *Notch* receptors or *CBF1*, we were able to show the expression of *Notch* receptors and ligands through the more sensitive method of RT-PCR. Next we sought to use two reporter mouse lines to look for active Notch signalling in the adult retina and to confirm Notch signalling in cell populations known to localize Notch signalling components in the embryonic and early postnatal retina.

3.2.1 The Notch Transgenic Reporter mouse line shows reporter expression in the embryonic, early postnatal, and adult retina.

Immunohistochemistry for EGFP was performed at E14.5, P1.5, and P40 on Notch Transgenic Reporter (NTR) mice and wild type littermates (Figure 12). Coronal whole head sections at E14.5 had high levels of reporter expression in the nasal epithelium (12A, arrowhead). In the E14.5 retina EGFP reporter expression was detectable albeit at lower levels than the nasal epithelium (12A,E). At higher magnification, EGFP was visible throughout the embryonic neural retina (12B,F), with some cells expressing higher levels of reporter than others (12B, arrows). At P1.5, EGFP expression was present throughout the NBL and within the GCL (12C,G), again with some cells

expressing higher levels than others (12C, arrows). In the adult retina (P40), expression was observed in a specific subset of cells (12D,H), although there was some background fluorescence in the outer plexiform layer and outer segments of the photoreceptors that was seen in both the transgenic and wild type controls. A subset of bipolar cells (arrow) as well as a subset of amacrine cells (arrowhead) expressed EGFP reporter. Dendritic processes were seen in both the ON and OFF sublamina within the inner plexiform layer (IPL). Many ganglion cells within the GCL also expressed EGFP reporter (open arrowhead).

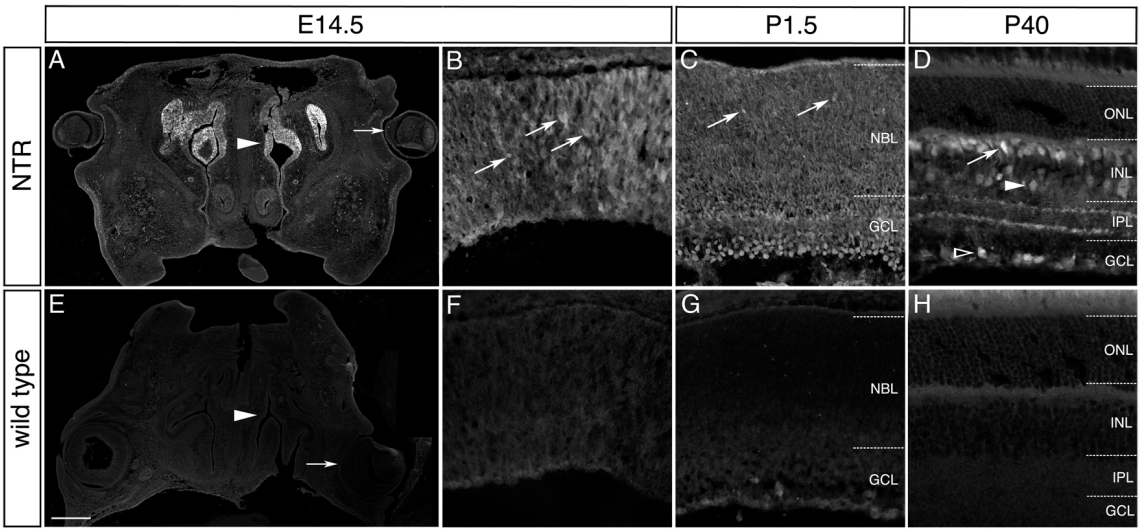

Figure 12. EGFP reporter expression for Notch signalling is present in early development, down-regulated postnatally, and detected in a subset of cells in the adult retina. EGFP is visible in the nasal epithelium in a coronal section of an E14.5 embryo **(A,E)**. At higher magnification, EGFP is visible throughout the developing E14.5 neural retina **(B,F)**, with some cells expressing higher levels of reporter than others **(B,** arrows**)**. At P1.5 **(C,G)**, EGFP expression is detected in cells (arrows), in the neuroblastic layer (NBL) and in the ganglion cell layer. In the adult P40 retina **(D,H)**, EGFP expression is restricted to a subset of bipolar cells (arrow), some amacrine cells (arrow head), and some ganglion cells (open arrowhead). Scale bar = 600 μm in A,E, 100 μm in B,F, and 80 μm in C,G, and 50 μm in D,H.

Many cell-type specific markers exist that enable one to distinguish distinct cell types within the retina (Table 5). To determine which cell types in the inner nuclear layer were expressing EGFP, the cell-type specific markers calbindin and Chx10 were examined (Figure 13). Calbindin is a cell marker for horizontal cells and a subset of amacrine cells. EGFP did not colocalize with horizontal cells expressing calbindin (13A-C, arrowheads), or with any calbindin expressing amacrine cells (13A-C, arrows). Chx10 is a cell marker for all bipolar cells. Reporter expression showed colocalization with many Chx10 positive cells (13D-F, arrows), but some Chx10 positive cells did not have detectable EGFP expression (13D-F, arrowheads).

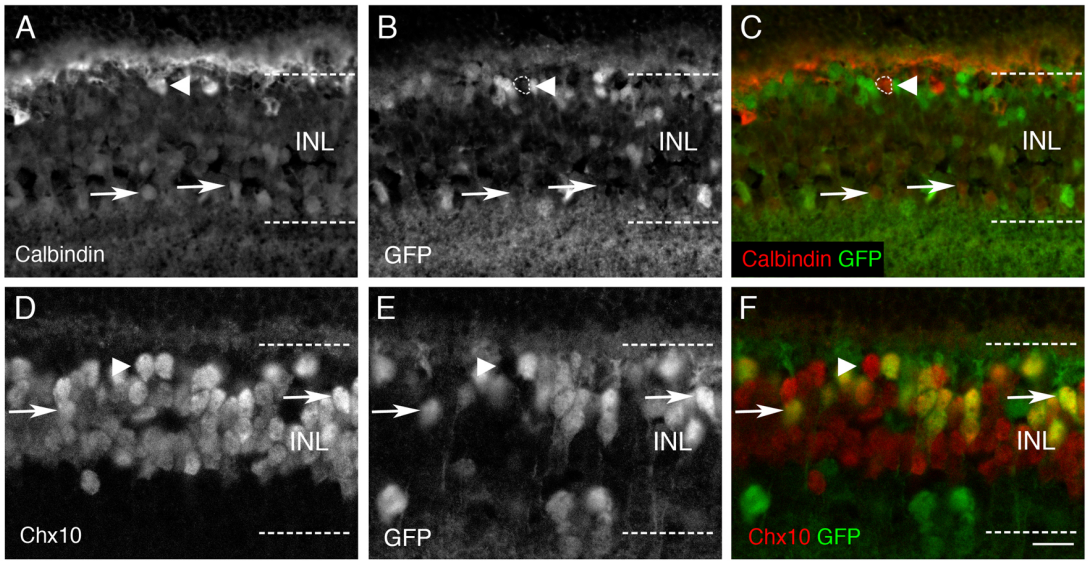

Figure 13. EGFP is present in bipolar cells but not horizontal cells in the adult retina. EGFP does not colocalize with calbindin, a marker for horizontal (arrowhead) and a subset of amacrine (arrow) cells **(A-C)**. EGFP in the inner nuclear layer (INL) shows strong colocalization with Chx10 (arrows), a cell marker for all bipolar cell interneurons **(D-F)**, but not all bipolar cells express EGFP (arrowhead). Scale bar = 30 μm.

There are at least 10 subtypes of retinal bipolar cells in the mouse retina (Ghosh et al., 2004). To determine which subtypes were expressing EGFP, we examined a number of cell markers which identify subsets of bipolar cell types (Figure 14). Recoverin, a marker for photoreceptors as well as cone type 2 OFF bipolar cells colocalized with some EGFP positive cells (14A-C, arrow), although not all recoverin positive bipolar cells were EGFP positive (14A-C, dashed lines). Vsx1, a marker for cone type 2 OFF and cone type 7 ON bipolar cells showed colocalization in some cells (14D-F, arrow), but most cells that were Vsx1 positive did not show colocalization (14D-F, dashed lines). PKCα, a marker for rod ON bipolar cells showed colocalization with many EGFP positive cells (14G-I, arrow), although not all cells positive for PKCα expressed EGFP (14G-I, dashed lines), and there were some bipolar cells positive for EGFP that were not positive for PKCα (14G-I, arrowhead). Cabp5, a marker for rod ON, cone type 3 OFF, and cone type 5 ON bipolar cells was present in some(14J-L, arrow) but not all (14J-L, dashed lines) EGFP positive cells. In all images, there were examples of cells within the inner part of the INL, which according to morphology and location were presumed to be amacrine cells (14, open

arrowheads), although this was not confirmed by immunohistochemistry in the NTR mouse line.

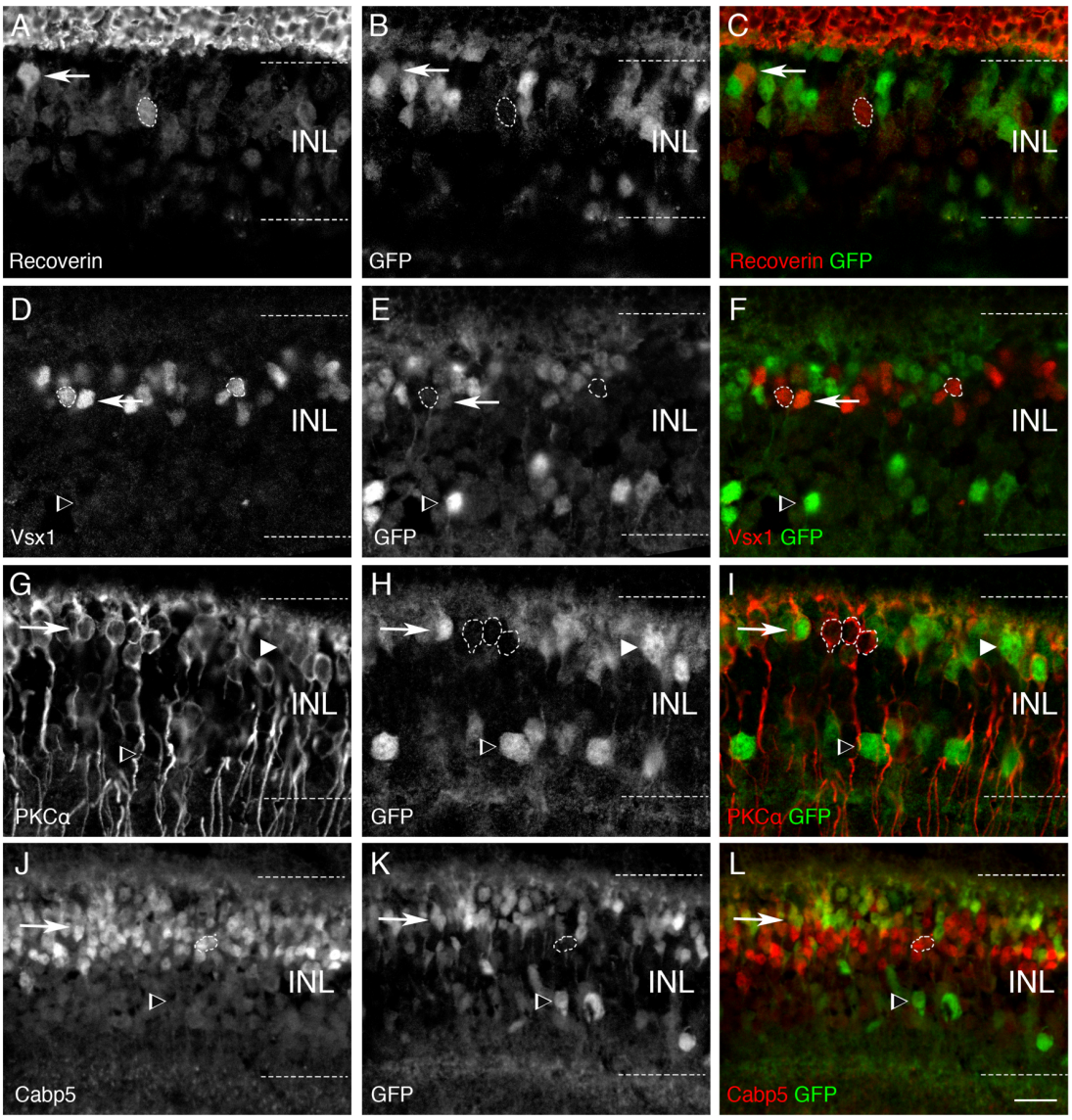

Figure 14. EGFP is present in a subset of rod ON and cone type 2 OFF bipolar cells. EGFP colocalization (arrow), is seen with recoverin, a cell marker for cone type 2 OFF bipolar cells **(A-C)**. Some weak EGFP colocalization is seen with Vsx1, a cell marker for cone type 2 OFF and type 7 ON bipolar cells **(D-F)**. Strong EGFP colocalization is seen with PKCα, a rod ON bipolar marker **(G-I)**. EGFP colocalization is seen with Cabp5, a cell marker for rod ON, cone type 3 OFF, and cone type 5 ON bipolar cells **(J-L)**. EGFP is detected in a subset of amacrine cells (open arrowheads). Scale bar = 30 μm.

Taken together, this data indicates that in the NTR mice, EGFP is detected in a large subset of the rod ON and cone type 2 OFF bipolar cells, a subset of amacrine cells, and a subset of ganglion cells in the adult retina. EGFP is also detected in the E14.5 retina, and in the ganglion cell layer and NBL of the P1.5 retina.

3.2.2 The CBFRE:H2B-Venus reporter for Notch signalling shows venus expression in neural embryonic tissue, and in a subset of cells in the early postnatal and adult retina

After breeding the NTR mice for two to three generations, the transgene became silenced, a problem that Jackson Laboratories had also reported (www.jax.org). Since the silencing occurred before we were able to perform proof of principle experiments to verify the presence of Notch signalling in the retina, we sought another transgenic reporter mouse line. The CBFRE:H2B-Venus line chosen because the transgene design was based on the NTR mice. The endogenous levels of H2B-Venus were strong enough to detect with confocal microscopy without the use of immunoshistochemistry. An adult sample was stained using the same GFP antibody that was used for the NTR mice, but no additional reporter positive cells were detected (data not shown).

Mice that were hemizygous for the CBFRE:H2B-Venus transgene were examined at E14.5, P1.5, and P40 time points and compared to non-transgenic wild type littermates (Figure 15). At E14.5 in coronal sections of the embryonic head, venus expression could be seen in the ventricular zone (15A,E, arrow) and in a disperse manner throughout the brain. Within the eye, venus expression was detected in the RPE as well as in the lens epithelium and proliferative zone of the lens (L) (15B,F). At this time point no venus expression was detected within the neural retina (R) (15B,F). At P1.5, there was a subset of venus positive cells in the apical region of the NBL (15C,G, arrows), as well as in a subset of cells in the innermost region of the NBL (15C,G, arrowheads). Venus expression was also detected in a large number of cells within the GCL at P1.5 (15C,G, open arrowheads). At P40, there was an increased number of cells in which venus was detected (15D,H), including all cells in the ONL and a large number of cells in the basal INL. Venus was also detected in a large number of cells within the GCL.

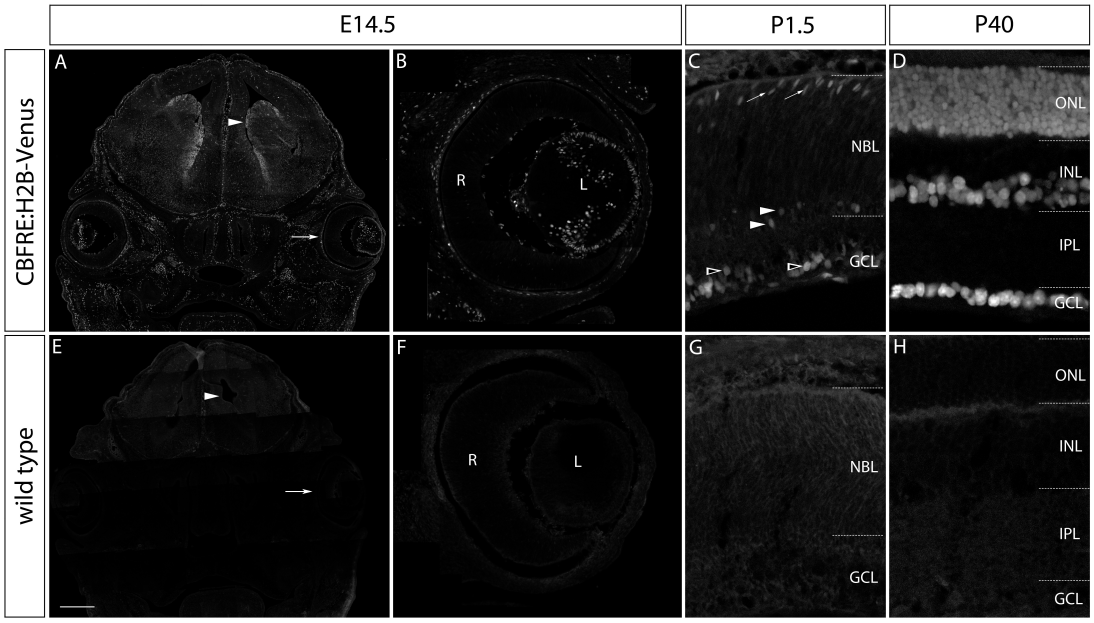

Figure 15. CBFRE:H2B-Venus reporter expression for Notch signalling is not present in the embryonic retina, is upregulated in the early postnatal stages, and expressed in photoreceptors, amacrine, and ganglion cells in the adult retina. At E14.5 venus is visible in the ventricle (arrowhead), and in the retinal pigmented epithelium (RPE, arrow) and lens of the eye **(A,E)**. At higher magnification, venus is visible in the lens epithelium of the lens (L) and in the RPE, but not in the neural retina (R) **(B,F)**. At P1.5 venus positive cells are visible at the apical edge of the neuroblastic layer (NBL) (arrows), and the basal edge (arrowheads), and in the ganglion cell layer (GCL) (open arrowheads) **(C,G)**. At P40, venus is visible in the outer nuclear layer (ONL), in the ventral part of the inner nuclear layer (INL), and in the GCL **(D,H)**. Scale bar = 600 μm in A and E, 60 μm in B,C, F and G, and 50 μm in D and H.

To determine which cell types in the INL expressed venus, syntaxin, a marker for amacrine cells, and Brn3b, a marker for ganglion cells were used (Figure 16). Venus was detected in a large number of syntaxin positive cells (16A-C, arrows), but there were examples of syntaxin positive cells that did not express venus (16A-C, dashed lines). Many cells that were positive for Brn3B were positive for venus expression (16D-F, arrows), although there were a small number of Brn3b positive cells which did not express venus (16D-F, dashed lines).

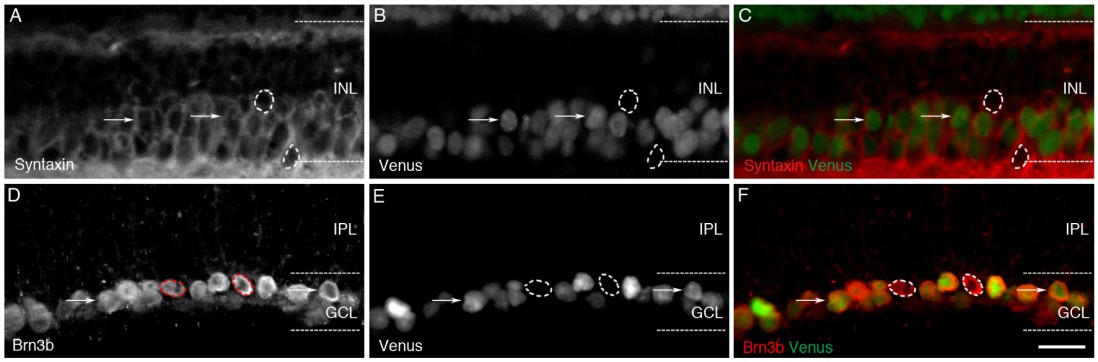

Figure 16. CBFRE:H2B-Venus reporter is present in amacrine and ganglion cells. At P40, venus colocalization (arrows) is seen with syntaxin, a cell marker for amacrine cells **(A-C)**. Colocalization is also seen with Brn3b, a cell marker for ganglion cells **(D-F)**. Scale bar = 25µm.

To examine whether any other cell types besides amacrine, ganglion and photoreceptors were present in the venus positive population a series of bipolar cell markers and a horizontal cell marker were used (Figure 17). No bipolar cells immunolabelled by the pan-bipolar marker Chx10 co-expressed venus (17A-C). This was confirmed with immunostaining for other bipolar cell makers including the rod ON bipolar marker PKCα (17D-F) and the rod ON, cone type 3 OFF and cone type 5 ON bipolar marker Cabp5 (17G-I). Calbindin staining (17J-L), revealed that no horizontal cells or the subset of amacrine cells labelled by calbindin were venus positive.

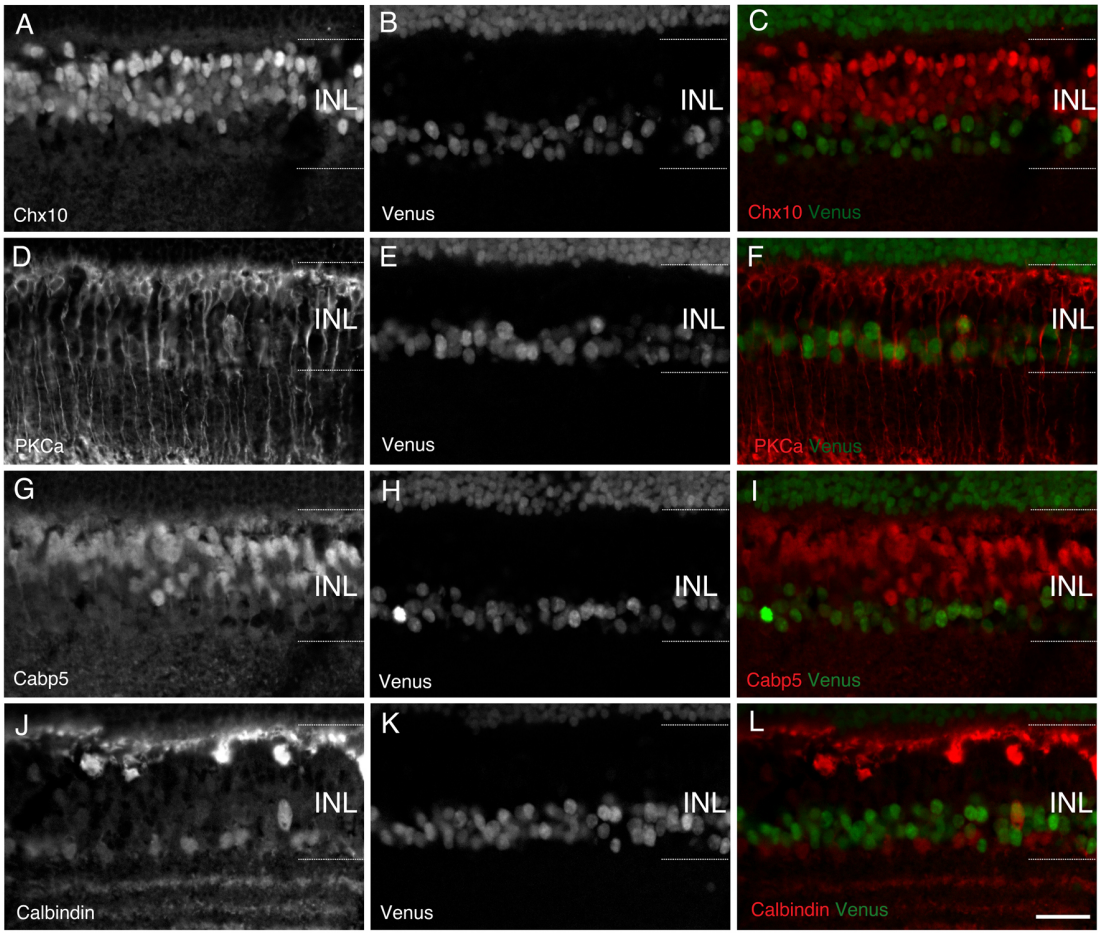

Figure 17. CBFRE:H2B-Venus reporter is not present in bipolar or horizontal cells. Venus did not colocalize with Chx10 **(A-C)**, a cell marker for all bipolar cells, PKCα **(D-F)**, a cell marker for rod ON bipolar cells, cabp5 **(G-I)**, a cell marker for rod ON, cone type 3 OFF, and cone type 5 ON bipolar cells , or calbindin **(J-L)**, a cell marker for horizontal cells and a subset of amacrine cells. Scale bar = 50 µm.

3.2.3 The specificity of the CBFRE:H2B-Venus reporter mouse line could not be confirmed by Notch inhibition.

Due to the different expression patterns that we observed in the retinas of the two mouse lines, we sought to verify the specificity of the venus expression for Notch signalling. To do this, we cultured retinal explants from CBFRE:H2B-Venus transgenic mice with DAPT, a well characterized Notch small molecule inhibitor, which prevents the γ-secretase cleavage, thereby preventing the release of the Notch intracellular domain (NICD) (Nelson et al., 2006). Explants were cultured from P0.5-P2.5 and from P1.5-P5.5

in culture media containing either 10 μM DAPT dissolved in DMSO, or in a control condition with DMSO. At the end of the culturing period, explants were fixed, sectioned, and examined for morphology and venus fluorescence intensity (Figure 18). Explants cultured with DAPT from P0.5-P2.5 had similar tissue morphology to control explants, with no disruption to the NBL or GCL (18A-F). Explants cultured with DAPT from P1.5-P5.5 showed drastic morphological differences from the DMSO control explants. DAPT explants contained numerous rosettes (18G-I, arrowheads) which disrupted the inner and outer nuclear layer organization compared to the control explants (18J-L). The Shapiro-Wilk test revealed that the DAPT measurements were not normally distributed therefore the non-parametric Wilcoxon rank sum test was performed. There was no significant difference between the average venus fluorescence intensities for the explants cultured with DAPT and DMSO from P0.5-P2.5 ($W = 34$, $p = 0.261$), or explants cultured with DAPT from P1.5-5.5 ($W = 88$, $p = 0.378$). Although there was no significant difference between the average intensity values – likely due to the small sample size - the explants cultured from P1.5-5.5 show a large increase in average venus fluorescence when cultured with DAPT (18M).

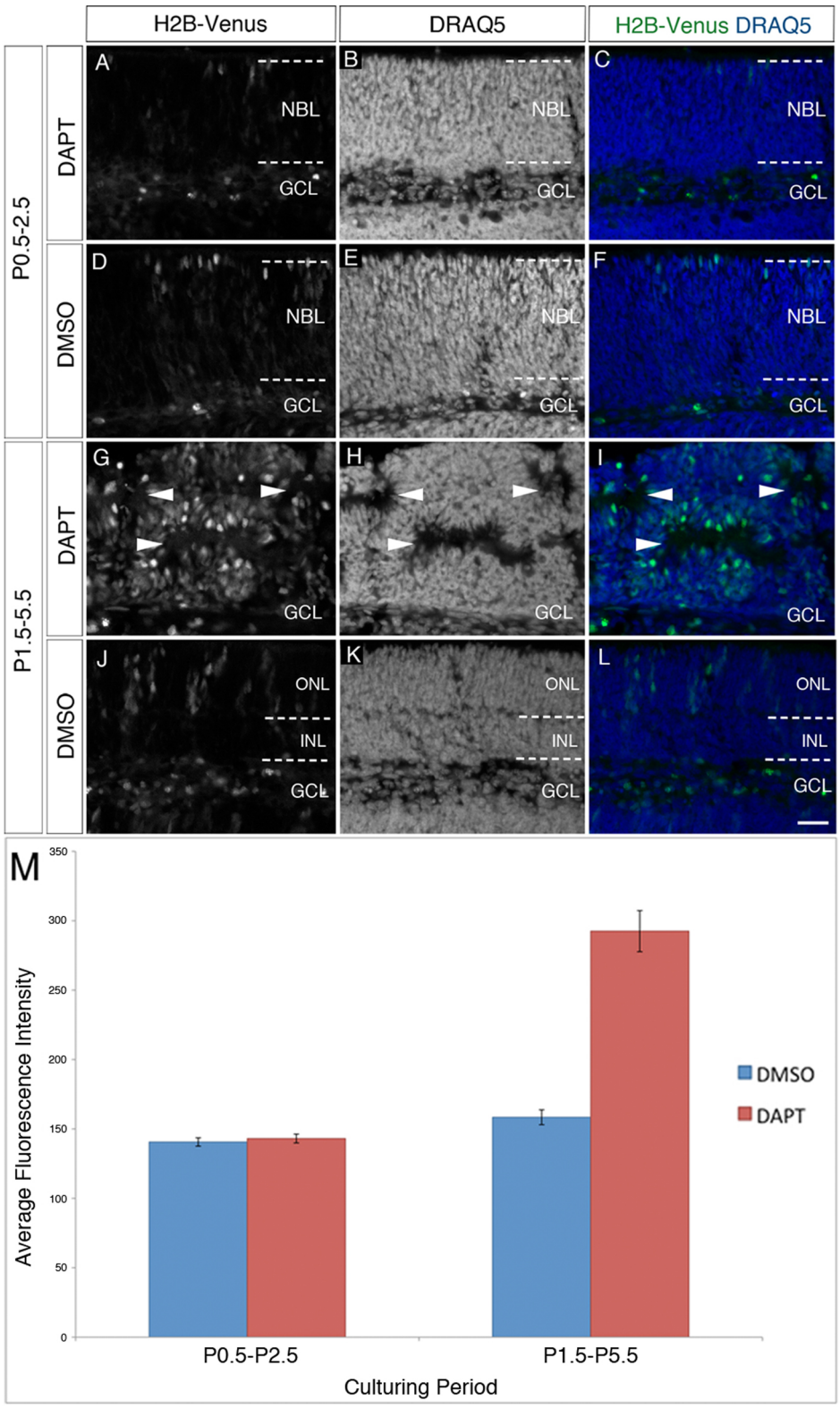

Figure 18. CBFRE:H2B-Venus levels are increased by long-term but not short-term application of DAPT. Explants cultured from P0.5-P2.5 in the Notch inhibitor DAPT **(A-C)** did not show a significant difference in venus fluorescence when compared to control explants

cultured for the same time in DMSO **(D-F)**. Explants cultured from P1.5-P5.5 in DAPT, displayed a disruption of cell adhesion in the form of rosettes (arrowheads), and a loss of defined inner and outer nuclear layers, characteristic of Notch inhibition and showed a non-significant increase in venus fluorescence when compared to control explants cultured for the same time in DMSO **(J-L)**. Scale bar = 60 μm. Quantification revealed no significant difference in the average venus fluorescence intensity **(M)** when explants were cultured with the Notch inhibitor DAPT when compared to those cultured with DMSO from P0.5-P2.5 ($W = 34$, $p = 0.261$). Explants cultured in DAPT from P1.5-P5.5 had a non-significant increase in average fluorescence when compared to explants cultured in DMSO ($W = 88$, $p = 0.378$).

Cumulatively, data from the CBFRE:H2B-Venus revealed venus reporter expression in photoreceptors, most amacrine cells, and most ganglion cells in the adult retina. There was no venus expression in the E14.5 embryonic neural retina, although there was expression in the RPE and other brain regions. At P1.5, there was venus expression in the outermost and innermost regions of the NBL, as well as in the ganglion cell layer. Furthermore, we were unable to show decrease of venus signal when we inhibited Notch signalling in the early postnatal retina.

Chapter 4: Discussion

The main objective of this study was to characterize Notch signalling in the adult retina. mRNA expression was examined using RT-PCR as well as *in situ* hybridization for various components of the Notch signalling pathway. To further characterize endogenous Notch signalling activity, we used two transgenic mouse lines which are designed to report for canonical Notch signalling. Although any expression in the adult retina of *Notch* was below the threshold of detection by *in situ* hybridization, RT-PCR results indicated that the receptors *Notch1, Notch2,* the ligands *Jagged1, and Jagged2,* as well as the *CBF1* were all present.. The transgenic reporter mouse lines revealed two different expression patterns and the specificity of these reporters could not be verified, however the expression of the NTR mouse line closely agrees with previous expression studies performed by Ahmed et al., (1995). If the faithfulness of this reporter can be verified, these results indicate a possible role for Notch signalling in the adult retina.

4.1 Notch signalling component *mRNA* expression in the retina

4.1.1 RT-PCR

RT-PCR has been previously used to examine Notch expression in the retina. Ahmed *et al.,* (1995) were able to observe *Notch1* expression in the retina not only during development, but throughout postnatal development and in the adult retina. Single cell RT-PCR has also revealed expression of Notch2 in some ganglion cells and in a photoreceptor cell (Roesch et al., 2008). Expression of the downstream signalling bHLH transcription factor *Hes1* has also been seen throughout retina development and in the adult retina (Tomita et al., 1996). All of these results support our findings that mRNA for Notch signalling components are found it the adult retina.

Similarly, my RT-PCR results revealed that Notch signalling components were expressed throughout retinal development as well as in the adult retina. It should be noted that any band intensity comparisons within the same primer set are only semi-quantitative. Furthermore, no quantitative comparisons can be made between different

primer sets because different primers may have different affinities for cDNA sequences. *Notch1* and *Notch2* were expressed in the retina throughout development and in the adult. Only a very weak band was seen for *Notch3* expression in the adult retina when compared to earlier developmental time points, suggesting that any Notch activity in the adult retina is likely due to Notch1 or Notch2. Expression for *Jagged1*and *Jagged2* was seen in both throughout development and in the adult retina, but the *Delta-like ligands1* and *4* although both strongly expressed early in postnatal development, were downregulated in the adult retina. *CBF1* was also detected at lower levels in the adult than in the early postnatal stages.

RT-PCR lacks cellular resolution and theoretically even one copy of mRNA could be enough to produce a PCR band. The cellular composition of the tissue used for RT-PCR needs to be taken into account because there are several non-neuronal cell populations in the retina that also express Notch signalling components. Notch1 as well as the ligands Jagged1, Delta-like ligand1 and Delta-like ligand4 have been shown to be present in the vasculature of the retina throughout development (Hofmann and Luisa Iruela-Arispe, 2007). Furthermore, in the mature retina, Jag1 and Delta-like ligand4 are present in the vessels and the endothelial cells of the mature arteries (Hofmann and Luisa Iruela-Arispe, 2007). Müller glia have also been shown to express Notch2 throughout development and also in the adult (Roesch et al., 2008; Zhu et al., 2013). Therefore, we cannot rule out that using RT-PCR, the detection of mRNA for Notch signalling components could be due to non-neuronal cells found in the retina rather than from expression in the neural retina.

4.1.2 *In situ* hybridization

In situ hybridization using probes for *Notch1*, *Notch2,* and *CBF1* revealed expression throughout development. Strong expression for *Notch1* and *Notch2* has been previously shown around the ventricles in the VZ/SVZ throughout embryonic development (Higuchi et al., 1995). This gave us confidence in the specificity of our probes since we saw a strong signal for both our *Notch* probes in the VZ/SVZ of our E14.5 sections. The expression patterns of the retinas at E14.5 was also very similar to that seen in previous studies (Lindsell et al., 1996; Bao and Cepko, 1997). At P1.5, *Notch1* expression is

similar to what was seen at P5 by Bao and Cepko (1997), with high signal detection within the dividing neuroblastic layer (NBL). We were able to detect *Notch2* in the GCL in addition to the NBL at P1.5, however Bao and Cepko (1997) were only able to detect *Notch2* in the ciliary body by P5. The lack of a definitive *in situ* signal in the adult retina despite the fact that embryonic expression is consistent with the published data, suggests that if there is *Notch* expression in the adult retina it is below the threshold of detection by this method. To date, only one study has shown adult retinal *in situ* hybridization expression for *Notch*. Ahmed et al., was able to detect *Notch1* signal in the INL and the GCL of the adult rat retina using an *in situ* probe designed to hybridize to a similar region in the mRNA as our probe (1995). However, other studies have been unable detect signal for *Notch* past P5 (Bao and Cepko, 1997).

Robust signal was seen with the sense *in situ* hybridization probes that were designed to serve as negative controls. The nucleotide sequences for the sense probes are in the same orientation as the target mRNA and therefore should not hybridize with the mRNA. Since we do not have a proper negative control using a sense probe, this limits the degree of certainty we can have with our anti-sense probe results. There are several reasons why we may have seen this high signal with the sense probes. Firstly, the wash stringency that we used was not extremely high. Probes that were specifically bound should not be affected by increasing the wash stringency, but we should be able to reduce the amount of signal seen in the sense probes. To increase stringency, a decreased salt concentration in the SSC buffer should be used and the temperature of these washes should be increased. Because we ran an experiment where we performed the entire *in situ* hybridization procedure except for adding the probe, we know that the sense signal is not due to non-specific secondary antibody binding, or to an erroneous alkaline-phosphatase colour reaction. At this point we cannot rule out the possibility that the signal we are detecting with the sense probes is real. It has been shown in some tissues that antisense mRNA transcripts can be produced in some situations, and can function as regulatory elements (Coker et al., 1998). Due to the size of the probes, and because we ran the probe sequence through BLAST, it is highly unlikely that they are binding to non-related mRNAs with complementary sequences.

4.2 Transgenic Notch reporter mouse lines reveal different Notch signalling signals in the adult and developing retina

4.2.1 Expression patterns of Notch reporter throughout development

The two transgenic mouse lines that we examined in this study did not produce the same reporter expression throughout development, or in the adult retina. In the E14.5 retina, in the NTR mouse line EGFP was detected throughout the embryonic retina, whereas in the CBFRE:H2B-Venus mice, venus was only present in the RPE and not present at all in the neural retina. At P1.5, although both transgenic lines had reporter expression in the ganglion cell layer, while EGFP was detected throughout the NBL, venus was only present in a subset of cells in the outermost and innermost NBL. Finally, in the adult retina (P40), EGFP was detected in rod ON and cone type 2 OFF bipolar cells and in a subset of amacrine and ganglion cells whereas venus was found in the entire outer nuclear layer, in most amacrine cells, and most ganglion cells. Due to the conflicting cellular localization of the Notch reporters, the expression pattern of Notch signalling using this method was not conclusive.

The expression pattern seen at E14.5 using *in situ* hybridization, both in this study and in previous studies (Lindsell et al., 1996; Bao and Cepko, 1997), reveals that *Notch1* mRNA is present in the neural retina, and *Notch2* mRNA in the RPE at this time point. As there is no venus detected in the neural retina at E14.5, this leads us to question the faithfulness of the CBFRE:H2B-Venus reporter, especially in the retina. However, venus is expressed in the RPE and in the developing lens at the time point, both places where it should be expressed (Lindsell et al., 1996; Bao and Cepko, 1997; Saravanamuthu et al., 2012). The NTR mouse line reporter expression at E14.5 more closely resembles that of the *in situ* studies, therefore it is more likely that this line is accurately reporting Notch signalling at embryonic time points.

Ahmed *et al.,* provided evidence for Notch1 expression in the adult retina similar to what we detected using the NTR mouse line. Using a human-anti-Notch1 antibody they were able to show staining in the INL and GCL in both adult human and rat retinas

(1995). This data was supported by *in situ* hybridization results mentioned in the previous section. However, the immunohistochemistry performed in this experiment used immunoperoxidase staining, which makes use of an enzymatic reaction that can cause high background signal levels. The only other indication for the possible expression of Notch in the adult retina comes from studies which show that Notch1 and Notch2 are present in Müller glia in the adult retina (Roesch et al., 2008; Zhu et al., 2013), but this is not consistent with the expression pattern for either of the Notch reporters. Therefore, although Notch in the adult retina has not been widely studied, again our NTR mouse line most closely matches what has been previously described for Notch expression.

The Notch knockdown experiments were unable to show a reduction in venus fluorescence as a result of Notch signalling inhibition via the small molecule inhibitor DAPT (Figure 18). When retinas were cultured for two days in DAPT, there was no significant difference in the average fluorescence values of these retinas when compared to control retinas. It is likely however, that this culture period was not sufficient to knock down Notch signalling, as by the second day of culture the retinas did not exhibit any rosette structures that are characteristic of retinas cultured in DAPT (Nelson et al., 2007). Furthermore, the greater than 24-hour half life of venus means that this likely would not have been a sufficient amount of time to see a reduction in fluorophore even if Notch signalling had been inhibited. When retinas were cultured from P1.5 until P5.5, we observed an unexpected increase in average fluorescence values, which according to the reporter construct should indicate an increase in Notch signalling. Although this increase was found to be not statistically significant, there was a high variance between samples, likely due to the small sample size of fluorescence measurements. Images taken after 4 days cultured with DAPT clearly show an increase in venus positive cells when compared to control retinas, and the characteristic rossettes are present as well. This indicates that the CBFRE:H2B-Venus is not a faithful reporter for Notch signalling.

There are some issues associated with using DAPT as a Notch signalling inhibitor. DAPT is a γ-secretase inhibitor. Although the γ-secretase cleavage is crucial to the release of the NICD from the Notch receptor and therefore to the binding of NICD to the

CBF1 responsive element in the transgene, γ-secretase activity is not only involved in the Notch signalling pathway. γ-secretase is known to be involved in the cleavage of at least 60 different proteins (De Strooper and Annaert, 2010), including other substrates which also affect neuronal differentiation (Yang et al., 2010). This drug may cause phenotypic changes that we attribute to Notch signalling which are in fact due to inhibiting other signalling pathways. Although this may be part of the reason that we do not see a downregulation of venus reporter, even in studies that showed other phenotypic changes due to DAPT application were still able to show a downregulation of Notch signalling at the same time (Yang et al., 2008), suggesting that in this case the reporter was probably not faithfully reporting Notch in the retina.

Other alternatives for Notch inhibition besides DAPT do exist. Compound E {(S,S)-2-[2-(3,5- Difluorophenyl)-acetylamino]-N-(1-methyl-2-oxo-5- phenyl-2,3-dihydro-1H-benzo [e] [1,4] diazepin-3-yl)- propionamide} has been shown to be more specific in its γ-secretase inhibition and targets Notch signaling over other amyloid precursor proteins more specifically than DAPT, and at lower concentrations (Yang et al., 2008). In future studies, Compound E might prove to be a better γ-secretase inhibitor to use in retina culture systems than DAPT and could be used to verify the upregulation of the venus inhibitor that we observed in this study.

Dominant negative approaches for Notch signaling might also be an option. A CBF1 dominant negative construct has been developed, which directly binds to and inhibits CBF1 from interacting with DNA (Kato et al., 1996; Kato et al., 1997). However, this dominant negative is a plasmid-based construct, and electroporation in the retina is only possible between the date of birth of the pup and P1.5. Since the plasmid preferentially enters into cells that are actively dividing (Matsuda and Cepko, 2004), and since cell birth in the retina is divided into early born and late born cell types (Marquardt and Gruss, 2002), this limits the cell populations in which Notch signaling can be inhibited using this method, mainly to a proportion of bipolar and photoreceptor cells. This excludes the ganglion cells, a cell population that was consistently reporter positive in both transgenic cell lines and which showed *in situ* hybridization expression of *Notch* at P1.5.

A study characterizing the CBFRE:H2B-Venus line has recently been accepted for publication (Nowotschin et al., 2013). Here, the authors attempted to show the faithfulness of the reporter. They were able to show some evidence for upregulation of reporter activity by the electroporation of NICD into the visceral ectoderm at E6.5, a tissue which does not have normal Notch signal or expression (Nowotschin et al., 2013). However, the authors were unable to show a knockdown of signalling due to inhibition of Notch signalling (S. Nowotschin, personal communication). In a screen of many tissues, they found other regions where Notch was known to be expressed but was not detected, but they attribute this to positional effects of the transgene. They also saw some ectopic expression of the reporter where it is not known to be seen, and speculate that this is due to the long half-life of the venus fluorophore.

4.2.2 Problems associated with Notch reporter transgenic lines

Both Notch reporter lines were created by pronuclear injection of a plasmid into a fertilized oocyte. Although this is a fairly standard method for generating a transgenic mouse, there are several caveats associated with this approach (reviewed by Matthaei, 2007; Gama Sosa et al., 2010). First, transgenes often insert into genomic DNA as concatemers, with multiple copies of the transgene arranged in tandem. This can lead to an unpredictable copy number of the transgene, which may affect the level of expression. Furthermore, the insertion site of the plasmid is completely random. Because of this, the transgenes are susceptible to positional effects. These may include unexpected expression levels due to endogenous enhancers present in the DNA sequence near to the insertion site, or epigenetic effects due to chromatin silencing. Indeed, this unpredictable insertion site has proven to be advantageous for retinal studies, and recently retina labelling patterns in transgenic reporter mice designed to report for genes important to other neural systems, some of which not known to be expressed in the retina, have been documented (Siegert et al., 2009). Many of these mice have actually proven to be extremely helpful as they often label distinct populations of retinal cell types (Münch et al., 2009).

There are several potential problems specific to the transgene construction for both of these reporter lines. The transgene is built around responsiveness of the CBF1 responsive element. Rather than the NICD binding directly to the transgene reporter, CBF1 protein needs to be present in the nucleus to act as a mediator between both the NICD and the transgene. Furthermore, CBF1 acts as both a transcriptional repressor and activator (Fortini and Artavanis-Tsakonas, 1994; Kovall, 2007). When it is present in the transgenic animal, in the absence of NICD it would act not only to repress the expression of downstream Notch target genes, but also the fluorescent reporter. However, since the transgene contains a basal SV40 promoter, the possibility exists that if CBF1 is not present to repress its activity, there could be some low levels of reporter expression in these cells in the absence of NICD. This could be especially problematic in the CBFRE:H2B-Venus line because the venus fluorophore is extremely bright and even low levels can be detected (Sarkar et al., 2009) Furthermore the construction of this transgene doesn't take into account the fact that DNA binding might be regulated in cell types or at different developmental time points by epigenetic changes (reviewed by Jones and Takai, 2001). Just because NICD is present (which would lead to reporter activation), the actual binding sites upstream the inhibitory bHLH genes might not be activated by NICD.

A similar problem with using these transgenic reporter lines for developmental studies is that the expression patterns are affected by the stability of the fluorophore. The half-life for EGFP is at least 24 hours (Li et al., 1998), and venus is even more stable (Sarkar et al., 2009). This means that even after Notch signalling has ceased, the reporter will continue to be detected for at least 24 hours. This temporal limitation could definitely impact studies of such a tightly regulated and dynamic signalling pathway such as Notch.

4.3 A possible role for Notch signalling in the adult retina

As a role for Notch in the adult neural retina has not been explored, at this point a possible functional role for Notch is only speculation. That being said, a few studies have come out in the last couple of years that open the possibility of such a role. Although Notch was traditionally thought to be involved largely in development and stem

cell maintenance, it has recently been implicated in the adult brain, especially in its role in synaptic plasticity (Wang et al., 2004).

Notch has been shown to be directly regulated by synaptic plasticity genes in the brain including Activity-regulated cytoskeleton-associated gene (Arc) (Alberi et al., 2011). *Arc* mRNA is known to accumulate at sites of high synaptic activity, particularly when NMDA receptors are active (Lyford et al., 1995). Arc itself has been shown to modulate AMPA receptor trafficking (Shepherd et al., 2006) and is present at sites of LTP (Alberi et al., 2011). Furthermore, Notch1 and Arc directly interact, and both are upregulated in response to neuronal activity (Alberi et al., 2011). Recent studies have revealed light-induced (Jones et al., 2012) and activity dependent (Xia et al., 2007) synaptic plasticity in retinal ganglion cells. Since light-induced plasticity has been shown to be due to changes in AMPA receptor composition ganglion cells (Jones et al., 2012), it is possible that Arc and perhaps Notch1 may play roles in this process.

It has been suggested that Arc is expressed in at least some retinal neuronal sub-types. During a screen of transgenic reporter mouse strains constructed using Bacterial artificial chromosome (BAC) constructs with reporters under the control of various gene promoters expression of an Arc reporter in the adult retina was characterized (Siegert et al., 2009). They found that reporter was present in the A17 subset of amacrine cells (Siegert et al., 2009). This is of particular interest for us because although there are no A17 specific antibodies, based on location and morphology, it is likely that at least some of the amacrine cells we observed positive EGFP expression in using our NTR line were of this population. Furthermore, A17 amacrine cells are known to form reciprocal synapses with rod ON bipolar cells (Grimes et al., 2010), another cell population that strongly expressed the EGFP reporter. However, since cone type 2 OFF bipolar cells and a large subset of ganglion cells also expressed the reporter, and as rod ON bipolar cells do not synapse directly with ganglion cells (Kolb, 1979), if the NTR mouse line is faithfully reporting Notch activity in the retina, there is clearly more than one retinal signalling pathway involved.

4.4 Conclusions and Future Directions

This research has shown that there may be a possible role for Notch signalling in the adult retina. Although were unable to show the cellular colocation of mRNA for the Notch signalling components by *in situ* hybridization, RT-PCR revealed the expression of *Notch1*, *Notch2* and the ligands *Jagged1* and *Jagged2* in the adult retina. Examination of two separate Notch reporter transgenic mouse lines revealed two completely different expression patterns, although the specificity of these reporters could not be verified.

Further work is needed to determine the presence and localization of Notch signalling components in the adult retina. Although the NTR mouse line has been put on hold and is no longer distributed by Jackson laboratories, they are re-deriving a new strain of this mouse from another lab {Tg(CP-EGFP)25Gaia/ReyaJ} and are hoping to release it in October 2013. Ideally, this line should be obtained and the specificity of the reporter expression for Notch signalling in the retina should be verified using a signalling inhibitor such as DAPT or compound E.

To increase the cellular resolution of the RT-PCR results, single-cell RT-PCR could be performed using the two Notch reporter mouse lines similar to what was described by Roesch and colleagues (2008). The retinas would be dissociated and cells could be isolated based on their GFP/venus expression. If the cells are fixed they can also be stained with markers for various retina cell types to determine which cells are expressing the reporter. Single cell RT-PCR could confirm the specificity of the Notch reporters, because in theory, fluorescent cells would have an upregulated expression of *Notch* receptors compared to non-fluorescent cells. Since the CBF1 responsive element can integrate signalling from any of the Notch receptors, this would help us to resolve which Notch receptor(s) (Notch1-3) are responsible for the signalling in a given cell.

The *in situ* hybridizations could also be optimized, and perhaps the detection signal strength could be increased by using multiple probes for the same receptor in a given hybridization. By having multiple probes which bind to slightly different locations on the

nucleotide sequence we may be able to increase the signal strength. Furthermore, probes were designed and constructed for *Notch3*, and the Notch ligands *Jagged1* and *Jagged2* as well as *Delta-like ligand1, 3*, and *4* (See Table 4). These probes could be used to investigate a possible role for these ligands and receptor in the adult retina.

If the expression pattern of Notch can be verified, further research will need to be done to establish a possible role for Notch in the adult retina. If the NTR line can be verified to be faithful for Notch signalling, it can be used to examine a possible role in light-dependent activation of a signalling cascade. For example, light deprivation studies could be performed to see if EGFP expression can be downregulated in response to light deprivation. Furthermore, regardless of whether the transgenic mouse lines can be verified, a role for Notch in synaptic plasticity could be conducted in a manner similar to that performed by Jones *et al.,* (2012) but in the presence of a small molecule Notch inhibitor to see if the light-induced plasticity can be downregulated in the absence of Notch signalling.

Although there are many unanswered questions, the research presented in this thesis represents and exciting possibility for a previously unknown role for Notch signalling in the adult retina.

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